Glutamate, Glutamine, and GABA as Substrates for the Neuronal and Glial Compartments After Focal Cerebral Ischemia in Rats • Editorial Comment

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Glutamate, Glutamine, and GABA as Substrates for the Neuronal and Glial Compartments After Focal Cerebral Ischemia in Rats

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Background and Purpose—Even though the utilization of substrates alternative to glucose may play an important role in the survival of brain cells under ischemic conditions, evidence on changes in substrate selection by the adult brain in vivo during ischemic episodes remains very limited. This study investigates the utilization of glutamate, glutamine, and GABA as fuel by the neuronal and glial tricarboxylic acid cycles of both cerebral hemispheres after partially reversible focal cerebral ischemia (FCI).

Methods—Right hemisphere infarct was induced in adult Long-Evans rats by permanent occlusion of the right middle cerebral artery and transitory occlusion of both common carotid arteries. (1,2-$^{13}C_2$) acetate was infused for 60 minutes in the right carotid artery immediately after carotid recirculation had been re-established (1-hour group) or 23 hours later (24-hour group). Extracts from both cerebral hemispheres were prepared and analyzed separately by ^{13}C nuclear magnetic resonance and computer-assisted metabolic modeling.

Results—FCI decreased the oxidative metabolism of glucose in the brain in a time-dependent manner. Reduced glucose oxidation was compensated for by increased oxidations of (^{13}C) glutamate and (^{13}C) GABA in the astrocytes of the ipsilateral hemispheres of both groups. Increased oxidative metabolism of (^{13}C) glutamine in the neurons was favored by increased activity of the neuronal pyruvate recycling system in the 24–hour group.

Conclusions—Data were obtained consistent with time-dependent changes in the utilization of glutamate and GABA or glutamine as metabolic substrates for the glial or neuronal compartments of rat brain after FCI. **(*Stroke*. 1998;29:1048-1057.)**

Key Words: cerebral ischemia, focal ■ cerebral metabolism ■ middle cerebral artery occlusion ■ neuronal damage ■ stroke, experimental

Some of the metabolic mechanisms underlying the development of ischemic brain damage have been investigated using highly enriched cultures of neural cells[1–4] or cerebral cortex slices.[5,6] However, the metabolic events occurring in vivo in the neuronal and glial compartments of the adult brain during the development of ischemic damage remain less understood.

The mammalian brain contains essentially two different metabolic compartments: the glial and the neuronal. Classic radiolabeling experiments and more recent ^{13}C NMR evidence have shown that these two compartments differ in their utilization of particular substrates, their metabolic products, and the presence of characteristic enzymes.[7–10] The glial compartment uses glucose or acetate as its main substrates, is characterized by the presence of glutamine synthase, and produces glutamine, which is transferred to the neuronal compartment and metabolized. The neuronal compartment uses glucose and glial glutamine as its main substrates, is characterized by the presence of high glutaminase and GABA

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decarboxylase activities, and releases glutamate and GABA, which are transferred to the glial compartment and metabolized.[11] In addition, glial lactate has been proposed recently as an important substrate for the neuronal compartment, at least in neural cell cultures of fetal or neonatal origin during neuronal activation.[12] The metabolisms of neurons and glial cells interact closely, competing for glucose as a primary substrate, and using the glutamate, glutamine, and GABA produced in the other cell type as an alternative substrate for oxidation in the tricarboxylic acid cycle. Thus, several substrates are available to neurons and glial cells in situ, allowing to compensate deficits in one substrate with increased utilization of others.

