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Astrocytes may play a role in the etiology of absence epilepsy: A comparison between immature GAERS not yet expressing seizures and adults

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Neuronal–astrocytic interactions in 1-month-old Genetic Absence Epilepsy Rats from Strasbourg (GAERS) before the occurrence of seizures are compared to those in non-epileptic rats (NERs) and in adult GAERS expressing epilepsy. Animals received [1-^{13}C]glucose and [1,2-^{13}C]acetate, preferential substrates of neurons and astrocytes, respectively, and extracts from cerebral cortex, subcortex and cerebellum were analyzed by NMR spectroscopy. Increased mitochondrial metabolism took place in the cortical neurons of immature and adult GAERS and therefore does not seem to be a consequence of the occurrence of absence seizures. Glutamine supply to GABAergic neurons was reduced in cortex and subcortex in young GAERS, as reflected by increased glutamine content and decreased 13C-labeling of GABA. In the brain of immature GAERS, interactions between glutamatergic neurons and astrocytes appeared normal whereas increased astrocytic metabolism took place in adult GAERS, suggesting that astrocytic alterations could possibly be the cause of seizures.

Keywords: ^{13}C NMR spectroscopy; Neurons; Astrocytes; Genetic model of childhood absence epilepsy; GAERS

Introduction

Genetic Absence Epilepsy Rats from Strasbourg (GAERS) represent an isomorphic, predictive and homologous model of childhood absence epilepsy (Danober et al., 1998; Meeren et al., 2005). In this polygenetic model (Rudolf et al., 2004), all animals display seizures within a cortico-thalamic loop, consisting in behavioral arrest with twitching of vibrissae. Seizures are associated with bilateral 5- to 9-Hz synchronous spike and wave discharges (SWDs). Conversely to humans, in GAERS, SWDs appear only after full electrocortical maturation at around 35–40 days and persist throughout adult life (Danober et al., 1998).

Absence seizures are thought to result from a possible imbalance in glutamatergic and GABAergic neurotransmission (Danober et al., 1998). However, it is not clear why absence seizures in the GAERS start only around 35–40 days after birth. In adult GAERS, local cerebral glucose metabolism was up-regulated in almost every single brain region. Conversely, in immature GAERS, this metabolic increase was limited to limbic regions and to the structures belonging to the remote circuit of seizure control and was not occurring in the thalamo-cortical regions exhibiting SWDs (Nehlig et al., 1998). Likewise, decreased protein amounts of glutamate transporters were found in 30-day-old compared to adult GAERS and a strain of inbred non-epileptic rats, NERs (Dutuit et al., 2002). At the same time, increased amounts of the astrocyte-specific protein glial fibrillary acidic protein (GFAP) were found before and after the age of occurrence of seizures in GAERS compared with NERs (Dutuit et al., 2000). On the basis of these age-related differences, we decided to investigate neuronal–astrocytic interactions before the onset of absence seizures in GAERS, to see if early disturbances in neuronal and astrocytic metabolism might underlie the generation of absence seizure.

Recently, we reported that neuronal and astrocytic glutamate metabolism was enhanced in cortex, while GABA levels were decreased in cortex and thalamus of GAERS compared with NERs. These changes in glutamatergic and GABAergic neurotransmission may underlie the occurrence of spontaneous seizures in this model (Melo et al., 2006). ^{13}C nuclear magnetic resonance (NMR) spectroscopy which was used in the study by Melo et al. (2006) is an excellent tool to analyze neuronal glial interactions in the brain (Sonnewald and Kondziella, 2003). To study whether dysregulation of astrocyte–neuron interactions precedes the onset of absence epilepsy, 30-day-old GAERS and NERs were injected with [1-^{13}C]glucose and [1,2-^{13}C]acetate. At the acetyl CoA level glucose is metabolized more in the neuronal than in the astrocytic TCA cycle

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(Qu et al., 2000) whereas acetate is selectively taken up by astrocytes (Waniewski and Martin, 1998).

Materials and methods

Animals

Eight male GAERS (63th generation) and 11 male NERs (57th generation), 30-day-old, originating from the Strasbourg breeding colony, were used in the experiment. The animals were maintained at 22 °C room temperature under a 12-h/12-h normal light/dark cycle (lights on at 7:00 a.m.) with food and water *ad libitum*. All animal experimentation was performed in accordance with the rules of the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the French Department of Agriculture (License No. 67–97).

Both groups of rats were given i.p. injections of sodium [1,2-^{13}C]acetate (504 mg/kg, 0.6 M solution) and [1-^{13}C]glucose (543 mg/kg 0.3 M solution; 99% ^{13}C enriched, Cambridge Isotope Laboratories, Woburn, MA, USA). Fifteen minutes after the injection, the animals were decapitated and their heads were snap-frozen in liquid nitrogen. The brains were removed and the cerebral cortex, subcortex and cerebellum were dissected out, weighed and frozen at −80 °C. The cerebral cortex samples consisted of the upper half of the cortex to the horizontal midline of the brain and were mainly frontal and somatosensory cortices, both the parietal and motor areas. Because of the great difficulty to dissect out the different parts of the subcortical areas without thawing that would clearly modify the concentrations of some labile metabolites and also because of the small size of the brains at 30 days (the limit for measurement of metabolites is 20 mg tissue), the subcortex sample was taken as a whole. It included the thalamus, hypothalamus and parts of hippocampus.

