Glutamat og GABA:
Hovedaktører i nevronal metabolisme

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PREFACE AND ACKNOWLEDGEMENTS

This thesis presents experimental work carried out from June 2001 until August 2006 at the Norwegian University of Science and Technology (NTNU). The studies have been done at the Department of Neuroscience at The Faculty of Medicine and was supported by NTNU, the Central Norway Regional Health Authority (Helse Midt-Norge)/ St. Olavs Hospital, Trondheim University Hospital and the Norwegian Research Council. An initial two month student summer grant inspired me to pursue the medical student research program (forskerlinjen). I was the first female to graduate from this program in June of 2005, when I also finished my medical degree. Finally, in August 2005, I officially started on my PhD. My supervisors have been Professor Ursula Sonnewald and from August 2005 also dr.scient. Hong Qu. I am grateful for all their help and enthusiasm! I also wish to thank my other co-authors for important contributions to this research, especially Professor Arne Schousboe and Associate Professor Helle Waagepetersen at the Danish University of Pharmaceutical Sciences.

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SUMMARY

Disturbance of neuronal metabolism has implications for a number of neurological and psychiatric conditions, and enhanced knowledge of this is important in developing new methods for treating such disorders. The present research was undertaken to aid understanding of diseases related to disturbance in glutamate and γ-amino butyric acid (GABA) metabolism.

Two different types of neuronal cell cultures were used in these studies; one containing GABAergic neurons of cerebral neocortical origin and one containing cerebellar neurons. The latter consists primarily of glutamatergic granule neurons in addition to ~6 % GABAergic neurons and a small number of astrocytes. Metabolism was studied by ^{13}C magnetic resonance spectroscopy (MRS) and mass spectrometry (MS) after adding ^{13}C-labeled precursors ([1-^{13}C]glucose, [U-^{13}C]glutamate or [U-^{13}C]glutamine) to the medium of these cultures. High performance liquid chromatography (HPLC) was used to quantify different amino acids in cell extracts and medium. The amount of protein in the cultures was determined to assess cell damage.

In the cerebellar neuronal cultures, GABA was present in surprisingly large amounts compared to neocortical GABAergic cultures. ^{13}C MRS experiments showed that GABA was actively synthesized throughout the culture period by the subpopulation of glutamate decarboxylase (GAD) positive (GABAergic) neurons and subsequently distributed to the other cells in the culture, i.e. to the granule neurons. The function of GABA in these glutamatergic neurons still remains uncertain; however, roles as neurotrophic and neuroprotective agent as well as substrate for energy production have been suggested.

As shown previously, both glutamate and glutamine were shown to be excellent precursors for intermediary metabolism in cerebellar neurons. However, it was concluded that glutamate was preferred over glutamine, suggesting that these neurons rely more on reuptake of released glutamate than of supply of glutamine from astrocytes for glutamate homeostasis. This is not surprising when considering the cerebellar structure, with few astrocytes compared to neurons and a relatively large distance between astrocyte and synapse.

Exposure of cerebellar cultures to 50 μM kainic acid (KA), a potent glutamate agonist, which is known to eliminate vesicular release of GABA in these cultures, only marginally affected glutamate and GABA metabolism, whereas increasing the KA concentration to 0.5 mM led to a reduction of both GABA and glutamate metabolism compared to unexposed cultures. It was previously believed that treatment with 50 μM KA eliminated the GABAergic neurons in cerebellar cultures, and KA has therefore been added in order to obtain essentially pure glutamatergic granule cell cultures. Although KA treatment abolishes vesicular GABA release, the GABA synthesizing cells are not eliminated by this treatment and still produce GABA in substantial amounts.

Results from the present studies can only be understood in terms of inter- and intracellular compartmentation of metabolism. The main focus of metabolic compartmentation studies has been on the two compartments made up by neurons and astrocytes. One pathway previously believed to take place in the astrocytic but not in the neuronal compartment, is the pyruvate recycling pathway for complete tricarboxylic acid (TCA) cycle oxidation of glutamate. Despite this, in one of the present studies, such recycling was clearly present in both astrocytic and neuronal cultures from cerebellum.

LIST OF PAPERS

This thesis is based on the following publications:

Paper 1

Sonnewald U, Olstad E, Qu H, Babot Z, Cristòfol R, Suñol C, Schousboe A and Waagepetersen H. First direct demonstration of extensive GABA synthesis in mouse cerebellar neuronal cultures. *J Neurochem* (2004) 91, 796-803

Paper 2

Sonnewald U, Kortner TM, Qu H, Olstad E, Suñol C, Bak LK, Schousboe A and Waagepetersen HS. Demonstration of extensive GABA synthesis in the small population of GAD positive neurons in cerebellar cultures by the use of pharmacological tools. *Neurochem Int* (2006) 48, 572-578

Paper 3

Olstad E, Qu H and Sonnewald U. Glutamate is preferred over glutamine for intermediary metabolism in cultured cerebellar neurons. *J Cereb Blood Flow Metab* (2006) *in press*

Paper 4

Olstad E, Qu H and Sonnewald U. Long-term kainic acid exposure reveals compartmentation of glutamate and glutamine metabolism in cultured cerebellar neurons. *Neurochem Int* (2006) *in press*

Paper 5

Olstad E, Olsen GM, Qu H and Sonnewald U. Pyruvate recycling in cultured neurons from cerebellum. *J Neurosci Res* (2006) *in press*

ABBREVIATIONS

acetyl CoA	acetyl coenzyme A
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
AOAA	aminooxyacetic acid
ATP	adenosine triphosphate
CNS	central nervous system
DMEM	Dulbecco's minimum essential medium
EAAT	excitatory amino acid transporter
FCS	fetal calf serum
GABA	γ-amino-butyric acid
GABA-T	GABA aminotransferase
GAD	glutamate decarboxylase
GAT	GABA transporter
GC	gas chromatography
GDH	glutamate dehydrogenase
GLUT	glucose transporter
GS	glutamine synthetase
GSH	glutathione
GVG	γ-vinyl GABA
HPLC	high performance liquid chromatography
KA	kainic acid
MR	magnetic resonance
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
NMDA	N-methyl-D-aspartate
nOe	nuclear Overhauser effect
OAA	oxaloacetate
OPA	*o*-phthaldialdehyde
PAG	phosphate activated glutaminase
PC	pyruvate carboxylase
TCA	tricarboxylic acid

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1 INTRODUCTION

1.1 Medical Aspects of Neuronal Metabolism

Normal energy metabolism in the brain has several unusual features compared to other organs, and disturbance of this metabolism is considered important in many brain disorders (Balázs et al., 2006). One of the features of normal brain function is the **high metabolic rate**; in fact, the brain is one of the most metabolically active organs in mammals, illustrated by the fact that despite constituting modest 2 % of the total body mass, the brain accounts for an astounding 20 % of the resting body's oxygen consumption (McKenna et al., 2006a). This oxygen is almost exclusively used for oxidation of glucose (Sokoloff, 1960), the main energy source of the brain. Under extraordinary conditions, like prolonged starvation, the mature brain can adapt to using ketone bodies produced in the liver from fat to cover some of the energy needs (Stryer, 1995b). Nevertheless, the brain is not very flexible when it comes to energy substrates compared to other organs and is **critically dependent on aerobic metabolism of glucose** (Dugan and Kim-Han, 2006; McKenna et al., 2006a). Another feature is the **limited intrinsic energy stores** of the brain. Although some glycogen can be stored, mainly in astrocytes (Pfeiffer-Guglielmi et al., 2003; McKenna et al., 2006a), the brain has no significant energy reserve. It has been estimated that if glycogen was the only source of fuel, it would be consumed in a few minutes (McKenna et al., 2006a). Thus, the brain is dependent on a constant supply of glucose and oxygen via the blood.

The dependence of a constant blood supply carrying glucose and oxygen makes the brain particularly vulnerable to ischemic injury

(Dugan and Kim-Han, 2006). This is most often seen as a disruption of blood supply to a part of the brain caused by a thromboembolic occlusion of an intracranial artery, commonly known as a **stroke** (Smith, 2004). This is the most common neurological disorder in terms of both morbidity and mortality (De Girolami et al., 1999). When the blood flow, and thereby the energy supply, to the brain is impaired, ATP levels decreases, which in turn affects the active ion pumps, such as the Na^+/K^+ ATPase. The ion gradients over the cell membrane, and thus the membrane potential will be disrupted, and the neurons are depolarized (Smith, 2004; Balázs et al., 2006; Dugan and Kim-Han, 2006). This causes a cascade of events ultimately leading to cell death. With the reduction of cerebral blood flow in ischemia, the extracellular glutamate concentration is substantially elevated (Smith, 2004). This leads to excessive activation of excitatory amino acid receptors, in particular glutamate receptors, causing cell death, a mechanism referred to as **excitotoxicity** (Olney, 1978).

A role for excitotoxicity has been implicated in the etiology of many **neurodegenerative diseases**, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Mattson, 2003; Balázs et al., 2006). Excessive or prolonged activation of specific glutamate receptors results in a rise in intracellular Ca^{2+} concentration, triggering a cascade of intracellular events culminating in neurodegeneration. Different types of neurons have different vulnerability to excitotoxicity, depending on their receptors, Ca^{2+} permeability and ability to handle an increase in intracellular Ca^{2+} (Balázs et al., 2006). The glutamatergic N-methyl-D-aspartate (NMDA) receptors are the primary receptors activating excitotoxicity because of their high permeability to Ca^{2+}, although other glutamate receptors can initiate excitotoxicity by allowing excessive Ca^{2+} entry. Studies have shown that cytoplasmic Ca^{2+} is insufficient to cause

neuronal death in itself, and that mitochondrial Ca^{2+} accumulation is essential for excitotoxic cell death (Stout et al., 1998; Nicholls et al., 2003). Ca^{2+} causes mitochondria to generate reactive oxygen species, and this oxidative damage can initiate cell death. Diseases such as Alzheimer's disease, Parkinson's disease and ALS are accompanied by increased oxidative stress, and in these patients, neurons are more susceptible to excitotoxic death (Balázs et al., 2006). Thus, excitotoxicity contributes to oxidative stress, which in turn reduces the threshold for excitotoxicity, leaving cells more vulnerable to injury. This is one of the reasons why excitotoxicity contributes to many neurodegenerative diseases. Knowledge of regulation of glutamate receptors in Alzheimer's disease, Parkinson's disease and ALS have resulted in clinically efficacious drugs and new therapeutic medications are continually being developed (Mattson, 2003).

Another common neurological disorder is **epilepsy**, characterized by recurrent, spontaneously occurring seizures with symptoms caused by abnormal excessive or hypersynchronous neuronal activity in the brain (Blume et al., 2001; Fisher et al., 2005). The epileptic seizure is a pathophysiological process characterized by a synchronous activation of a large group of neurons in the brain. This may be caused by a disturbance in the fine-tuned balance between excitatory glutamatergic and inhibitory GABAergic neurotransmission, a theory supported by the fact that inhibition of γ-amino butyric acid (GABA) synthesis and administration of GABA antagonists and glutamate agonists induce seizures (Bradford, 1995; Hosford, 1995). Studies of glutamate and GABA contents in epileptogenic brain tissue have shown contradictory results. An increased level of glutamate compared to GABA in superfusates and microdialysates from hyperactive focal tissue was presented by Bradford, (1995), whereas Aasly et al. (1999) showed an increased GABA concentration in brain tissue from epilepsy surgery. This indicates that a high concentration

of GABA in tissue not necessarily provides protection against seizures. Activation of glutamate receptors is essential for seizure activity and mediates excitotoxic neuronal damage and death (Balázs et al., 2006). Antiepileptic drugs such as phenobarbital, phenytoin and carbamazepine work by suppressing excitability by different mechanisms.

Glutamate and glutamate receptors also appear to have a role in several non-degenerative neurological and psychiatric disorders. One example is **schizophrenia**, a psychiatric disorder characterized by psychosis, impaired perception or expression of reality (positive symptoms) and by significant social dysfunction (negative symptoms) (Morrison and Murray, 2005). For decades, theories and treatment of schizophrenia have focused on dopaminergic neurons. However, in recent years it has been suggested that glutamatergic neuro-transmission is also involved in the pathophysiology of this disease (Carlsson et al., 2001; Carlsson et al., 2004; Balázs et al., 2006; Kondziella et al., 2006). Numerous *in vivo* and *ex vivo* studies have shown disturbances of glutamate signaling in schizophrenia patients (for review, see de Bartolomeis et al., 2005). This supports the glutamate hypofunction theory, which focuses on the NMDA receptors. Pharmacological inhibition of these receptors leads to a state with positive and negative symptoms resembling those of schizophrenia (Rujescu et al., 2006). Levels of glutamine have been shown to be altered in patients experiencing their first episode of schizophrenia (Theberge et al., 2002), whereas in postmortem brain biopsies of schizophrenic patients, a reduction of glutamine synthetase (GS), the enzyme catalyzing the formation of glutamine from glutamate, was reported (Burbaeva et al., 2003). The recent advances in knowledge on glutamate involvement in schizophrenia pathophysiology pave the way for new pharmacological strategies in treating schizophrenia (de Bartolomeis et al., 2005; Balázs et al., 2006).

In order to understand the pathophysiological mechanisms, a premise for the development of pharmacological treatment of these diseases, basic research on neuronal metabolism is of importance.

1.2 The Cells of the Brain

The functional unit responsible for transmitting and processing information in the nervous system is the neuron (De Girolami et al., 1999; Augustine, 2004). These are cells anatomically and functionally specialized for transmission of electrical and chemical signals. However, in the cerebrum the neurons are greatly outnumbered by the other main cell type of the brain, the glia (Nedergaard et al., 2003). During phylogenetic development, the glia to neuron ratio has increased at the same time as the cerebrum and especially the cerebral cortex has expanded in size (Karlen and Krubitzer, 2006). The human brain has the largest neocortical surface relative to brain size and the highest glia to neuron ratio of all land mammals, which can suggest that glial cells play important roles in higher cognitive functions (Nedergaard et al., 2003). In contrast to the cerebrum, the cerebellum is one of the most evolutionary primitive brain regions. In the cerebellum, the neurons greatly outnumber the glial cells (Andersen et al., 1992). This is because of the numerous glutamatergic granule cells, in fact this single cell type is by far the most numerous neuronal cell type in the brain. It has been calculated that the human cerebellum consists of approximately 105×10^9 granule cells (Andersen et al., 1992), whereas the number of neurons in the neocortex is approximately 20×10^9 (Pakkenberg and Gundersen, 1997; Gredal et al., 2000).

1.2.1 Neurons and Neurotransmission (Augustine, 2004)

The intracellular signal of the neuron is an electric impulse caused by ion movement across the cell membrane. This **action potential** propagates from its point of initiation at the cell body and runs down the axon to the nerve ending, where the neuron forms synapses with other cells, either neurons or effector cells (muscle- or glandular cells). In the nerve ending the electrical signal is transformed to a chemical signal consisting of **neurotransmitters**, which lead the signal to the next cell.

Chemical transmission between neurons involves **synthesis**, **storage**, **release**, **receptor binding**, and **inactivation** (including uptake or reuptake) of the transmitter substance. The neurotransmitter is first formed and stored in vesicles where it is protected from enzymatic degradation. When the neuronal cell membrane is depolarized by an action potential, the vesicles release the transmitter to the synaptic cleft. The transmitter molecules diffuse passively in the synaptic cleft between the two cells and bind to receptors typically on the postsynaptic cell. Receptor binding leads to a change in the cell membrane's permeability to one or more ions, and the membrane potential of the postsynaptic cell can temporarily be changed. An excitatory impulse will cause membrane **depolarization** and decrease the membrane potential, whereas an inhibitory impulse will lead to membrane **hyperpolarization** and increase the membrane potential. The direction of the change in membrane potential is determined by the neurotransmitter and the receptor it binds to. Since most neurons are innervated by thousands of synapses, the postsynaptic effects of each active synapse can be added together in space and time, and determine whether the postsynaptic neuron will generate a new action potential or not.

Excitatory impulses are mainly transferred through the neurotransmitter **glutamate**. Binding of glutamate to receptors on

the postsynaptic neuron brings it closer to the threshold for triggering of an action potential. Inhibitory impulses are mainly caused by the neurotransmitter **GABA**. Under influence of GABA, the postsynaptic neuron will be brought further away from the action potential threshold, and thus GABA works against the formation of an action potential.

There are two main types of **receptors**, ionotropic, ligand-gated ion channels, and metabotropic receptors. On the **ionotropic** receptors, the binding site is located on the ion channel itself, and these receptors therefore transfer fast postsynaptic signals. The **metabotropic** receptors have an indirect connection between binding site and ion channel through second messengers. These receptors have a modulating effect by increasing or decreasing the probability for an action potential to be triggered by the sum of postsynaptic signals. In addition to receptors on the postsynaptic neuron, there are autoreceptors responding to the neurotransmitter released from the neuron itself and modulating release or synthesis.

After receptor binding the transmitter is inactivated, either actively (through enzymatic degradation (e.g. acetylcholine), reuptake into the presynaptic neuron or uptake in glia) or passively (by diffusion).

Chemical neurotransmission thus involves five steps; synthesis, storage, release, receptor binding and inactivation, each a potential target for pharmacological modulation.

1.2.2 Glia

Historically, glial cells were considered a type of passive connective tissue, which provided structural support to the neurons, which were considered to be the only true functional cells of the brain. Today, glial cells are recognized as partners to neurons in virtually

every function of the brain, and as participants in the pathophysiology of the dysfunctional or diseased brain (Nedergaard et al., 2003).

There are three main types of glial cells in the brain; **microglia**, **oligodendrocytes** and **astrocytes** (the last two are sometimes referred to as macroglia). Microglia are derived from macrophages and serve a phagocytic function in the brain. Oligodendrocytes produce myelin in the central nervous system (CNS). Myelin consists of multiple layers of oligodendrocyte membranes wrapped concentrically around one or more axons, acting like insulation allowing the action potentials to be conducted at high speed. The astrocytes' main task is regulation of the chemical environment of the brain. These glial cells have endfeet surrounding the blood vessels in the brain. The astrocytes interact with the vasculature to form a gliovascular network, which has been subject for intense research activity the past decade (Nedergaard et al., 2003). It has been suggested that astrocytes influence the integrity of the blood-brain barrier consisting of the endothelial cells connected with tight junctions (Ransom et al., 2003). This barrier keeps many substances from entering the brain, and is one of the ways the brain is protected against potentially harmful substances. Astrocytes also envelop synapses in the CNS, preventing neuroactive transmitters from moving freely in the brain, and play an important role in inactivation of these and other substances through efficient uptake and conversion into other substances. These glial cells also play a significant role in supplying neurons with a number of metabolites and precursors for amino acid neurotransmitters. This is described in the following section.

1.2.3 Neuronal-Glial Interaction and Compartmentation

In this thesis, metabolism is studied *in vitro* in cell cultures consisting of mainly one cell type (Hertz et al., 1985). By analyzing

the metabolites in different cell types separately, useful information can be provided. However, it is important to acknowledge that the *in vivo* situation is different from *in vitro*. Brain tissue is a metabolically heterogeneous system including two distinct compartments consisting of neurons and glia (van den Berg et al., 1969; Berl and Clarke, 1983; McKenna et al., 2006a). There is an extensive exchange of metabolites between the two cell types, and this is essential for normal brain function.

A component of the compartmentation is that astrocytes contain a **different set of enzymes** than neurons. They can therefore supply neurons with substrates the neurons themselves are unable to synthesize. **Pyruvate carboxylase** (PC) is for example present only in glia (Yu et al., 1983; Shank et al., 1985), and this enables these cells to convert pyruvate to oxaloacetate (OAA), which is part of the tricarboxylic acid (TCA) cycle. Neurons are depending on a flux of precursors for TCA cycle intermediates from astrocytes. Without this the TCA cycle in neurons would be drained of carbon atoms because neurons have no net synthesis of TCA intermediates, and by releasing the neurotransmitters glutamate and GABA carbon atoms derived from the cycle are lost.

Another astrocyte specific enzyme is **glutamine synthetase** (GS), and thus glutamine is only produced in astrocytes (Norenberg and Martinez-Hernandez, 1979), but is exported to a great extent to neurons, where it is an important precursor for amino acids, such as glutamate and GABA (Schousboe et al., 1977; Sonnewald et al., 1993; Schousboe, 2003; McKenna et al., 2006a). This constitutes the basis for the **"glutamate-glutamine-GABA cycle"** (Berl and Clarke, 1969; van den Berg and Garfinkel, 1971; Benjamin and Quastel, 1975; Berl and Clarke, 1983; for review see Bak et al., 2006), which is discussed later, and in detail in paper 3.

1.3 Transport and Metabolism of Glucose, Glutamate and GABA

1.3.1 Glucose

As mentioned, the brain is one of the most metabolically active organs in mammals, and glucose is the brain's **main energy source** (McKenna et al., 2006a). Delivery of glucose from the blood to the brain requires transport across the blood-brain barrier. This is facilitated by glucose transporter proteins (GLUTs). Three of these proteins have been established as cell specific transporters in mammalian brain (Vannucci et al., 1997). Firstly, two isoforms of GLUT1, the 55 kDa and 45 kDa isoforms, which are primarily detected in endothelial cells of the blood-brain barrier and in astrocytes, respectively (Maher et al., 1994; Maher, 1995). Secondly, GLUT3, which is a neuronal glucose transporter and lastly GLUT5, which is exclusively expressed in microglia of the human and rat brain (Payne et al., 1997).

Inside the cells, glucose ($C_6H_{12}O_6$) is eventually converted to carbon dioxide (CO_2) and water (H_2O) in three phases, this oxidation generates energy in the form of ATP (Stryer, 1995c; McKenna et al., 2006a). In **glycolysis**, occurring in the cytoplasm of the cell, glucose is divided into two C_3-fragments in the form of pyruvate. The latter can be converted to lactate, alanine or acetyl coenzyme A (acetyl CoA), which can be processed in the **TCA cycle**. This cycle takes place in mitochondria, and produces reducing equivalents for oxidative phosphorylation. In addition to energy production, the TCA cycle also supplies carbon skeletons for the synthesis of metabolites such as glutamate and GABA. The last phase of metabolism, the one generating the most ATP, occurs in the inner membrane of the mitochondria and is called the **electron transport chain**. In this aerobic catabolism of one glucose molecule in the brain a total of 36 molecules of ATP are produced (Stryer, 1995c).

1.3.2 Glutamate

Glutamate is an **excitatory amino acid** mediating fast excitatory synapse responses in the CNS (Storm-Mathisen et al., 1983; Fonnum, 1984). It is widespread in all of the CNS and the brain contains large amounts, about 5-15 mmol per kg wet weight, depending on the region (Schousboe, 1981). In addition to being the most important excitatory neurotransmitter, glutamate has an important metabolic function.

Glutamate does not cross the blood-brain barrier, and is thus produced from glucose within the brain itself (Gruetter et al., 1994; McKenna et al., 2006a). There are mainly two mechanisms for **synthesis** of glutamate. The amino acid can be formed from the TCA cycle intermediate α**-ketoglutarate** by transamination (catalyzed by one of the aminotransferases, most commonly aspartate aminotransferase (ASAT) or alanine aminotransferase (ALAT)) or reductive amidation (catalyzed by glutamate dehydrogenase (GDH)). The other mechanism of glutamate synthesis is conversion from **glutamine** synthesized in glial cells and exported to neurons where it enters mitochondria, where the enzyme phosphate activated glutaminase (PAG) catalyzes the reaction (Kvamme et al., 2000; Kvamme et al., 2001). Regulation of the transmitter pool of glutamate and the availability of this pool is based on an elaborate interaction between neurons and glia.

After synthesis, glutamate is stored in synaptic vesicles in high concentrations and released to the synapse after increase in intracellular calcium following depolarization of the nerve ending. The **release** is modulated by a metabotropic auto-receptor on the presynaptic neuron. The concentration of glutamate in the synapse can rise from 2-5 μM before release to as much as 50-100 μM after depolarization.

There are both ionotropic and metabotropic glutamate **receptors**. The ionotropic glutamate receptors located on the postsynaptic neuron are divided into three classes; the **NMDA** receptor, the **AMPA** receptor and the **KA** receptor. These subtypes are named after the first synthetic agonists, which bound strongly and relatively specific to them, N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, and kainic acid, respectively. Binding of glutamate to one of these receptors can lead to depolarization of the membrane of the postsynaptic neuron.

In papers 1 and 4, cell cultures were exposed to the potent glutamate agonist kainic acid (**KA**), binding to the KA and AMPA classes of ionotropic receptors (Lerma, 1998). KA injection has been used as an epilepsy model and the effects of KA have previously been studied both in animals and cell cultures, increasing the knowledge of the epileptogenesis (Ben-Ari and Cossart, 2000). Animals injected with KA (systemic or intracerebral) have seizures resembling complex partial epileptic seizures (Ben-Ari, 1985; Sperk, 1994; Bradford, 1995; Muller et al., 2000; Qu et al., 2003). The synchronized neuronal hyperactivity starts in the CA3-region of the hippocampus and spreads to other limbic structures. The seizures are followed by cell loss comparable to the cell loss seen in patients with temporal lobe epilepsy (Nadler, 1981). Some time after the injection (weeks to months), the animals develop spontaneous epileptic seizures, thus they develop epilepsy (Ben-Ari, 1985; Leite et al., 2002).

In cell cultures, KA has shown effects on survival of neurons; however, these effects are not fully understood (Balázs et al., 1990; Kato et al., 1991; Jensen et al., 1999; Drian et al., 2001). The complexity is illustrated by KA having a trophic effect with increased survival of cerebellar neurons in culture at low doses, whereas high doses are toxic to these cells (Balázs et al., 1990). Studies have also shown that KA has different, even opposite, effects on neurons in

different developmental stages (Frandsen and Schousboe, 1990; Drian et al., 2001;). In papers 1 and 4, KA effects on cell survival and metabolism in neuronal cultures were studied.

The receptors are, as previously mentioned, named after their synthetic agonists. It is of great value that also glutamate receptor **antagonists** are known. This makes selective inhibition of receptors possible. In papers 3, 4 and 5, cell cultures were incubated in medium containing [U-^{13}C]glutamate in order to study glutamate metabolism. In these experiments glutamate receptor antagonists DNQX (6,7 dinitroquinoxaline-2,3(1H,4H)-dione, an AMPA/kainate-selective glutamate receptor antagonist), and D-AP5 (D-2-amino-5-phosphonopentanoic acid, which inhibits the NMDA receptor), were added to the incubation medium of the cell cultures to avoid toxic effects of glutamate during incubation (Frandsen et al., 1989).

As mentioned, a high glutamate concentration has neurotoxic effects, and it is of critical importance to keep the extracellular glutamate concentrations low. Glutamate receptors are widespread, and can be found on most of the cellular elements (dendrites, nerve endings, neuronal cell bodies as well as glial cells) in the brain. After release glutamate can diffuse out of the synaptic cleft and interact with glutamate receptors in other locations than the postsynapse, and it is therefore important to remove the transmitter from the cleft after release. This is mainly done by uptake through sodium dependent **glutamate transporters** in the cell membranes of astrocytes surrounding the synapse (for review, see Danbolt, 2001). Five distinct high affinity subtypes of glutamate (excitatory amino acid) transporters are at present identified; EAAT1 (GLAST), EAAT2 (GLT), EAAT3 (EAAC), EAAT4 and EAAT5. EAAT1 and EAAT2 are responsible for most of the glutamate uptake, and until recently they were believed to be found exclusively on astroglia. However, Danbolt et al. (2006) reported that in hippocampal slices, about 15 % of EAAT2 was

distributed in nerve terminals and axons, and that neuronal glutamate reuptake through these was quantitatively significant. EAAT3 is present in several types of neurons as well as in glia, particularly in oligodendrocytes (Conti et al., 1998). EAAT4 is expressed mainly in the purkinje cells of the cerebellum, while EAAT5 is found in the retina (Arriza et al., 1997).

Glutamate taken up by astrocytes can be metabolized to glutamine by the above mentioned astrocyte specific enzyme glutamine synthetase (GS). Glutamine can then be released from the astrocytes and taken up in the nerve ending of the glutamatergic neuron, where it once again is converted to glutamate by the enzyme PAG. Thus, a recycling of the neurotransmitter called the **glutamate-glutamine cycle** based on neuronal-glial interaction occurs (see above). This cycle is the main subject of paper 3 and is illustrated in Figure 1.1.
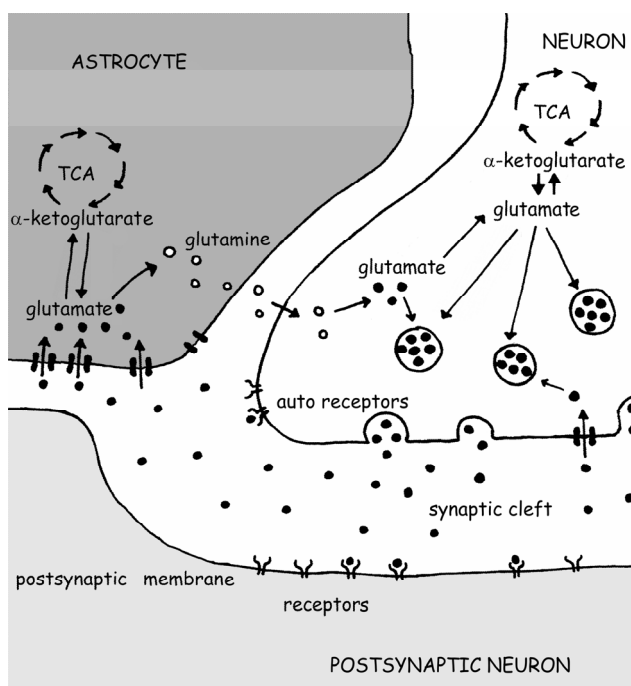
FIGURE 1.1 Glutamate is formed from α-ketoglutarate in the TCA cycle and from glutamine synthesized in astrocytes. After release to the synaptic cleft, glutamate is taken up in astroglia and converted to glutamine, which can be exported back to neurons, where it can be converted to glutamate again. This glutamine-glutamate cycle thus involves both neurons and glia.

Glutamate can also be converted to α-ketoglutarate, which can be further processed in the TCA cycle for production of energy or intermediate metabolites in both astrocytes and neurons.

1.3.3 GABA

GABA is, like glutamate, an amino acid neurotransmitter, but whilst glutamate is excitatory, GABA is the most abundant **inhibitory** neurotransmitter in the brain (Storm-Mathisen, 1974; Storm-Mathisen et al., 1983). GABA is mainly formed by decarboxylation of glutamate, a process catalyzed by the enzyme **glutamate decarboxylase** (GAD), which exists in two isoforms, GAD_{65} and GAD_{67}. GAD_{65} appears to be targeted to membranes and axonal regions including nerve endings, and has been hypothesized to preferentially synthesize GABA for vesicular release (Waagepetersen et al., 1999; Waagepetersen et al., 2001), whereas GAD_{67} is more widely distributed throughout the cell. GAD has been detected in various GABAergic neurons, but also in glutamatergic hippocampal granule cells (Schwarzer and Sperk, 1995; Gutierrez and Heinemann, 2006). However, the role of GABA in these cells is yet to be understood. The study of GABA in glutamatergic cerebellar neurons is discussed in papers 1, 2, 3 and 4.

It should be noted that there are other possible pathways of GABA synthesis. It can be formed from **putrescine** in two ways; by oxidative deamination catalyzed via diamine oxidase and by transformation into monoacetylputrescine which then undergoes deamination via monoamine oxidase. However, this GABA synthesis pathway has been shown to be insignificant in the brain (Seiler, 1980). Also in paper 2, it was shown that GABA in cerebellar neuronal cultures was not synthesized by this pathway.

When the presynaptic GABAergic neuron is depolarized, GABA is released from vesicles to the synaptic cleft by exocytosis (Augustine, 2004). The transmitter molecules cross the cleft by

passive diffusion and are bound to receptors on the postsynaptic neuron. The most important postsynaptic GABA receptor is the **GABA$_A$-receptor**, an ionotropic receptor where the GABA binding sites are located on the alpha subunits. When GABA is bound to the receptor, Cl$^-$ ions flow into the cell, and the postsynaptic membrane is hyperpolarized (Augustine, 2004). The GABA$_A$ receptor is a target for a number of **pharmacological agents**, for example benzodiazepines and various anesthetics. Auto regulation of GABAergic neurons is mainly mediated through metabotropic GABA$_B$ receptors in the presynaptic cell membrane. A third receptor, the presynaptic ionotropic GABA$_C$ receptor is also described.

The effect of GABA is rapidly terminated by **reuptake** of the transmitter into the presynaptic neuron and to a lesser degree uptake by surrounding astrocytes (Schousboe, 1981; Borden, 1996; Schousboe, 2003) via GABA transporters (GAT). Four subtypes of transporters have so far been identified; GAT-1, primarily present on GABAergic neurons and to a lesser extent in astrocyte membranes, GAT-2, GAT-3 and the low affinity subtype BGT-1. The antiepileptic agent tiagabine inhibits GAT-1 (Borden, 1996), and thus increases the GABA concentration in the synaptic cleft, making more GABA available to the receptors. GABA taken up in the nerve terminal can be stored in vesicles and used again. Another option for intracellular GABA is conversion via **GABA aminotransferase** (GABA-T) to succinic semialdehyde, which is subsequently oxidized to succinate in the GABA shunt (Balázs et al., 1970). GABA-T can be inhibited by γ-vinyl-GABA (GVG) and aminooxyacetic acid (AOAA) (Wu and Roberts, 1974; Lippert et al., 1977). AOAA can also inhibit GAD and a number of transaminases when present in high concentration (Wu and Roberts, 1974). In paper 2, GVG and AOAA were added to the medium of cerebellar neuronal cultures.

Succinate formed from GABA can be utilized for energy production or formation of intermediate metabolites in the TCA cycle, for example α-ketoglutarate which can be converted to glutamate and glutamine (the latter only in astrocytes). Glutamine can be transferred from the astrocyte back to the neuron, where it can be converted to glutamate in the mitochondria via the enzyme PAG. Glutamate can also be converted to α-ketoglutarate and thus enter the TCA cycle of the neuron or be transformed into GABA again by the enzyme GAD. Figure 1.2 shows the **GABA recycling**, and its involvement of both neurons and astrocytes (Sonnewald et al., 1993).
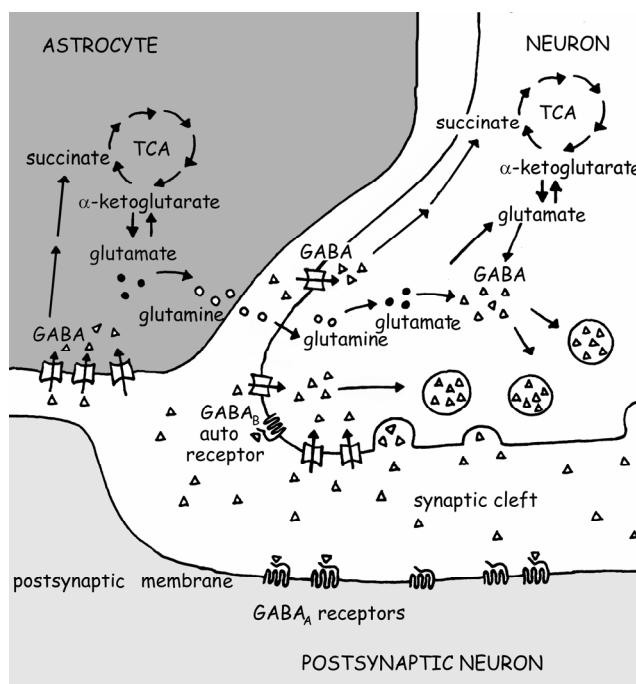
FIGURE 1.2 GABA is produced in neurons from glutamate, which either comes from α-ketoglutarate in the TCA cycle or from glutamine transferred from astroglia. After release to the synaptic cleft, GABA is taken up in neurons and glia via transporter proteins. Inside the neuron, the transmitter can be stored in vesicles and be re-used, or succinate from GABA can be metabolized in the TCA cycle of both neurons and astrocytes.