The aim of this study was to investigate the use of glutamate, glutamine, and GABA as alternative substrates to glucose by the neuronal and glial compartments in both rat brain hemispheres after partially reversible FCI. To this end we used a combination of previously validated ^{13}C NMR and

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Selected Abbreviations and Acronyms

Ac = acetate
AcCoA = acetyl-CoA
Cr = creatine
PCr = phosphocreatine
FCI = partially reversible focal cerebral ischemia
GABA = γ-aminobutyryc acid
Gln = glutamine
Glc = glucose
Glu = glutamate
HPLC = high-performance liquid chromatography
Ino = myo-inositol
Lac = lactate
MABP = mean arterial blood pressure
NAA = N-acetyl-aspartic acid
NMR = nuclear magnetic resonance
OAA = oxalacetate
Succ = succinate
TSP = 2,2′-3,3′ tetradeutero trimethyl-silyl-propionate

mathematical modelling techniques.[13–15] The approach used (1,2-$^{13}C_2$) acetate as a substrate and computer-assisted analysis of extracts prepared from the ipsilateral and contralateral hemispheres, 1 hour or 24 hours after the ischemic insult. It has been shown that after FCI, glutamate and GABA behave, in addition to their role as excitatory or inhibitory transmitters, as true alternative substrates to glucose in the glial compartment of the in situ brain, while glutamine assumes a similar role in the neuronal compartment. These alterations in substrate selection in both compartments are time dependent and affect both the ipsilateral and contralateral hemispheres.

Materials and Methods

Animals, Surgical Procedures, and Experimental Design

The experimental protocols used in this study were approved by appropriate institutional review committees, and meet the guidelines of the responsible governmental agency. Long-Evans female rats (250±25 g, n=10), receiving rat chow and water ad libitum, were subjected to focal cerebral ischemia after an overnight fasting period. Anesthesia was induced with 2.5 mL/kg body weight of a mixture of ketamine hydrochloride (25 mg/mL), diazepam (2 mg/mL), and atropine (0.1 mg/mL) injected intraperitoneally, and prolonged when necessary with one third of the initial dose. Animals were allowed to breathe unassisted. The surgical procedure was essentially that described by Chen et al in 1986[16] and Liu et al in 1989,[17] with the modifications previously reported.[18,19] Briefly, under a surgical microscope, a small craniectomy was made over the main trunk of the right middle cerebral artery and above the rhinal fissure. The right middle cerebral artery was permanently ligated just before its bifurcation between the frontal and parietal branches. During this occlusion period, the right external carotid artery was cannulated retrogradely, and the catheter was placed near the origin of the internal carotid artery.[20,21] Both common carotid arteries were then exposed and clamped for 90 minutes. Restoration of carotid blood flow was directly observed in all cases, after removal of the clamps. Then, the animals were infused with (1,2-$^{13}C_2$) acetate solution (32 μmol · min^{-1} · 100 g body wt^{-1}, pH 7.2) for 60 minutes, either immediately after carotid blood flow was reestablished (1-hour group, n=5) or 23 hours later (24-hour group, n=5). The right femoral artery was cannulated during surgery and subsequent infusion, allowing continuous monitoring of arterial pressure. At the end of the infusion, 0.3-mL blood samples were drained from both jugular veins and from the femoral artery to determine arterial and venous blood gas values, pH, and glucose. Body temperature was maintained at 37±0.5°C during the experiment with a servo-controlled rectal probe heating pad. An additional thermistor probe was placed in the region of the middle cerebral artery under the temporal muscle and over the cerebral cortex, while brain temperature was maintained at 36±0.5°C with a tungsten lamp located over the head. At the end of the infusion, the head of the animals was funnel-frozen with liquid nitrogen. The ipsilateral and contralateral hemispheres were dissected separately while still at low temperature, powdered under liquid nitrogen, and extracted separately with methanol/HCl/perchloric acid.[22] Extracts were lyophilized to dryness and resuspended in D_2O (99.9%D) before high resolution ^{13}C and ^{1}H NMR.