Tissue and plasma extraction

Each frozen tissue sample was homogenized in 1 ml ice-cold 7% perchloric acid and centrifuged for 10 min at 4 °C and 4000×*g*. The supernatants were transferred into new tubes and the precipitates were re-extracted with 0.5 ml perchloric acid and centrifuged again for 10 min at 4 °C and 4000×*g*. The supernatants were combined. The tubes were kept on ice at all times possible. Each sample was neutralized with 2 M KOH, centrifuged, lyophilized and stored at −20 °C.

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

Lyophilized samples were dissolved in 200 µl 99% D_2O and pH was adjusted to values between 6.8 and 7.0. The samples were transferred into NMR microtubes (Shigemi Inc., PA, USA). Proton-decoupled ^{13}C NMR spectra from cerebral cortex and cerebellum samples were accumulated on a BRUKER DRX500 spectrometer. Spectra from subcortex samples were obtained on a BRUKER600 spectrometer using a Bruker BioSpin CryoProbe (BRUKER Analytik GmbH, Rheinstetten, Germany). The following acquisition parameters were applied; 30° pulse angle, acquisition time of 1.3 s and a relaxation delay of 0.5 s. The number of scans was typically 15,000 for cerebral cortex and cerebellum and 20,000 for subcortex. Some spectra were also broad band decoupled only during acquisition to avoid nuclear Overhauser effect and accompanied by a relaxation delay of 20 s to achieve fully relaxed spectra. From several sets of spectra correction factors were obtained and applied to the integrals of the individual peaks.

^{1}H NMR spectra were acquired, on the same spectrometers, with the following acquisition parameters: 90° pulse angle, an acquisition time of 1.36 s and a relaxation delay of 10 s, 320 scans were accumulated for each sample. Water suppression was achieved by applying a low-power presaturation pulse at the water frequency.

Data and statistical analysis

Relevant peaks in the ^{13}C and ^{1}H NMR spectra were identified and integrated using XWINNMR software. The amounts of ^{13}C were quantified from the integrals of the peak areas, using ethylene glycol as internal standard. Amounts of metabolites were quantified using relevant peaks in the ^{1}H spectra which were integrated using ethylene glycol as an internal standard, and corrected for number of protons. All results are expressed as mean ± SD of 8 GAERS and 11 NER. All concentrations were corrected for tissue weight. Statistical differences between the two groups were analyzed using unpaired two-tailed Student's *t*-test. The level of significance was set at $p < 0.05$.

Glutamine–glutamate–GABA cycle

Even though only neurons transmit electrical signals, astrocytes may influence the threshold of neuronal firing and synchronization by regulating the extracellular milieu in the synaptic cleft (Tian et al., 2005). Glutamate is released from glutamatergic neurons during neurotransmission and cleared away from the synaptic cleft by uptake via glutamate transporters mostly into surrounding astrocytes (Danbolt, 2001). Once inside the astrocytes, glutamate is converted (i) to α-ketoglutarate by glutamic acid dehydrogenase (GDH) (EC 1.4.1.2) or transaminases or (ii) to glutamine by the astrocyte specific enzyme glutamine synthetase (GS) (EC 6.3.1.2) (Norenberg and Martinez-Hernandez, 1979). Thereafter glutamine may be released to neurons where it is re-converted to glutamate by the enzyme phosphate-activated glutaminase (PAG) (EC 3.5.1.2). Finally glutamate can be converted to α-ketoglutarate and enter the TCA cycle or be reloaded into synaptic vesicles. In GABAergic neurons glutamate can be converted to GABA, a reaction catalyzed by glutamic acid decarboxylase (GAD) (EC 4.1.1.15). These co-operations between neurons and astrocytes are termed the glutamine–glutamate–(GABA) cycle and are necessary because of the differential distribution of certain enzymes and transporters in neurons and astrocytes (Berl and Clarke, 1969, 1983).

Metabolic fate of [1-^{13}C]glucose and [1,2-^{13}C]acetate

To interpret the results from the ^{13}C NMR spectra (see typical example in Fig. 1), one has to know how [1-^{13}C]glucose and [1,2-^{13}C]acetate are metabolized in neurons and astrocytes (Fig. 2). Under normal conditions, the major metabolic fuel for all brain cells is glucose. [1-^{13}C]Glucose is transported into neurons and astrocytes where it can be converted to [3-^{13}C]pyruvate via glycolysis for generation of energy or metabolites. [3-^{13}C]Pyruvate has several fates; it can be (i) converted to [3-^{13}C]lactate, (ii) transaminated to [3-^{13}C]alanine or (iii) enter the TCA cycle as [2-^{13}C]acetyl CoA via the enzyme pyruvate dehydrogenase (PDH)