2 OBJECTIVES

Disturbance of neuronal metabolism has implications for a number of neurological and psychiatric conditions, and enhanced knowledge of this will hopefully lead to new methods for treating such disorders. The present research was undertaken to aid understanding of diseases related to disturbance in glutamate and GABA metabolism.

The specific questions addressed were the following:

Glutamate and glutamine
- It is known that glutamate and glutamine serve as substrates for intermediary metabolism in cerebellar neurons. Is there a substrate preference between these two amino acids?
- Is glutamate and glutamine metabolism in cerebellar neurons affected by long-term exposure to KA?
- The pyruvate recycling pathway has been shown to operate in astrocytes. Is it also active in cultured neurons from cerebellum?

GABA
- Is GABA present in cerebellar neuronal cultures, and if so, how is the concentration compared to that in neocortical neuronal cultures?
- If GABA is present in these neurons, how does it get there; is it taken up from serum in the medium or is it synthesized by the cerebellar neurons (GABAergic and/or glutamatergic)?
- If it is synthesized, what is the mechanism and time course throughout the culturing period for this synthesis?
- Does long-term KA exposure affect GABA synthesis in these cultures?

3 METHODS

3.1 Neuronal Cell Cultures

Cell cultures represent an important *in vitro* method in neurobiology, and primary cultures of neurons from cerebral cortex and cerebellum from mice are frequently used as models for studying basic physiological mechanisms as well as pathological conditions and pharmacological intervention (Schousboe et al., 1985). Primary cultures are prepared by taking cells directly from an organism, in contrast to cultures from cell lines which originate from one individual cell or a group of cells, often from tumors. The advantage of primary cultures is that they consist of "normal" diploid cells and thus their properties and metabolism more closely resembles that of the corresponding cells *in vivo* than do cell lines (Hertz et al., 1985).

In order to obtain viable cells, timing is crucial. Tissue must be at the developmental stage which favors cultivation of the preferred cell type. For neuronal cultures, the tissue must be at a proliferating or early post-mitotic stage (Hertz et al., 1985). The reason for this is that older neurons with established axons and dendrites will be more vulnerable to mechanical damage during the culture preparation. Different CNS cells are ready for cultivation at different ontogenetic stages. In mice, neurogenesis is nearly completed at the time of birth, with a few exceptions, one of them being interneurons in cerebellar cortex. Granule neurons are such cerebellar interneurons which develop approximately from day two until 15 after birth. The cerebellar neuronal cultures, consisting of about 90 % glutamatergic granule neurons, are therefore prepared from tissue taken from seven-day-old mice (Messer, 1977; Schousboe et al., 1989). A photomicrograph of cerebellar neurons in culture is shown in Figure

3.1. Neuronal cultures from cerebral cortex consisting mainly of GABAergic interneurons, are established with tissue from 15-day-old mouse fetuses (Drejer et al., 1987; Hertz et al., 1989).
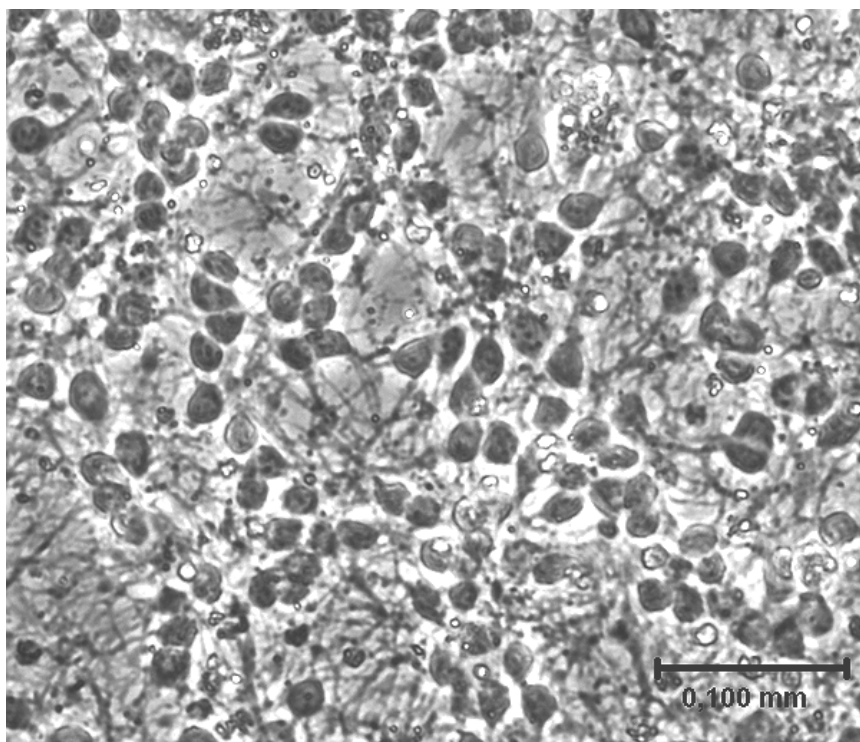

FIGURE 3.1 Photomicrograph of cerebellar neurons cultured for seven days. The majority of the cells are glutamatergic granule neurons characterized by their small size and the presence of granules in the cell body. The bar represents 0.100 mm.

In the present studies, cerebellar neuronal cultures are used in all papers, whereas neocortical cultures in addition are used in paper 1. The cultures are prepared by dissecting out the brain region of interest, i.e. cerebral cortex or cerebellum. The tissue then undergoes a multiple step purification, first it is finely cut with a razorblade. Subsequently the tissue is trypsinized followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. The steps of chemical and mechanical division result in single cells in suspension, which is transferred to a Dulbecco's minimum essential medium (DMEM). The medium contains 31 mM glucose and 10 %

(v/v) fetal calf serum (FCS), which has been through heat inactivation of the complement system. The cell suspension is seeded in poly-D-lysine coated Petri dishes after adjustment of cell density based on cell counting. Poly-D-lysine has an electrostatic attraction of negatively charged cell membranes, which gives high affinity for neurons and makes it easier for these cells to attach to the dishes. The cultures are incubated at 37 °C in 95 % atmospheric air with 5 % CO_2.

The presence of glia in neuronal cultures is unfortunate because these cells will proliferate and thus displace the neurons. In order to reduce the content of non-neuronal cells in the culture, the cytotoxic chemical cytosine arabinoside is added to the culture medium 24-48 hours after preparation. The proliferation of dividing cells like glia will be inhibited by this treatment, whereas neurons are at a post-mitotic stage and not dividing at this point in time, and will therefore not be affected (Hertz et al., 1985). Despite the cytotoxic treatment, some glial cells are present in the neuronal cultures. Approximately 5 % of the cells in the cerebellar neuronal cultures are glial cells (Messer, 1977).

In the present studies the cell cultures were exposed to different chemical substances and extracted after various days *in vitro* as described in the papers. In the extraction procedure, the cultures are divided into three fractions; medium, cell extract and protein. Different parameters were analyzed in medium and cell extract, and the protein amount was quantified as described later.

3.2 Identification of Metabolites and Metabolic Pathways by MRS

3.2.1 MRS in Neurobiological Research

Magnetic resonance spectroscopy (MRS) is a method that can be used to detect metabolites and map metabolic pathways in cells. It has a number of advantages in studies of cell metabolism. The atomic nuclei most frequently used in metabolic MR research are ^{1}H, ^{31}P and ^{13}C (for review, see Bachelard and Badar-Goffer, 1993).

^{1}H and ^{31}P have a high natural abundance, and are often used for studying differences in concentration of biological compounds under different metabolic conditions. In contrast to these nuclei, ^{13}C has a natural abundance of only 1.1 %. This makes detection difficult, and ^{13}C MRS is of limited use in studies of endogenous metabolites unless the compounds occur in large amounts. The low natural abundance of ^{13}C can, however, be used as an advantage in the study of metabolic pathways (Cerdan and Seelig, 1990; Bachelard and Badar-Goffer, 1993; Sonnewald et al., 1994). ^{13}C-labeled precursors can be added to cell cultures or be injected into animals or humans, and MRS can be used to detect and quantify ^{13}C atoms and their position in different metabolites are detected and quantified. Thus, metabolic pathways can be monitored with little background interference from endogenous metabolites. As a result, ^{13}C MRS is an important tool in analyzing brain metabolism and the metabolic trafficking between different cellular compartments.

3.2.2 Basic MR Theory (Derome, 1987; Hornak, 1997)

The background for magnetic resonance spectroscopy is the phenomenon of nuclear magnetic resonance. MR was first discovered in 1946 by Felix Bloch and Edward Purcell, and for this work they were jointly awarded the Nobel price in physics in 1952 (Hornak, 1996). The phenomenon is based on the nuclear magnetic momentum of the

atom, and the nuclear resonance arises when the nuclei of certain atoms are situated in a static magnetic field and in addition are exposed to an oscillating magnetic field.

Only those nuclei which possess the quality called "spin" can experience this phenomenon. Individual unpaired electrons, protons and neutrons possess spins of ½. This means that spin can have values that are multiples of ½, and spin can be positive or negative. Two or more particles with spin in opposite direction can neutralize the observable effect of the spin, and these particles will not be detectable by MRS. The nucleus of the ^{12}C-atom (which constitutes most of the natural carbon) has a spin of 0, and cannot be detected, whereas the nucleus of the ^{13}C-atom contains six protons and seven neutrons, and has a net spin of ½.

Nuclei with spin behave like small magnets, which point in different directions. If an externally applied magnetic field is imposed, the nuclei will orientate themselves with respect to the direction of the field to minimize their energy and point in one of two possible directions, either in the same direction as the magnetic field, which is the lower energy position (E_1) or opposite to the magnetic field (antiparallel), which is a position of higher energy (E_2). The nuclei will precess around its own axis with a certain frequency called the Larmor frequency.

In addition to the static magnetic field (B_0), an oscillating magnetic field (B_1) in the form of electromagnetic waves (radio waves) is applied perpendicular to B_0. This adds energy to the system, and makes some of the nuclei in the low energy position change to the high energy position, as illustrated in Figure 3.2. This excitation can only happen if the frequency of the radio waves matches the energy difference, ΔE, between the two energy levels. The energy difference and thus the resonance frequency, is different for different nuclei, there can only be resonance for one type of nucleus at the

time. ^{13}C has a resonance frequency of 10.71 MHz per Tesla, where Tesla (T) denotes the strength of the magnetic field.
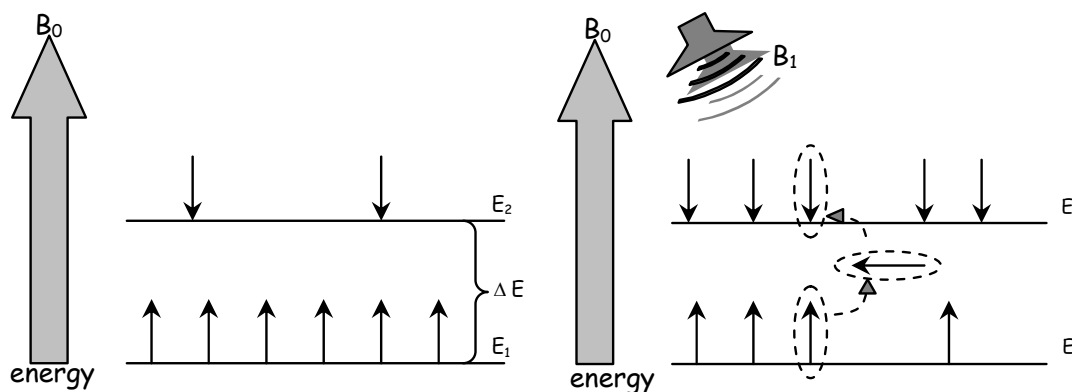

FIGURE 3.2 In a static magnetic field, B_0, the ^{13}C nuclei will behave like small magnets, illustrated by arrows. They will orientate themselves in a position with low energy, E_1, or in a position with high energy, E_2. The difference between the two energy levels is ΔE. When energy in the form of radio waves is added, B_1, some of the spins will be excited and change direction to the high energy position, as shown on the right.

When the oscillating magnetic field B_1 is turned off, the system is in a high energetic, unstable state. The system will return to the equilibrium state in a process called spin relaxation, where the excited spins are restored to their low energy position. In this process electric current is generated in a detection coil as a signal called the Free Induction Decay (FID). The procedure of applying electromagnetic waves with the right frequency is repeated numerous times, and the FID signals are stored in a computer. The FID spectra are acquired in the time domain and cannot be analyzed directly. Through the mathematical operation known as the Fourier transformation, the FID spectra are therefore converted into MR spectra in the frequency domain. Under the right circumstances the area under each peak in the MR spectra is directly proportional to the number of nuclei, and thus to the concentration of the different compounds. In the present experiments, lyophilized cell extracts were redissolved in D_2O containing 0.10% ethylene glycol as an internal standard. The MR

analyses were done using different instruments; details are given in papers 1 and 5.

3.2.3 ^{13}C MRS (Derome, 1987; Hornak, 1997)

The resonance frequency of the ^{13}C nucleus is determined by the strength of the magnetic field, as described previously (10.71 MHz per Tesla). In addition, the structure of the molecule containing the ^{13}C-atom and the atoms surrounding the ^{13}C influence the resonance frequency. This means that there are slightly different Larmor frequencies for the same nuclear type in different positions within a molecule. The reason for this is that the electrons also work as magnets which affect the nuclei. The electrons in the chemical bonds give rise to magnetic fields which can locally modify the external magnetic field. The carbon nucleus will for example have a higher affinity for the electrons than the hydrogen nucleus in a C-H bond. The carbon nucleus is referred to as shielded, and the resonance frequency of the ^{13}C nucleus is decreased. In a C=O bond, the situation is opposite, the oxygen nucleus has the highest electron affinity, the ^{13}C nucleus becomes unshielded and the Larmor frequency is increased. The fact that different carbon atoms will have a slightly different frequency because of their chemical environment is called chemical shift. This makes it possible to distinguish between different metabolites and also different nuclei within each metabolite as they appear in specific locations in the spectrum. This can be seen in Figure 3.3, showing an MR spectrum of cell extracts from cerebellar neuronal cultures incubated in medium containing [U-^{13}C]glutamate.

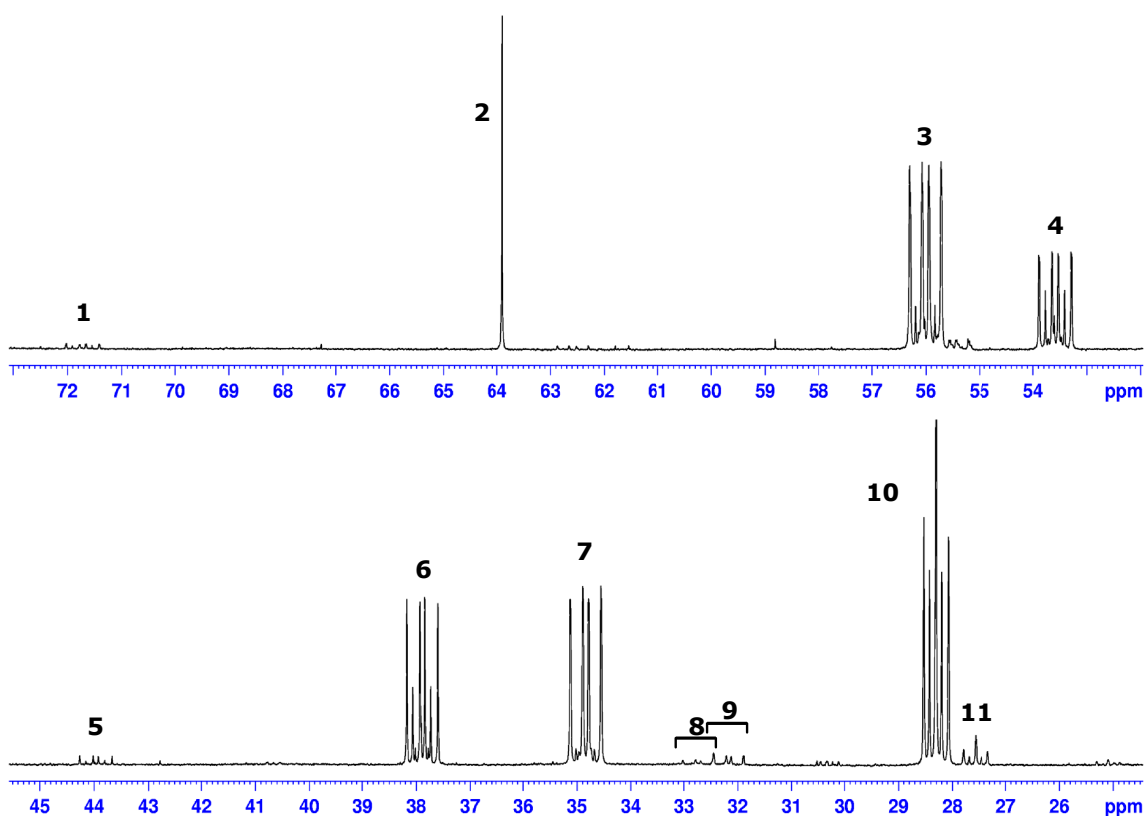FIGURE 3.3 A ^{13}C NMR spectrum of cell extracts from cerebellar neuronal cultures incubated in medium containing [U-^{13}C]glutamate, for details see paper 5. Peak assignments: (1) malate C-2, (2) ethylene glycol (internal standard), (3) glutamate C-2, (4) aspartate C-2, (5) malate C-3, (6) aspartate C-3, (7) glutamate C-4, (8) glutamate C-4 in glutathione, (9) glutamine C-4, (10) glutamate C-3, (11) glutamine C-3.

Looking at the MR spectrum in Figure 3.3 it can be seen that the peaks have different configurations, most of them are multiplets consisting of more than one peak with different heights. This is because magnetic nuclei also are influenced by surrounding magnetic nuclei. This can be a nucleus of the same (homonuclear coupling) or a different kind (heteronuclear coupling). If a ^{13}C atom only has ^{12}C neighboring atoms, it is observed as a single peak (a singlet) in the spectrum. However, if it has one or two ^{13}C neighboring atoms, it will be represented as a doublet, a triplet or a doublet of doublets. The splitting occurs because the labeled neighbors will influence the first

^{13}C atom, changing the spin-spin coupling constant (J) or the frequency separation, between the different peaks in a multiplet. This is illustrated in Figure 3.4 taken from paper 5 of a part of an ^{13}C MR spectrum from cell extracts of cerebellar neurons after incubation with [U-^{13}C]glutamate, for details see paper 5.
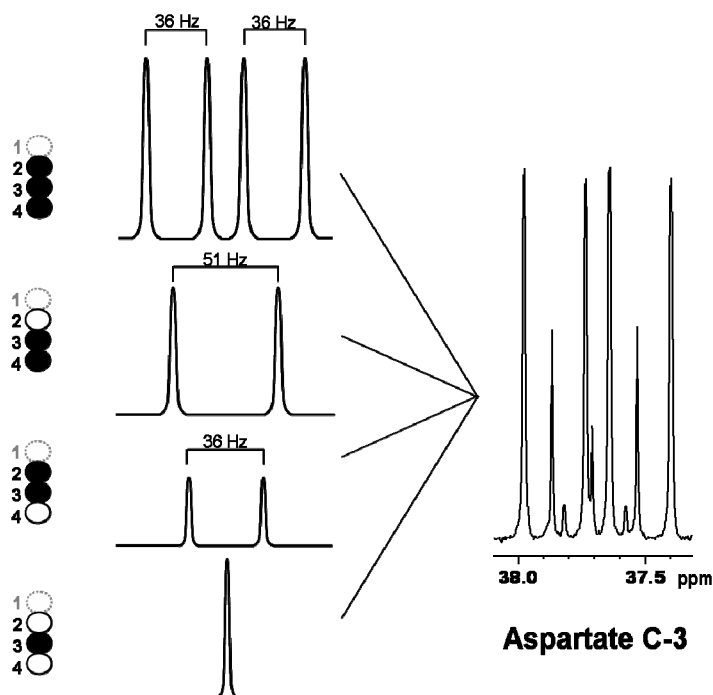

FIGURE 3.4 Part of a ^{13}C MR spectrum of cell extracts from cerebellar neuronal cultures incubated in medium containing [U-^{13}C]glutamate, for details see paper 5. The aspartate C-3 multiplet and the isotopomers responsible for the configuration of the peaks are shown. The effect of homonuclear ^{13}C-^{13}C coupling can be seen by the difference in coupling constants (given in Hz). ● represents ^{13}C and ○ represents ^{12}C atoms. No information can be obtained about labeling in the C-1 position indicated by broken gray lines.

^{13}C nuclei will also be affected by neighboring protons (heteronuclear coupling), because these nuclei posses spin. This leads to splitting of the peaks in an MR spectrum. To avoid this splitting, the protons are exposed to radio waves around their Larmor frequency, so that the same number of protons are in the low (E_1) as in the high (E_2) energy position, and by this operation the spectra become proton decoupled. When energy is added to the system to decouple the

protons, ΔE for the ^{13}C nucleus increases and some of the carbon peaks (those representing ^{13}C atoms bound to protons) in the spectrum appear artificially large. This is called the nuclear Overhauser effect (nOe), and when quantifying the peaks, the values must be adjusted for this effect.

By adding ^{13}C labeled precursors such as [1-^{13}C]glucose, [U-^{13}C]glutamate or [U-^{13}C]glutamine to neuronal cell cultures, the cells will incorporate the ^{13}C-compounds in their metabolism. Using ^{13}C MRS, the different metabolites in which ^{13}C has been incorporated can be identified and quantified. In addition the method can be used to distinguish between ^{13}C-labeling in different positions in the metabolites.

3.3 Mass Spectrometry

3.3.1 Detection of ^{13}C Labeling in Metabolites by MS

Mass spectrometry (MS) coupled to a separation method can also be used to obtain information about ^{13}C labeling in different metabolites (Biemann, 1962). The advantage of using MS is that it is far more sensitive than MRS. In cell extracts from cerebellar neurons incubated for two hours with [U-^{13}C]glutamate or [U-^{13}C]glutamine it was possible to detect labeling in glutamate, glutamine, GABA and aspartate in addition to the TCA intermediates malate, succinate, fumarate and citrate. When six of the same cell extract samples were pooled together and analyzed by ^{13}C MRS, only labeling in glutamate, glutamate incorporated into glutathione, glutamine, aspartate, and occasionally malate was seen (Figure 3.3). On the other hand, the disadvantage of MS compared to MRS is that it only gives the percent distribution of different masses (M (the mass of the parent ion), M+1 (the mass of the parent ion plus 1 unit of molecular weight (Dalton)

corresponding to one atom of ^{13}C), M+2, M+3, etc.) of the metabolite isotopomers, whereas the position of the ^{13}C atoms within the molecule is not detected by this method as it is by MRS.

3.3.2 Basic GC/MS Theory (McMaster and McMaster, 1998)

Mass spectrometry is often used in combination with gas chromatography (GC/MS). In experiments described in papers 3, 4 and 5, cell extract samples were lyophilized, redissolved in 10 mM HCl, adjusted to pH<2 and dried under atmospheric air. The amino acids were extracted into an organic phase of ethanol and benzene and dried again under atmospheric air before derivatization with MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide) + 1% t-BDMS-Cl (*tert*-butyldimethylchlorosilane) as described by Mawhinney et al. (1986). The cell extract sample is then injected into the injection port of the GC, where it is immediately vaporized and carried to the column by the carrier gas. It is important that the carrier gas is inert and does not react with the sample or column, and for this reason helium was used in the present studies. The column used was a capillary column coated with silica (Varian WCOT fused silica 25 m x 0.25 mm ID coating CP-Sil 5CB-MS). The various components in the cell extract sample travel through the column at different speeds based on their chemical and physical characteristics (mass, shape, interaction with column surface, etc.), and they are separated. Each component ideally produces a specific peak which appears in the chromatogram after a characteristic retention time.

After separation of the different metabolites in the cell extracts by GC, MS is used to separate molecules of the same metabolite with different masses (M, M+1, M+2, etc.), i.e. different isotopomers of each metabolite. The gas carrying the separated metabolites is let into the ionization chamber where a beam of electrons is accelerated with a high voltage. The molecules in the sample are shattered into ionized

fragments upon collision with the high voltage electrons. The charged fragments are electrically focused into an intense ion beam which enters the quadrupole analyzer. The electrically charged poles of the quadrupole create an electromagnetic field, and the ion beam is forced into a corkscrew, three-dimensional sine wave. Across the quadrupole rods a combined field of direct current and an oscillating radio frequency signal is applied. This interrupts the paths of all ions except for those with one specific mass to charge ratio. A mass spectrum is obtained by scanning through the mass range of interest over time. When using the instrument's SCAN mode, the whole mass range is scanned. However, when knowing which masses to look for, the instrument is set to scan over a very small mass range, the selected ion monitoring (SIM) mode. The narrower the mass range the more specific the SIM assay. The method used in the present studies was developed using the SCAN mode for analyzing standard solutions of individual compounds to determine the retention time and the masses of interest for the compounds. When this was done, a SIM method was set up with retention time windows in which the instrument was set to scan over a few masses in order to enhance sensitivity. After being selected in the quadrupole, the charged particles travel in a curved path towards the detector, and on the way the charge is amplified through collisions with the detector surface.

The computer linked to the GC/MS instrument gives a plot of relative abundance against the mass to charge ratio value of the ions. An example of two gas chromatograms and mass spectra is shown in Figure 3.5. The peaks are integrated and the percentage of mono-, double-, triple labeling etc. in a compound is calculated after correction for natural abundance determined in a standard solution of unlabeled compounds. However, as mentioned earlier, this method does not differentiate between isotopomers containing the same number of ^{13}C atoms in different positions.
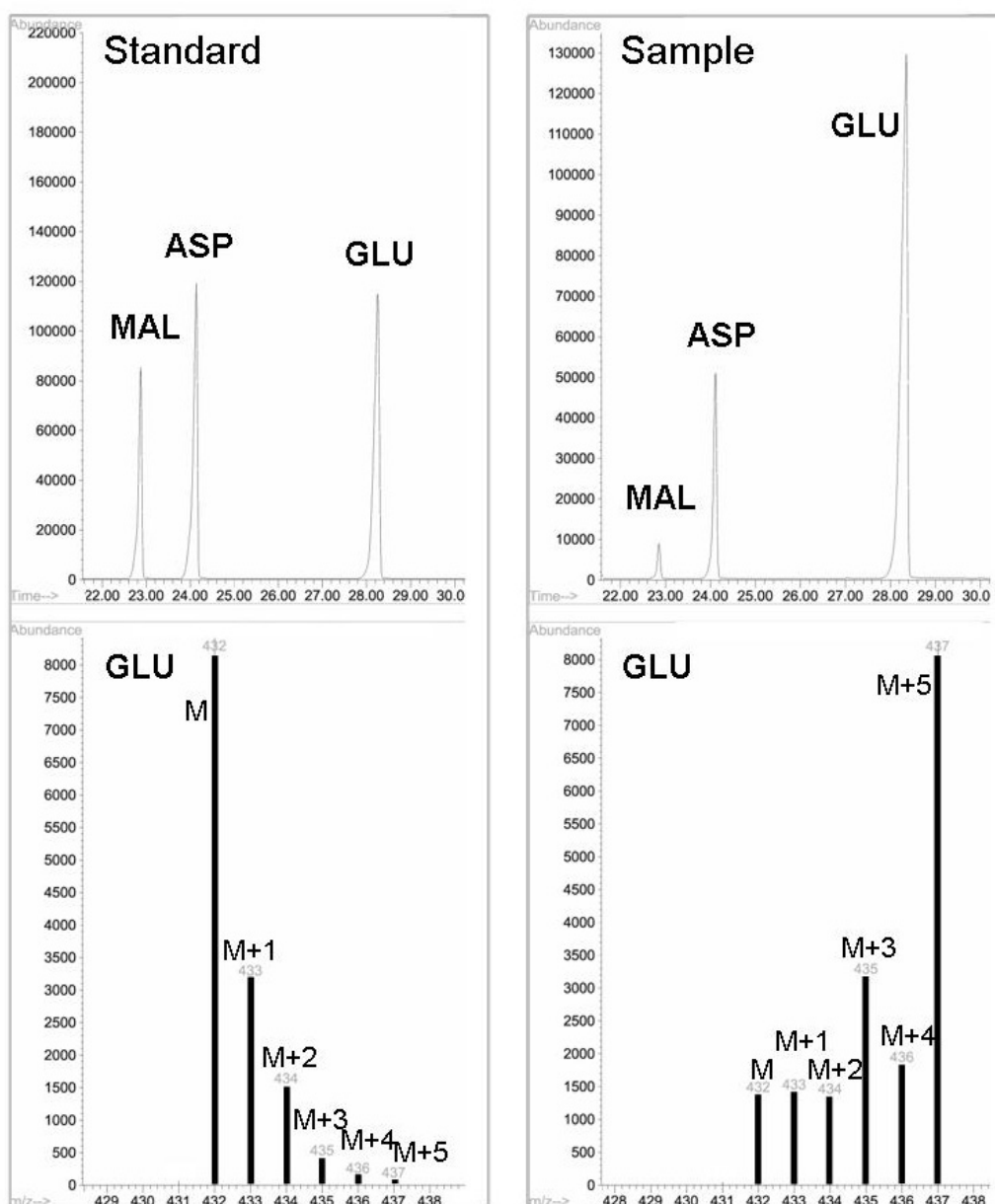
FIGURE 3.5 Parts of gas chromatograms (top) and mass spectra (bottom) from a standard solution of unlabeled compounds (left) and a sample of cell extract from cerebellar neuronal cultures incubated for two hours in medium containing 0.25 mM [U-^{13}C]glutamate, for details see paper 3. The chromatograms show the malate, aspartate and glutamate peaks, and the mass spectra show masses M (unlabeled) to M+5 (uniformly labeled) for glutamate.

3.4 ^{13}C Labeling Patterns

Understanding the labeling patterns from ^{13}C labeled precursors involves knowledge about cell metabolism. This can be found in a biochemistry textbook, for example the one written by Stryer (1995a)

3.4.1 Labeling from [1-^{13}C]glucose

In papers 1 and 2, neuronal cell cultures prepared for MRS analysis were cultured in medium containing [1-^{13}C]glucose for the whole culture period. Glucose is the most important substrate for neuronal metabolism, and the metabolites made from this labeled glucose, will contain ^{13}C and thus be detectable by ^{13}C MRS. In order to interpret the MR-spectra and understand the results obtained from these spectra, it is necessary to know the relevant metabolic conversions of [1-^{13}C]glucose. This is illustrated in Figure 3.6.
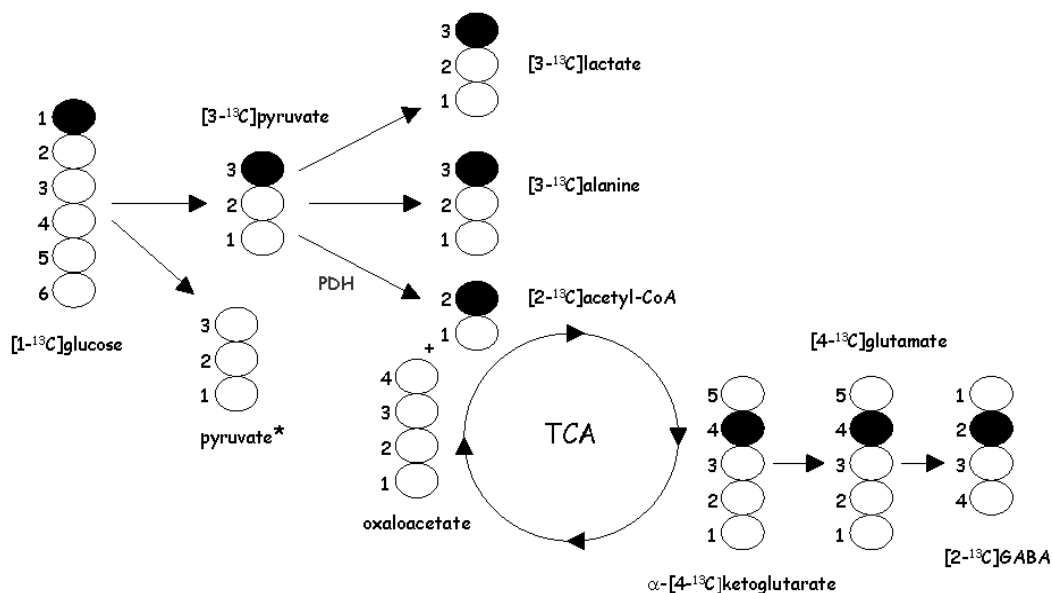

FIGURE 3.6 Metabolism of [1-^{13}C]glucose in neurons. ● represents ^{13}C and ○ represents ^{12}C atoms. PDH is the enzyme pyruvate dehydrogenase which catalyzes the reaction from pyruvate to acetyl-CoA. *Unlabeled pyruvate will have the same conversions as [3-^{13}C]pyruvate, but the products will not be detectable by ^{13}C MRS.

Through glycolysis, [1-^{13}C]glucose is converted to two pyruvate molecules. One of them will contain a ^{13}C-atom in the third position ([3-^{13}C]pyruvate), whereas the other one will contain only ^{12}C-atoms (the natural abundance of ^{13}C of 1.1 % is not taken into consideration). [3-^{13}C]pyruvate can be converted to [3-^{13}C]lactate or [3-^{13}C]alanine. Alternatively, [3-^{13}C]pyruvate may enter the tricarboxylic acid cycle via pyruvate dehydrogenase (PDH) as [2-^{13}C]acetyl-CoA. In the TCA cycle, [2-^{13}C]acetyl-CoA is combined with oxaloacetate (OAA) and converted through several steps to α-ketoglutarate with ^{13}C-labeling in the C-4 position, which may leave the TCA cycle and form [4-^{13}C]glutamate, which in turn can be converted to [2-^{13}C]GABA.