High Resolution ^{13}C and ^{1}H NMR Spectroscopy of Brain Extracts

High resolution ^{13}C NMR spectra (90.56 MHz, 22°C, pH 7.4) of neutralized perchloric acid extracts of individual hemispheres, were obtained at 8.4 T with a Bruker AM-360 NMR spectrometer (Bruker Analytik GMBH). Acquisition conditions were as follows: 60° pulses, 18.5-KHz sweep width, 64K word computer memory (1.769-second acquisition time), total recycle time of 5.7-seconds, and approximately 22 000 scans. Broad band de-coupling (1 watt average forward power) was applied only during the acquisition. Chemical shifts were calibrated with the resonance of a 10% (vol/vol) dioxane solution (67.4 ppm) placed in a coaxial capillary.

High-resolution ^{1}H NMR spectra (360.1 MHz, 22°C) of the extracts used for ^{13}C NMR spectroscopy were acquired using the following: 90° pulses, 3968 Hz spectral width, 16K computer memory (1.3-second acquisition time), 128 transients for each spectrum, and 20-second relaxation delay. The residual water signal was attenuated with a 3-second presaturating pulse applied with the decoupler on the water resonance. Chemical shifts were referenced to internal TSP. ^{1}H and ^{13}C NMR assignments were made by comparison with published values.[13,23]

Computer-Assisted Simulation of ^{13}C NMR Spectra From (1,2-$^{13}C_2$) Acetate Metabolism in Both Hemispheres of Rat Brain After FCI

Computer-assisted interpretation of the ^{13}C NMR spectra from extracts of each cerebral hemisphere, in both time groups, was performed using the METASIM program version 2.1.[14] METASIM simulates the ^{13}C NMR spectrum produced by the metabolism of a given ^{13}C-labeled substrate in a user-defined metabolic network consisting of metabolite pool sizes and interconnecting fluxes (Figure 1). The network consists of two main cerebral compartments, namely neuronal and glial, with extracellular spaces for plasma and interstitial fluid. The neuronal compartment groups both glutamatergic and GABAergic neurons, whereas the glial compartment contains both astroglia and oligodendroglia.

Under steady state conditions the METASIM program calculates the probability of formation (or disappearance) of individual ^{13}C isotopomers of the metabolites located in the network nodes, using an algorithm based on the recursive definition of input-output equations.[24] Calculated ^{13}C isotopomer populations are used to compute and display the resulting ^{13}C NMR spectrum as the weighted sum of the individual ^{13}C isotopomer contributions, using predefined chemical shifts and ^{13}C-^{13}C coupling constants for each carbon. The METASIM curve fits an experimental spectrum by automatically optimizing the values of pool sizes and relative metabolic fluxes converging on the nodes of the specified network using an iterative, nonlinear least-squares procedure. Briefly, the following procedure was followed for flux and pool size optimization. Simulations of the ^{13}C multiplet structures of all the observable carbons of glutamate, glutamine, and GABA in both cerebral hemispheres were performed with METASIM, starting with the values for pool sizes and relative fluxes previously validated in the normoxic brain.[14,15] Progressive modifications of these starting values generated ^{13}C NMR spectra that were virtually identical to the experimental ones (c.f. Figure 5). Finally, the set of refined parameter values was automatically optimized by curve fitting the ^{13}C multiplets of the experimental