If α-[4-^{13}C]ketoglutarate does not leave the cycle, it will (after several steps) appear as [2-^{13}C]oxaloacetate (OAA) or [3-^{13}C]oxaloacetate (because succinate, one of the intermediate compounds between α-ketoglutarate and OAA in the TCA cycle, is a symmetrical molecule). ^{13}C-labeled OAA can be converted to [2-^{13}C]aspartate or [3-^{13}C]aspartate by transamination, or condense with a new acetyl-CoA-molecule, labeled or unlabeled with ^{13}C (from labeled or unlabeled pyruvate), and make a second turn in the TCA cycle. If ^{13}C-labeled OAA reacts with unlabeled acetyl-CoA, the resulting labeling (after several steps) in glutamate and GABA is [2-^{13}C]- and [3-^{13}C]glutamate and [3-^{13}C]- and [4-^{13}C]GABA. If ^{13}C-labeled OAA reacts with [2-^{13}C]acetyl-CoA, [2,4-^{13}C]- and [3,4-^{13}C]glutamate and [2,4-^{13}C]- and [2,3-^{13}C]GABA are formed. The labeling [1-^{13}C]glucose in glutamate and GABA after one and two turns in the TCA cycle is shown in Figure 3.7.

After more turns in the TCA cycle and reactions between molecules with and without ^{13}C-atoms in different positions, the possibilities are many for ^{13}C-labeling of the different metabolites, and

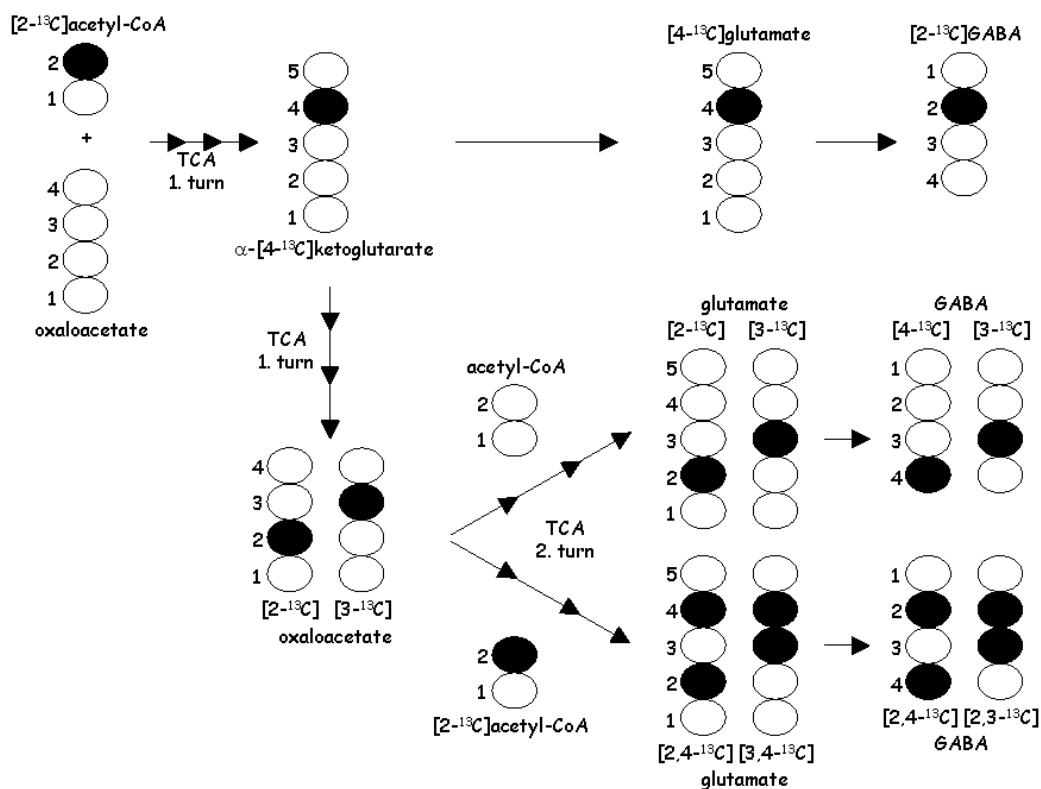the picture becomes more complicated than shown in Figures 3.6 and 3.7.

FIGURE 3.7 Labeling of ^{13}C in glutamate and GABA from [1-^{13}C]glucose in the first and second turn of the TCA cycle. • represents ^{13}C and ○ represents ^{12}C atoms.

3.4.2 Labeling from [U-^{13}C]glutamate and [U-^{13}C]glutamine

In papers 3, 4 and 5, neuronal cell cultures from cerebellum were incubated in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine. When taken up by the neurons, the latter can be converted into the former, and from here on the labeling patterns are the same for the two precursors. [U-^{13}C]glutamate can together with cysteine and glycine form the tripeptide glutathione (GSH). The labeled glutamate incorporated in glutathione can be identified by ^{13}C MRS; its peaks will appear in a different location in the spectrum than free glutamate (Figure 3.3). Another possibility for [U-^{13}C]glutamate

is conversion into [U-¹³C]GABA catalyzed by the enzyme GAD. [U-¹³C]GABA could not be detected in cell extracts by MRS after two hours incubation in medium containing [U-¹³C]glutamate. However, using MS, the M+4 isotopomer of GABA (representing [U-¹³C]GABA) was detected. A third option for [U-¹³C]glutamate is the formation of α-[U-¹³C]ketoglutarate, which is metabolized in the TCA cycle. After several steps in the TCA cycle, labeled α-ketoglutarate is turned into [U-¹³C]oxaloacetate, which can condense with unlabeled acetyl CoA to form [3,4,5,6-¹³C]citrate. The resulting glutamate isotopomer (after several steps) is [1,2,3-¹³C]glutamate formed from α-[1,2,3-¹³C]ketoglutarate. This first turn for [U-¹³C]glutamate in the TCA cycle is shown in Figure 3.8.
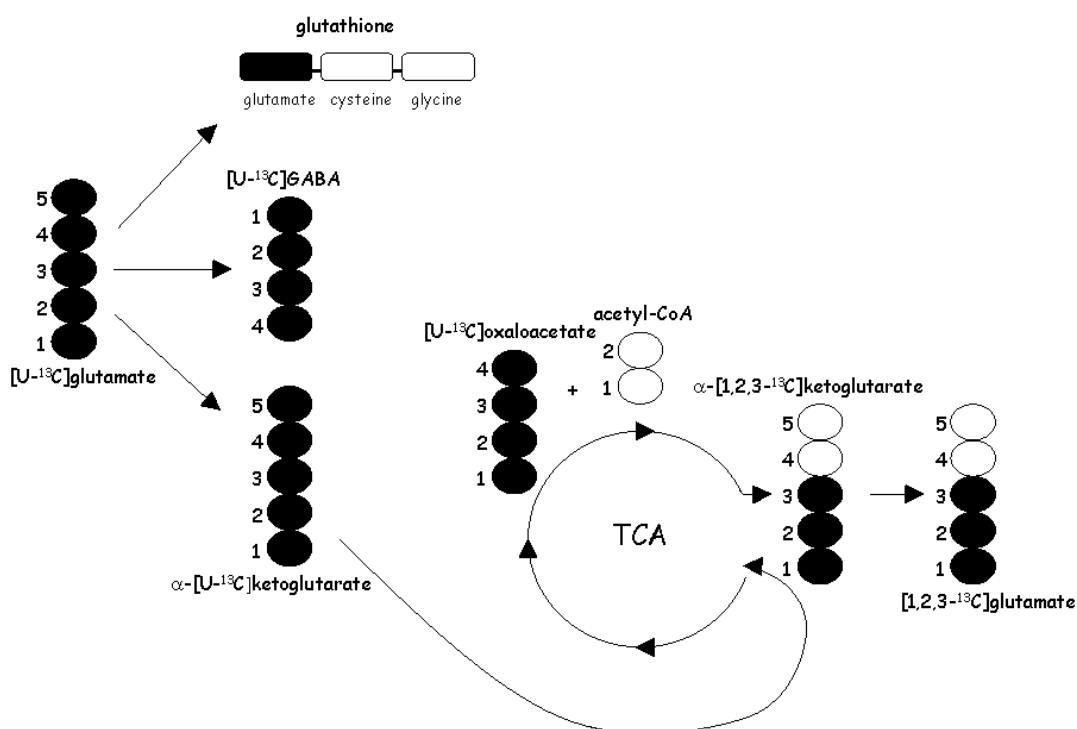

FIGURE 3.8 Metabolism of [U-¹³C]glutamate in neurons. ● represents ¹³C and ○ represents ¹²C atoms. Glutathione is a tripeptide, and the black box is representing labeled glutamate, while cysteine and glycine are amino acids without ¹³C labeling, represented by white boxes.

If α-[1,2,3-^{13}C]ketoglutarate does not leave the TCA cycle as [1,2,3-^{13}C]glutamate, but continues its voyage in the cycle, ^{13}C labeling is distributed amongst the TCA cycle intermediates as presented in Figure 3.9.

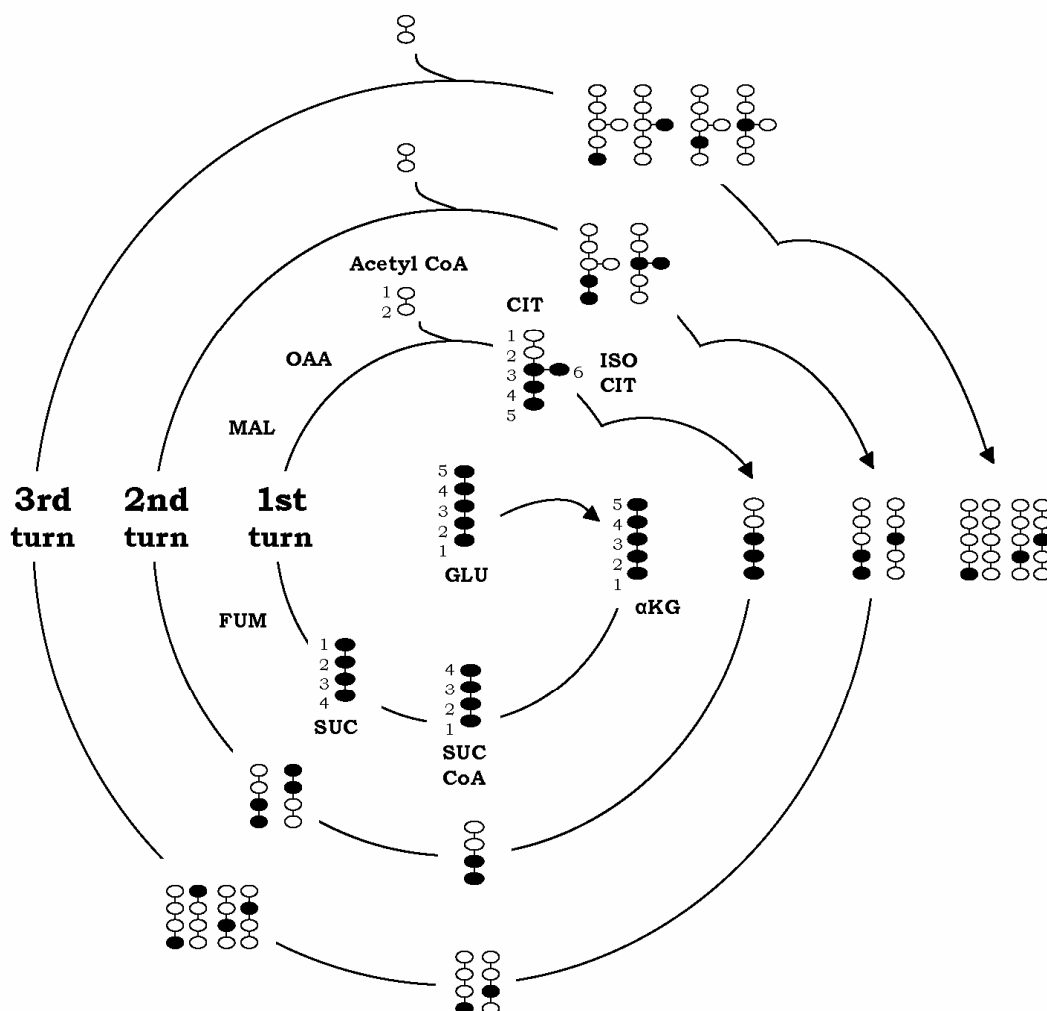FIGURE 3.9 Schematic representation of possible isotopomers of metabolites arising from [U-^{13}C]glutamate or [U-^{13}C]glutamine via the three first turns in the TCA cycle in neurons: ● represents ^{13}C and ○ represents ^{12}C atoms. For clarity, the labeling of fumarate, malate, OAA and isocitrate is left out; the three first compounds are labeled in the same manner as succinate and the latter as citrate, although the numbering of the C atoms differs. GLU: glutamate; αKG: α-ketoglutarate; SUC-CoA: succinyl-CoA; SUC: succinate; FUM: fumarate; MAL: malate; OAA: oxaloacetate; CIT: citrate; ISOCIT: isocitrate.

From labeled intermediates of the first TCA cycle turn, [U-^{13}C]aspartate and [U-^{13}C]lactate can be formed, the former from transamination of [U-^{13}C]OAA and the latter derived from [U-^{13}C]OAA or [U-^{13}C]malate. The presence of [U-^{13}C]lactate implies that also [U-^{13}C]pyruvate is present in the cells, and as mentioned, this compound can enter the TCA cycle through acetyl CoA. Pyruvate derived from TCA cycle intermediates re-entering the cycle as acetyl CoA constitutes the pyruvate recycling pathway. In paper 5, it was shown that this recycling, previously believed to be astrocyte specific in cell cultures (Håberg et al., 1998; Waagepetersen et al., 2002), also take place in cerebellar neurons. In this case acetyl CoA is ^{13}C labeled, and this gives rise to particular labeling patterns in metabolites derived from the TCA cycle. The labeled isotopomers in glutamate and aspartate resulting from TCA cycle activity involving labeled and unlabeled acetyl CoA are shown in paper 5.

After more turns in the TCA cycle and entry of unlabeled or labeled acetyl CoA condensing with different isotopomers of OAA, the possibilities are many for ^{13}C-labeling of the different metabolites, and the picture becomes more complicated than shown in the illustrations.

3.5 Identification and Quantification of Amino Acids by HPLC

High Performance Liquid Chromatography (HPLC) is a type of chromatography which in the present studies was used to quantify different amino acids in cell extracts and medium. The amino acids were pre-column derivatized with *o*-phthaldialdehyde and subsequently separated on a ZORBAX SB-C18 (4.6 × 250 mm, 5 μm) column from Agilent using a phosphate buffer (50 mM, pH = 5.9) and a solution of methanol (98.75 %) and tetrahydrofuran (1.25 %) as eluents (Geddes and Wood, 1984). A gradient of the two eluents was

used to obtain a faster and more optimal separation. The separated amino acids were detected with fluorescence and quantified by comparison to a standard curve derived from standard solutions of amino acids run after every twelve samples. An example of an HPLC chromatogram is presented in Figure 3.10.
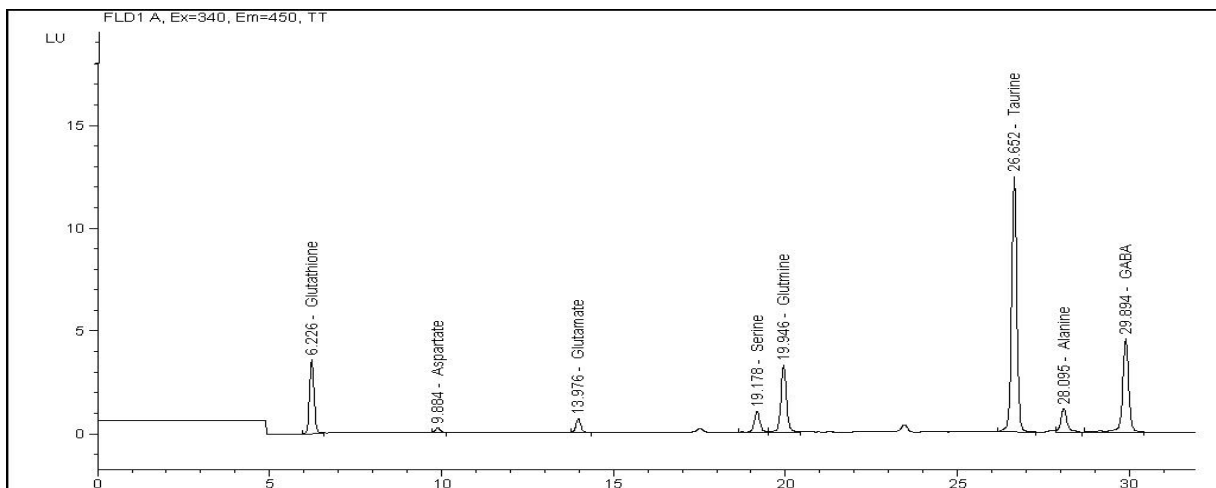

FIGURE 3.10 An HPLC chromatogram showing peaks representing glutathione and different amino acids in solution eluted at different retention times, the x axis shows time in minutes, and the y axis shows fluorescence intensity.

3.6 Protein Quantification

The amount of protein in the cell cultures was determined using the Pierce BCA protein assay. Bicinchoninic acid (BCA) is a water-soluble sodium salt and a sensitive, stable and specific reagent for the copper ion Cu^+. When samples containing protein are treated with BCA protein assay reagent, protein reduces Cu^{2+} to Cu^+ in an alkaline solution, and a purple colored product is then formed by the interaction between two BCA-molecules and one copper ion (Smith et al., 1985). This product is water soluble and exercises strong absorbance at 570 nm. The amount of protein is determined by measuring absorbance on a spectrophotometer and comparing to standard samples made from bovine serum albumin.

4 SUMMARY OF PAPERS

Paper 1

Neocortical and cerebellar neurons were cultured in medium containing $[1-^{13}C]$glucose for seven days to evaluate neuronal metabolism.

In the cerebellar cultures consisting mainly of glutamatergic granule cells, a surprisingly **extensive content and ^{13}C labeling of GABA** was seen. The intracellular amount of GABA in these cultures was 20 ± 4 nmol/mg protein compared to 32 ± 2 nmol/mg protein in cultures of neocortical neurons (predominantly GABAergic). GABA labeling was similar in the two types of cultures.

The cerebellar neurons contained only **6 % glutamate decarboxylase (GAD)-positive neurons** as shown using immunolabeling of GAD_{67}, whereas a dense network of neurons in the neocortical cultures stained positively for GAD_{67}.

Exposure of the cerebellar cultures to 50 μM KA, which is known to eliminate vesicular release of GABA, only marginally affected GABA labeling and cellular content and had no effect on the number of GAD_{67} positive neurons in the cerebellar cultures. However, **KA exposure eliminated a massive punctate immunostaining** observed in control cultures. It can be postulated that this staining represents GAD close to vesicles, and that this synthesis is eliminated by 50 μM KA. Increasing the KA concentration to 0.5 mM in the culture medium for seven days led to a reduction of GABA labeling and content compared to cerebellar cultures not exposed to KA.

Although it is likely that this large capacity for GABA synthesis resides in the relatively few GAD-positive neurons, it seems unlikely that they could contain the large GABA content in the cultures. Therefore it must be concluded that **the newly synthesized GABA is redistributed among the majority of the cells in these cultures, i.e. the glutamatergic neurons**. The function of GABA in these neurons is yet to be understood.

Paper 2

The **time course of glutamate and GABA synthesis** and degradation in cerebellar neuronal cultures (maintained *in vitro* for 1-13 days) was investigated in this study. Moreover, the enzymes involved in GABA synthesis in these cultures were probed pharmacologically.

The intracellular amount of **GABA increased the first five days** before decreasing, whereas **glutamate was constant until day six and thereafter decreased**. Furthermore, GABA content in the medium increased with time in culture, indicating release from cells in a non-depolarization dependent manner. Formation of labeled GABA after incubation with either [1-^{13}C]glucose or [U-^{13}C]glutamine demonstrated GABA synthesis during the first three days *in vitro*, and synthesis after one week was shown by labeling from [U-^{13}C]glutamine added on day seven. Thus, there was a **continuous GABA synthesis and degradation throughout the culture period** in cerebellar neuronal cultures.

Adding aminooxyacetic acid (AOAA, 10 μM), an inhibitor of transaminases and other pyridoxalphosphate dependent enzymes including GABA-T, to the culture medium caused an increase in intracellular GABA and a decrease in glutamate. The specific GABA-T inhibitor γ-vinyl GABA also increased GABA, but had no effect on glutamate content. In the presence of 10 μM AOAA, GABA labeling from [U-^{13}C]glutamine was not affected, ruling out the putrescine pathway for GABA synthesis. Increasing the AOAA concentration to 0.5 mM led to a decrease in GABA, presumably due to a partial inhibition of GAD. In order to block both GAD and the transaminases completely, 5 mM AOAA was used, this abolished GABA labeling. The results indicate that **GABA synthesis in cerebellar cultures is catalyzed by GAD and takes place in the subpopulation of GAD positive cells**. In the presence of 5 mM AOAA glutamate and protein content in the cultures was reduced, most likely through inhibition of transaminases involved in glutamate synthesis as well as those involved in the malate-aspartate shuttle resulting in impaired oxidative metabolism of glucose.

Paper 3

In this study the role of the **glutamate-glutamine cycle** in cultures of cerebellar neurons was evaluated.

Cells were incubated in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine in the presence and absence of unlabeled glutamine and glutamate, respectively. **Both [U-^{13}C]glutamate and [U-^{13}C]glutamine were shown to be excellent precursors** for synthesis of neuroactive amino acids and TCA cycle intermediates. Labeling from [U-^{13}C]glutamate was higher than from [U-^{13}C]glutamine in all metabolites measured. The presence of [U-^{13}C]glutamate plus unlabeled glutamine in the experimental medium led to labeling very similar to that from [U-^{13}C]glutamate alone. However, incubation in medium containing [U-^{13}C]glutamine in the presence of unlabeled glutamate almost abolished labeling of metabolites. Thus, **glutamate was the preferred substrate** for intermediary metabolism in cerebellar neurons. It can be concluded that the **cerebellar neurons rely more on reuptake of glutamate than supply of glutamine from astrocytes** for glutamate homeostasis. This is not surprising when considering the cerebellar structure, with few astrocytes compared to neurons and a relatively large distance between astrocyte and synapse.

Label distribution indicating TCA cycle activity showed more prominent cycling from [U-^{13}C]glutamine than from [U-^{13}C]glutamate, showing **compartmentation of metabolism**.

Labeling of succinate from [U-^{13}C]glutamate or [U-^{13}C]glutamine was lower than of the other TCA cycle intermediates. This could be caused by the unlabeled carbon skeleton of GABA (synthesized in GAD positive neurons during the culture period and distributed throughout the culture) entering the TCA cycle in granule cells as succinate. Thus, a possible function of GABA in glutamatergic neurons is as a substrate for energy production, the results indicate an **active role of the GABA shunt** in these cultures.

Paper 4

The long-term effects of the glutamate agonist kainic acid (KA) on glutamate and glutamine metabolism in cerebellar neurons were investigated in this study. Neurons were cultured in medium containing 0.05 or 0.50 mM KA for seven days and subsequently incubated in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine.

The **protein amount and number of cells was greatly reduced in cultures exposed to 0.50 mM** compared to those exposed to 0.05 mM KA.

Glutamine consumption was not affected by KA concentration, whereas **high KA led to decreased glutamate consumption**. This confirmed the role of KA as an inhibitor of glutamate transport reported earlier.

Neurons cultured with 0.50 mM KA and incubated with [U-^{13}C]glutamate contained decreased amounts of glutamate, aspartate and GABA compared to those cultured with 0.05 mM KA. Incubation of cells exposed to 0.50 mM KA with [U-^{13}C]glutamine led to an increased amount of glutamate compared to cells exposed to 0.05 mM KA, whereas the intracellular amounts of aspartate and GABA was unaffected by KA concentration.

Furthermore, **mitochondrial metabolism of α-[U-^{13}C]keto-glutarate derived from [U-^{13}C]glutamate and [U-^{13}C]glutamine was significantly reduced by 0.50 mM KA**.

Intracellular compartmentation was illustrated by the fact that TCA cycling of the carbon skeleton from [U-^{13}C]glutamine was more pronounced than that from [U-^{13}C]glutamate. Moreover, cycling of the carbon skeleton from [U-^{13}C]glutamate subsequently used to form GABA was affected by KA, whereas cycling of the carbon skeleton from [U-^{13}C]glutamine was not, showing compartmentation of the GABAergic cellular population. Finally, **intercellular compartmentation** was evident because the carbon skeleton from [U-^{13}C]glutamate had a higher turnover in the GABAergic than in the glutamatergic compartment which constitute these cultures.

Paper 5

In this study the **pyruvate recycling** pathway for complete oxidation of glutamate was investigated in cultures of astrocytes and neurons* from cerebellum. The two types of cultures were incubated in medium containing [U-^{13}C]glutamate. Previous cell culture studies have reported pyruvate recycling taking place in astrocytes, but it has not been detected in neurons.

Using mass spectrometry, the M+4 mass in glutamate and glutamine and M+3 in aspartate are the only isotopomer masses resulting from TCA cycle activity involving pyruvate recycling that can be distinguished from those derived from TCA cycling using unlabeled acetyl CoA. **Atom percent excess of M+4 in glutamate was similar for astrocytes and neuron-enriched cultures**. However, the latter showed more recycling in glutamine (synthesized in the small fraction of astrocytes) than the pure astrocyte cultures, whereas the reverse was the case for aspartate. In fact, the atom percent excess of the isotopomer representing pyruvate recycling in glutamine was slightly but significantly higher than that in glutamate in the neuron-enriched cultures.

In order to verify pyruvate recycling in neurons, cell extracts were analyzed using ^{13}C MRS, and **recycling was clearly detectable in glutamate and aspartate**. The reason why such recycling in neurons was detected now, but not earlier could be the enhanced sensitivity of the cryo MR probe used in the present experiment.

It can be concluded that **pyruvate recycling is taking place in neurons as well as in astrocytes**.

*Because these cultures have been shown to contain a small number of astrocytes in addition to the neurons, they are referred to as "neuron-enriched" in this publication. It should be noted that they are identical to the cultures called granule cell cultures and cerebellar neuronal cultures in other parts of this thesis and in other publications.

5 DISCUSSION

GABA in cerebellar neuronal cultures

Cell cultures are excellent model systems for the study of specific aspects of cellular function. Neuronal cultures have been used to obtain information about regional differences in the brain, and the neurotransmitter phenotype has been of great interest. Two types of neuronal cultures have been used in these studies in our laboratory; neocortical and cerebellar.

The GABAergic nature of neocortical neuronal cultures has been shown in various studies (Yu et al., 1984; Drejer et al., 1987; Belhage et al., 1993; Waagepetersen et al., 2001). Upon depolarization of the cell membrane, these cells release GABA in a Ca^{2+}-dependent fashion. GABA labeling from ^{13}C labeled glucose has been reported in cortical neuronal cultures (Sonnewald et al., 1991; Sonnewald et al., 1993; Waagepetersen et al., 1998). In addition to GABA, these cultures contain substantial amounts of glutamate, which is not surprising because glutamate is the direct precursor of GABA and also takes part in cell metabolism.

On the other hand, neuronal cultures prepared from dissociated cerebella of seven-day-old rats and mice have been shown to express both GABAergic and glutamatergic characteristics, due to the fact that they primarily consist of cerebellar glutamatergic granule cells with a minor contribution of GABAergic stellate and basket neurons (Thangnipon et al., 1983; Schousboe et al., 1989). Upon depolarization of the cell membrane, these cells show vesicular release of both glutamate and GABA (Pearce et al., 1981; Drejer et al., 1982; Palaiologos et al., 1988; Palaiologos et al., 1989; Belhage et al., 1992; Damgaard et al., 1996). Different explanations for the presence of GABA and GABA release in these cultures have been suggested.

One suggestion has been that GABA is synthesized and released by the small number of GABAergic neurons in these cultures (Damgaard et al., 1996). This is in concurrence with the "one neuron one neurotransmitter" hypothesis. The same hypothesis makes it difficult to accept the presence of GABA (thought to be a neurotransmitter only) in glutamatergic cells. However, there have been reports of the presence of GABA in glutamatergic neurons both *in vivo* and *in vitro* (Sandler and Smith, 1991; White et al., 1994; Schwarzer and Sperk, 1995; Lehmann et al., 1996; Jaffe and Figueroa, 2001; Sperk et al., 2003; Gutierrez and Heinemann, 2006). Jaffe and Figueroa (2001) reported GABA release from granule cells in the olfactory bulb, and showed that this partly was due to reversing of GABA transporters. Lehmann et al. (1996) showed immunocytochemical localization of GABA immunoreactivity in dentate granule cells, both in control rats and in rats, in which epilepsy had been induced through kindling. Sandler and Smith (1991) demonstrated coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus, whereas White et al. (1994) found the same in striatal projection neurons in rats. An explanation for this has been that GABA is taken up from the surrounding (*in vivo*) or from serum in the medium (*in vitro*) by these glutamatergic neurons.

Stating that GABA is present in neuronal cultures from the cerebellum, leads to the question of whether GABA is actively synthesized in these cultures. This was investigated in paper 1, where [1-^{13}C]glucose was present in the culture medium for the entire incubation period (seven days). ^{13}C MRS analyses showed an extensive GABA labeling from [1-^{13}C]glucose which proves that GABA is actively synthesized by the cells from glucose via glycolysis and TCA cycle. The amount and labeling of GABA in neuronal cerebellar cultures were surprisingly high compared to cultures of neocortical

neurons, which almost entirely consist of GABAergic neurons (Hertz et al., 1985).

In order to find out more about GABA synthesis in cerebellar neuronal cultures, GAD immunostaining was done by a collaborating research group. Results from this are also presented in paper 1. Immunostaining showed that approximately 6 % of neurons in the cerebellar cultures exhibit GAD-like immunostaining, and there was pronounced punctuate staining in the cell processes of these cells. This is in compliance with the earlier mentioned studies reporting a minor contribution of GABAergic stellate and basket neurons in these cultures (Thangnipon et al., 1983; Schousboe et al., 1989).

Various studies have shown that by exposing cerebellar neuronal cultures to 50 μM KA, vesicular release of GABA is eliminated (Drejer and Schousboe, 1989; Simmons and Dutton, 1992; Damgaard et al., 1996). KA treatment has thus been thought to eradicate the GABAergic neurons and has been used in order to obtain pure glutamatergic granule cell cultures (Drejer and Schousboe, 1989). The belief that KA eliminates GABAergic neurons in the cerebellar cultures has been supported by the fact that GABAergic neurons in neocortical cultures are vulnerable to KA toxicity at low concentrations as assessed by measurement of lactate dehydrogenase leakage (Frandsen and Schousboe, 1990).

In paper 1, addition of 50 μM KA to the cell culture medium essentially eliminated the punctate immunostaining most likely corresponding to GABA-containing vesicles, whereas the number of GAD_{67}-positive cells remained unchanged. GABA labeling and cellular content of GABA was only marginally affected. This indicates that KA treatment does not eliminate the GABAergic neurons in cerebellar cultures, as previously believed, but rather alters the GABAergic neurons so that their vesicular GABA release is terminated.

It can be discussed whether the GABAergic neurons in cerebellar cultures can account for all the GABA present in these cultures. Calculations based on previously published data suggest that the 6 % GABAergic cells are not sufficient to contain all the GABA detected in the cell extract from these cultures (see paper 1). The granule cells most likely also contain a share of the detected GABA, the size of this share and the function of GABA in these glutamatergic cells is unknown. A discussion of GABA function in granule cells is found in papers 1 and 2. In paper 3 it was proposed that the GABA distributed from GABAergic to glutamatergic neurons enters the TCA cycle as succinate and is used for energy metabolism. This was suggested because a dilution of labeling was seen in succinate compared to the other TCA intermediates after incubation with [U-^{13}C]glutamate or [U-^{13}C]glutamine.

Another question arises when GABA synthesis in cerebellar neuronal cultures is established; what is the time course of GABA synthesis in these cultures? MRS studies have shown that short time incubation with [U-^{13}C]glutamate and [1-^{13}C]glucose leads to labeling of GABA in cortical neurons (Westergaard et al., 1995; Waagepetersen et al., 1998). Using similar incubation conditions with both [U-^{13}C]glutamate and [U-^{13}C]glucose for cerebellar neurons did not lead to MRS detectable labeling of GABA (Qu et al., 2000; Waagepetersen et al., 2000). However, using mass spectrometry, a more sensitive method, labeled GABA was detected in the cerebellar neurons after incubation with [U-^{13}C]glutamate (Qu et al., 2000). Labeling of GABA from [U-^{13}C]glutamate and [U-^{13}C]glutamine was also detected by MS in papers 3, 4 and 5. In paper 1, the cultures were given labeled glucose not only for a short time, but for the entire culturing period. This led to significant GABA labeling in both neocortical and cerebellar cultures. Thus, it is clear that GABA is present, but, since labeling after short exposure is very small (Qu et

al., 2000), it appears that there is a much slower turnover of GABA in cerebellar neurons compared to cortical GABAergic neurons.

Investigations of the time course of GABA formation and degradation in cerebellar neuronal cultures is presented in paper 2, in which cultures were extracted after 1-13 days *in vitro* and amounts of GABA and glutamate in cell extracts and media were quantified by HPLC. The experiments showed that the amount of GABA increased the first five days before decreasing. Protein increased until day four, whereas glutamate was constant until day six and thereafter decreased. The study demonstrated GABA synthesis in the cultures since the amount of GABA more than doubled during the first five days in culture. This was accompanied by an increase in the amount of protein. It should be noted that the number of neurons does not increase after the brain tissue is removed from the animals, on the contrary approximately 50% die (Westergaard et al., 1991). Thus, the increase in protein reflects cell growth and possibly differentiation. After day five the amount of GABA decreased slightly until the end of the culture period (day 13). The prominent increase of GABA during the first week in culture may reflect its functional importance during differentiation (Belhage et al., 1998; Waagepetersen et al., 1999). Experiments using [U-^{13}C]glutamine and [1-^{13}C]glucose presented in the same paper showed that there was a continuous GABA synthesis and degradation throughout the culture period in cerebellar neuronal cultures.

In paper 2, pharmacological agents were used in order to identify the enzymes involved in GABA synthesis in the cerebellar neuronal cultures. The presence of the specific GABA-T inhibitor γ-vinyl-GABA (GVG) in the medium led to an increase in intracellular GABA content compared to untreated cultures. This was also seen in cultures where aminooxyacetic acid (AOAA) was added. In low concentrations (10 μM), AOAA has been shown to block GABA-T, and

thus inhibit the conversion of GABA to succinic semialdehyde and subsequently succinate (Wallach, 1961; Schousboe et al., 1974). Cell cultures exposed to AOAA were therefore expected to have higher GABA concentrations than control cultures analogous to the effect of GVG. The experiments showed that this indeed was the case, an increase in GABA concentration was observed in the AOAA group compared to the control group. In addition, exposure of cerebellar neuronal cultures to 10 μM AOAA led to an increase in glutamate content compared to untreated control cultures. This is because this concentration of AOAA also inhibits other transaminases besides GABA-T, among them transaminases responsible for glutamate synthesis (Kihara and Kubo, 1989). Transaminases are also involved in the malate aspartate shuttle, and synthesis of glutamate has been shown to be dependent of the function of this shuttle in cerebellar neuronal cultures (Palaiologos et al., 1988). The fact that GABA was increased in the presence of AOAA, rules out that the putrescine pathway is responsible for GABA synthesis in these cultures, because this pathway also involves transamination (Seiler, 1980). GABA synthesis in these cultures is thus likely to be catalyzed by GAD. This was supported by using 5 mM AOAA on some cultures in paper 2, a concentration that blocks both GAD and transaminases completely (Wu and Roberts, 1974; Kihara and Kubo, 1989), and that abolished GABA labeling from [U-^{13}C]glutamine completely.

In conclusion, the present studies have shown that GABA is present and is actively synthesized during the entire culture period in cerebellar neuronal cultures. The enzyme GAD which catalyzes the conversion of glutamate to GABA is most likely responsible for the GABA synthesis and this takes place in the ~6 % GAD positive cells in the cultures. GABA content and labeling from [1-^{13}C]glucose is not affected by the presence of 50 μM KA in the culture medium, even though vesicular GABA release is eliminated by this treatment. After

synthesis, GABA is released to the medium in a non-depolarization dependent manner, and is probably taken up by the granule neurons constituting the majority of the neurons in these cultures. The function of GABA in these glutamatergic neurons still remains uncertain; however roles of neurotrophic and neuroprotective agent as well as substrate for energy production have been suggested.

Compartmentation of metabolism

The brain is a highly heterogeneous organ on various levels. Macroscopically this can be seen as a difference between different parts of the brain, as for example between the cerebrum and the cerebellum. Moving closer, the brain regions can be divided into gray and white matter, and these subdivisions can be further separated into the type of cellular elements constituting them; mainly neurons and glia. As mentioned in the introduction, there are subdivisions of these cell types as well. Thus, it is to be expected that brain metabolism is compartmentalized, and this has indeed been shown in several studies from the beginning of the 1960s, first by Berl, Clarke, Lajtha and Waelsch (for review, see Hertz, 2004). They injected radioactively labeled glutamate intracisternally, and observed that a small pool of this glutamate was rapidly used to synthesize glutamine. This "small compartment has been shown to be made up by glia, most likely predominantly or exclusively astrocytes. The neurons have been shown to be unable to synthesize glutamine and constitute the "large compartment" (Berl and Clarke, 1983; Hertz, 2004; McKenna et al., 2006a). The main focus in studies of brain compartmentation has thus been on the two compartments made up by neurons and astrocytes.

In the first study of brain compartmentation when injecting radioactive glutamate (Berl et al., 1961 as cited in Hertz, 2004) the labeling of glutamine was higher than that of the precursor glutamate. This was because the activity of the glutamine-forming glutamate

pool, into which the precursor selectively entered, was "diluted" by unlabeled glutamate from other pools (Hertz, 2004). The same was the case with other precursors (Waelsch et al., 1964; Berl and Frigyesi, 1969). However, glucose injections did not cause more labeling of product (glutamine) than precursor (glutamate), suggesting that it entered both compartments equally well (Hertz, 2004). A few years later, van den Berg and Garfinkel (1971) did simulation studies of metabolite flow and inter-compartmental trafficking of compounds. GABA was shown to be formed in one compartment and predominantly degraded in another, thus there was a flow of GABA between compartments. This was balanced by a glutamine flow going in the other direction (van den Berg and Garfinkel, 1971). An analogous glutamate-glutamine cycle was suggested by Benjamin and Quastel (1975).

In all the papers in this thesis, evidence of compartmentation of metabolism has been seen. This may seem surprising since cell cultures consisting of predominantly one cell type have been used. However, the cerebellar neuronal culture model system has been shown to be well suited for studying metabolic compartmentation. Firstly because of the resemblance to the *in vivo* situation both in respect to glutamate and glutamine metabolizing enzymes (Drejer et al., 1985). Secondly, the cultures show similarities to the in vivo situation due to expression of both GABAergic as well as glutamatergic characteristics (Pearce et al., 1981; Hertz et al., 1985; Hertz and Schousboe, 1987; Drejer and Schousboe, 1989; Kovacs et al., 2003). The glutamatergic granule cells dominate quantitatively in the cultures, just as in the cerebellum in vivo (Drejer and Schousboe, 1989; Andersen et al., 1992). In addition, the cultures contain about 6 % GABAergic stellate and basket neurons, and a small number of glial cells (Messer, 1977; Drejer et al., 1985; Damgaard et al., 1996,

paper 1). Thus, cellular compartmentation of metabolism can readily be studied in these cultures.

In paper 1, cerebellar neurons were cultured in medium containing [1-^{13}C]glucose. From the first turn in the TCA cycle, [4-^{13}C]glutamate and [2-^{13}C]GABA can be formed as shown in Figure 3.6. In calculating the cycling ratio of the two amino acids, the ratio of the product GABA was higher than that of glutamate (results not shown in the paper). This is a classical example of compartmentation analogous to that seen by (Berl et al., 1961). The same type of compartmentation was seen in paper 5 by the atom percent excess of the isotopomer derived from recycling in glutamine being higher than that in glutamate in the neuronal cultures.

In paper 2, it was shown that GABA synthesis in cerebellar neuronal cultures takes place in a compartment constituted by the subpopulation of GAD positive GABAergic neurons present in these cultures. In paper 4, a further compartmentation of the GABAergic compartment was reported. This was evident from the fact that cycling of the carbon skeleton from [U-^{13}C]glutamate subsequently used to form GABA was affected by KA, whereas cycling of the carbon skeleton from [U-^{13}C]glutamine was not. Since the cerebellar cultures consist of two types of GABAergic neurons, stellate and basket neurons, it is not surprising that the GABAergic compartment shows further compartmentation.

The compartmentation of metabolism discussed so far has been due to differential metabolism in different cell types; glia and neurons, glutamatergic and GABAergic neurons, and also subtypes of GABAergic neurons. This intercellular compartmentation is partly due to the fact that different cell types contain different enzymes. Although most enzymes involved in cell metabolism are present in all cell types, some are cell specific. Examples are the presence of GAD in the GABAergic, but not the glutamatergic neurons in the cerebellar

cultures (paper 1) and that of GS and PC in astrocytes, but not in neurons (Norenberg and Martinez-Hernandez, 1979; Yu et al., 1983; Shank et al., 1985). The latter two enzymes enable astrocytes to convert glutamate to glutamine and pyruvate to OAA, whereas neurons are unable to make these conversions. Another metabolic pathway believed to be astrocyte specific is the pyruvate recycling pathway for complete oxidation of glutamate in the TCA cycle (Håberg et al., 1998; Waagepetersen et al., 2002). However, experiments presented in paper 5 proved this belief wrong by showing pyruvate recycling in cultured cerebellar neurons.

Compartmentation has also been shown in monocultures consisting of one type of cells (Westergaard et al., 1995; Waagepetersen et al., 1998; Waagepetersen et al., 2006). This can be due to differential intracellular distribution of enzymes between cytosol and mitochondria as proposed in paper 1 to explain the differential effect of KA on labeling in alanine and lactate. Moreover, intracellular compartmentation of processes that occur exclusively in or on mitochondria, such as conversion of glutamine to glutamate by the enzyme PAG located in the inner mitochondrial membrane (Kvamme et al., 2000; Kvamme et al., 2001) have also been reported. This is called mitochondrial heterogeneity, and is presumably caused by differential distribution of enzymes in different mitochondria (McKenna et al., 2000; McKenna et al., 2006b). This was seen from results presented in papers 3 and 4 since TCA cycling of the carbon skeleton from [U-^{13}C]glutamine was more pronounced than that from [U-^{13}C]glutamate. This is in accordance with intracellular compartmentation previously reported in cultured cortical neurons, where TCA cycle metabolism of the carbon skeleton subsequently used in formation of GABA was higher from [U-^{13}C]glutamine than from [U-^{13}C]glutamate (Westergaard et al., 1995).

From the minute scale of metabolic compartmentation between different cell types, different organelles (cytosol and mitochondria) inside one cell type and between different organelles (mitochondria) inside one individual cell, it is time to change perspective and view the findings in the present papers on a larger scale. As mentioned in the introduction, there are several obvious differences between the cerebrum and the cerebellum, one being the glia to neuron ratio. Thus, it is not surprising that this has implications for the neuron glial interactions resting on neuron glial compartmentation and the extensive exchange of metabolites between the two cell types. One of the most studied pathways is the glutamate-glutamine-GABA cycle (Berl and Clarke, 1969; van den Berg and Garfinkel, 1971; Berl and Clarke, 1983; for review, see Bak et al., 2006). Although the glutamine-GABA cycle was first to be described (van den Berg and Garfinkel, 1971), it has been believed to be of less importance than the glutamate-glutamine cycle due to the fact that a smaller part of the neurotransmitter released from GABAergic neurons is taken up by surrounding astrocytes than that released from glutamatergic neurons (Schousboe et al., 1977; Danbolt, 2001; Schousboe, 2003). The glutamate-glutamine cycle was the subject of paper 3, in which it was shown that glutamatergic cerebellar neurons rely more on reuptake of glutamate than supply of glutamine from astrocytes for glutamate homeostasis, and thus it can be proposed that the glutamate-glutamine cycle is of less importance to the cerebellum than in cortex. This reminds us of how highly heterogeneous the brain is and that extrapolation of findings from one region of the brain or from one cell type to another should be exercised with care.

Future perspectives

The present studies have been performed using cell cultures. This offers a simplified system for metabolic studies. Even though the

enzymes involved in glutamate metabolism in these cultures have been shown to be similar to those *in vivo* (Drejer et al., 1985; Larsson et al., 1985), it should be taken into consideration that there still are many differences between the *in vitro* and *in vivo* situations. The main weakness is perhaps that the cultures are established using immature cells of mainly one type that are taken out of their structural context. The importance of testing findings from cell culture work by different methods and comparing them to *in vivo* studies cannot be emphasized enough. With this said, the detailed information about metabolic fluxes in the intact functioning brain obtained during the last decade using new technology like *in vivo* MRS, would not have been possible without the knowledge on metabolic pathways and compartmentation obtained using isolated cell types (Hertz, 2004). Perhaps the biggest challenge lies in combining the methodical tools to reach a higher level of knowledge. It therefore remains to be seen whether the findings from the present studies have implications *in vivo*.

6 CONCLUSIONS

The questions addressed in the objectives section can now be answered:

Glutamate and glutamine

❖ It is known that glutamate and glutamine serve as substrates for intermediary metabolism in cerebellar neurons. Is there a substrate preference between these two amino acids?

➢ As shown previously, both glutamate and glutamine were shown to be excellent precursors for intermediary metabolism in cerebellar neurons (papers 3 and 4), however, in paper 3 it was concluded that glutamate is preferred over glutamine.

❖ Is glutamate and glutamine metabolism in cerebellar neurons affected by long-term exposure to KA?

➢ In paper 1 it was shown that a KA concentration of 50 μM present in the culture medium of cerebellar neurons did not affect the glutamate metabolism in these cells. However, increasing the KA concentration in the medium to 0.50 mM, led to a decrease in intermediary metabolism of both glutamate and glutamine (paper 4). Thus, KA does affect glutamate and glutamine metabolism, at least when present in a high concentration (>50 μM)

❖ The pyruvate recycling pathway has been shown to operate in astrocytes. Is it also active in cultured neurons from cerebellum?

➢ Despite results from previous cell culture studies, which have shown pyruvate recycling in astrocytes but not in neurons, this

pathway was clearly present in both astrocytic and neuronal cultures from cerebellum in experiments presented in paper 5.

GABA

❖ Is GABA present in cerebellar neuronal cultures, and if so, how is the concentration compared to that in neocortical neuronal cultures?

➢ Yes, GABA was present in both the GABAergic and glutamatergic neurons in cerebellar cultures (papers 1 and 2). In paper 1, the average content of intracellular GABA was 20 ± 4 nmol/mg protein in cerebellar and 32 ± 2 nmol/mg protein in neocortical cultures.

❖ If GABA is present in these neurons, how does it get there; is it taken up from serum in the medium or is it synthesized by the cerebellar neurons (GABAergic and/or glutamatergic)?

➢ The GABA content in cerebellar neurons cannot be the result of uptake from the medium alone, because ^{13}C MRS analyses presented in paper 1 showed incorporation of ^{13}C label in GABA from [1-^{13}C]glucose, i.e. GABA is actively synthesized in these cultures. In paper 2 it was shown that GABA is synthesized by the subpopulation of GAD positive neurons present in these cultures and distributed to the other cells in the culture. This was supported by findings in paper 3, suggesting an active role of the GABA shunt in these cultures.

❖ If it is synthesized, what is the mechanism and time course throughout the culturing period for this synthesis?

➢ GABA in cerebellar neurons is synthesized mainly by decarboxylation of glutamate catalyzed by GAD. It seems unlikely that the putrescine pathway contributes to the

synthesis. Experiments presented in paper 2 showed that the amount of GABA in cell extract of cerebellar neuronal cultures more than doubled during the first five days in culture. After day five the amount decreased slightly until the end of the culture period (day 13).

❖ Does long-term KA exposure affect GABA synthesis in these cultures?

➢ Treatment with 50 μM KA only marginally affected cellular content and labeling from [1-^{13}C]glucose of GABA compared to untreated cerebellar neuronal cultures. However, the phenotype of the GAD positive neurons seems to be changed (paper 1). Exposure to 0.50 mM KA for seven days led to differential effects on GABA content in the cells depending on medium was changed (paper 4) or not (paper 1) and in the presence of glutamate or glutamine in the fresh medium (paper 4). In conclusion, there are indications that 50 μM KA present in the medium during the culture period does not affect metabolism of cerebellar neurons, whereas 0.50 mM has complex effects on GABA synthesis in these cultures.

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First direct demonstration of extensive GABA synthesis in mouse cerebellar neuronal cultures

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Demonstration of extensive GABA synthesis in the small population of GAD positive neurons in cerebellar cultures by the use of pharmacological tools

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Abstract

Cultures of dissociated cerebella from 7-day-old mice were maintained in vitro for 1–13 days. GABA biosynthesis and degradation were studied during development in culture and pharmacological agents were used to identify the enzymes involved. The amount of GABA increased, whereas that of glutamate was unchanged during the first 5 days and both decreased thereafter. The presence of aminooxyacetic acid (AOAA, 10 μM) which inhibits transaminases and other pyridoxal phosphate dependent enzymes including GABA-transaminase (GABA-T), in the culture medium caused an increase in the intracellular amount of GABA and a decrease in glutamate. The GABA content was also increased following exposure to the specific GABA-T inhibitor γ-vinyl GABA. From day 6 in culture (day 4 when cultured in the presence of AOAA) GABA levels in the medium were increased compared to that in medium from 1-day-old cultures. Synthesis of GABA during the first 3 days was demonstrated by the finding that incubation with either [1-^{13}C]glucose or [U-^{13}C]glutamine led to formation of labeled GABA. Synthesis of GABA after 1 week in culture, when the enzymatic machinery is considered to be at a more differentiated level, was shown by labeling from [U-^{13}C]glutamine added on day 7. Altogether the findings show continuous GABA synthesis and degradation throughout the culture period in the cerebellar neurons. At 10 μM AOAA, GABA synthesis from [U-^{13}C]glutamine was not affected, indicating that transaminases are not involved in GABA synthesis and thus excluding the putrescine pathway. At a concentration of 5 mM AOAA GABA labeling was, however, abolished, showing that glutamate decarboxylase, which is inhibited at this level of AOAA, is responsible for GABA synthesis in the cerebellar cultures. In conclusion, the present study shows that GABA synthesis is taking place via GAD in a subpopulation of the cerebellar neurons, throughout the culture period.
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Keywords: Cerebellar granule neurons; GABA-transaminase; Glutamate

1. Introduction

Neuronal phenotypes are classically connected to expression of specific enzymes and other entities such as the vesicular glutamate transporters for glutamatergic neurons (Fremeau et al., 2004). Characteristics of GABAergic neurons are GABA transporters in vesicles and plasma membranes and glutamate decarboxylase (GAD), the main GABA synthesizing enzyme (Saito et al., 1974; Borden, 1996; Chaudhry et al., 1998). GABA may also be formed via the putrescine pathway; however, this pathway appears to be operational mostly during early development (Seiler, 1980). Degradation of GABA takes place via GABA-transaminase (GABA-T) and succinate semialdehyde dehydrogenase, which are not only characteristics of GABAergic neurons, but are ubiquitously present in neurons and astrocytes throughout the brain (McGeer et al., 1983).

Abbreviations: AOAA, aminooxyacetic acid; DIV, days in vitro; GABA, γ-aminobutyric acid; GABA-T, GABA-transaminase; GAD, glutamate decarboxylase; GVG, γ-vinyl GABA; MCL, molecular carbon labeling; PAG, phosphate activated glutaminase; TCA, tricarboxylic acid

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Dissociated cultures of cerebella consist of a majority of neurons with glutamatergic and some with GABAergic phenotype since both glutamate and GABA are released in a Ca^{2+} dependent manner upon depolarization of the cell membranes (Pearce et al., 1981; Drejer et al., 1982, 1987; Drejer and Schousboe, 1989). However, the neuronal characteristics of cerebellar cultures can be influenced by exposure of the cells to kainic acid (KA). This is reflected by the finding that vesicular release of GABA can be essentially eliminated by culturing the cells in the presence of 50 µM KA (Drejer and Schousboe, 1989; Damgaard et al., 1996). In spite of this it has been demonstrated that the ability of these cultures to synthesize GABA is not affected by KA (Sonnewald et al., 2004). Immunohistochemistry using a GAD_{67} antibody demonstrated GAD-like immunostaining in ~6% of the cell bodies and additionally punctate fluorescence was observed in the processes throughout the cultures grown in the absence of KA. Exposure of the cerebellar neurons to KA (50 µM) eliminated the punctate staining but had no effect on the GAD-like immunostaining in cell bodies. Replacing KA with a glutamate transport inhibitor to induce excitotoxicity led to reduced levels of GAD and the vesicular GABA transporter (Kovacs et al., 2003). Surprisingly, the cellular content of GABA in 7-day-old cerebellar cultures was similar to that observed in cultures of cortical neurons of the same age which contain a dense layer of GAD positive neurons (Sonnewald et al., 2004).

It is intriguing that GABA synthesis in these cerebellar cultures after a 7-day culturing period reached a magnitude comparable to that observed in neocortical neurons, since the intensity of GAD_{67} immunofluorescence was considerably lower in the cerebellar neurons. Little is known about the time course of GABA synthesis in the latter cultures. Results presented in this study describe the metabolism of GABA and glutamate in cerebellar cultures. It has been shown that, during the course of a week, both cerebellar and cortical neurons in culture develop an enzymatic machinery analogous to that of the brain in vivo (Drejer et al., 1985; Larsson et al., 1985). Hence, most experiments were done on days 3, 5 and 7 in culture. Cerebellar neurons were cultured in the presence of 50 µM KA and [1-^{13}C]glucose or [U-^{13}C]glutamine to monitor GABA synthesis. Glutamine may serve as precursor for GABA involving either GAD or the putrescine pathway. To probe the involvement of GABA degrading enzymes in the maintenance of GABA homeostasis, experiments were performed using 10 µM aminooxyacetic acid (AOAA) which inhibits GABA-T, other transaminases and other pyridoxal phosphate dependent enzymes or 100 µM γ-vinyl GABA (GVG) to selectively inhibit GABA-T (Schousboe et al., 1974; Lippert et al., 1977). In order to inhibit GAD completely, a concentration of 5.0 mM AOAA was used (Wu and Roberts, 1974).

2. Materials and methods

2.1. Materials

Plastic tissue culture dishes were purchased from Nunc A/S (Roskilde, Denmark), fetal calf serum from Seralab Ltd. (Sussex, UK) and culture medium from GIBCO BRL, Life Technologies A/S (Roskilde, Denmark). 7-day-old mice (NMRI) were purchased from Møllegaard Breeding Center (Ejby, Denmark) or were obtained from the animal facility at the Department of Pharmacology, The Danish University of Pharmaceutical Sciences. [1-^{13}C]Glucose and [U-^{13}C]glutamine (98%+ enriched) were from Cambridge Isotopes Laboratories (Woburn, MA, USA). KA was from Sigma Chemical Co. (St. Louis, MO, USA) or Tocris Cookson Inc. (Ellisville, MO, USA). All other chemicals were of the purest grade available from regular commercial sources.

2.2. Cerebellar neurons and culture conditions

All animal procedures were conducted according to national regulations. Cerebellar cells were isolated from cerebella of 7-day-old mice (Schousboe et al., 1989). The brain tissue was trypsinized followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. Cells were suspended (3×10^6 cells/ml) in a slightly modified Dulbecco's minimum essential medium (DMEM) containing 28 mM glucose and 0.45 mM glutamine, 50 µM KA and 10% (v/v) fetal calf serum and seeded in poly-D-lysine coated culture dishes or flasks. Six-well plates were used for HPLC and 25 cm^2 flasks for liquid chromatography mass spectrometry (LC–MS) analyses. In some cultures, [1-^{13}C]glucose (28 mM) or [U-^{13}C]glutamine (0.45 mM) were used instead of unlabeled substrate. Some cultures were exposed to 10 µM or 0.5 mM AOAA or 100 µM GVG from day 0. Cytosine arabinoside (20 µM) was added after 24–48 h to prevent astrocyte proliferation (Schousboe et al., 1989). After the indicated number of days in vitro (DIV), medium was removed and cells were washed with 0.9% saline and extracted with 70% (v/v) ethanol, followed by centrifugation at $3000 \times g$ for 5 min. The supernatants were lyophilized and stored at −20 °C. In some cases media were collected, deproteinized with ethanol (70% (v/v) final concentration), lyophilized and stored at −20 °C. Cellular protein in the ethanol pellets was determined after re-dissolving in 1 M KOH at 37 °C for 30 min, using the Pierce BCA protein assay with bovine serum albumin as standard.

2.3. Acute exposure to AOAA

In order to investigate the ability of AOAA to inhibit GAD completely, cultures were treated with 5.0 mM AOAA which was added to the medium on day 7 in vitro. After 30 min, half (2.5 ml) of the culture medium was removed and 1 ml fresh serum-free DMEM was added. This medium contained pyruvate (5.0 mM, final concentration), [U-^{13}C]glutamine (0.5 mM, final concentration) and AOAA (5.0 mM, final concentration). After 3 h, the cultures received 8 µl of [U-^{13}C]glutamine (219 mM) to the incubation media (3.5 ml) to preserve an adequate amount of the labeled precursor in the medium and the incubation was continued for another 3 h. Control cultures were treated identically except that AOAA was not added. At the end of the incubation period, the medium was removed and the cells were washed twice with ice-cold PBS prior to extraction using 70% (v/v) ethanol. The procedures for centrifugation and protein analysis were as described above. The cell extracts were analyzed for percent labeling employing LC–MS.

2.4. HPLC and LC–MS analyses

Glutamate and GABA were quantified in cell extracts and in some cases GABA was quantified in the culture media by high performance liquid chromatography (HPLC) analysis using fluorescence detection, after pre-column derivatization with o-phthaldialdehyde (Geddes and Wood, 1984). All LC–MS analyses were performed using a Shimadzu LCMS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. The Phenomenex EZ:faast amino acid analysis kit for LC–MS was used for analysis of labeling in glutamate and GABA.

2.5. Data analysis

Percent ^{13}C (atom percent excess) was determined for $M + 1$ (the mass of the parent ion (M) plus 1 unit of molecular weight (Dalton) corresponding to 1 atom of ^{13}C), $M + 2$, $M + 3$, $M + 4$ plus in the case of glutamate $M + 5$ after correction for natural abundant ^{13}C as described by Biemann (1962). To obtain a

measure of total incorporation of ^{13}C label, the average percent of labeled carbon atoms for each metabolite was calculated, i.e. percent molecular carbon labeling (MCL, for further details see Bak et al. (2006)). The [U-^{13}C] labeling (percent of total amount) of GABA and glutamate is the atom percent excess for $M + 4$ and $M + 5$, respectively, following subtraction of natural abundances. All results are presented as means ± S.D. Differences between cultures of different developmental stages or exposed to various pharmacological agents were analyzed statistically with one-way ANOVA followed by the LSD (least significant difference) post hoc test for multiple comparison or Student's t-test for two groups, and $p < 0.05$ was considered statistically significant.

3. Results

As a measure of cell growth the amount of protein in the cultures was shown to almost double over a period of 4 days in vitro and this level was maintained until day 13 (Fig. 1). Treatment of the cultures with AOAA (10 μM) had no effect on the protein content (Fig. 1).

The developmental patterns of the amounts (nmol/well) of glutamate and GABA in the cerebellar neurons maintained in the presence or absence of 10 μM AOAA during the culture period (13 days) are shown in Fig. 2A and B. The amount of glutamate was constant until day 6 in culture after which it was decreasing with time until day 10 in culture. In the presence of AOAA the amount of glutamate was lower than that of age matched untreated cultures from days 4 to 9. The amount of GABA more than doubled till day 5 and decreased subsequently to a level similar to that at the beginning of the culture period. In the presence of AOAA the GABA levels were significantly increased compared to the age matched untreated cultures from day 4 and throughout the culture period.

Fig. 3 shows the amount of protein (mg/well, A) and the GABA (B) and glutamate (C) contents (nmol/mg protein) in cerebellar neurons cultured for 3, 5 and 7 days. Culturing the cells in medium containing GVG (100 μM) had no effect on the protein content, whereas that of glutamate was slightly higher on day 5 and lower on day 7 compared to the age matched untreated cultures. The GABA content was increased in the presence of GVG at days 5 and 7 in culture compared to age matched untreated cultures. Culturing in the presence of 0.5 mM AOAA
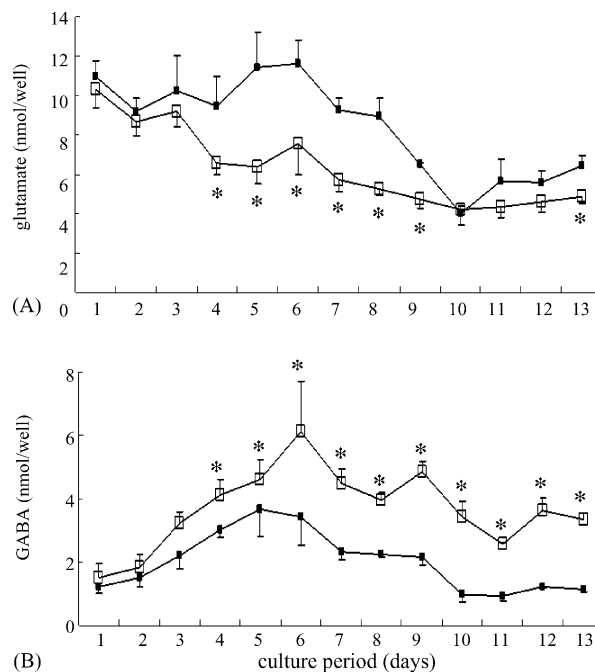

Fig. 2. The amount (nmol/well) of glutamate (A) and GABA (B) in cell extracts of cerebellar neurons. The cells were seeded in 6-well plates and maintained in medium containing KA (50 μM) in the presence or absence of AOAA (10 μM) for 13 days as described in Section 2. Results are means ± S.D. of 3–6 samples. Filled squares represent the control cells, and open squares the AOAA treated cultures. Statistically significant differences between the different days in vitro (controls) were analyzed using ANOVA followed by the LSD (least significant difference) post hoc test, and $p < 0.05$ was considered significant. For the development in culture of glutamate contents values for DIV 7–13 were significantly reduced compared to DIV 1–6, and for GABA DIV 3–6 were significantly higher than DIV 1 and DIV 7–13 significantly lower than DIV 6. No difference for GABA was found between DIV 5 and 6. Likewise, differences between control cultures and AOAA treated cells were assessed using the same procedure and statistically significant differences ($p < 0.05$) are indicated by asterisks.

led to decreased amounts of protein and glutamate at all days investigated and the GABA content was decreased on days 3 and 5 compared to age matched untreated cells.

Fig. 4 shows the GABA concentration (nmol/ml) in media from neurons cultured in the absence or presence of 10 μM AOAA for 1, 4 and 6 days. For comparison the GABA concentration in medium (without cells) kept in the incubator for 3 and 6 days was determined and the average value is shown in Fig. 4. It is necessary to determine the GABA concentration in medium without cells since fetal calf serum contains GABA and also to evaluate the effect of incubation at 37 °C for several days. The concentration of GABA in the medium of cells maintained for 1 and 4 DIV was the same as that in medium without cells. At 6 DIV the GABA concentration in the medium was higher than that at 1 and 4 DIV. In the presence of AOAA the GABA concentration was increased already on day 4 compared to medium from AOAA treated cultures on day 1 and it was increased further on day 6 reaching a level significantly higher than that of the age matched untreated cultures.

The MCL (see Section 2) of GABA and glutamate in cerebellar neurons cultured in media containing either [1-^{13}C]glucose or [U-^{13}C]glutamine is presented in Table 1.

Fig. 1. The amount of protein (mg/well) in cerebellar neurons. The cells were seeded in 6-well plates and maintained in medium containing KA (50 μM) in the presence or absence of AOAA (10 μM) for 13 days as described in Section 2. Results are means ± S.D. of 3–6 samples. Filled squares represent the control cells, and open squares the AOAA treated cultures. Differences between days 1 or 2 and subsequent days in vitro were analyzed using ANOVA followed by the LSD (least significant difference) post hoc test, and $p < 0.05$ was considered statistically significant. Protein at DIV 3–13 was statistically significantly different from DIV 1 and 2 regardless of the presence of AOAA.
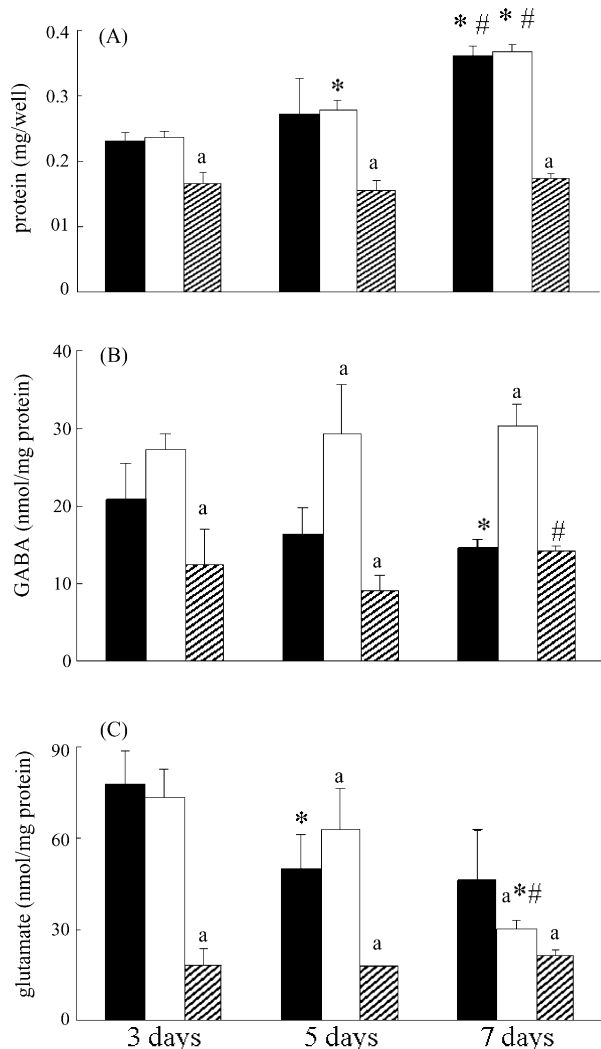
Fig. 3. The amount (mg/well) of protein (A), and contents (nmol/mg protein) of GABA (B) and glutamate (C) in cerebellar neurons cultured in 6-well plates. Cells were cultured in medium containing KA (50 μM) in the presence or absence of GVG (100 μM) or AOAA (0.5 mM) as described in Section 2. Results are means ± S.D. of 3–8 samples. Filled bars represent control cells. Open and hatched bars represent GVG and AOAA treated cultures, respectively. Statistically significant differences between treated and untreated (control) cultures as well as between cells cultured for different number of days were analyzed using ANOVA followed by the LSD (least significant difference) post hoc test, and $p < 0.05$ was considered significant. (a) Significantly different from the untreated cells on the same day, (*) significantly different from the corresponding cultures on day 3, and (#) significantly different from the corresponding cultures on day 5.

When [1-^{13}C]glucose was present in the medium, the MCL of GABA was increased at 7 compared to 3 DIV, regardless of the presence of 10 μM AOAA. MCL of GABA was lower in AOAA treated compared to untreated cultures kept for 3 or 7 DIV. No differences were observed in the MCL of glutamate from [1-^{13}C]glucose between AOAA treated and untreated cultures maintained for 3 or 7 DIV. However, an increase in the MCL of glutamate was observed in cerebellar neurons cultured for 7 days compared to 3 days. Following 3 days in culture in medium containing [1-^{13}C]glucose and AOAA the MCL of glutamate was higher than that of GABA whereas in the untreated cultures no difference was observed. After 7 days in culture in medium
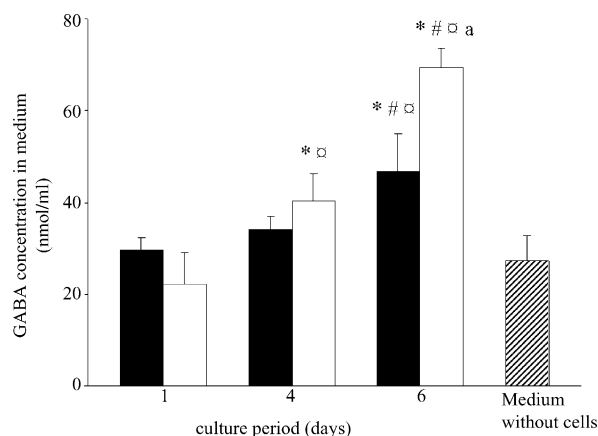
Fig. 4. The concentration (nmol/ml) of GABA in medium from cerebellar neurons cultured in 6-well plates and maintained in medium (2 ml) containing KA (50 μM) in the presence or absence of AOAA (10 μM). Results are corrected for evaporation and are expressed as means ± S.D. of 3–5 samples. Filled bars represent untreated cultures (control) and open bars AOAA treated cultures. The hatched bar represents the average of the GABA concentration in media kept in the incubator for 3 or 6 days. It was possible to use the average since no difference was found comparing media kept for 3 and 6 days. Statistically significant differences between AOAA treated and not-treated (control) cultures as well as between cells cultured for different number of days were analyzed using ANOVA followed by the LSD (least significant difference) post hoc test, and $p < 0.05$ was considered significant. (*) Significantly different from the corresponding cultures at 1 DIV, (#) significantly different from the corresponding cultures at 4 DIV, (¤) significantly different from medium without cells, and (a) significantly different from control cultures at the same DIV.