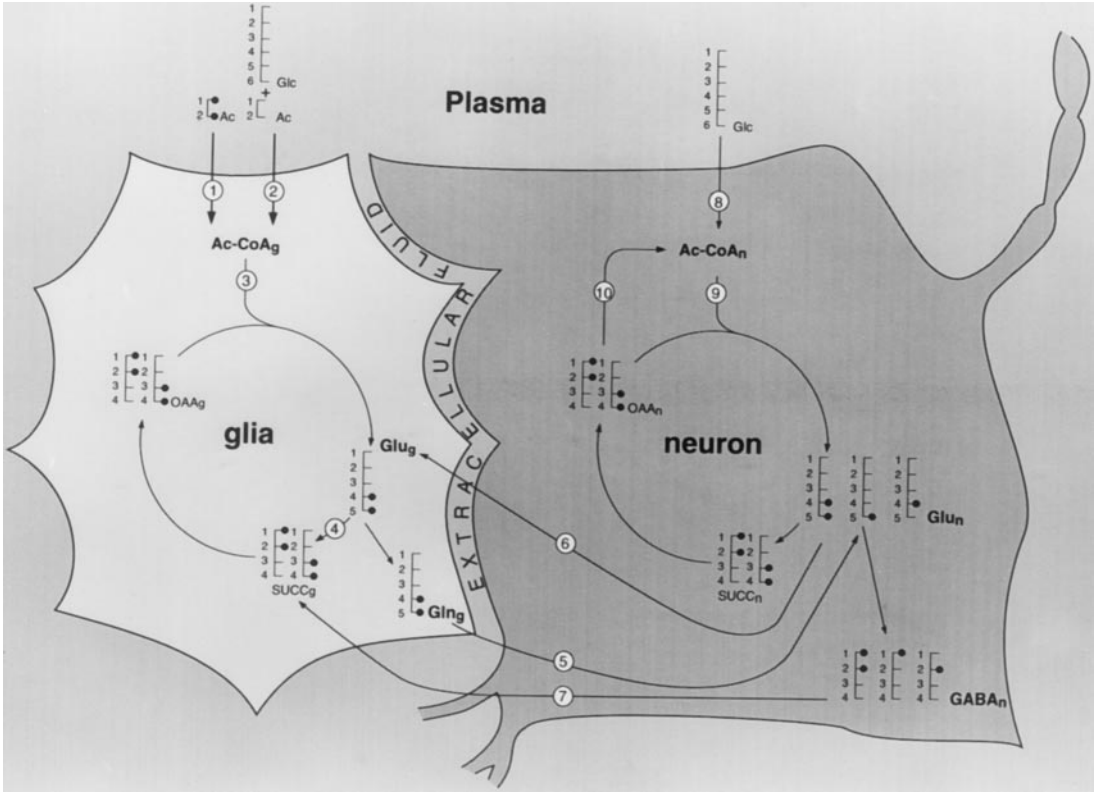

Figure 1. Metabolic network used to simulate cerebral metabolism of (1,2-$^{13}C_2$) acetate in both hemispheres of rat brain after FCI. The network consists of fluxes (circled numbers) and metabolite nodes (abbreviated names). Two metabolic compartments associated with the glial (left) or neuronal (right) environments exist in the brain. Infused (1,2-$^{13}C_2$) acetate (flux 1) is activated to (1,2-$^{13}C_2$) acetyl-CoA only in the glial compartment, where it is diluted with unlabeled acetyl-CoA from unlabeled glucose (flux 2), originating the glial acetyl-CoA pool (Node $AcCoA_g$). $AcCoA_g$ enters the glial cycle (flux 3) labeling the small glutamate pool (Node Glu_g) and the glutamine pool (Gln_g). The glial compartment also metabolizes glutamate (flux 6) and GABA (flux 7) produced in the neurons (Nodes Glu_g and $SUCC_g$, respectively). The neuronal tricarboxylic acid cycle uses unlabeled glucose (flux 8) and some glial glutamine (flux 5) as substrates (Nodes $AcCoA_n$ and Glu_n, respectively). $AcCoA_n$ enters the neuronal cycle (flux 9) labeling the large neuronal glutamate pool (Node Glu_n), which is the main precursor of GABA. Only the glial compartment contains glutamine synthase activity.[11] The neuronal compartment contains the glutamate decarboxylase and most of the glutaminase activity as well as the pyruvate recycling system (flux 10). The METASIM program was used for the simulations.[14,15]

spectra to those of the simulated ones. More detailed descriptions of METASIM have been provided.[14,25,26]

Other Determinations

MABP was recorded in the femoral artery during the infusion using a pressure transducer (Schiller). Once the acetate solution had been infused, P_{O_2}, P_{CO_2}, and pH were determined in samples from arterial and venous blood taken from the femoral artery and jugular veins, respectively, using a clinical automatic analyzer (Nova Biomedical Analyzer). Glucose was determined spectrophotometrically with an NADP-coupled assay.[27] Total amino acid concentrations were measured by reverse-phase HPLC.[28] The Student's *t* test for unpaired values was used to assess statistical significance ($P<0.05$, $P<0.01$, and $P<0.001$) between the groups.

Materials

(1,2-$^{13}C_2$) acetate (99.9% ^{13}C) and D_2O (99.9% D) were purchased from ISOTEC Inc. The rest of the reagents were of the highest purity available from Sigma Chemical Co.

Results

Physiological Parameters of Arterial and Venous Blood After FCI

Table 1 shows physiological parameters measured in arterial and venous blood in the course of FCI. Measurements performed in the right and left jugular veins gave very similar results, and the mean value was taken as representative of venous blood in every animal. MABP remained within normal values before and during the carotid occlusion period, but decreased slightly in the post-occlusion period. After the infusion of (1,2-$^{13}C_2$) acetate, arteriovenous differences in glucose were larger in the 1-hour group (1.2 ± 0.12 mmol/L) than in the 24-hour group (0.7 ± 0.07 mmol/L). Venous pH was lower in the 1-hour group than in the 24-hour group. Similar metabolic changes have been reported previously.[29]

Effect of FCI on the ^{1}H NMR Spectra of Extracts From Both Hemispheres of Rat Brain After (1,2-$^{13}C_2$) Acetate Infusion

Figure 2 shows representative ^{1}H NMR spectra obtained from extracts of the contralateral (left) and ipsilateral (right) cerebral hemispheres from rats infused with (1,2-$^{13}C_2$) acetate in the 1-hour (top) and 24-hour (bottom) groups. Spectra obtained from the ipsilateral hemispheres showed higher Lac/NAA ratios (1.32 ± 0.12 and 0.78 ± 0.02) than the contralateral hemispheres (0.27 ± 0.055 and 0.20 ± 0.03). The latter Lac/NAA ratios are similar to those previously reported

TABLE 1. Physiological Parameters in Arterial and Venous Blood of Rats in the Course of FCI Followed by Infusion of (1,2-$^{13}C_2$) Acetate

	1-Hour Group		24-Hour Group	
Parameter	Femoral	Jugular	Femoral	Jugular
MABP pre-carotid occlusion, mm Hg	95.4±4.7	...	99.7±4.0	...
MABP during carotid occlusion, mm Hg	98.6±5.5	...	96.48±3.7	...
MABP post-carotid, occlusion, mm Hg	84.1±3.9	...	88.1±3.8	...
Glucose, mmol/L	9.8±0.8	8.6±1.05	12.3±1.5	11.5±1.6
Po_2, mm Hg	97.2±8.7	50.5±4.8	99.0±8.4	55.7±3.5
Pco_2, mm Hg	46.0±3.9	52.7±2.9	47.5±1.9	51.9±1.8
pH	7.4±0.03	7.35±0.04	7.4±0.05	7.4±0.03

MABP was determined during the occlusion of both common carotid arteries. The remaining measurements were performed in samples from arterial and venous blood at the end of the infusion with (1,2-$^{13}C_2$) acetate.

for normoxic brains.[30] ^{1}H NMR spectra from the ipsilateral hemispheres also showed more intense signals for the acetate methyl group (resonance 2) than those from the contralateral hemispheres, a situation that was specially apparent in the 24-hour group. In this case, ^{13}C satellites corresponding to the methyl signals of (1,2-$^{13}C_2$) acetate were clearly visible (arrows). This observation indicates that (1,2-$^{13}C_2$) acetate accumulates in the ipsilateral hemispheres, probably because of reduced oxidation in the tricarboxylic acid cycle. No other significant alterations could be detected in the remaining metabolites observable by ^{1}H NMR. Amino acid analysis revealed no significant difference in the total concentrations of glutamate, glutamine, and GABA between the ipsilateral and contralateral hemispheres of the 1-hour and 24-hour groups (not shown).