Table 1
The MCL (%) of GABA and glutamate from [1-^{13}C]glucose and [U-^{13}C]glutamine in cerebellar neurons cultured for 3 and 7 DIV

Amino acid	MCL (%)	
	[1-^{13}C]Glucose	[U-^{13}C]Glutamine
GABA		
3 Days		
AOAA	19.7 ± 0.2[a]	12.1 ± 0.4
Control	23.6 ± 0.8	10.9 ± 1.3
7 Days		
AOAA	26.1 ± 0.3[a,b]	4.2 ± 0.2[a,b]
Control	28.8 ± 0.2[b]	1.3 ± 0.3[b]
Glutamate		
3 Days		
AOAA	23.2 ± 0.1[c]	7.0 ± 1.3[c]
Control	23.1 ± 0.8	5.7 ± 1.8[c]
7 Days		
AOAA	28.3 ± 0.4[b,c]	0.8 ± 0.3[b,c]
Control	27.9 ± 0.1[b,c]	1.1 ± 0.5[b]

Cerebellar neurons were cultured in 25 cm^2 flasks in media containing KA (50 μM) in the presence or absence of AOAA (10 μM) using [1-^{13}C]glucose or [U-^{13}C]glutamine as described in Section 2. The average labeling of molecular carbon (MCL) in percent (see Section 2) of GABA and glutamate is shown ± S.D. of four cultures. Statistically significant differences between experimental conditions and culture ages were analyzed using ANOVA followed by the LSD (least significant difference) post hoc test, and $p < 0.05$ was considered statistically significant.
[a] Significantly different from the corresponding control cultures.
[b] Significantly different from similarly treated cultures at 3 DIV.
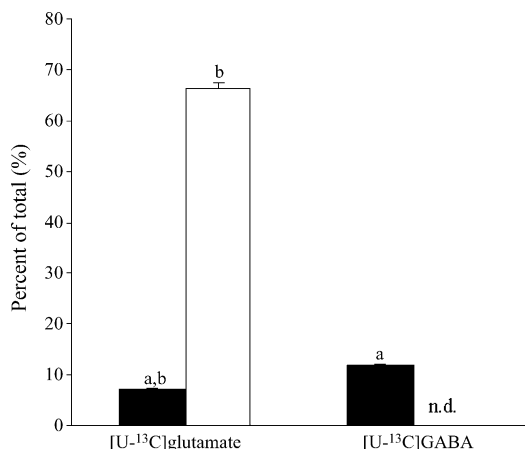[c] Significantly different from the percent ^{13}C labeling in GABA in cultures treated identically.

Fig. 5. The [U-^{13}C] labeling (percent of total amount) of GABA and glutamate in cerebellar neurons (7 DIV) incubated in medium containing [U-^{13}C]gluta-mine in the presence or absence of AOAA (5.0 mM) as detailed in Section 2. Results are means ± S.D. of 4 to 5 samples. Filled bars represent the untreated and open bars the AOAA treated cells. Statistically significant differences between these were analyzed using the unpaired two tailed Student's t-test, and $p < 0.05$ was considered statistically significant. (a) Significantly different from the corresponding AOAA treated cultures, (b) significantly different from [U-^{13}C]GABA in cultures maintained under similar conditions, and n.d. means not detectable.

containing [1-^{13}C]glucose the MCL of glutamate was slightly higher in AOAA treated than that observed for GABA and the opposite relationship was observed in untreated cultures. When [U-^{13}C]glutamine was present in the medium from the beginning of the culture period, the MCL of both GABA and glutamate was decreased in cells cultured for 7 compared to 3 DIV. The MCL of GABA was higher than that of glutamate regardless of the experimental conditions, except at day 7 in cultures not exposed to AOAA. Furthermore, the MCL of GABA was increased in the AOAA treated cells in 7-day-old cultures but unchanged in 3-day-old cultures. This is in contrast to cells cultured in the presence of [1-^{13}C]glucose, in which label was decreased in the presence of AOAA both at 3 and 7 DIV. However, as shown in Fig. 5, when AOAA (5 mM) was added to the medium on day 7, conversion of [U-^{13}C]glutamine to [U-^{13}C]glutamate took place whereas no [U-^{13}C]GABA was detected. In these cultures pyruvate was added to the culture medium together with [U-^{13}C]glutamine to ensure TCA cycle metabolism and cell survival in the presence of AOAA which prevents a continuous oxidation of glucose due to the inhibition of the malate aspartate shuttle (McKenna et al., 2006). Cell viability was checked by microscopic inspection of the cultures and cell morphology was not affected by the AOAA treatment (results not shown).

4. Discussion

The present study demonstrates GABA synthesis via GAD in cerebellar neurons throughout the first week in culture. The cellular content of GABA in the cerebellar neurons more than doubled during the first 5 days in culture, whereas that of glutamate remained unchanged. This was accompanied by an increase in the amount of protein. It should be noted that the number of neurons does not increase after the brain tissue is removed from the animals and subsequently seeded in the culture dishes. Actually, approximately 50% of the seeded cells die (Westergaard et al., 1991). Thus, the increase in protein reflects cell growth and possibly differentiation. The enzymatic machinery of cerebellar neurons after 1 week in culture is comparable to that observed in the brain in vivo (Drejer et al., 1985). After day 5 in culture the amount of GABA and glutamate inside the cells decreased slightly until the end of the culture period (day 13). However, GABA content in the medium increased with time. This indicates that GABA is released from the cerebellar cells in a non-depolarization dependent manner, presumably via reversal of transporters and may reflect its functional importance during differentiation (Belhage et al., 1985; Waagepetersen et al., 1999). Furthermore, GABA release is a prerequisite for its neurotrophic action and may also play a role in neuroprotection.

Synthesis of GABA during the first 3 days was demonstrated by the finding that incubation with either [1-^{13}C]glucose or [U-^{13}C]glutamine led to formation of labeled GABA. It may be conceivable that GABA synthesis could be especially prominent during the first few days in culture since it has been reported that in hippocampus, GAD$_{67}$ is expressed in the mossy fibers of the developing rat brain, whereas in adults, GAD$_{67}$ was no longer detectable, unless seizures were induced (Maqueda et al., 2003). To investigate the possibility that GABA synthesis is not only taking place during the early phase of the culturing period but continues also during later stages, [U-^{13}C]glutamine was added on day 7 in culture and both GABA and glutamate labeling was pronounced. Furthermore, when [1-^{13}C]glucose was present in the culture medium for 3 and 7 days, labeling on day 7 was clearly higher than on day 3. Labeling from [U-^{13}C]glutamine under the same conditions was less pronounced than that from glucose, which may reflect that the amount of [U-^{13}C]glutamine available in the medium was insufficient to sustain neuronal metabolism for 7 days. That this may be the case is supported by the finding that when [U-^{13}C]glutamine was added on day 7, GABA labeling was much higher.

To investigate the enzymatic pathways responsible for GABA synthesis and degradation, enzyme inhibitors were added to the culture medium. The enzyme responsible for degradation of GABA, GABA-T, is inhibited by GVG (Lippert et al., 1977). As expected (Gram et al., 1988), GVG at a concentration of 100 μM increased the GABA content of the cultures. GVG had no effect on the protein content which may indicate that the GABA concentration even in the absence of GVG was adequate for maintenance of normal neuronal growth. This is supported by the finding that GABA at 50 μM, a value comparable to that observed in the present study, acts as a trophic factor in the development of cerebellar neuronal cultures (Hansen et al., 1984). The transaminase inhibitor AOAA was used at 10 μM, a concentration sufficient to inhibit mainly GABA-T (Schousboe et al., 1974) but also other transaminases (Kihara and Kubo, 1989). As expected, the amount of GABA increased with time even more than in the absence of AOAA whereas the glutamate concentration decreased compared to that in untreated cells. The latter finding may be compatible with the previous demonstration that

biosynthesis of neurotransmitter glutamate in the glutamatergic neurons in these cultures is dependent upon the function of the malate aspartate shuttle which involves transamination (Palaiologos et al., 1988). Glutamate formed via transamination seems to be important for GABA labeling from [1-^{13}C]glucose (for pathway see Brenner et al. (2005)) since such labeling was decreased in the presence of 10 μM of AOAA. Conversion of α-ketoglutarate to glutamate is mostly achieved by transamination, which will be blocked by AOAA. However, glutamate dehydrogenase is also present in the cerebellar neurons (Zaganas et al., 2001) and can convert α-ketoglutarate to glutamate. The efficiency of this process is evident from the unchanged glutamate labeling in the presence of 10 μM AOAA. This, together with a decreased GABA labeling, points towards compartmentation of glutamate metabolism, indicating GABA synthesis in a different cellular compartment from where the majority of glutamate synthesis is taking place.

There are two known pathways for conversion of glutamine to GABA. One is called the putrescine pathway (Seiler, 1980) which involves transamination and the other conversion of glutamine to glutamate and subsequent decarboxylation to GABA which is referred to as the GAD pathway. Labeling of GABA from [U-^{13}C]glutamine was not affected by 10 μM AOAA, indicating that transamination is not involved in the process. Thus, it appears likely that the GAD pathway is responsible for GABA synthesis from glutamine in the cerebellar cultures. This, together with the fact that metabolism of glutamate and GABA seems compartmentalized as mentioned above, indicates that GABA synthesis takes place in the ~6% GAD positive cells observed by Sonnewald et al. (2004).

An increase in the AOAA concentration from 10 μM to 0.5 mM led to a pronounced decrease in the GABA and glutamate content as well as the amount of protein at 3 and 5 DIV. As mentioned above, in the presence of the transaminase blocker, the decrease in glutamate content was expected. The large decrease in GABA may to a certain extent be explained by partial inhibition of GAD in the presence of 0.5 mM AOAA (Wu and Roberts, 1974) and the decrease in protein content may to some extent reflect the decrease in the availability of the neurotrophic agent GABA. However, it is conceivable that an impaired oxidative metabolism of glucose played a prominent role in the large decrease of the protein content since this metabolism is dependent upon the malate aspartate shuttle which was inhibited by 0.5 mM AOAA (Kauppinen et al., 1987). An impaired glucose metabolism will affect neurons and therefore it may indirectly contribute to the decrease in glutamate as well as GABA contents. Interestingly, on day 7 in culture, the GABA content was not different from that of age matched untreated cells, whereas glutamate and the protein content were lower.

To obtain further information about GABA synthesis, AOAA was used at a concentration of 5 mM, which is expected to block both GAD and transaminases completely (Wu and Roberts, 1974; Kihara and Kubo, 1989). Indeed, GABA labeling from [U-^{13}C]glutamine was totally abolished even though glutamate was extensively labeled.

In conclusion, the present results show that GABA synthesis is taking place via GAD in a subpopulation of the cerebellar neurons, throughout the culture period. Labeling of GABA occurs from both [1-^{13}C]glucose and [U-^{13}C]glutamine and can be blocked by AOAA. Moreover, it is confirmed that net synthesis of glutamate is dependent on the activity of the malate aspartate shuttle.

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Glutamate is preferred over glutamine for intermediary metabolism in cultured cerebellar neurons

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Glutamate is preferred over glutamine for intermediary metabolism in cultured cerebellar neurons

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The glutamate–glutamine cycle is thought to be of paramount importance in the mature brain; however, its significance is likely to vary with regional differences in distance between astrocyte and synapse. The present study is aimed at evaluating the role of this cycle in cultures of cerebellar neurons, mainly consisting of glutamatergic granule cells. Cells were incubated in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine in the presence and absence of unlabeled glutamine and glutamate, respectively. Cell extracts and media were analyzed using high-performance liquid chromatography (HPLC) and gas chromatography combined with mass spectrometry (GC/MS). Both [U-^{13}C]glutamate and [U-^{13}C]glutamine were shown to be excellent precursors for synthesis of neuroactive amino acids and tricarboxylic acid (TCA) cycle intermediates. Labeling from [U-^{13}C]glutamate was higher than that from [U-^{13}C]glutamine in all metabolites measured. The presence of [U-^{13}C]glutamate plus unlabeled glutamine in the experimental medium led to labeling very similar to that from [U-^{13}C]glutamate alone. However, incubation in medium containing [U-^{13}C]glutamine in the presence of unlabeled glutamate almost abolished labeling of metabolites. Thus, it could be shown that glutamate is the preferred substrate for intermediary metabolism in cerebellar neurons. Label distribution indicating TCA cycle activity showed more prominent cycling from [U-^{13}C]glutamine than from [U-^{13}C]glutamate. Labeling of succinate was lower than that of the other TCA cycle intermediates, indicating an active role of the γ-amino butyric acid shunt in these cultures. It can be concluded that the cerebellar neurons rely more on reuptake of glutamate than supply of glutamine from astrocytes for glutamate homeostasis.
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Introduction

Glutamate is a multipurpose amino acid in the mature central nervous system. It is not only the major excitatory neurotransmitter, in addition it takes part in transamination and thus nitrogen homeostasis and is the precursor for other important molecules, including the main inhibitory neurotransmitter, γ-amino butyric acid (GABA). Although glutamate is ubiquitous in all parts of the central nervous system and present in large amounts in the brain, it is of critical importance that brain gluta-

mate homeostasis is strictly controlled. The extracellular concentration of glutamate needs to be kept low, both to increase the signal-to-noise ratio in binding of transmitter substance in the synaptic cleft and to prevent excitotoxicity caused by excessive excitation of glutamate receptors and subsequent cell injury or death (for references, see Daikhin and Yudkoff, 2000). Rapid transport of glutamate from the synaptic cleft is performed through several types of specific transporter proteins, and uptake into astrocytes surrounding the synapse is believed to be more important than reuptake into the presynaptic neuron (Schousboe *et al*, 1977; Danbolt, 2001). Thus neurons experience a net loss of glutamate, which must be replenished by astrocytes because of the lack of the anaplerotic enzyme pyruvate carboxylase in neurons (Shank *et al*, 1985). This constitutes the basis for the pathway known as the glutamate–glutamine cycle (Berl and Clarke, 1983) in which

neurotransmitter released from neurons is taken up by surrounding astrocytes, where it is converted to glutamine by the glial-specific enzyme glutamine synthetase (Norenberg and Martinez-Hernandez, 1979). Glutamine is not neuroactive and can move in the extracellular space without interfering with receptors, and is the most abundant amino acid in blood and cerebral spinal fluid with a concentration of ∼0.5 mmol/L (Grill *et al*, 1992; White *et al*, 2004). Glutamine uptake into neurons is mediated by different general amino-acid transporters (Su *et al*, 1997; Dolinska *et al*, 2004). In neurons, glutamine can be converted into glutamate by the enzyme phosphate activated glutaminase and act as precursor for restoring the neurotransmitter pool, completing the glutamate–glutamine cycle. Indeed, several studies have confirmed the importance of glutamine as precursor for neurotransmitter glutamate (for a review, see Peng *et al*, 1993). As described, the cycle necessitates extensive interaction between neurons and astrocytes. This will, however, vary because of differences in the number of glial cells per neuron and also in respect to how closely the astrocytes envelop the synapses. Thus, it can be expected that the importance of the glutamate–glutamine cycle varies with location in the brain.

The present study is aimed at evaluating the role of the glutamate–glutamine cycle in cultured cerebellar neurons. It has been shown that after 7 days *in vitro*, these mainly glutamatergic cultures express glutamate and glutamine metabolizing enzymes analogous to the brain *in vivo* (Drejer *et al*, 1985). The cells were incubated in medium containing [U-¹³C]glutamate or [U-¹³C]glutamine in the presence and absence of unlabeled glutamine and glutamate, respectively. High-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analysis of cell extracts and media revealed that both [U-¹³C]glutamate and [U-¹³C]glutamine were excellent precursors for synthesis of neuroactive amino acids and tricarboxylic acid (TCA) cycle intermediates in these cultures. However, glutamate was shown to be the preferred substrate.

Materials and methods

Materials

NMRI mice were obtained from Taconic M&B (Copenhagen, Denmark). Plastic tissue culture dishes were purchased from Nunc A/S (Roskilde, Denmark) and fetal calf serum from Seralab Ltd. (Sussex, UK). Culture medium, glutamate receptor antagonists DNQX (6,7-dinitroquinoxaline-2,3-dione) and D-AP5 (D-2-amino-5-phosphonopentanoic acid) were from Sigma Chemical Co. (St Louis, MO, USA). [U-¹³C]Glutamate and [U-¹³C]glutamine were from Cambridge Isotope Laboratories (Woburn, MA, USA), and the GC/MS derivatization reagent MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide) + 1%

t-BDMS-Cl (*tert*-butyldimethylchlorosilane) was purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

Cell Cultures

Cerebellar neurons were isolated and cultured from 7-day-old mice as described by Schousboe *et al* (1989). Briefly, tissue was trypsinized followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. Cells were suspended (2.5×10^6 cells/ml) in a modified Dulbecco's minimum essential medium containing 24.5 mmol/L KCl, 31 mmol/L glucose, 7 μmol/L *p*-aminobenzoic acid, 0.05 mmol/L kainic acid, and 10% (v/v) fetal calf serum, and seeded in poly-D-lysine coated Petri dishes (2 ml/35 mm). After 48 h in culture, 20 μmol/L (final concentration) cytosine arabinoside was added to the medium to prevent astrocytic proliferation.

Experiments Using [U-¹³C]Glutamate and [U-¹³C]Glutamine for Gas Chromatography/Mass Spectrometry Analysis

The culture medium was removed from 7-day-old cultures and the cells were incubated for 2 h at 37°C in 2 ml serum-free experimental medium (prepared without glutamine) containing 3 mmol/L glucose and either no additions (for HPLC analysis), [U-¹³C]glutamate (0.25 mmol/L), [U-¹³C] glutamine (0.50 mmol/L), [U-¹³C]glutamate (0.25 mmol/L) plus unlabelled glutamine (0.50 mmol/L) or [U-¹³C] glutamine (0.50 mmol/L) plus unlabelled glutamate (0.25 mmol/L). To avoid toxic effects of glutamate during the incubation period, two glutamate receptor antagonists DNQX (25 μmol/L), a selective antagonist at the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainate receptor subtypes, and D-AP5 (100 μmol/L), an *N*-methyl-D-aspartate (NMDA) antagonist, were also present in the experimental medium (Frandsen *et al*, 1989). After 2 h, the experimental medium was collected and the cells were washed twice with cold phosphate-buffered saline and extracted with 70% (v/v) ethanol. The cell extracts were scraped off the dishes and centrifuged at 10,000*g* for 15 min to separate the metabolites from the insoluble proteins. The supernatants (cell extracts) were divided into two parts, one directly analyzed with HPLC and the other lyophilized for subsequent sample preparation for GC/MS analysis. Cellular protein in the ethanol pellets was determined after dissolving in 1 mol/L KOH at 37°C for 60 mins, using the Pierce BCA (Pierce, Rockford, IL, USA) protein assay with bovine serum albumin as standard.

High-Performance Liquid Chromatography

Amino acids in cell extracts and experimental media were quantified by HPLC on a Hewlett Packard 1100 system (Agilent Technologies, Palo Alto, CA, USA). The amino acids were precolumn derivatized with

o-phthaldialdehyde (Geddes and Wood, 1984) and subsequently separated on a ZORBAX SB-C18 ($4.6 \times 250\,mm$, $5\,\mu m$) column from Agilent using a phosphate buffer ($50\,mmol/L$, $pH = 5.9$) and a solution of methanol (98.75%) and tetrahydrofuran (1.25%) as eluents. The separated amino acids were detected with fluorescence and compared with a standard curve derived from standard solutions of amino acids run after every 12 samples.

Gas Chromatography/Mass Spectrometry

Lyophilized cell extracts were redissolved in HCL ($10\,mmol/L$), adjusted to $pH < 2$ with $6\,mol/L$ HCL, and dried under atmospheric air. The amino acids were extracted into an organic phase of ethanol and benzene and dried again under atmospheric air before derivatization with MTBSTFA in the presence of 1% *t*-BDMS-Cl (Mawhinney *et al*, 1986). The samples were analyzed on a Hewlett Packard 5890 Series II gas chromatograph linked to a Hewlett Packard 5972 Series mass spectrometer.

Data Analysis

Peaks from MS spectra were integrated, and atom percent excess (^{13}C) of glutamate, glutamine, GABA, succinate, malate, aspartate, and citrate was determined after calibration using unlabeled standard solutions (Biemann, 1962). Results from HPLC quantification of amino acids in cell extracts were combined with values of atom percent excess obtained from GC/MS to give nmol/mg protein of different ^{13}C labeled isotopomers of glutamate, aspartate, and GABA. Consumption of [U-^{13}C]glutamate and [U-^{13}C]glutamine was calculated by subtracting the remaining amounts of the two amino acids measured in the experimental medium from the amounts added followed by correction for the amount of cellular protein in the culture. Results are presented as means $\pm$ s.d. Differences between groups were analyzed statistically using one-way analysis of variance followed by the least significant difference *post hoc* test, and $P < 0.05$ was considered statistically significant.

Results

Cerebellar neurons were incubated in medium with different additions: no addition (control group), [U-^{13}C]glutamate, [U-^{13}C]glutamine, [U-^{13}C]glutamate plus glutamine, and [U-^{13}C]glutamine plus glutamate (see Materials and methods). Since HPLC analysis does not distinguish between isotopomers, the latter two groups were combined in Table 1, which shows the cellular content of selected amino acids in cerebellar neurons and the consumption of glutamate and glutamine. Compared with control, adding glutamate to the experimental medium led to increased intracellular levels of aspartate, glutamate, glutamine, and GABA. Cultures incubated with glutamine contained increased levels of GABA corresponding to the increase seen when glutamate was added. Aspartate and glutamate also increased as a response to glutamine addition, but not to the same extent as when glutamate was added. As expected, intracellular glutamine concentration also increased when glutamine was added to the medium. When both glutamate and glutamine were added to the experimental medium, aspartate, glutamate, and glutamine levels were increased more than when the two amino acids were added individually, whereas GABA content was increased to the same extent as when glutamate and glutamine were added alone. Quantification of glutamate and glutamine in the experimental media showed that glutamate consumption was much higher than glutamine consumption despite the medium concentration of glutamine being twice that of glutamate. Glutamate consumption was unaffected by the presence of glutamine, whereas the consumption of glutamine was reduced by nearly 50% when glutamate was added to the medium.
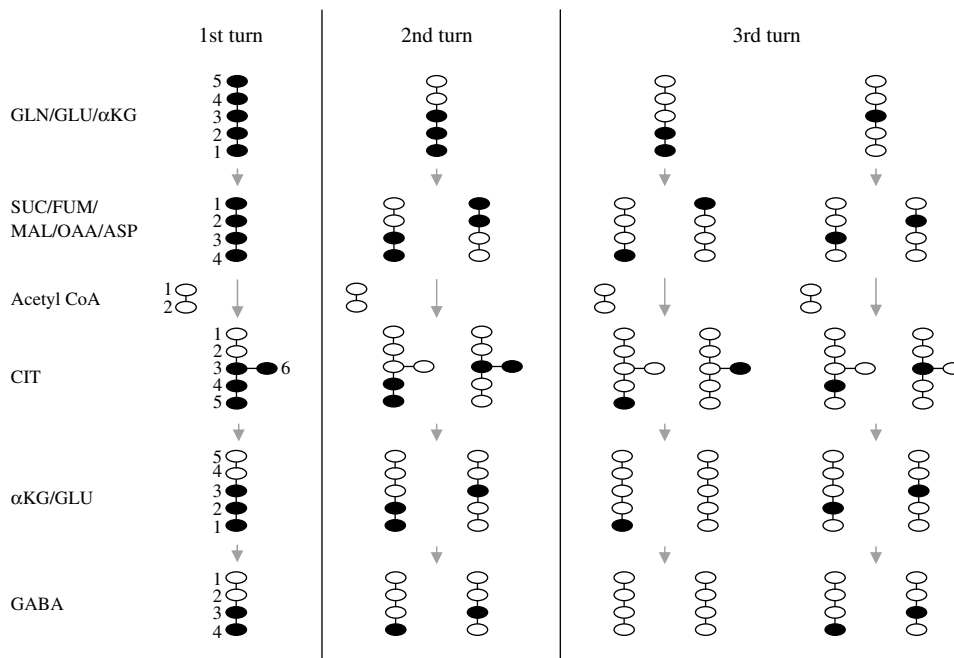
[U-^{13}C]Glutamate or [U-^{13}C]glutamine from the experimental medium enters neurons through specific transporter proteins. Once inside the cells, [U-^{13}C]glutamine can be converted to [U-^{13}C]glutamate, which can be decarboxylated to uniformly labeled GABA in GABAergic neurons, or in all cells,

Table 1 Cellular content of selected amino acids (nmol/mg protein) in extracts of cultured cerebellar neurons and consumption of glutamate and glutamine (μmol/mg protein/2 h) from the experimental medium

	Cellular content (nmol/mg protein)				Consumption (μmol/mg protein/2 h)	
	Aspartate	*Glutamate*	*Glutamine*	*GABA*	*Glutamate*	*Glutamine*
Ctr	31 ± 1	88 ± 3	3 ± 1	8 ± 1	—	—
Glu	231 ± 12^a	445 ± 16^a	20 ± 2^a	11 ± 1^a	1.5 ± 0.1	—
Gln	$76 \pm 6^{a,b}$	$134 \pm 10^{a,b}$	$57 \pm 6^{a,b}$	10 ± 2^a	—	0.7 ± 0.1
Glu+Gln	$278 \pm 20^{a,b,c}$	$529 \pm 31^{a,b,c}$	$84 \pm 5^{a,b,c}$	11 ± 1^a	1.6 ± 0.1	0.4 ± 0.1^c

Cerebellar neurons were incubated for 2 h in medium containing $3\,mmol/L$ glucose, $25\,\mu mol/L$ DNQX, and $100\,\mu mol/L$ D-AP5 and either no addition (Ctr (control), $n = 3$), $0.25\,mmol/L$ glutamate (Glu, $n = 6$), $0.50\,mmol/L$ glutamine (Gln, $n = 6$), or $0.25\,mmol/L$ glutamate plus $0.50\,mmol/L$ glutamine (Glu+Gln, $n = 12$), for details see Materials and methods. Results are presented as means $\pm$ s.d., and $P < 0.05$ was considered statistically significant.
[a] Different from the Ctr group.
[b] Different from the Glu group.
[c] Different from the Gln group.

Figure 1 Schematic representation of possible isotopomers of various metabolites arising from [U-^{13}C]glutamate or [U-^{13}C]glutamine via the three first turns in the TCA cycle in neurons: ● represents ^{13}C and ○ represents ^{12}C atoms; GLN: glutamine; GLU: glutamate; αKG: α-ketoglutarate; SUC: succinate; FUM: fumarate; MAL: malate; OAA: oxaloacetate; ASP: aspartate; CIT: citrate; GABA: γ-amino butyric acid.

be turned into α-[U-^{13}C]ketoglutarate and be metabolized in the TCA cycle for energy production and metabolite synthesis. The ^{13}C label from the precursor's carbon skeleton will in the latter case be distributed among the TCA cycle metabolites and GABA as illustrated in Figure 1.

The labeling of glutamate, GABA, succinate, malate, aspartate, and citrate as detected by GC/MS is presented in Figure 2 as atom percent excess (% labeling). When [U-^{13}C]glutamate is turned into α-[U-^{13}C]ketoglutarate and enters the TCA cycle, [1,2,3-^{13}C]glutamate ($M+3$) is formed after one turn. This isotopomer results from condensation of uniformly labeled oxaloacetate (OAA) with unlabeled acetyl-CoA forming [3,4,5,6-^{13}C]citrate (Figure 1), which can be turned into [1,2,3,6-^{13}C]isocitrate, α-[1,2,3-^{13}C]ketoglutarate and finally into [1,2,3-^{13}C]glutamate. After another turn in the TCA cycle, half of the glutamate formed will be [1,2-^{13}C]glutamate ($M+2$) and half [3-^{13}C]glutamate ($M+1$). After a third turn, 25% of glutamate will be unlabeled, whereas 75% will be labeled either in the 1, 2, or 3 position ($M+1$) (Figure 1).

From [U-^{13}C]glutamate entering the TCA cycle via α-[U-^{13}C]ketoglutarate, uniformly labeled succinate, malate, and OAA are formed (Figure 1), and OAA can be transaminated into aspartate. All of these compounds have four C atoms, and their [U-^{13}C] isoforms have the mass $M+4$ and are presented in Figure 2. [U-^{13}C]OAA can, as already mentioned, condense with unlabeled acetyl-CoA to form [3,4,5,6-^{13}C]citrate with six carbon atoms, four of

which are ^{13}C ($M+4$). Hence, the mass $M+4$ represents the first turn in the TCA cycle for all these compounds. In the next turn, they will all contain two labeled C atoms and appear as $M+2$, and in the third turn, they will contain one labeled C atom ($M+1$) (Figure 1).

As shown in Figure 2, labeling from [U-^{13}C]glutamate (column A) and [U-^{13}C]glutamine (column B) was substantial, with glutamate giving the highest percent labeling in all metabolites analyzed. When [U-^{13}C]glutamate and unlabeled glutamine were added to the experimental medium (column C), percent label decreased only slightly compared with when [U-^{13}C]glutamate was added alone (column A). However, when [U-^{13}C]glutamine and unlabeled glutamate were added to the medium, labeling was almost abolished for all metabolites (column D). Figure 2 shows that labeling of glutamate and malate, aspartate and citrate was high (>65% from [U-^{13}C]glutamate and 40% to 60% from [U-^{13}C]glutamine (in the absence of glutamate)), whereas that of succinate was much lower (<30% labeling from glutamate and <10% from glutamine). Although the percent labeling in succinate was lower, the pattern was the same as for the other TCA metabolites.

GABA can be formed from glutamate in GABA ergic neurons, which constitute about 6% of cerebellar neuronal cultures (Sonnewald *et al*, 2004). GABA formed directly from [U-^{13}C]glutamate is uniformly labeled ($M+4$). From [1,2,3-^{13}C]glutamate (after one turn in the TCA cycle), [3,4-^{13}C]GABA is formed ($M+2$). Another turn in the TCA cycle for
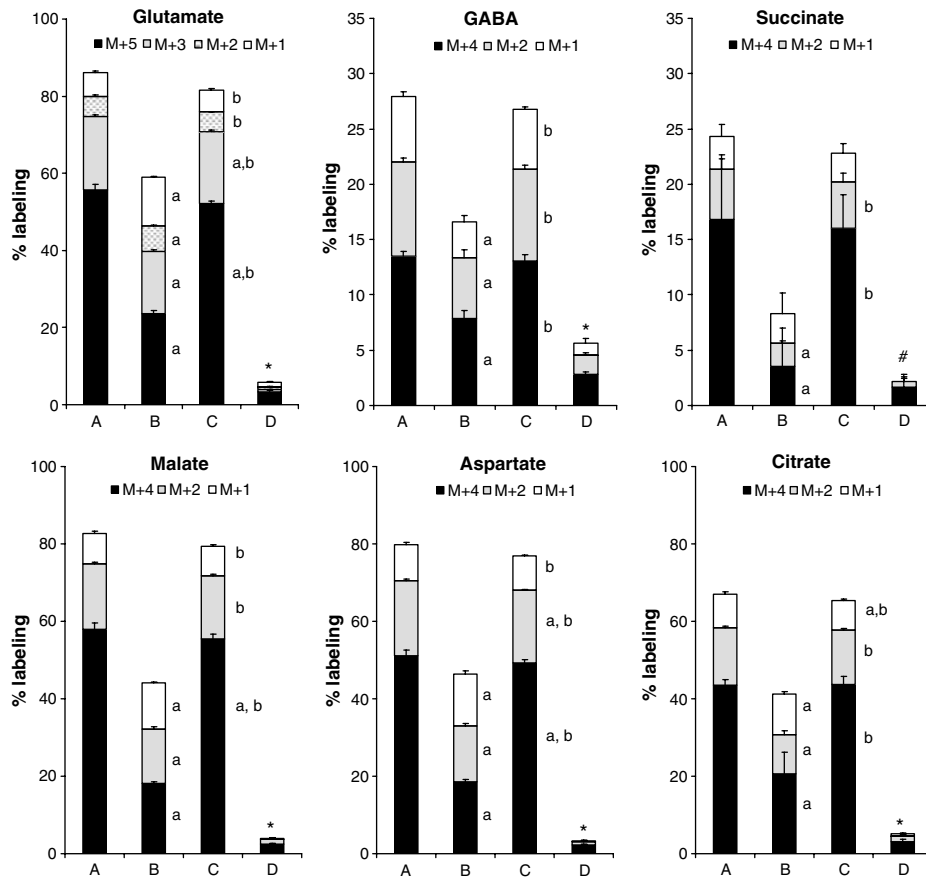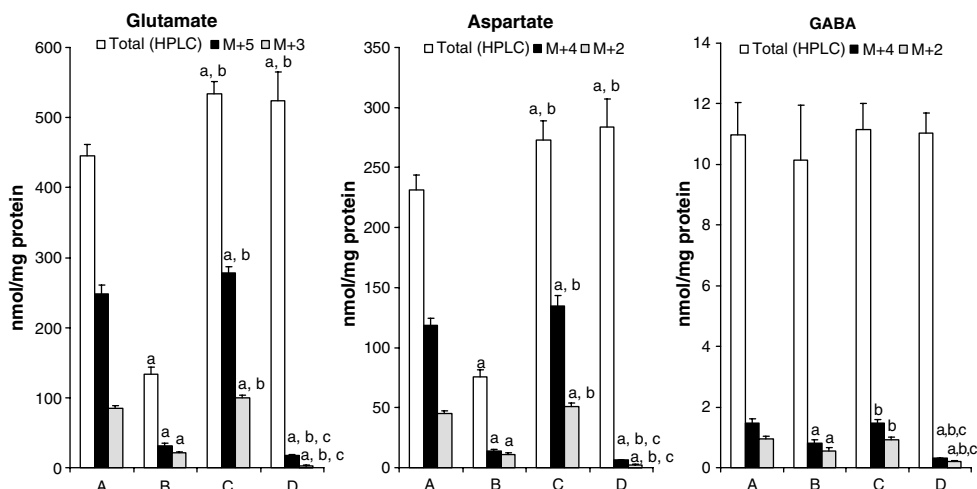
Figure 2 Percent labeling in glutamate, GABA, succinate, malate, aspartate and citrate as detected by GC/MS in cell extracts of cultured cerebellar neurons after incubation with [U-^{13}C]glutamate (column A), [U-^{13}C]glutamine (column B), [U-^{13}C]glutamate plus unlabeled glutamine (column C) or [U-^{13}C]glutamine plus unlabeled glutamate (column D), for details see Materials and methods. Results are presented as means + s.d. in atom percent excess, and $P < 0.05$ was considered statistically significant. *All masses are different from the corresponding masses in the other groups. #$M + 4$ is different from the groups incubated with [U-^{13}C]glutamate with and without glutamine, the other masses are different from all other groups. [a]Different from the corresponding mass in the A group; [b]different from the corresponding mass in the B group; [c]different from the corresponding mass in the C group.

the glutamate carbon skeleton and subsequent formation of GABA will result in labeling in one C atom in GABA ($M+1$), from both [1,2-^{13}C] and [3-^{13}C]glutamate, [4-^{13}C] and [3-^{13}C]GABA, respectively. The same two isotopomers are also formed after three turns in the TCA cycle (Figure 1). Figure 2 shows that the labeling of GABA analyzed by GC/MS was lower than that of glutamate, malate, aspartate, and citrate. Total labeling from [U-^{13}C]glutamate was ~30% regardless of the presence of unlabeled glutamine. Significantly less (17%) GABA was labeled from [U-^{13}C]glutamine alone, and this was further reduced in the presence of unlabeled glutamate (6%).

Intracellular amounts of glutamate, aspartate, and GABA as quantified by HPLC are shown in Figure 3. The amounts of glutamate and aspartate varied considerably with addition of glutamate or glutamine in the experimental medium, but followed the same pattern, whereas GABA concentration was independent of glutamate and glutamine content in medium. Results from mass spectrometry provide information about percent labeling, which combined with information about amounts of metabolites gives quantitative data. Amounts of different ^{13}C labeled isotopomers of glutamate, aspartate, and GABA from the first two turns in the TCA cycle are shown in Figure 3. The intracellular amount of uniformly labeled glutamate was 248 nmol/mg protein after incubation with [U-^{13}C]glutamate. This was reduced to 32 and 17 nmol/mg protein when glutamine was the labeled precursor, in the absence and presence of unlabeled glutamate, respectively. When labeled glutamate was added in the presence of unlabeled glutamine, the amount of intracellular uniformly labeled glutamate was, however, increased to 279 nmol/mg protein. The same trend was seen in [U-^{13}C]aspartate, although the amounts were ~50% of those of [U-^{13}C]glutamate. These two excitatory amino acids also showed similarities in the next turn in the TCA cycle. GABA had a much lower labeling from both labeled precursors than glutamate and aspartate, labeling from [U-^{13}C]glutamine being lower than that from [U-^{13}C]glutamate.

Figure 3 The amounts of glutamate, aspartate, and GABA (white bars), the amounts of $M+5$ ([U-^{13}C]glutamate), $M+4$ ([U-^{13}C]aspartate, [U-^{13}C]GABA) (black bars) and the amounts of $M+3$ ([1,2,3-^{13}C]glutamate), $M+2$ ([1,2-^{13}C] and [3,4-^{13}C]aspartate) and $M+2$ ([3,4-^{13}C]GABA) (grey bars) in cell extracts of cultured cerebellar neurons after incubation with [U-^{13}C]glutamate (column A), [U-^{13}C]glutamine (column B), [U-^{13}C]glutamate plus unlabeled glutamine (column C), or [U-^{13}C]glutamine plus unlabeled glutamate (column D), for details see Materials and methods. Results are presented as means + s.d. in nmol/mg protein, and $P < 0.05$ was considered statistically significant. [a]Different from the corresponding metabolite in the A group; [b]different from the corresponding metabolite in the B group; [c]different from the corresponding metabolite in the C group.

Discussion

Metabolism of glutamate and glutamine in the brain is closely connected via the glutamate–glutamine cycle, in which neurotransmitter glutamate taken up from the synaptic cleft by astrocytes is converted to glutamine and transported back to neurons as a precursor for the neurotransmitter pool (Berl and Clarke, 1983). Similar mechanisms operate in the glutamate–glutamine–GABA cycle (van den Berg and Garfinkel, 1971; Sonnewald et al, 1993b). However, reuptake into the presynaptic neuron is believed to be the preferred mechanism for removal of GABA from the synapse (Schousboe, 2003). To which extent glutamine and thus this cycle is important for cultured cerebellar neurons has been explored in the present study by comparing [U-^{13}C]glutamate and [U-^{13}C]glutamine as substrates for intermediary metabolism. In our model, addition of glutamine to the neuronal cultures mimics astrocyte–neuronal interactions in the brain, with the advantage of isolating neuronal from glial metabolism and thus making interpretation of results unambiguous.

[U-^{13}C]Glutamate and [U-^{13}C]Glutamine Metabolism

Glutamine has been shown to be an excellent precursor for the neurotransmitters glutamate and GABA, both in cultured cerebellar neurons (Waagepetersen et al, 2005), cortical neurons (Westergaard et al, 1995), and in freshly isolated cortical synaptosomes from rat brain (Yudkoff et al,

1989; Sonnewald and McKenna, 2002). Label from [U-^{13}C]glutamine was also found in glutamate and GABA in the present study. Moreover, labeling of TCA cycle intermediates and aspartate was detected, showing that the carbon skeleton of [U-^{13}C]glutamine entered the TCA cycle. The presence of isotopomers from subsequent turns also showed that the carbon skeleton stayed in the TCA cycle. This confirms that the glutamate–glutamine cycle does not operate in a stochiometric fashion (McKenna et al, 1993, 1994; Sonnewald et al, 1993a) and that glutamine is readily oxidized by cerebellar cultures for energy.

Exogenous glutamate has also been used to label neuronal metabolites. Westergaard et al (1995) showed that in cultured cortical neurons, which are predominantly GABAergic, incubation with [U-^{13}C]glutamate gave high enrichment in aspartate in addition to labeling of GABA. Incubating cerebellar neurons in medium containing [U-^{13}C]glutamate in the present study led not only to uniformly labeled intracellular aspartate and glutamate, but to some extent, the carbon skeleton also stayed in the TCA cycle for several turns. This is in accordance with similar studies of cerebellar neurons analyzed with magnetic resonance spectroscopy (Sonnewald et al, 1996; Santos et al, 2006). Using the more sensitive method mass spectrometry, extensive labeling of TCA cycle intermediates and GABA was also detected in the present study. Labeling of GABA in cerebellar cultures has earlier been shown by Qu et al (2000). The present study confirms that [U-^{13}C]glutamate serves as an excellent precursor for intermediary metabolism in cultured cerebellar neurons.

As shown in the previous paragraphs, both [U-13C]glutamine and [U-13C]glutamate are well suited as precursors for neurotransmitter formation and substrates for neuronal intermediary metabolism. However, in the present study [U-13C]glutamate gave a higher percent labeling than [U-13C]glutamine in all metabolites, including GABA. Sonnewald and McKenna (2002) found that [U-13C]glutamate was superior in labeling of aspartate, whereas GABA labeling was only observed from [U-13C]glutamine, and not from [U-13C]glutamate when unlabeled glutamine was present, in cortical synaptosomes. Although [U-13C]glutamate was a better precursor for GABA than [U-13C]glutamine in the present study, glutamine was a relatively better precursor for GABA than for the other metabolites investigated, indicating that GABA synthesis occurs in a separate compartment (see below) consistent with findings in cortical synaptosomes (Sonnewald and McKenna, 2002). Westergaard et al (1995) showed that in primary cultures of cortical neurons labeling of aspartate, GABA, and [1,2,3-13C]glutamate was very similar from [U-13C]glutamine and [U-13C]glutamate. Thus, not surprisingly, GABAergic neurons in the cerebellum appear to have a different substrate preference for GABA synthesis than GABAergic neurons in cerebral cortex. In cultured cortical neurons, TCA cycle metabolism of the carbon skeleton subsequently used in formation of GABA was more pronounced from [U-13C]glutamine than from [U-13C]glutamate (Westergaard et al, 1995). This was also observed in the present study in cultured cerebellar neurons. With [U-13C]glutamate as the precursor, approximately 60% of the labeled succinate, malate, aspartate, and citrate was uniformly labeled and thus derived from the first turn, $\sim 20\%$ was from the second and $\sim 10\%$ from the third turn in the TCA cycle. When [U-13C]glutamine was present in the experimental medium, these numbers were $\sim 40\%$, $\sim 30\%$, and $\sim 25\%$, respectively, and similar results were seen in GABA. Thus, cycling from glutamine was more prominent than that from glutamate. The fact that mitochondrial metabolism of the carbon skeleton of endogenous glutamate derived from glutamine and glutamate taken up into the cells differed could be due to differences in the distribution of the enzyme phosphate activated glutaminase catalyzing the formation of glutamate from glutamine. This compartmentation could perhaps be related to intracellular mitochondrial heterogeneity (Westergaard et al, 1995; Sonnewald et al, 1998). Interestingly, label distribution indicating TCA cycling of the carbon skeleton subsequently converted into GABA was similar whether [U-13C]glutamate or [U-13C]glutamine was the precursor present in the experimental medium, showing intercellular compartmentation in addition to the intracellular compartmentation mentioned above.

To evaluate the preference for glutamate and glutamine as substrates for intermediary metabolism in cultured brain cells or synaptosomes, both substrates have to be present in the medium simultaneously. When Sonnewald and McKenna (2002) incubated synaptosomes with [U-13C]glutamate in the presence of unlabeled glutamine, the carbon skeleton from [U-13C]glutamate entered the TCA cycle and labeled aspartate and [1,2,3-13C]glutamate, but not GABA. However, label from [U-13C]glutamine in the presence of unlabeled glutamate was incorporated into GABA, but not aspartate. This is consistent with what has been reported in cortex for the two precursors applied separately (Westergaard et al, 1995), showing that the compartmentation is maintained under more physiological conditions with both substrates present (Sonnewald and McKenna, 2002). The importance of glutamine as a precursor and thus the importance of the glutamate–glutamine–GABA cycle for GABA synthesis is surprising since reuptake of GABA into the presynapse is believed to be prominent (Schousboe, 2003). Neurotransmitter glutamate, however, is thought to be removed from the synaptic cleft mainly by uptake into astrocytes (Schousboe et al, 1977; Danbolt, 2001). On the basis of this, it can be assumed that the importance of the glutamate–glutamine cycle should be greater for the synthesis of neurotransmitter glutamate than for GABA. However, results from the present study suggest otherwise. Consumption of glutamate in the cerebellar neurons was twice that of glutamine when given alone and together with glutamine, even though the glutamine concentration in the medium was twice as high as that of glutamate. Most importantly, glutamine consumption was reduced by nearly 50% in the presence of glutamate compared with when glutamate was not present in the experimental medium. Thus, glutamate could substitute for glutamine, but the reverse was not the case. Labeling of intracellular metabolites was only slightly reduced when neurons were incubated in medium containing [U-13C]glutamate in the presence of unlabeled glutamine compared with that in the absence of glutamine. Surprisingly, when unlabeled glutamate was present together with [U-13C]glutamine, labeling was almost abolished in all metabolites measured. The carbon skeleton of [U-13C]glutamate and [U-13C]glutamine will, in the TCA cycle, not only be distributed into the metabolites mentioned above, but also be converted to $^{13}CO_2$, which is not detected by the experimental setup used in the present study. As mentioned above, the carbon skeleton of glutamine stayed in the TCA cycle longer than that of glutamate and thus it is likely that a higher percentage of glutamine compared with that of glutamate was converted to $^{13}CO_2$.

Considering the results presented, it can be postulated that the glutamate–glutamine cycle is of less importance for neurons in the cerebellum than what has been described for cerebral cortical neurons. This is further supported by the fact that

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granule neurons are by far the most abundant cells in the rat cerebellum (265×10^6) and outnumber glia (2.2×10^6) in the granule layer significantly (Korbo et al, 1993). A similar quantitative neuronal dominance has been reported in human cerebellum (Andersen et al, 1992). Thus, in the cerebellar cortex, glial processes do not envelop all synapses. Indeed, it has been shown that around synapses between the parallel fibers, the axons of granule neurons, and interneuron dendrites, astrocyte processes are lacking (Danbolt, 2001). Therefore, the distance from the synapse to the nearest glial cell is quite large and, in addition, the relative number of glutamate transporters on these astrocytes is low (Danbolt, 2001). However, it has been shown that in the cerebellar granule cell layer, the density of mRNA for a neuronal glutamate transporter was very high (Velaz-Faircloth et al, 1996). Considering the relatively large distance between synaptic regions of cerebellar granule cells and astrocytes and the high level of glutamate transporters on these neurons, it can be argued that reuptake of glutamate must be important for granule neurons. This has indeed been shown by Waagepetersen et al (2005), who reported that the intracellular pool of glutamate in cerebellar neurons was dependent on reuptake of extracellular glutamate.

The 'Partial Tricarboxylic Acid Cycle'

Battaglioli and Martin (1990) showed that in synaptosomes, aspartate synthesis was strongly stimulated by glutamate and glutamine, but the stimulation by glutamate was greatest. Similarly, in the present study, an increase in intracellular glutamate concentration was always accompanied by a corresponding increase in that of aspartate. The intracellular glutamate concentration was highest in the cells incubated with both glutamate and glutamine, followed by those incubated with glutamate alone, and thereafter cells incubated with glutamine, which also led to an increase compared with control. The coupling between glutamate and aspartate can be explained by the fact that cerebellar neurons have a high activity of glutamate dehydrogenase and aspartate amino transferase (Drejer et al, 1985; Westergaard et al, 1991; Zaganas et al, 2001). Thus, entry of glutamate via α-ketoglutarate into the TCA cycle and conversion of oxaloacetate to aspartate is very efficient in the cerebellum and energy is obtained from this so-called 'partial TCA cycle' (Hertz et al, 1991; Sonnewald and McKenna, 2002).

GABA Shunt in Cerebellar Neurons

The cerebellar neuronal cultures consist primarily of glutamatergic granule cells with a minor contribution of GABAergic stellate and basket neurons, that is, GABAergic as well as glutamatergic

characteristics are expressed in these cultures (Pearce et al, 1981; Hertz et al, 1985; Hertz and Schousboe, 1987; Drejer and Schousboe, 1989; Kovacs et al, 2003). Thus, cultures of dissociated cerebellum constitute an excellent model system for the in vivo situation, in which the association of GABAergic with glutamatergic neurons can be investigated. The major enzyme responsible for GABA synthesis in brain, glutamate decarboxylase, was shown to be present in 6% of cultured cerebellar neurons (Sonnewald et al, 2004) indicating the presence of two distinct cell types and thus, at least two cellular compartments. In the present study, the amount of intracellular GABA in the cultures was increased to the same extent by all experimental conditions, compared with control cultures. This is in contrast to the results obtained for aspartate, which increased with increasing glutamate concentration, as mentioned above, showing cellular compartmentation. It has been shown that the glutamate decarboxylase positive neurons, which constitute the GABAergic compartment, produce GABA during the whole culture period and that GABA is distributed throughout the whole culture (Sonnewald et al, 2006). In the present study, no labeled substrates were present in the medium during the 7-day culture period, thus GABA produced during this time was unlabeled. When medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine was added to the cultures for the 2-h incubation period, labeled GABA could be formed only in the small number of GABAergic cells. Hence, most GABA in the cultures in general, and in the cerebellar granule neurons in particular, was unlabeled at the time of culture extraction.

GABA can be catabolized via the GABA shunt, in which the carbon skeleton of GABA enters the TCA cycle after conversion to succinate. Interestingly, percent labeling of succinate was very similar to that of GABA and much lower than that of the other TCA cycle intermediates measured. The number of GABAergic neurons in the cultures is small, and thus it seems unlikely that the succinate content in these neurons could account for the dilution of labeling in the total succinate pool. It can be assumed that catabolism of GABA and entry of the carbon skeleton of GABA into the TCA cycle through the GABA shunt is taking place in the glutamatergic cells from unlabeled GABA. Hence, a possible function of GABA in the glutamatergic neurons is the ability to produce a separate succinate pool for potential energy production in the TCA cycle, suggesting a special role for the GABA shunt in the cerebellum.

Conclusion

Through the results presented, it could be shown that glutamate is preferred over glutamine as a substrate for intermediary metabolism in cultured

cerebellar neurons. It can be concluded that these neurons rely more on reuptake of glutamate than supply of glutamine from astrocytes for glutamate homeostasis.

Acknowledgements

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Long-term kainic acid exposure reveals compartmentation of glutamate and glutamine metabolism in cultured cerebellar neurons

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Long-term kainic acid exposure reveals compartmentation of glutamate and glutamine metabolism in cultured cerebellar neurons

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Abstract

Glutamate neurotoxicity is implicated in most neurodegenerative diseases, and in the present study the long-term effects of the glutamate agonist kainic acid (KA) on cerebellar neurons are investigated. Primary cell cultures, mainly consisting of glutamatergic granule neurons, were cultured in medium containing 0.05 or 0.50 mM KA for 7 days and subsequently incubated in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine. The amount of protein and number of cells were greatly reduced in cultures exposed to 0.50 mM KA compared to those exposed to 0.05 mM KA. Glutamine consumption was not affected by KA concentration, whereas that of glutamate was decreased by high KA, confirming reduction in glutamate transport reported earlier. Neurons cultured with 0.50 mM KA and incubated with glutamate contained decreased amounts of glutamate, aspartate and GABA compared to those cultured with 0.05 mM KA. Incubation of cells exposed to 0.50 mM KA with glutamine led to an increased amount of glutamate compared to cells exposed to 0.05 mM KA, whereas the intracellular amounts of aspartate and GABA remained unaffected by KA concentration. Furthermore, mitochondrial metabolism of α-[U-^{13}C]ketoglutarate derived from [U-^{13}C]glutamate and [U-^{13}C]glutamine was significantly reduced by 0.50 mM KA. The results presented illustrate differential vulnerability to KA and can only be understood in terms of inter- and intracellular compartmentation.

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Keywords: ^{13}C labeling; GABA; Glutamatergic neurons; Kainic acid; Mass spectrometry; Tricarboxylic acid cycle

1. Introduction

Neurodegenerative diseases, characterized principally by the progressive loss of neurons, can affect various brain regions and include a number of different diagnoses. Typically, the pattern of neuronal loss is selective, affecting one or more groups of neurons, while leaving others intact. Ataxia-telangiectasia is an example of a degenerative disorder, with predominantly cerebellar neuronal degeneration with selective loss of GABAergic Purkinje and glutamatergic granule cells (Crawford, 1998; De Girolami et al., 1999). Selective destruction of one or more groups of neurons is also seen after exposure to different toxic substances. In the cerebellum,

granule neurons are very sensitive to methyl mercury, thiophene and 2-chloropropionic acid, whereas Purkinje cells are sensitive to ethanol and diphenylhydantoin (Fonnum and Lock, 2000; Sonnewald et al., 2002). Glutamate homeostasis and metabolism is of supreme importance in the brain. However, the balance between physiological and pathological signaling is delicate, and thus glutamate neurotoxicity is implicated in most neurodegenerative diseases. It has been shown that the glutamate agonist kainic acid (KA) exerts toxic effects after systemic or intracranial injection in rodents. Systemic KA injections lead to seizures and subsequent neuronal cell loss, and has therefore been widely used as an epilepsy model (Ben-Ari, 1985; Bradford, 1995; Schwarzer and Sperk, 1995; Müller et al., 2000; Qu et al., 2003). Injection of KA directly into cerebella of hamsters and rats has been shown to selectively damage neurons which receive synaptic input from granule cells, while the granule cells themselves were spared (Herndon and Coyle, 1977); leading to the hypothesis that KA exerts its toxic effects through glutamate receptors. In

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contrast, Lovell and Jones (1980), found destruction of a substantial number of granule cells in mouse cerebellar cortex with sparing of many Purkinje cells after injection of KA in mouse cerebellar cortex. In cell culture experiments, KA has been shown to cause both positive and negative effects on survival of neurons from this area (Balázs et al., 1990; Frandsen and Schousboe, 1990; Kato et al., 1991; Jensen et al., 1999; Drian et al., 2001). Furthermore, it has been reported that KA exposure led to disturbed maturation of GABA$_A$ receptor subunit expression in these neurons (Engblom et al., 2003).

Although several authors have investigated neurotoxic effects of short time exposure to KA, little is known about long-term effects on neuronal metabolism. Thus, the purpose of the present study was to investigate the effects of long-term exposure of cerebellar neurons to KA with respect to glutamate and glutamine metabolism. Cerebellar neurons were cultured in medium containing 0.05 or 0.50 mM KA for the whole 7-day culture period, and subsequently incubated for 2 h in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine. High performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analyses were performed on media and cell extracts, and cellular protein was quantified. The cerebellar neuronal culture model system is well suited for this kind of metabolic study because after 7 days in vitro, the cultures express glutamate and glutamine metabolizing enzymes analogous to that of the brain in vivo (Drejer et al., 1985). Moreover, the cultures show similarities to the in vivo situation due to expression of both GABAergic as well as glutamatergic characteristics (Pearce et al., 1981; Hertz et al., 1985; Hertz and Schousboe, 1987; Drejer and Schousboe, 1989; Kovacs et al., 2003). The glutamatergic granule cells dominate quantitatively in the cultures, just as in the cerebellum in vivo (Drejer and Schousboe, 1989; Andersen et al., 1992). In addition, the cultures contain about 6% GABAergic stellate and basket neurons, and a small number of glial cells (Messer, 1977; Drejer et al., 1985; Damgaard et al., 1996; Sonnewald et al., 2004). Thus, cellular compartmentation of metabolism can readily be studied in these cultures. Furthermore, the blood–brain-barrier, which normally excludes glutamate and glutamine from entering the brain, is bypassed in this system.

2. Experimental procedures

2.1. Materials

NMRI mice were obtained from Taconic M&B (Copenhagen, Denmark). Plastic tissue culture dishes were purchased from Nunc A/S (Roskilde, Denmark) and fetal calf serum (FCS) from Seralab Ltd. (Sussex, UK). Culture medium, kainic acid, glutamate receptor antagonists DNQX (6,7-dinitroquinoxaline-2,3-dione) and D-AP5 (D-2-amino-5-phosphonopentanoic acid) were from Sigma Chemical Co. (St. Louis, MO, USA). [U-^{13}C]Glutamate and [U-^{13}C]glutamine were from Cambridge Isotope Laboratories (Woburn, MA, USA), and the GC/MS derivatization reagent MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide) + 1% t-BDMS-Cl (tert-butyldimethylchlorosilane) was purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

2.2. Cell cultures

Cerebellar neurons were isolated and cultured from 7-day-old mice as described by Schousboe et al. (1989). Briefly, the tissue was trypsinized followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. Cells were suspended (2.75×10^6 cells/ml) in a modified Dulbecco's minimum essential medium (Hertz et al., 1982) containing 24.5 mM KCl, 31 mM glucose, 7 μM p-aminobenzoic acid, 10% (v/v) FCS and either 0.05 or 0.50 mM kainic acid, and seeded in poly-D-lysine coated Petri dishes (2 ml/35 mm). After 48 h in culture, 20 μM (final concentration) cytosine arabinoside was added to the medium to prevent astrocytic proliferation.

2.3. Microscopy

Cell morphology was assessed using a Nikon Eclipse TE 2000-S inverted phase-contrast microscope (Nikon, Inter Instruments AS, Norway). The microscope was equipped with a SPOT RT Digital Camera (SPOT Diagnostic Instruments, Sterling Heights, MI, USA), and photomicrographs of cells were taken after 7 days in vitro.

2.4. Experiments using [U-^{13}C]glutamate and [U-^{13}C]glutamine

The culture medium containing 0.05 or 0.5 mM KA was removed from 7-day-old cultures and the cells were incubated for 2 h at 37 °C in 2 ml serum free medium prepared without glutamine, containing 3 mM glucose and either no additions (control, for HPLC analysis), [U-^{13}C]glutamate (0.25 mM) or [U-^{13}C]glutamine (0.50 mM). KA was not present in this incubation medium. In order to avoid the toxicity of glutamate during the incubation, two glutamate receptor antagonists DNQX (25 μM), an AMPA/kainate-selective antagonist, and D-AP5 (100 μM), an NMDA antagonist, were also present in the incubation medium (Frandsen et al., 1989). After the incubation period, medium was removed for HPLC analysis and the cells were washed twice with cold phosphate buffered saline and extracted with 70% (v/v) ethanol. The cell extracts were scraped off the dishes and centrifuged at $10,000 \times g$ for 15 min to separate the metabolites from the insoluble proteins. The supernatants were divided into two parts, one directly analyzed with HPLC; the other lyophilized for subsequent sample preparation for GC/MS analysis.

2.5. Protein quantification

Cellular protein in the ethanol pellets was determined after dissolving in 1 M KOH at 37 °C for 60 min, using the Pierce BCA (Pierce, Rockford, IL, USA) protein assay with bovine serum albumin as standard.

2.6. HPLC

Amino acids in cell extracts and medium were quantified by HPLC on a Hewlett Packard 1100 system (Agilent Technologies, Palo Alto, CA, USA). The amino acids were pre-column derivatized with o-phthaldialdehyde (Geddes and Wood, 1984) and subsequently separated on a ZORBAX SB-C18 (4.6 mm × 250 mm, 5 μm) column from Agilent using a phosphate buffer (50 mM, pH 5.9) and a solution of methanol (98.75%) and tetrahydrofurane (1.25%) as eluents. The separated amino acids were detected with fluorescence and quantified by comparison to a standard curve derived from standard solutions of amino acids run after every twelve samples.

2.7. GC/MS

Lyophilized cell extracts were redissolved in HCl (10 mM), adjusted to pH < 2 with 6 M HCl and dried under atmospheric air. The amino acids were in multiple steps extracted into an organic phase of ethanol and benzene and dried again under atmospheric air before derivatization with MTBSTFA in the presence of 1% t-BDMS-Cl (Mawhinney et al., 1986). The samples were analyzed on a Hewlett Packard 5890 Series II gas chromatograph linked to a Hewlett Packard 5972 Series mass spectrometer. Atom percent excess (^{13}C) of glutamate, glutamine, GABA and aspartate was determined after calibration using unlabeled standard solutions (Biemann, 1962).

2.8. Metabolic fate of [U-^{13}C]glutamate and [U-^{13}C]glutamine

Incubating cerebellar neurons in medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine and subsequent analysis of cell extracts by mass spectrometry can provide useful information about the metabolism in these cells. In order to interpret the mass spectrometry data, it is necessary to know the metabolic fate of the two labeled precursors. [U-^{13}C]Glutamate or [U-^{13}C]glutamine from the incubation medium enters the neurons through transporter proteins. Once inside the cells, [U-^{13}C]glutamine can be converted to [U-^{13}C]glutamate. The latter can be decarboxylated into uniformly labeled GABA in GABAergic neurons, or it can be converted into α-[U-^{13}C]ketoglutarate and be metabolized in the tricarboxylic acid (TCA) cycle for energy production and metabolite synthesis. The ^{13}C label from the precursor's carbon skeleton will in the latter case be distributed among the TCA cycle metabolites as illustrated in Fig. 1. From the first turn in the TCA cycle, all metabolites will be uniformly labeled to the oxaloacetate (OAA) step. [U-^{13}C]OAA can be transaminated to [U-^{13}C]aspartate and leave the TCA cycle, or continue cycling by condensing with unlabeled acetyl-CoA to form [3,4,5,6-^{13}C]citrate. In the TCA cycle, [3,4,5,6-^{13}C]citrate is converted into [1,2,3,6-^{13}C]isocitrate and α-[1,2,3-^{13}C]ketoglutarate as one turn in the TCA cycle is completed. From α-[1,2,3-^{13}C]ketoglutarate leaving the TCA cycle, [1,2,3-^{13}C]glutamate and [3,4-^{13}C]GABA can be derived. If α-[1,2,3-^{13}C]ketoglutarate stays in the cycle (Fig. 1), [1,2-^{13}C]succinyl-CoA is formed. From [1,2-^{13}C]succinyl-CoA, two possible isotopomers of succinate, [1,2-^{13}C]- or [3,4-^{13}C]succinate arises due to the symmetrical nature of this molecule. Fumarate, malate, OAA and aspartate formed from [1,2-^{13}C]- or [3,4-^{13}C]succinate will all be present in both isoforms. After introduction of another unlabeled acetyl-CoA into the TCA cycle and subsequent exit of

labeled α-ketoglutarate after two completed turns in the TCA cycle, the isotopomers [1,2-^{13}C]- and [3-^{13}C]glutamate, and [3-^{13}C]- and [4-^{13}C]GABA can be derived. Further oxidative metabolism of labeled α-ketoglutarate in the cycle results in isotopomers labeled in either one of the four C positions for succinate, fumarate, malate, OAA and aspartate (Fig. 1). The third turn in the TCA cycle is concluded by the formation of α-ketoglutarate singly labeled in C-1, C-2 or C-3 position with the possibility to form [1-^{13}C]-, [2-^{13}C]- and [3-^{13}C]glutamate or [3-^{13}C]- and [4-^{13}C]GABA.

2.9. Data analysis

Peaks from mass spectra were integrated, and atom percent excess values were calculated for glutamate, aspartate and GABA by comparison with unlabeled standard solutions. Atom percent excess values for masses larger than M (i.e. $M + 1$, $M + 2$, etc.) were added to give percent ^{13}C enrichment of the different compounds (Table 1). Results from HPLC quantification of the above mentioned amino acids in cell extracts were combined with values of atom percent excess obtained from MS to give nmol/mg protein of the uniformly labeled compounds and of the isotopomers from the first, second and third turn of the TCA cycle (Table 2). Setting the percent enrichment presented in Table 1 to 100% made it possible to compare the groups incubated with [U-^{13}C]glutamate or [U-^{13}C]glutamine directly with respect to which extent of glutamate and GABA was derived without TCA cycle activity and from precursors from consecutive turns of the TCA cycle, the latter also including aspartate. Consumption of [U-^{13}C]glutamate and [U-^{13}C]glutamine was calculated by subtracting the amounts of the two amino acids measured in the incubation media after 2 h from the amounts added. Results are presented as mean ± standard
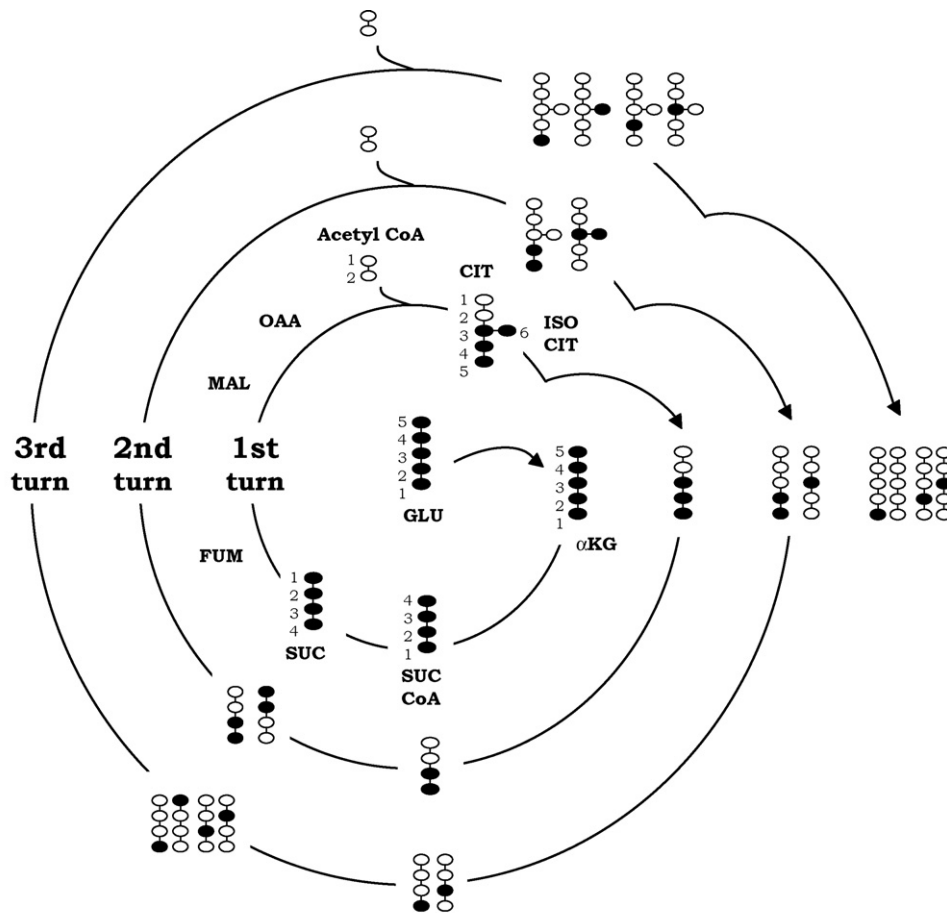

Fig. 1. Schematic representation of possible isotopomers of metabolites arising from [U-^{13}C]glutamate or [U-^{13}C]glutamine (after conversion to [U-^{13}C]glutamate) via three turns in the TCA cycle: (●) ^{13}C and (○) ^{12}C atoms. The labeling of fumarate, malate and OAA is the same as that of succinate, whereas isocitrate labeling is the same as that of citrate, although the numbering of the C atoms differs. GLU: glutamate; αKG: α-ketoglutarate; SUC-CoA: succinyl-CoA; SUC: succinate; FUM: fumarate; MAL: malate; OAA: oxaloacetate; CIT: citrate; ISOCIT: isocitrate.

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Table 1
Percent ^{13}C enrichment in glutamate, aspartate and GABA in cell extracts of cerebellar neurons cultured in medium containing 0.05 or 0.50 mM kainic acid (KA)

	Glutamate (% ^{13}C)	Aspartate (% ^{13}C)	GABA (% ^{13}C)
glu			
0.05 mM KA	90.6 ± 0.5	85.4 ± 0.4	29.2 ± 0.8
0.50 mM KA	92.1 ± 0.4[a]	86.3 ± 0.7[a]	28.7 ± 3.1
gln			
0.05 mM KA	58.8 ± 1.2	48.5 ± 1.0	17.2 ± 1.2
0.50 mM KA	64.7 ± 1.4[a]	52.9 ± 1.8[a]	20.7 ± 1.7[a]

On day 7 in vitro, the culture medium was removed and the cells were incubated for 2 h in medium containing 0.25 mM [U-^{13}C]glutamate (glu, $n = 6$) or 0.50 mM [U-^{13}C]glutamine (gln, $n = 6$), for details see Section 2. Results are presented as mean % ± S.D., and $p < 0.05$ was considered significant after analysis using Student's t-test.

[a] Different from the corresponding cells cultured in medium containing 0.05 mM KA.

deviation (S.D.). Differences between more than two groups were analyzed statistically using one-way analysis of variance (ANOVA) followed by the LSD (least significant difference) post hoc test, and between two groups using Student's t-test, $p < 0.05$ was considered significant.

3. Results

3.1. Neuronal survival and cell morphology

Cerebellar neurons were cultured with medium containing 0.05 or 0.50 mM KA for 7 days before incubation in fresh medium containing either no additions (control group), [U-^{13}C]glutamate or [U-^{13}C]glutamine (see Section 2). The amount of protein in the cultures was not affected by different incubation conditions (results not shown); however, protein amount in cultures exposed to medium with 0.50 mM KA for the whole 7-day culture period was significantly decreased compared to cultures exposed to medium with 0.05 mM KA, as shown in Fig. 2A. The number of cells per culture dish was visibly reduced, whereas the morphology of single cells as assessed by microscopy was unaffected by KA concentration (Fig. 2B).