Effects of FCI on the ^{13}C NMR Spectra of Extracts From Both Hemispheres of Rat Brain After (1,2-$^{13}C_2$) Acetate Infusion

Figure 3 depicts the aliphatic portion of representative proton-decoupled ^{13}C NMR spectra of extracts from both cerebral hemispheres of rats infused with (1,2-$^{13}C_2$) acetate, either 1 hour after FCI (top) or 24 hours later (bottom). Resonances from the C2, C3, and C4 carbons of glutamate and glutamine

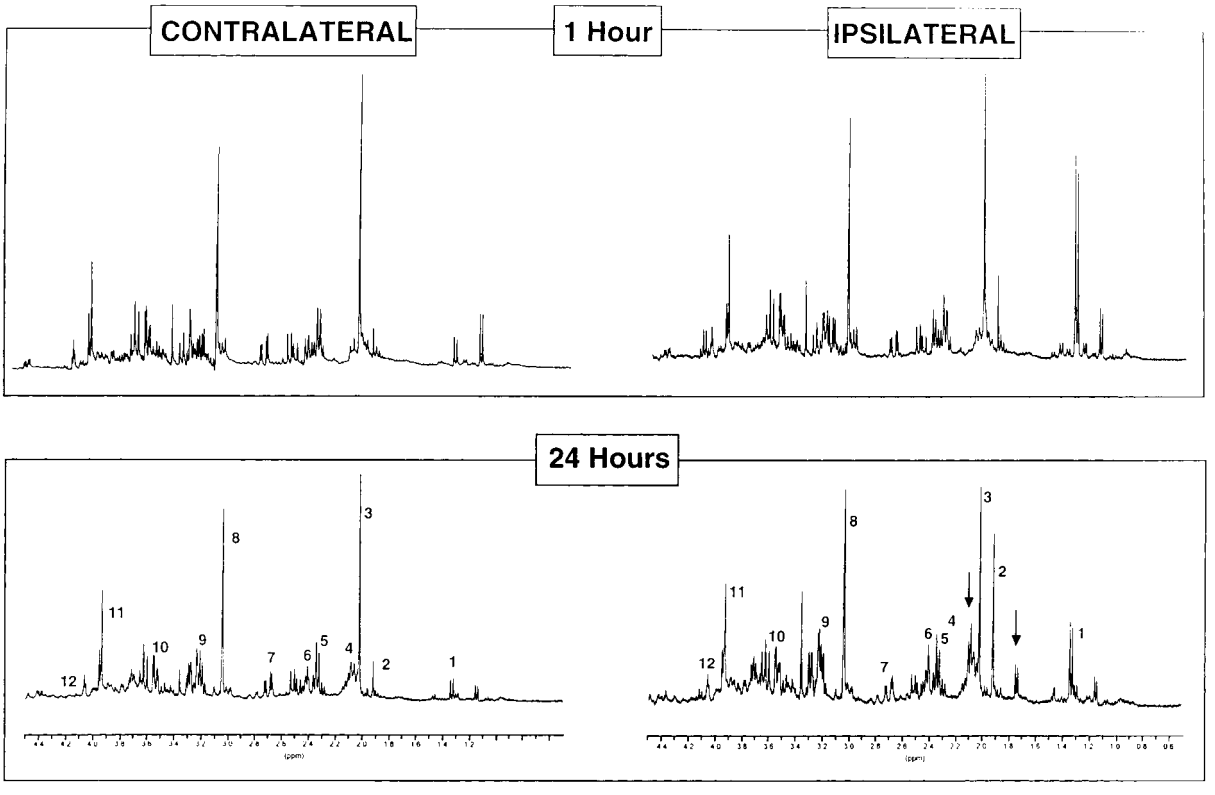

Figure 2. Representative ^{1}H NMR spectra (360.13 MHz) of extracts from both hemispheres of rat brain infused with (1,2-$^{13}C_2$) acetate, 1 hour or 24 hours after FCI. Permanent occlusion of the middle cerebral artery was performed in the right (ipsilateral) hemisphere. Only the aliphatic portion of the spectra is shown. 