3.2. Intracellular concentrations and consumption of amino acids

The cellular content of selected amino acids in cultured cerebellar neurons is presented in Fig. 3. Compared to control, intracellular concentrations of glutamate and aspartate were increased after incubation in medium with [U-^{13}C]glutamate and to a lesser extent after incubation in medium with [U-^{13}C]glutamine in cultures exposed to either 0.05 or 0.50 mM KA compared to cells incubated in plain medium (control group). Cells exposed to the high KA concentration, had a lower glutamate and aspartate content after incubation in control medium and medium containing [U-^{13}C]glutamate compared to cultures exposed to low KA. On the other hand, after incubation with medium containing [U-^{13}C]glutamine, high KA led to an increase in glutamate content compared to the low KA group, whereas the intracellular aspartate concentration after incubation with medium containing [U-^{13}C]glutamine remained unaffected by KA concentration. The intracellular concentration of aspartate followed that of glutamate, and was approximately 50% of that of glutamate under all conditions. Incubating neurons with medium containing [U-^{13}C]glutamate or [U-^{13}C]glutamine increased cellular content of glutamine in both KA groups compared to control. Incubation in medium with [U-^{13}C]glutamine gave a higher intracellular glutamine level than incubation in the presence of [U-^{13}C]glutamate. Cultures incubated in control medium had the same glutamine content whether cultured with 0.05 or 0.50 mM KA, whereas culturing cells in medium with the high KA concentration led to decreased glutamine content after incubation in the presence of [U-^{13}C]glutamate and increased glutamine content after incubation in medium with [U-^{13}C]glutamine compared to low KA. The amount of GABA in cells cultured in the presence of 0.05 mM KA and incubated with [U-^{13}C]glutamate or [U-^{13}C]glutamine was increased to the same extent compared to control. Culturing cells in the presence of 0.50 mM KA led to increased intracellular concentration of GABA after incubation in plain medium (control group), whereas incubation in medium with [U-^{13}C]glutamate led to reduced intracellular GABA content

Table 2
Cellular content of labeled glutamate, aspartate and GABA (nmol/mg protein) derived from different turns in the TCA cycle in cerebellar neurons cultured in medium containing 0.05 or 0.50 mM kainic acid (KA)

	Glutamate (nmol/mg protein)				Aspartate (nmol/mg protein)				GABA (nmol/mg protein)			
	Total label	Uniformly	First turn	Second turn	Total label	First turn	Second turn	Third turn	Total label	Uniformly	First turn	Second/third turn
glu												
0.05 mM KA	404 ± 16	248 ± 13	85 ± 4	47 ± 3	198 ± 10	118 ± 6	45 ± 3	22 ± 2	3.2 ± 0.3	1.5 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
0.50 mM KA	300 ± 34[a]	211 ± 22[a]	50 ± 7[a]	23 ± 3[a]	107 ± 16[a]	71 ± 11[a]	20 ± 3[a]	8 ± 1[a]	2.3 ± 0.2[a]	1.2 ± 0.1[a]	0.6 ± 0.1[a]	0.3 ± 0.1[a]
gln												
0.05 mM KA	79 ± 6	32 ± 3	22 ± 1	18 ± 1	37 ± 3	14 ± 1	11 ± 1	10 ± 1	1.7 ± 0.3	0.8 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
0.50 mM KA	103 ± 9[a]	45 ± 5[a]	28 ± 2[a]	20 ± 2[a]	42 ± 4[a]	17 ± 2[a]	12 ± 1	11 ± 1	1.7 ± 0.3	0.8 ± 0.1	0.5 ± 0.1	0.3 ± 0.1

The amounts of differently labeled isotopomers of glutamate, aspartate and GABA in cell extracts of cerebellar neurons incubated in medium with 0.25 mM [U-^{13}C]glutamate (glu, $n = 6$) or 0.50 mM [U-^{13}C]glutamine (gln, $n = 6$) for 2 h, for details see Section 2. Results are presented as mean ± S.D. in nmol/mg protein, and $p < 0.05$ was considered statistically significant.

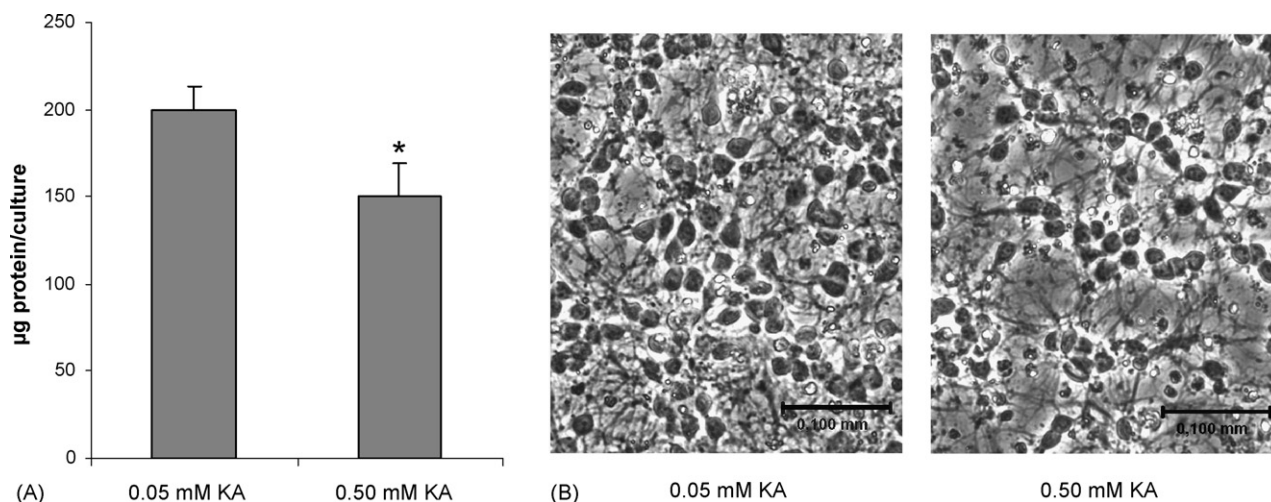[a] Different from the corresponding cells cultured in medium containing 0.05 mM KA.

Fig. 2. The amount of protein in cultures (A) and photomicrographs (B) of cerebellar neurons cultured in medium containing 0.05 or 0.50 mM kainic acid (KA) in 35 mm Petri dishes. On day 7 in vitro, the culture medium was removed, and the cells were incubated for 2 h in medium containing either no additions, 0.25 mM [U-^{13}C]glutamate or 0.50 mM [U-^{13}C]glutamine, for details see Section 2. The protein amount was not affected by different incubation conditions, thus cultures from different incubation conditions are presented together. Results are mean ± S.D. in μg protein per culture ($n = 12$ in each group), and $p < 0.05$ was considered statistically significant. Bars in photomicrographs represent 0.100 mm. *Different from the group cultured in medium containing 0.05 mM KA.
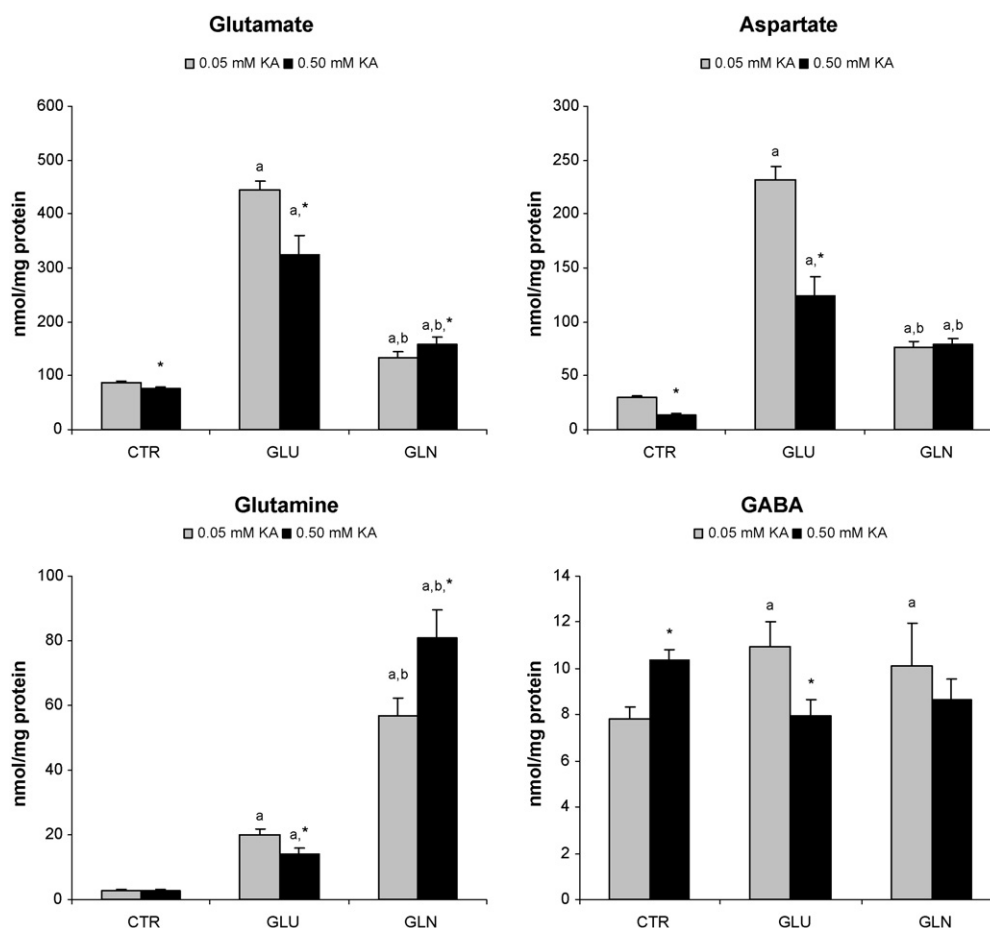
Fig. 3. Cellular content of glutamate, aspartate, glutamine and GABA detected by HPLC in extracts of cultured cerebellar neurons. Cells were cultured in medium containing either 0.05 or 0.50 mM kainic acid (KA). On day 7 in vitro, the culture medium was removed, and the cells were incubated for 2 h in medium with either no additions (ctr, $n = 3$), 0.25 mM [U-^{13}C]glutamate (glu, $n = 6$), 0.50 mM [U-^{13}C]glutamine (gln, $n = 6$), for details see Section 2. Results are presented as mean ± S.D. in nmol/mg protein, and $p < 0.05$ was considered statistically significant. [a]Different from the ctr group; [b]Different from the glu group; *Different from the corresponding group cultured in medium containing 0.05 mM KA.
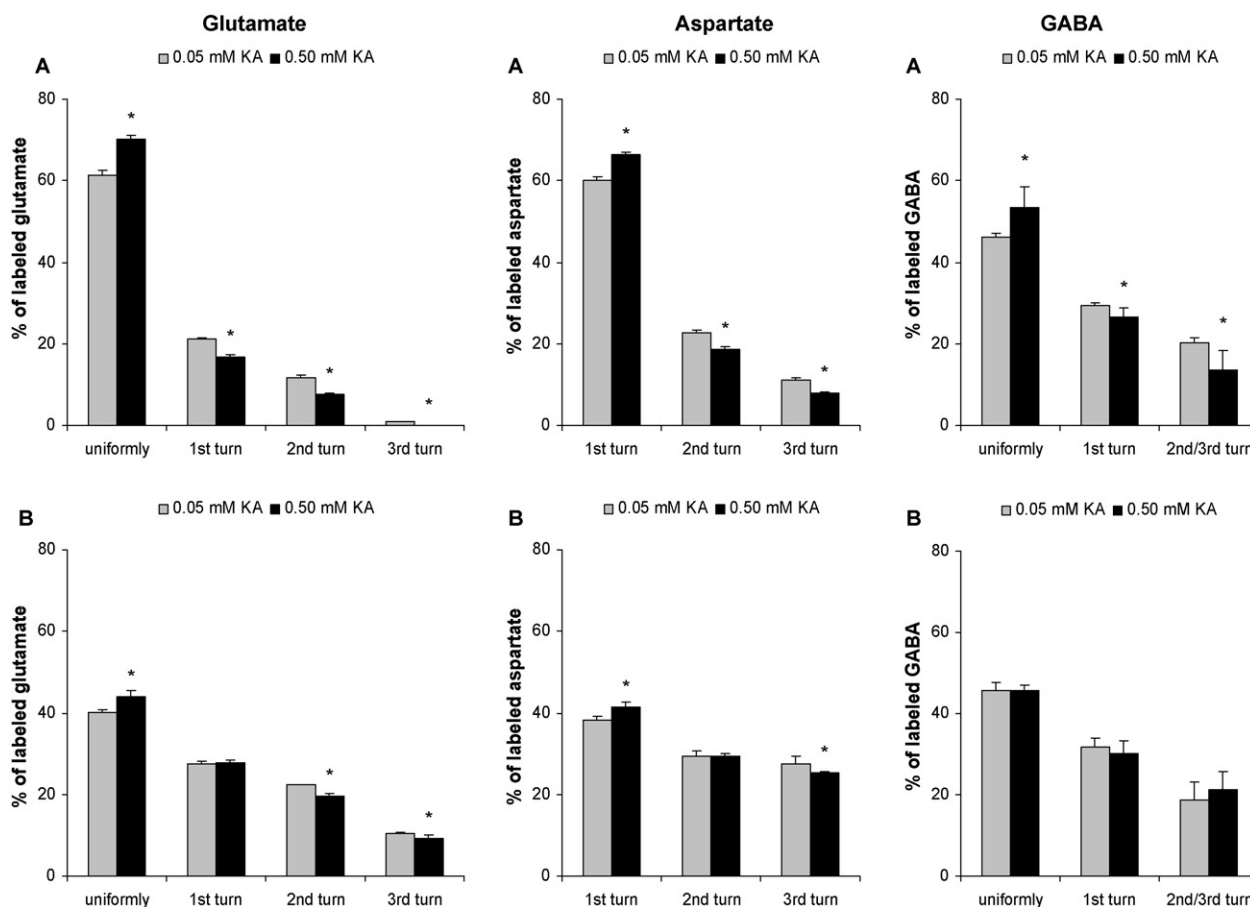
Fig. 4. Distribution of ^{13}C label in glutamate, aspartate and GABA detected by mass spectrometry analysis of cell extracts of cultured cerebellar neurons after incubation with 0.25 mM [U-^{13}C]glutamate (A, $n = 6$) or 0.50 mM [U-^{13}C]glutamine (B, $n = 6$). Cells were cultured in medium containing either 0.05 mM or 0.50 mM kainic acid (KA), for details see Section 2. Note that uniformly labeled glutamate and GABA is presented, and that GABA from the second and third turn is presented together due to the fact that the same isotopomers arise from both turns. Results are presented as mean ± S.D. in percent of the total labeled compound, and $p < 0.05$ was considered statistically significant. *Different from the corresponding group cultured in medium containing 0.05 mM KA.

compared to cells cultured in the presence of 0.05 mM KA. Incubation in medium with [U-^{13}C]glutamine led to similar intracellular GABA concentrations regardless of KA exposure during the culture period.

The cells' consumption of [U-^{13}C]glutamate or [U-^{13}C]glutamine was determined by quantifying the amounts of the two precursors in the incubation media and subtracting these from the amounts added. Cultures exposed to 0.50 mM KA and incubated in medium containing [U-^{13}C]glutamate, consumed approximately half of the amount of glutamate compared to cultures exposed to 0.05 mM KA, 734 ± 184 nmol/mg protein versus 1537 ± 55 nmol/mg protein during the 2 h incubation, respectively. In contrast, glutamine consumption by neurons incubated in medium containing [U-^{13}C]glutamine was not affected by KA concentration. The consumption of glutamine was 719 ± 59 nmol/mg protein in the low KA group and 627 ± 162 nmol/mg protein per 2 h in the high KA group.

3.3. ^{13}C labeling of metabolites

In Table 1, percent ^{13}C enrichment in glutamate, aspartate and GABA in cell extracts after incubation in medium with [U-^{13}C]glutamate or [U-^{13}C]glutamine is presented. In general, in glutamate and aspartate, percent enrichment after incubation in the presence of [U-^{13}C]glutamate was very high (>85%), and higher than after incubation in medium with [U-^{13}C]glutamine (approximately 45–65%). In GABA, percent enrichment was much lower, but also in this metabolite, the enrichment was higher after incubation in medium with [U-^{13}C]glutamate than with [U-^{13}C]glutamine, ~30% and ~20%, respectively. Cells cultured in the presence of 0.50 mM KA and incubated in medium containing [U-^{13}C]glutamate had a higher percent enrichment in glutamate and aspartate, but not in GABA, compared to cells cultured in the presence of 0.05 mM KA and incubated in medium with [U-^{13}C]glutamate. On the other hand, cells exposed to 0.50 mM KA and incubated in the presence of [U-^{13}C]glutamine, had a higher percent enrichment in all metabolites than cells cultured in medium containing 0.05 mM KA and incubated in medium with [U-^{13}C]glutamine.

In order to investigate the effect of KA on metabolism, ^{13}C labeling from [U-^{13}C]glutamate and [U-^{13}C]glutamine arising from different turns in the TCA cycle was calculated. In Fig. 4 percent labeling from [U-^{13}C]glutamate and [U-^{13}C]glutamine in uniformly labeled glutamate and GABA and also glutamate, aspartate and GABA from precursors derived from the three

first turns of the TCA cycle is presented. Cells cultured in the presence of 0.05 mM KA and incubated in medium with [U-^{13}C]glutamate contained ~60% uniformly labeled glutamate, i.e. un-metabolized precursor. Approximately 20% was [1,2,3-^{13}C]glutamate derived from α-[1,2,3-^{13}C]ketoglutarate formed after one turn in the TCA cycle, and ~10% was [1,2-^{13}C] or [3-^{13}C]glutamate from labeled α-ketoglutarate formed after two TCA cycle turns (see Fig. 1). Less than 1% of the labeled glutamate was formed from labelled α-ketoglutarate which had been through three full turns in the TCA cycle. Culturing cells in medium containing 0.50 mM KA before incubation with [U-^{13}C]glutamate, led to a different distribution of intracellular labeled glutamate. In this case, approximately 70% was un-metabolized [U-^{13}C]glutamate, whereas ~17% and ~8% was from labelled α-ketoglutarate after one and two TCA cycle turns, respectively. Only 0.1% of the labeled glutamate was formed after three full turns in the TCA cycle. A larger percentage of the labeled glutamate in the cells was un-metabolized, and thus a smaller percentage was derived from the different TCA cycle turns, in neurons cultured in medium with a high KA concentration compared to neurons cultured in medium with a low KA concentration. Cells cultured in medium containing 0.05 mM KA and incubated with [U-^{13}C]glutamine, showed a more even label distribution in glutamate derived from intermediates from different turns in the TCA cycle. In this case, uniformly labeled glutamate constituted ~40% of the labeled glutamate in the cells, and ~30%, ~20% and ~10% was formed after one, two and three TCA turns, respectively, indicating more prominent TCA cycling of α-ketoglutarate derived from glutamine than of that derived from glutamate. The metabolic effect of culturing neurons in high KA followed by incubation in medium with [U-^{13}C]glutamine was similar to that seen in cells incubated in the presence of [U-^{13}C]glutamate, but less pronounced. The distribution of label in aspartate formed from [U-^{13}C]glutamate and [U-^{13}C]glutamine was similar to that in glutamate. Moreover, the effect of KA on aspartate labeling was similar in the glutamate and glutamine groups. However, distribution of label in GABA after culturing cells in medium containing 0.05 mM KA and incubation in the presence of [U-^{13}C]glutamate or [U-^{13}C]glutamine was similar to that from [U-^{13}C]glutamine in glutamate and aspartate. Neurons cultured in the presence of 0.50 mM KA and incubated in medium with [U-^{13}C]glutamate showed similar KA effects in GABA as in glutamate and aspartate. However, neurons incubated with [U-^{13}C]glutamine showed no differences in labeling distribution of GABA whether the cells were cultured in medium containing 0.05 or 0.50 mM KA.

In Table 2, the effects of kainic acid on intracellular content of glutamate, aspartate and GABA and the effects on metabolism as detected in label distribution representing different TCA cycle turns, are combined (see Section 2). Cells cultured in medium containing 0.05 mM KA and subsequently incubated with [U-^{13}C]glutamate had a higher amount of labeled glutamate, aspartate and GABA than cells cultured with the same KA concentration, but incubated with [U-^{13}C]glutamine. Culturing neurons in medium with a high KA concentration led to a

reduction of total label and uniformly labeled glutamate, aspartate and GABA, as well as that derived from precursors from all TCA cycle turns, after incubation with [U-^{13}C]glutamate compared to neurons cultured in medium with a low KA concentration. In contrast, neurons cultured in the presence of high KA and subsequently incubated with [U-^{13}C]glutamine, contained higher levels of total label and uniformly labeled glutamate and that derived from precursors from all TCA cycle turns compared to neurons cultured with low KA. The amounts of total labeled and uniformly labeled aspartate were increased in the high KA compared to the low KA group, whereas labeling from the second and third turn of the TCA cycle was unaffected by KA. Labeling of GABA from [U-^{13}C]glutamine also remained unaffected by KA concentration during the culture period.

4. Discussion

The present study was conducted to elucidate the long-term effects of KA with special emphasis on glutamate and glutamine metabolism in cultured cerebellar neurons. As discussed below, KA exerted neurotoxic effects and decreased intermediary metabolism in these cells.

4.1. KA neurotoxicity

It has been shown in several studies that KA can act as a neurotoxic agent (Seil et al., 1979; Ben-Ari, 1985; Balázs et al., 1990; Schwarzer and Sperk, 1995). However, positive effects of KA has also been reported. In order to illustrate the complexity of KA effects on cells, it can be pointed out that exposure to low KA concentrations enhances survival of cerebellar neurons in culture with a maximal positive effect at ~0.05 mM KA, whereas high concentrations (>0.20 mM) are toxic to these cells (Balázs et al., 1990). In the present study, we chose to expose the cells in the control group to 0.05 mM KA, since this has been shown to enhance cell survival (Balázs et al., 1990) and give the purest glutamatergic preparation (Drejer and Schousboe, 1989; Sonnewald et al., 2004). Even though Engblom et al. (2003) reported disturbed maturation of GABA$_A$ receptor subunit expression in cerebellar neuronal cultures after exposure to 0.05 mM KA, glucose metabolism remained unaltered by this treatment (Sonnewald et al., 2004). In addition to the KA effects on cell survival mentioned above, KA is a potent agonist at the kainate and AMPA subclasses of ionotropic glutamate receptors (Lerma, 1998). Hence, administration of KA causes an acute excitatory response by direct receptor activation (Ben-Ari, 1985; Sperk, 1994; Bradford, 1995; Qu et al., 2003). In the present study, cell protein was reduced by approximately 25% in the cultures exposed to 0.50 mM KA compared to those cultured with 0.05 mM KA, confirming a neurotoxic effect of high KA concentrations. In cerebellar cultures, selective KA toxicity has been used as a tool to obtain pure cerebellar granule cell cultures (Drejer and Schousboe, 1989). Drejer and Schousboe added 0.05 mM KA to the cultures from day 5 in vitro, and showed that evoked [^{3}H]GABA release from the cultures was abolished, whereas

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[^{3}H]-D-aspartate release was unaffected by this treatment. However, other studies have shown that GABAergic neurons in these cultures are not eliminated by long-term exposure to 0.05 mM KA from day 0 in vitro (Sonnewald et al., 2004, 2006). The present study confirmed previous findings that GABA is produced in the cultures despite the presence of a high KA concentration in the culture medium. In fact, GABA content in the control cultures (incubated without [U-^{13}C]glutamate or [U-^{13}C]glutamine) increased after exposure to 0.50 mM KA compared to 0.05 mM KA (see below).

4.2. KA effect on amino acids metabolism

Culturing cerebellar neurons with 0.50 mM KA for the whole culture period had profound effects on cellular content of amino acids in the present study. These effects were exacerbated when [U-^{13}C]glutamate was present in the incubation medium. The decrease in cellular content of all amino acids detected in cells cultured with 0.50 mM KA compared to those cultured with 0.05 mM KA could have been caused by a decrease in uptake of [U-^{13}C]glutamate into the neurons. This theory was supported by the ∼50% reduction of glutamate consumption in the high KA compared to the low KA group. In fact, KA has been shown to be a selective inhibitor of the high-affinity sodium dependent glutamate transporter EAAT2 (GLT) (Koch et al., 1999; Danbolt, 2001; Bridges and Esslinger, 2005). When cultures in the present study were incubated with [U-^{13}C]glutamine, the situation was very different than after incubation with [U-^{13}C]glutamate. In this case, exposure to a high KA concentration during the culture period led to an increase in cellular content of glutamine and glutamate compared to cells exposed to a low KA concentration, whereas the consumption of glutamine from the medium was not affected by KA concentration. Thus, glutamine uptake in these cells was not impaired as a response to KA exposure. An increased cellular content of glutamine and glutamate can reflect both increased uptake of the precursors and decreased metabolism. Indeed, turnover in the TCA cycle of the carbon skeleton from [U-^{13}C]glutamate and [U-^{13}C]glutamine was decreased in the neurons exposed to 0.50 mM KA compared to those exposed to 0.05 mM, as discussed in the following.

In the previous paragraph, it was mentioned that the high KA concentration reduced glutamate uptake by the cerebellar neurons in culture by approximately 50% compared to the low KA concentration. The intracellular glutamate concentration was, however, only reduced by ∼25%. It can be assumed that this discrepancy is due to decreased metabolism of intracellular glutamate or a shift in metabolism resulting in an increased production of endogenous glutamate. There are few studies on the effect of KA on metabolism in cultured neurons. However, several groups have used KA to probe mitochondrial viability. Boje and Skolnick (1992) exposed cultured rat cerebellar neurons to various amounts of KA which was added 12 h after culture start, and assessed mitochondrial viability after 8 days in vitro. Compared to cells cultured without KA, exposure to 0.05 mM KA led to a decrease in mitochondrial viability of approximately 25%, and exposure to 0.50 mM KA led to a

further 25% reduction in mitochondrial viability. This is in agreement with the present study which suggests that a high KA concentration leads to an impairment of mitochondrial function, shown by the presence of more un-metabolized [U-^{13}C]glutamate or [U-^{13}C]glutamine in cells cultured in medium containing 0.50 mM KA compared to 0.05 mM KA, and also by the labeling pattern indicating reduced TCA cycle activity.

4.3. GABA in cerebellar neuronal cultures

As mentioned earlier, the presence of GABA in cultured cerebellar neurons has been confirmed by the present study. Sonnewald et al. (2006) reported that this GABA is produced by the ∼6% GABAergic neurons in the culture and is distributed to the other neurons, hence the glutamatergic neurons in these cultures contain GABA. Olstad et al. (2006) proposed that a possible function of GABA in these cells is energy production through the GABA shunt, where the carbon skeleton of GABA enters the TCA cycle as succinate. Interestingly, it has been shown that KA injections in rodents can induce GABA formation by glutamatergic neurons in the hippocampus (Schwarzer and Sperk, 1995). It was suggested that the cells were enabled to produce GABA for protection against excessive excitation. In the present study, similar results were obtained. Culturing the predominantly glutamatergic neurons in the presence of a high KA concentration, and subsequently incubating them in fresh medium without [U-^{13}C]glutamate or [U-^{13}C]glutamine present (the control group), led to an increase in intracellular GABA concentration compared to neurons exposed to a low KA concentration. Whether this increase was due to increased GABA production in GABAergic neurons, decreased GABA catabolism or synthesis of GABA in glutamatergic cells remains to be determined.

4.4. Compartmentation of glutamate and glutamine metabolism

It is well established that glutamine serves the role as precursor for neurotransmitter glutamate and GABA, and also as energy substrate for intermediary metabolism in neurons (Westergaard et al., 1995; Sonnewald and McKenna, 2002; Waagepetersen et al., 2005; Olstad et al., 2006). In order to fulfill this task, glutamine has to be converted to glutamate. Thus, it could be assumed that providing cultured neurons with [U-^{13}C]glutamine or [U-^{13}C]glutamate as precursors would result in similar labeling patterns in metabolites. Several studies have shown that this, however, is not the case (Westergaard et al., 1995; Olstad et al., 2006). In a monoculture consisting of one type of neurons, this is evidence for intracellular compartmentation or mitochondrial heterogeneity. As mentioned, dissociated cell cultures from postnatal rodent cerebellum consist of glutamatergic granule neurons, with a small contribution of GABAergic stellate and basket neurons. Despite adding an anti-mitotic agent (cytosine arabinoside) to the cultures, a small number of glial cells are also present in the culture. Thus, the fact that more than one cell type are present in

these cultures, intercellular compartmentation can be observed. Since only the GABAergic neurons have been shown to be capable of producing GABA (Sonnewald et al., 2004, 2006) and the astrocytes glutamine (Norenberg and Martinez-Hernandez, 1979), it is possible to differentiate between intercellular and intracellular compartmentation in cultures of cerebellar neurons. Indeed, results from the present study illustrate both inter- and intracellular compartmentation. Intracellular compartmentation has previously been reported in cultured cortical neurons, where TCA cycle metabolism of the carbon skeleton subsequently used in formation of GABA was higher from [U-^{13}C]glutamine than from [U-^{13}C]glutamate (Westergaard et al., 1995). Also in the present study intracellular compartmentation was seen since TCA cycling of the carbon skeleton from [U-^{13}C]glutamine was more pronounced than that from [U-^{13}C]glutamate in all metabolites except GABA. In the latter, cycling from the two labeled precursors was similar, indicating that in the GABAergic compartment, the carbon skeleton from [U-^{13}C]glutamate has a higher turnover than in the glutamatergic compartment. Although the cycling from [U-^{13}C]glutamate and [U-^{13}C]glutamine was similar in the GABAergic compartment, results from the present study suggest compartmentation inside the GABAergic compartment. This is evident from the fact that cycling of the carbon skeleton from [U-^{13}C]glutamate subsequently used to form GABA was affected by KA, whereas cycling of the carbon skeleton from [U-^{13}C]glutamine was not. Since the cerebellar cultures consist of two types of GABAergic neurons, stellate and basket neurons, it is not surprising that the GABAergic compartment shows further compartmentation.

5. Conclusion

Long-term exposure of cells to 0.50 mM KA led to a profound loss of cerebellar neurons, i.e. a neurotoxic effect of KA was seen in this study. Decreased glutamate consumption caused by culturing cells in a medium containing a high KA concentration was consistent with earlier reports of KA being a glutamate transport inhibitor, whereas glutamine uptake remained unaffected by KA concentration during the culture period. Furthermore, mitochondrial metabolism of the carbon skeleton from both [U-^{13}C]glutamate and [U-^{13}C]glutamine was significantly reduced after long-term exposure to 0.50 mM KA compared to exposure to 0.05 mM KA. The results presented can only be understood in terms of inter- and intracellular compartmentation of metabolism, since TCA cycling of the carbon skeleton from [U-^{13}C]glutamine was more pronounced than that from [U-^{13}C]glutamate, and since the carbon skeleton from [U-^{13}C]glutamate had a higher turnover in the GABAergic than in the glutamatergic compartment which constitute these cultures.

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Pyruvate recycling in cultured neurons from cerebellum

Olstad E, Olsen GM, Qu H and Sonnewald U.

Pyruvate Recycling in Cultured Neurons From Cerebellum

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Pyruvate recycling is a pathway for complete oxidation of glutamate and cellular location, and the physiological significance of such recycling has been debated during the last decade. The present study was aimed at elucidating whether recycling takes place in neuron-enriched cultures of dissociated cerebella, consisting mainly of glutamatergic granule cells, some GABAergic neurons, and few astrocytes. These cultures and cultures of astrocytes from cerebellum were incubated in medium containing [U-^{13}C]glutamate, and cell extracts were analyzed by gas chromatography and mass spectrometry. Additionally, in the case of the neurons, a magnetic resonance (MR) spectrum was obtained. It could be shown that the atom percentage excess of the isotopomer representing pyruvate recycling in glutamate (M + 4) was similar for astrocytes and neuron-enriched cultures. However, the latter showed more recycling in glutamine (synthesized in the small fraction of astrocytes) than the pure astrocyte cultures, whereas the reverse was the case for aspartate. In fact, the atom percentage excess of the isotopomer representing pyruvate recycling in glutamine was slightly but significantly higher than that in glutamate in the neuron-enriched cultures. It can be concluded that pyruvate recycling is clearly present in neurons, and this was verified by MR spectroscopy.   © 2007 Wiley-Liss, Inc.

Key words: astrocytes; glutamatergic neurons; mass spectrometry; metabolism; tricarboxylic acid cycle

Pyruvate carboxylase located in astrocytes is thought to be the enzyme responsible for anaplerosis in brain (Patel, 1974), and carboxylation of pyruvate has been demonstrated in rat, mouse, and human brain (Lapidot and Gopher, 1994; Hassel et al., 1995; Öz et al., 2004; Patel et al., 2005; Melø et al., 2006). Previous experiments and results from cell cultures incubated in the presence of [U-^{13}C]glucose and 3-nitropropionic acid confirmed that pyruvate carboxylation takes place in astrocytes but not neurons (Yu et al., 1983; Shank et al., 1985; Waagepetersen et al., 2001; Qu et al., 2001). During development anaplerosis (filling up) is necessary, because concentrations of glutamate and glutamine in brain increase (Tkáè et al., 2003), whereas in adults anaplerosis is not self-evident. It is generally accepted that the adult brain has to replenish the

tricarboxylic acid (TCA) cycle when a four (or more) carbon unit such as glutamine or a lactate molecule derived from TCA cycle intermediates (malate or oxaloacetate) leaves the brain. Glutamine is indeed released from the brain (Grill et al., 1992), but it has not been shown that lactate from brain is TCA cycle derived. Another possibility is that a four (or more) carbon unit is degraded in the brain. Pyruvate recycling is such a pathway in which compounds such as glutamate, glutamine, or aspartate, which are originally derived from pyruvate carboxylation, can be degraded to pyruvate and reenter the TCA cycle as acetyl CoA (see Fig. 1). Previous studies have shown that pyruvate recycling takes place in rat brain, and initially the cellular location was thought to be neurons (Cerdan et al., 1990). However, cell culture studies pointed toward astrocytes as the site for recycling (Sonnewald et al., 1996a; Bakken et al., 1997a,b, 1998a; Håberg et al., 1998; Alves et al., 2000; Waagepetersen et al., 2002).

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The present study was undertaken to reinvestigate the cellular location of pyruvate recycling. By using ^{13}C labeled compounds and ^{13}C magnetic resonance spectroscopy (MRS), it is possible to monitor cellular metabolism and astrocyte–neuron interactions. Various ^{13}C–labeled substrates have been used to unravel different aspects of cerebral metabolism. We incubated cerebellar neurons and astrocytes with medium containing [U-^{13}C]glutamate. Analysis of neuronal cell extracts by MRS and the more sensitive method gas chromatography/mass spectrometry (GC/MS) revealed that pyruvate recycling takes place in neurons as well as in astrocytes.