1 indicates lactate H3; 2, Ac H2 (^{12}C bonded); 3, NAA H6; 4, Glu-Gln H3, H3′; 5, Glu H4, H4′; 7, NAA H3, H3′; 8, Cr (PCr) methyl; 9, trimethyl ammonium groups of choline derivatives; 10, Ino H1, H3; 11, Cr (PCr) methylene; and 12, Ino H2. Arrows indicate ^{13}C satellites of (1,2-$^{13}C_2$) acetate H2.

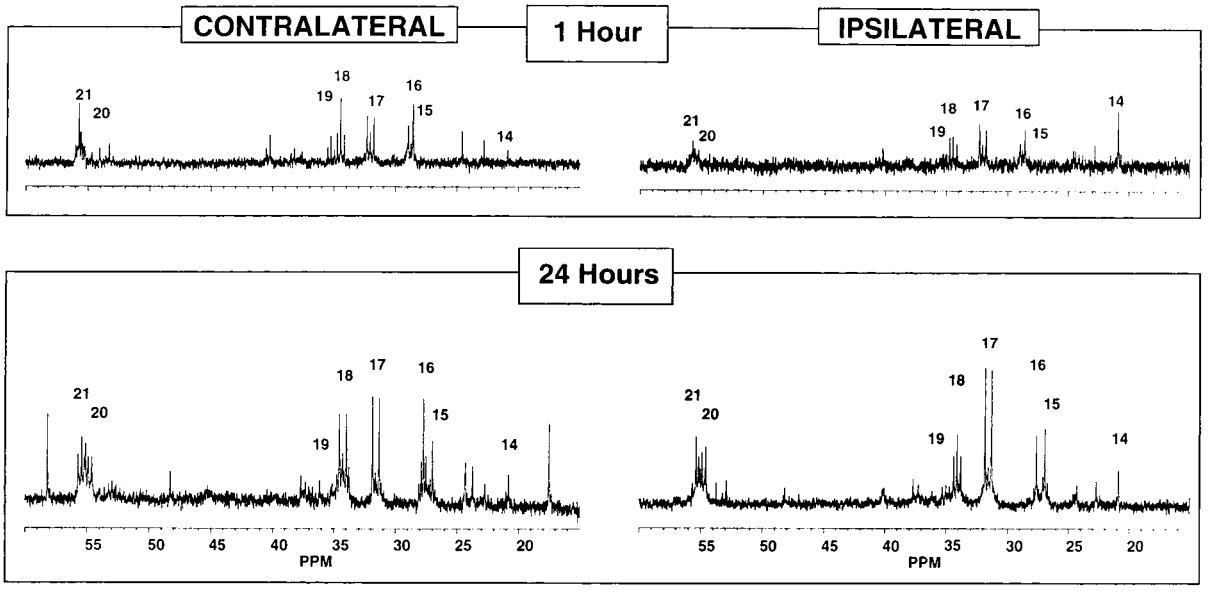

Figure 3. Representative proton decoupled ^{13}C NMR spectra (90.55 MHz) of extracts from both hemispheres of rat brain infused with (1,2-$^{13}C_2$) acetate, 1 hour and 24 hours after FCI. Permanent occlusion of the middle cerebral artery was performed in the right (ipsilateral) hemisphere. Only the aliphatic portion of the spectra is shown. 14 indicates lactate C3; 15, Gln C3; 16, Glu C3; 17, Gln C4; 18, Glu C4; 19, GABA C2; 20, Gln C2; and 21, Glu C2.