MATERIALS AND METHODS

Materials

NMRI mice were obtained from Taconic M&B (Copenhagen, Denmark). Plastic tissue culture dishes were pur–

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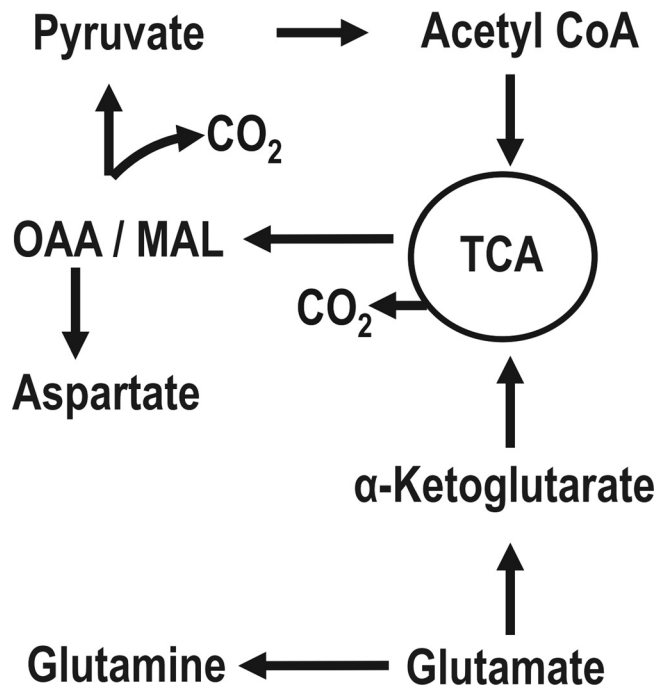
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Fig. 1. Schematic presentation of pyruvate recycling from glutamate, which can be amidated to glutamine or enter the TCA cycle as α-ketoglutarate and be converted to uniformly labeled succinate and after several steps malate (MAL) and oxaloacetate (OAA). The latter can further be transaminated into aspartate. If pyruvate recycling is active, malate via malic enzyme or oxaloacetate via phosphoenolpyruvate carboxykinase plus pyruvate kinase can be converted to pyruvate, which can enter the TCA cycle via acetyl CoA and give rise to unique labeling patterns in glutamate and aspartate.

chased from Nunc A/S (Roskilde, Denmark) and fetal calf serum (FCS) from Seralab Ltd. (Sussex, United Kingdom). Culture medium and glutamate receptor antagonists DNQX (6,7-dinitroquinoxaline-2,3-dione) and D-AP5 (D-2-amino-5-phosphonopentanoic acid) were from Sigma (St. Louis, MO). [U-^{13}C]glutamate (98%+ enriched) and 99.9% D_2O were from Cambridge Isotope Laboratories (Woburn, MA); ethylene glycol was from Merck (Darmstadt, Germany), and the GC/MS derivatization reagent MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide) + 1% t-BDMS-Cl (*tert*-butyldimethylchlorosilane) was purchased from Regis Technologies, Inc. (Morton Grove, IL). All other chemicals were of the purest grade available from regular commercial sources.

Cell Cultures

Neuron-enriched cerebellar cultures were isolated and cultured from 7-day-old mice as described by Schousboe et al. (1989). Briefly, the tissue was trypsinized, followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. Cells were suspended (2.75×10^6 cells/ml) in a modified Dulbecco's minimum essential medium (DMEM; Hertz et al., 1982), containing 24.5 mM KCl, 31 mM glucose, 7 μM p-aminobenzoic acid, 0.05 mM kainic acid, and

10% (v/v) fetal calf serum (FCS) and seeded in poly-D-lysine-coated Petri dishes (2 ml/35 mm). After 48 hr in culture, 20 μM (final concentration) cytosine arabinoside was added to the medium to prevent astrocytic proliferation. Experiments were performed on 7-day-old cultures.

Cerebellar astrocytes were cultured as described by Hertz et al. (1989). Briefly, cerebellum was taken from 7-day-old mice and passed through Nitex nylon netting (80-μm pore size) into DMEM containing 20% (v/v) FCS. Medium was changed 2 days after plating and subsequently twice per week, gradually changing to 10% FCS. From the third week, dibutyryl-cAMP was added to the medium to promote morphological differentiation of the astrocytes. Experiments were performed on 3-week-old cultures.

Experiments Using [U-^{13}C]Glutamate for MRS and GC/MS Analysis

The culture medium was removed, and the cells were incubated for 2 hr at 37°C in serum-free DMEM (prepared without glutamine) containing 3 mM glucose and [U-^{13}C] glutamate (neurons, 0.25 mM; astrocytes 0.5 mM). To avoid the toxicity of glutamate to neurons during the incubation, two glutamate receptor antagonists, DNQX (25 μM), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-selective antagonist, and D-AP5 (100 μM), an N-methyl-D-aspartate (NMDA) antagonist, were also present in the incubation medium of neurons (Frandsen et al., 1989). After the incubation period, the cells were washed twice with cold phosphate-buffered saline and extracted with 70% (v/v) ethanol. The cell extracts were scraped off the dishes and centrifuged at 10,000*g* for 15 min to separate the metabolites from the insoluble proteins. The supernatants (cell extracts) were divided into two parts; one was lyophilized for subsequent sample preparation for GC/MS analyses (six samples), and the remaining halves were pooled and lyophilized (one sample) for MR spectroscopy (neurons).

GC/MS

Lyophilized cell extracts were redissolved in HCl (10 mM), adjusted to pH <2 with 6 M HCl, and dried under atmospheric air. The amino acids were extracted into an organic phase of ethanol and benzene and dried again under atmospheric air before derivatization with MTBSTFA in the presence of 1% t-BDMS-Cl (Mawhinney et al., 1986). The samples were analyzed on a Hewlett Packard 5890 Series II gas chromatograph linked to a Hewlett Packard 5972 Series mass spectrometer.

MR Spectroscopy

The lyophilized sample consisting of six pooled cell extracts of neuron-enriched cultures was redissolved in D_2O. A proton decoupled 150.92-MHz ^{13}C MR spectrum was obtained on a Bruker 600 spectrometer using a Bruker Bio-Spin CryoProbe (Bruker Analytik GmbH, Rheinstetten, Germany). The spectrum was accumulated using a 30° pulse angle, an acquisition time of 1.08 sec, and a 0.5-sec relaxation delay. The number of scans was 129,024.

Possible isotopomers in glutamate and glutamine from the TCA cycle using unlabeled acetyl CoA
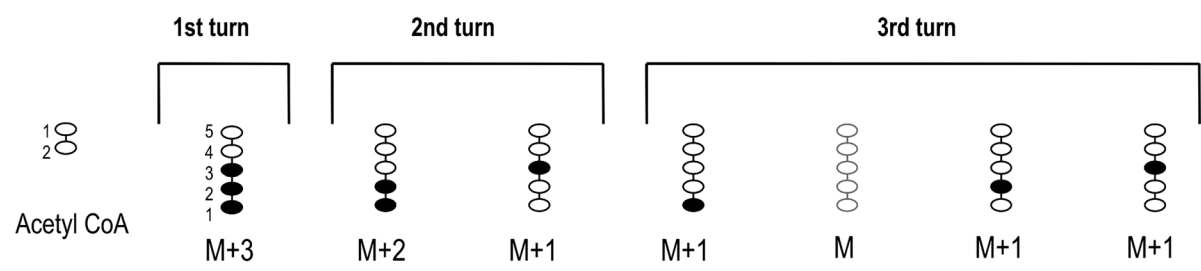
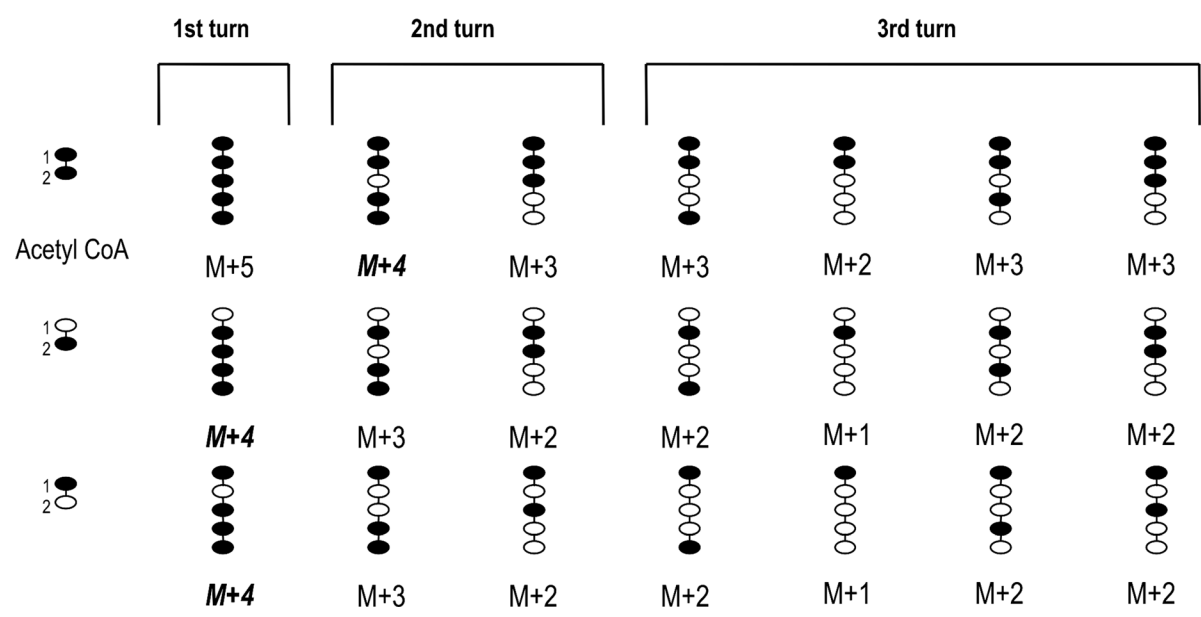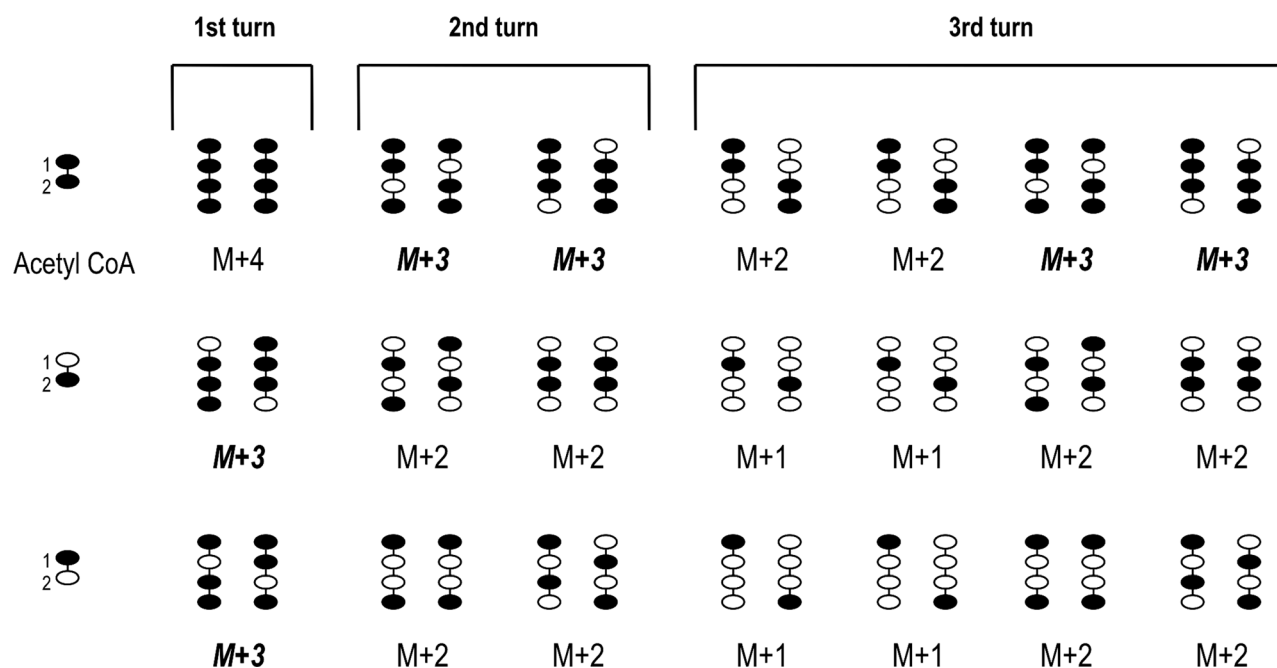## Possible isotopomers in glutamate and glutamine from the TCA cycle involving pyruvate recycling

Fig. 2. Schematic representation of possible isotopomers of glutamate and glutamine arising from [U-^{13}C]glutamate via the first turn in the TCA cycle (top) and pyruvate recycling (bottom): solid ovals represent ^{13}C, and open ovals represent ^{12}C atoms. The masses (M, M + 1, M + 2, etc.) of the different isotopomers are indicated, and the M + 4 isotopomers, which can only result from pyruvate recycling, are shown in boldface.

Data Analysis

Atom percentage excess (^{13}C) of glutamate, glutamine, and aspartate was determined after calibration with unlabeled standard solutions (Biemann, 1962). In the following, the sums of M + 1, M + 2, and M + 3 in glutamate or glutamine and that of M + 1, M + 2, and M + 4 in aspartate are used to describe TCA cycle activity, because these isotopomers are derived from the TCA cycle using unlabeled acetyl CoA. To describe pyruvate recycling, which is also part of TCA cycle activity, M + 4 in glutamate or glutamine and M + 3 in aspartate are used. It should be noted that this is a simplification in which the pyruvate recycling pathway is underestimated and the TCA cycle involving unlabeled acetyl CoA is overestimated (see Figs. 2, 3). The pyruvate recycling over TCA cycle activity (PR/TCA) ratio was calculated by divid-

ing atom percentage excess for glutamate or glutamine M + 4 (aspartate, M + 3) by the sum of TCA cycle derived isotopomers M + 1, M + 2, and M + 3 (aspartate, the sum of M + 1, M + 2, and M + 4). Results are presented as means ± SD. Differences between astrocytes and neurons were analyzed statistically by Student's t-test and differences in atom percentage excess of the corresponding isotopomers between glutamate, glutamine, and aspartate with one-way ANOVA followed by the LSD (least significant difference) post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

[U-^{13}C]glutamate present in the incubation medium can enter cells through specific transporter proteins. Once inside the cells, [U-^{13}C]glutamate has several

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Possible isotopomers in aspartate from the TCA cycle using unlabeled acetyl CoA
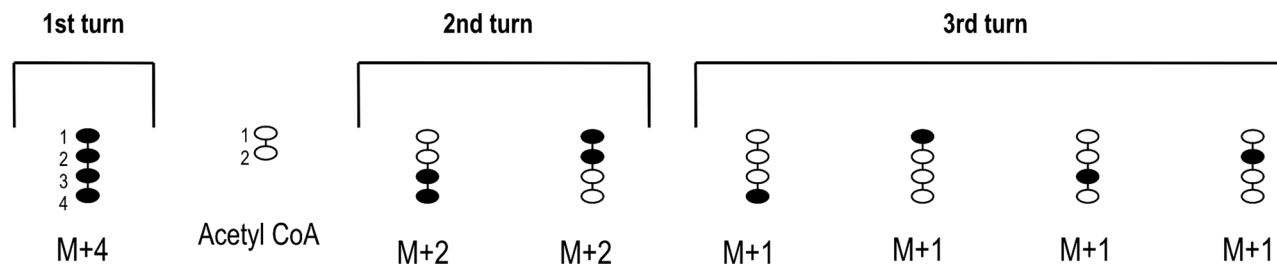
Possible isotopomers in aspartate from the TCA cycle involving pyruvate recycling

Fig. 3. Schematic representation of possible isotopomers of aspartate arising from [U-^{13}C]glutamate via the first turn in the TCA cycle (top) and pyruvate recycling (bottom): solid ovals represent ^{13}C, and open ovals represent ^{12}C atoms. The masses (M, M + 1, M + 2, etc.) of the different isotopomers are indicated, and the M + 3 isotopomers, which can only result from pyruvate recycling, are shown in boldface.

metabolic fates. Of interest for pyruvate recycling is the conversion into α-[U-^{13}C]ketoglutarate, which can enter the TCA cycle to be converted to uniformly labeled succinate and after several steps malate and oxaloacetate (OAA). The latter can also be transaminated to aspartate (Fig. 1). [U-^{13}C]oxaloacetate can stay in the cycle and condense with acetyl CoA to give rise, after several steps, to particular labeling patterns in glutamate and glutamine (Fig. 2) and aspartate (Fig. 3). However, if pyruvate recycling is active, malate via malic enzyme or OAA via phosphoenolpyruvate carboxykinase plus pyruvate kinase can be converted to pyruvate, which can enter the TCA cycle via acetyl CoA (Fig. 1) and give rise

to unique labeling patterns in glutamate (Fig. 2, bottom) and aspartate (Fig. 3, bottom). It should be noted that uniformly labeled glutamate is formed when [U-^{13}C]OAA condenses with [1,2-^{13}C]acetyl CoA, and this isotopomer cannot be distinguished from the [U-^{13}C]glutamate added to the medium.

With GC/MS, information about the percentage distribution of isotopomers with different masses (M, M + 1, M + 2, etc.) of each metabolite is obtained. However, the position of the ^{13}C atoms in the isotopomers is not readily available. From Figures 2 and 3, it can be seen that M + 4 in glutamate and glutamine and M + 3 in aspartate are the only isotopomer masses resulting from pyruvate

TABLE I. Percentage of Uniformly Labeled Isotopomers ([U-^{13}C], Glutamate, and Glutamine), Isotopomers Derived From the TCA Cycle Using Unlabeled Acetyl CoA (TCA) and From the TCA Cycle Involving Pyruvate Recycling (PR)★

	Glutamate			Aspartate		Glutamine		
	[U-^{13}C] (%)	TCA (%)	PR (%)	TCA (%)	PR (%)	[U-^{13}C] (%)	TCA (%)	PR (%)
Neurons	55.7 ± 1.5	30.4 ± 1.1 (0.15 ± 0.01)	4.6 ± 0.1	79.8 ± 0.4[b] (0.07 ± 0.001[b])	5.5 ± 0.1[b]	59.6 ± 1.1[b]	19.0 ± 0.9[b,c] (0.27 ± 0.03[b,c])	5.1 ± 0.5[b,c]
Astrocytes	62.9 ± 6.8[a]	18.2 ± 4.6[a] (0.33 ± 0.13[a])	5.8 ± 1.7	68.0 ± 2.1[a,b] (0.17 ± 0.04[ab])	11.3 ± 2.5[a,b]	46.1 ± 6.1[a,b]	9.7 ± 3.7[a,b,c] (0.31 ± 0.20)	2.5 ± 0.7[a,b,c]

★In parenthesis, the ratio of isotopomers derived from pyruvate recycling over those derived from TCA using unlabeled acetyl CoA is calculated for glutamate, aspartate, and glutamine in cultured neurons and astrocytes from cerebellum. Neuron-enriched and astrocyte cultures from cerebellum were incubated for 2 hr in medium containing 3 mM glucose, 25 μM DNQX, and 100 μM D-AP5 and 0.25 mM [U-^{13}C]glutamate (neurons, n = 6) or 0.5 mM (astrocytes, n = 6) [U-^{13}C]glutamate; for details, see Materials and Methods. The results are from GC/MS analyses, where [U-^{13}C] is represented by M + 5 (glutamate and glutamine), isotopomers derived from the TCA cycle using unlabeled acetyl CoA (TCA) by Σ(M + 1, M + 2 and M + 3) for glutamate and glutamine and Σ(M + 1, M + 2 and M + 4) for aspartate, and finally isotopomers from the TCA cycle involving pyruvate recycling are represented by M + 4 (glutamate and glutamine) and M + 3 (aspartate). Results are presented as means ± SD, and $P < 0.05$ was considered statistically significant.
[a]Different from the corresponding group in neurons.
[b]Different from atom percent excess of the corresponding glutamate isotopomers in the same cell type.
[c]Different from atom percent excess of the corresponding aspartate isotopomers in the same cell type.

recycling that can be distinguished from those from TCA cycling by GC/MS. By using this method, ^{13}C labeling was detected in glutamate, glutamine, GABA, aspartate, succinate, fumarate, malate, and citrate in neurons and astrocytes. For simplicity, only glutamate, glutamine, and aspartate labelings are shown, because they illustrate the point of this paper clearly. Glutamine (synthesized in astrocytes only) was detected both in the astrocytes and in the neuron-enriched cultures. In Table I, the percentage uniformly labeled glutamate and glutamine in neurons and astrocytes is presented. The fraction of uniformly labeled glutamate was higher in astrocytes than in neurons, whereas the opposite was the case for glutamine. Furthermore, the isotopomers derived from the TCA cycle using unlabeled acetyl CoA and those derived from pyruvate recycling in glutamate, aspartate, and glutamine are presented in Table I. It should be noted that some isotopomers from pyruvate recycling will also appear with the same number of ^{13}C atoms as those derived from the TCA cycle (see Figs. 2, 3). In addition, uniformly labeled isotopomers can also result from recycling. Therefore, the extent of pyruvate recycling is underestimated in Table I, insofar as this pathway is represented only by the M + 4 isotopomer in glutamate and glutamine and by M + 3 in aspartate. From Table I it is clear that atom percentage excess of the isotopomer from the TCA cycle is higher in neurons than astrocytes for all three amino acids, whereas pyruvate recycling in astrocytes and neuron-enriched cultures were similar in glutamate but not in glutamine and aspartate. The neuron-enriched cultures showed more atom percentage excess in the isotopomer derived from recycling in glutamine (synthesized in the small fraction of astrocytes) than in pure astrocyte cultures, whereas the reverse was the case for aspartate. In fact, the percentage isotopomer from recycling in glutamine was slightly but significantly higher than that in glutamate in the neuron-enriched cultures and lower in the astrocytes.

To visualize pyruvate recycling in a representative way, the ratio of M + 4 (PR) over the sum of M + 1, M + 2, and M + 3 (TCA) for glutamate and glutamine was calculated and is presented in parentheses in Table I. For aspartate, the uniformly labeled isotopomer resulting from the first TCA cycle turn (Fig. 3) is included in this ratio [(M + 3)/Σ(M + 1, M + 2 and M + 4)]. Approximately half of the aspartate in the cells (49% ± 9% astrocytes; 51% ± 1% neurons) was uniformly labeled. In evaluating the extent to which glutamate, glutamine, and aspartate were derived from pyruvate recycling compared with the extent to which isotopomers were derived from TCA cycle activity (PR/TCA ratio), it is clear that pyruvate recycling was more prominent in glutamate in astrocytes than in neurons (Table I). As mentioned, uniformly labeled aspartate was included in the calculation of PR/TCA in Table I, and this makes it evident that pyruvate recycling was much smaller than TCA cycle activity in this metabolite. Furthermore, the PR/TCA ratio for aspartate was much lower than the ratios for glutamate and glutamine in neurons, and lower than the ratio of glutamate in astrocytes (Table I). The PR/TCA ratio for glutamine was similar to that of glutamate in the astrocytes and similar in pure astrocyte cultures and in astrocytes present in neuron-enriched cultures.

With ^{13}C MR spectroscopy, it is possible to detect the position in which a particular compound is ^{13}C labeled in contrast to GC/MS, where only the extent of labeling is detectable under the conditions used in the present study. ^{13}C-labeled glutamate and aspartate were observed in the MR spectrum of extracts from neurons incubated with medium containing [U-^{13}C]glutamate (Fig. 4). To show enough detail, only the parts of the spectrum that contain glutamate C-4 and aspartate C-3 are shown. Looking at the glutamate C-4 frequency range presented in Figure 4A, peaks showing ^{13}C labeling in the C-4 posi-
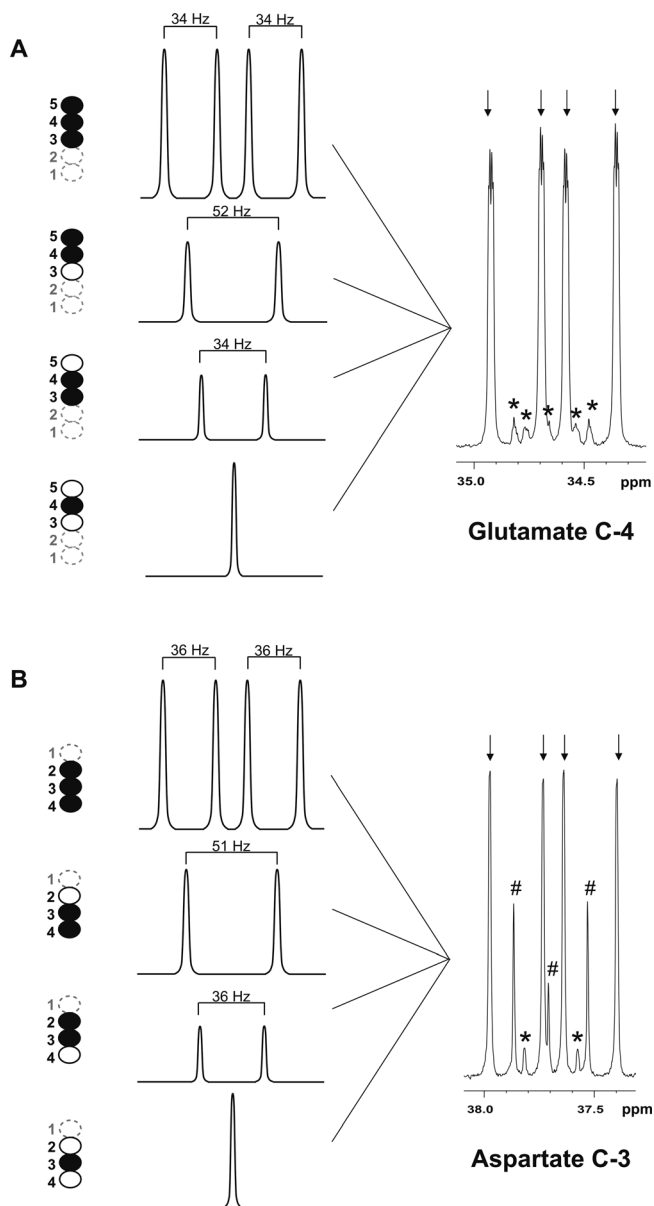
T1

F4

Glutamate C-4

Aspartate C-3

Fig. 4. Parts of the MR spectrum showing glutamate C-4 (**A**) and aspartate C-3 (**B**) and the isotopomers responsible for the configuration of the peaks: solid ovals represent ^{13}C, and open ovals represent ^{12}C atoms. Both peaks consist of a doublet of doublets (indicated by arrows) representing [3,4,5-^{13}C]glutamate and [2,3,4-^{13}C]aspartate, one doublet with approximately 50 Hz splitting from [4,5-^{13}C]glutamate (recycling, *) and [3,4-^{13}C]aspartate (TCA cycling, #), another doublet with approximately 35 Hz splitting from [3,4-^{13}C]glutamate (recycling, *) and [2,3-^{13}C]aspartate (recycling, *), and finally a singlet representing [4-^{13}C]glutamate (recycling, *) and [3-^{13}C]aspartate (TCA cycling, #). No information can be obtained about labeling in the C-1 and C-2 positions for glutamate and the C-1 position for aspartate indicated by dashed ovals.

tion also give information about neighboring positions C-3 and C-5, but not about the C-1 and C-2 positions. The peaks consist of a doublet of doublets (indicated by arrows) representing [3,4,5-^{13}C]glutamate, one doublet with 52 Hz splitting from [4,5-^{13}C]glutamate, another doublet with 34 Hz splitting from [3,4-^{13}C]glutamate, and finally a singlet representing [4-^{13}C]glutamate. Both doublets and the singlet arise from pyruvate recycling and are indicated by asterisks. The aspartate C-3 peaks (Fig. 4B) show a similar pattern with a doublet of doublets (arrows) representing [2,3,4-^{13}C]aspartate, one doublet with 51 Hz splitting representing [3,4-^{13}C]aspartate arising from TCA cycling (number sign), another doublet with 36 Hz splitting from [2,3-^{13}C]aspartate derived from pyruvate recycling (asterisks), and finally the [3-^{13}C]aspartate singlet from TCA cycling (number sign). [3,4,5-^{13}C]glutamate seen in the spectrum can be part of the precursor, [U-^{13}C]glutamate, whereas [2,3,4-^{13}C]aspartate can also be included in [U-^{13}C]aspartate derived from [U-^{13}C]glutamate via the first turn of the TCA cycle (Fig. 3). [3,4-^{13}C]aspartate and [3-^{13}C]aspartate are derived from the second and third turns of the TCA cycle, respectively (Fig. 3). [4,5-^{13}C]-, [3,4-^{13}C]-, and [4-^{13}C]glutamate are all derived from pyruvate recycling (Fig. 2). MR spectra of astrocyte extracts were not obtained; they have been published previously and showed recycling in glutamate and aspartate (Håberg et al., 1998).

DISCUSSION

Pyruvate recycling is a well-known metabolic pathway in the liver, but it was first detected in brain by Cerdan et al. (1990). In a later study by the same authors, the metabolism of [1,2-^{13}C]glucose and [U-^{13}C]-3-hydroxybutyrate was investigated in rat brain via ex vivo ^{13}C MR spectroscopy, taking advantage, in particular, of homonuclear [13]C-^{13}C spin coupling patterns. A quantitative analysis of the ^{13}C spectra demonstrated a cerebral pyruvate recycling system contributing maximally 17% of the pyruvate metabolism through the pyruvate dehydrogenase in brain (Künnecke et al., 1993). This recycling was believed to take place in neurons and not astrocytes, because it was apparent in glutamate but not in glutamine. In 1995, Hassel and Sonnewald reported partial pyruvate recycling in mouse brain astrocytes, because lactate formation from the TCA cycle was detected from [2-^{13}C]acetate but not [1-^{13}C]glucose. The enrichment of total brain lactate from [2-^{13}C]acetate reached approximately 1% in both the C-2 and the C-3 positions in fasted mice. It was calculated that this could account for 20% of the lactate formed in the glial compartment (Hassel and Sonnewald, 1995). Pyruvate recycling in brain was also studied in fasted rats receiving either an intraperitoneal or a subcutaneous injection of [1,2-^{13}C]acetate (Håberg et al., 1998). MR spectroscopic analysis of plasma showed larger amounts of [1, 2-^{13}C]acetate in the intraperitoneal group compared with the subcutaneous group, and this was coupled to more pyruvate recycling detected in the former group in glutamate and GABA. However, Lapidot and Gopher (1994) were not able to detect such recycling in rabbit brain.

Pyruvate recycling is a pathway for complete oxidation of glutamate in the TCA cycle. In cultured corti-

cal astrocytes, pyruvate formed from [U-^{13}C]glutamate was shown to reenter the TCA cycle after conversion to acetyl CoA, as demonstrated by the labeling patterns in aspartate C-2 and C-3, lactate C-2, and glutamate C-4, which provided evidence for pyruvate recycling in astrocytes (Håberg et al., 1998). Also, in neuron-enriched cultures from cerebellum, labeling of lactate was detected from [U-^{13}C]glutamate and [U-^{13}C]aspartate, but ^{13}C label was not shown to reenter the TCA cycle through acetyl CoA in these cells (Sonnewald et al., 1996b; Bakken et al., 1998a). Incubating cortical astrocyte cultures with [U-^{13}C]glutamine in the presence of glutamate or [U-^{13}C]aspartate also led to a small amount of pyruvate recycling in glutamate (Sonnewald et al., 1996a; Bakken et al., 1997b, 1998a). Recycling was also demonstrated in astrocytes using [3-^{13}C]glutamate but could not be shown in cortical GABAergic neurons (Waagepetersen et al., 2002). Hypoglycemia, a condition in which acetyl CoA production is reduced, could possibly lead to increased pyruvate recycling. However, recycling was abolished in astrocytes under this condition (Bakken et al., 1998b). It was hypothesized that cocultures of astrocytes and neurons could possibly have increased pyruvate recycling compared with astrocytes because of transfer of lactate produced by astrocytic mitochondria to neurons for glutamate synthesis. Surprisingly, experiments with [3-^{13}C]glutamate and neocortical cocultures did not show signs of pyruvate recycling even though astrocytes were present (Waagepetersen et al., 2002). However, recycling could be shown when cerebellar cocultures were superfused with medium containing [U-^{13}C]lactate or [U-^{13}C]glucose (Bak et al., 2006).

In the present study, cerebellar astrocytes were incubated with [U-^{13}C]glutamate, and cell extracts were analyzed by GC/MS. As expected, pyruvate recycling was detected in glutamate and aspartate. Recycling in aspartate was about twice as high as that evident in glutamate. This is due to the fact that [U-^{13}C]glutamate was added to the medium and constituted 63% of all glutamate in the astrocytes. The sum of the isotopomers from the TCA cycle and recycling was 24% of total glutamate (Table I). This was the case for 79% (Table I) of aspartate. Glutamine labeling via pyruvate recycling in astrocytes was much lower than that observed in glutamate. This was, however, due not to a lower ratio for pyruvate recycling/cycling in the TCA cycle but to the fact that, under the chosen incubation conditions, only 12% of glutamine was derived from mitochondrial metabolism compared with 24% of glutamate. The small percentage of glutamine derived from TCA cycle activity in astrocytes could explain why Cerdan et al. (1990) suggested that recycling took place in neurons, not astrocytes.

Neuron-enriched cultures from cerebellum are an excellent model for studying cerebellar metabolism, because they consist of a majority of glutamatergic with about 5% GABAergic neurons, mimicking the distribution in brain (Sonnewald et al., 2004a, 2006; Olstad

et al., 2006), and also astrocytes are present, though only very few. When these cultures were incubated in medium containing [U-^{13}C]glutamate, GC/MS analysis clearly showed pyruvate recycling in glutamate, aspartate, and glutamine. Furthermore, atom percentage excess of the isotopomer derived from pyruvate recycling in glutamate in astrocytes and neuron-enriched cultures was similar, thereby ruling out that the few astrocytes present in the neuron-enriched cultures were responsible for the recycling. When evaluating the extent to which glutamate was derived from pyruvate recycling compared with the extent to which isotopomers were derived from TCA cycle activity using unlabeled acetyl CoA, it is clear that pyruvate recycling was more prominent in glutamate in astrocytes than in neurons. However, this is due to the fact that in astrocytes less glutamate is derived from the TCA cycle than in neurons, whereas the percentage excess ^{13}C labeling for glutamate derived from pyruvate recycling (M + 4) was similar in astrocytes and neurons.

Aspartate showed a very low pyruvate recycling over TCA cycle activity ratio when all TCA-cycle-derived isotopomers, including uniformly labeled aspartate, were considered. However, comparing the atom percentage excess of the aspartate isotopomer derived from recycling and that of glutamate and glutamine, aspartate recycling was highest in both cell types.

The pyruvate recycling over TCA cycle activity ratio in glutamine (which is only synthesized in astrocytes) was similar to that in glutamate in the astrocytes and similar in pure astrocyte cultures and in neuron-enriched cultures containing some astrocytes. However, labeling from TCA cycle activity using unlabeled acetyl CoA was more pronounced in glutamine observed in neuron-enriched than in pure astrocyte cultures. Thus, there was more pyruvate recycling in glutamine from neuron-enriched than from astrocytic cultures. In fact, the atom percentage excess of the isotopomer derived from recycling in glutamine in the neuron-enriched cultures was slightly but significantly higher than that in glutamate in the same cell type. This could indicate that glutamate metabolism was compartmentalized in these neurons, as previously shown in various cell types (Waagepetersen et al., 1999; Qu et al., 1999, 2005; Eloqayli et al., 2002; Sonnewald et al., 2004b), and that glutamate derived from recycling was preferentially released from neurons in the neuron-enriched cultures to be converted to glutamine in the astrocytes. Alternatively, astrocytes might have a different metabolism in the presence of neurons than in a monoculture.

To verify pyruvate recycling in neurons, an MR spectrum was obtained of cell extracts, and recycling was clearly detectable in glutamate and aspartate. The reason why such recycling in neurons has not been detected earlier could be the enhanced sensitivity of the cryo-MR probe used in the present experiment. Taken together, these results clearly show significant pyruvate recycling in cerebellar neurons.

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ACKNOWLEDGMENTS

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290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
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302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
303. Valentina Maria do Rosario Cabral Iversen: MENTAL HEALTH AND PSYCHOLOGICAL ADAPTATION OF CLINICAL AND NON-CLINICAL MIGRANT GROUPS
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