and the C2 carbon of GABA were clearly detected in all spectra (see Figure legend for assignments). Even though infusion conditions were identical, intensities of the ^{13}C signals were lower in the 1-hour group than in the 24-hour group. In most cases, these ^{13}C resonances depicted an apparent triplet structure produced by the superposition of a singlet and a doublet. Singlets are derived from metabolites containing the observed ^{13}C bonded to ^{12}C neighbors, while doublets, and doublets of doublets, are derived from molecules containing the observed ^{13}C bonded to one or two ^{13}C neighbors, respectively.[13,14]

The multiplet structure of individual ^{13}C resonances is a direct consequence of the metabolic pathways that produce and degrade the observed metabolite carbon.[13–15,26] The main pathways affecting the relative intensities of the singlets, doublets, and multiplets observed in the resonances of glutamate, glutamine, and GABA carbons are as follows: the relative amounts of labeled and unlabeled acetyl-CoA entering the neuronal and glial tricarboxylic acid cycles and the relative exchanges of glutamate, glutamine, and GABA between the neuronal and glial compartments (c.f. Figure 1). A comparison of the ^{13}C NMR spectra shown in Figure 3 reveals that the relative proportions of singlets, doublets, or multiplets corresponding to the same carbon resonance are different in the ipsilateral and contralateral hemispheres, irrespective of the time elapsed after FCI. These results reveal differences in substrate selection between the two hemispheres. Of particular interest is the ratio of singlets to doublets in the glutamate C4 resonance (number 18 in Figure 3). This ratio provides an estimate of the relative flux through the pyruvate recycling system, with larger values of the ratio corresponding to higher relative fluxes through the recycling pathway.[13,14]

It is possible to obtain a more quantitative interpretation of the changes observed in the multiplet structures of Figure 3 in terms of metabolic fluxes using the METASIM program. Figure 4 illustrates this procedure with a comparison of representative experimental spectra (top) from the ipsilateral (right panels) and contralateral (left panels) hemispheres, with the METASIM simulations (bottom) obtained using the parameters indicated in the legend. Similar simulations were performed with five animals of the 1-hour group and five animals of the 24-hour group. Values of pool size and relative metabolic flux that best fit the model described in Figure 1 in these animals are summarized in Figure 5.

In the 1-hour group, the glial compartment of the ipsilateral hemispheres showed decreased acetate oxidation (flux 3) and increased oxidation of the (^{13}C) glutamate (flux 6) and (^{13}C) GABA (flux 7) imported from the neurons. The neuronal compartment showed increased oxidation of unlabeled glucose (flux 8) and decreased contribution of the pyruvate recycling system to the neuronal cycle (flux 10). The glial glutamate pool size decreased. There was a trend for a decreased glial glutamine pool size and increased neuronal glutamate pool size. These results are consistent with astrocytic swelling. Twenty-four hours later, acetate oxidation augmented (flux 1) in the glial compartment of the ipsilateral hemisphere and increased (^{13}C) glutamate oxidation (flux 6) was maintained. The enhanced glucose oxidation relative to pyruvate recycling seen in the ipsilateral neuronal compartment of the 1-hour group was not seen at 24 hours. Glutamine oxidation (flux 5) in the neuronal compartments of both hemispheres increased as compared with the 1-hour group. It should be mentioned that morphological studies indicate that the effects of focal cerebral ischemia are heterogeneous in the ipsilateral hemisphere. Heterogeneity includes the presence of a zone of well-perfused healthy tissue and a necrotic core surrounded by a penumbral zone with increasingly better perfusion.[18–20] Notably, heterogeneity has also been reported in the contralateral hemisphere.[18,19] The relative flux values

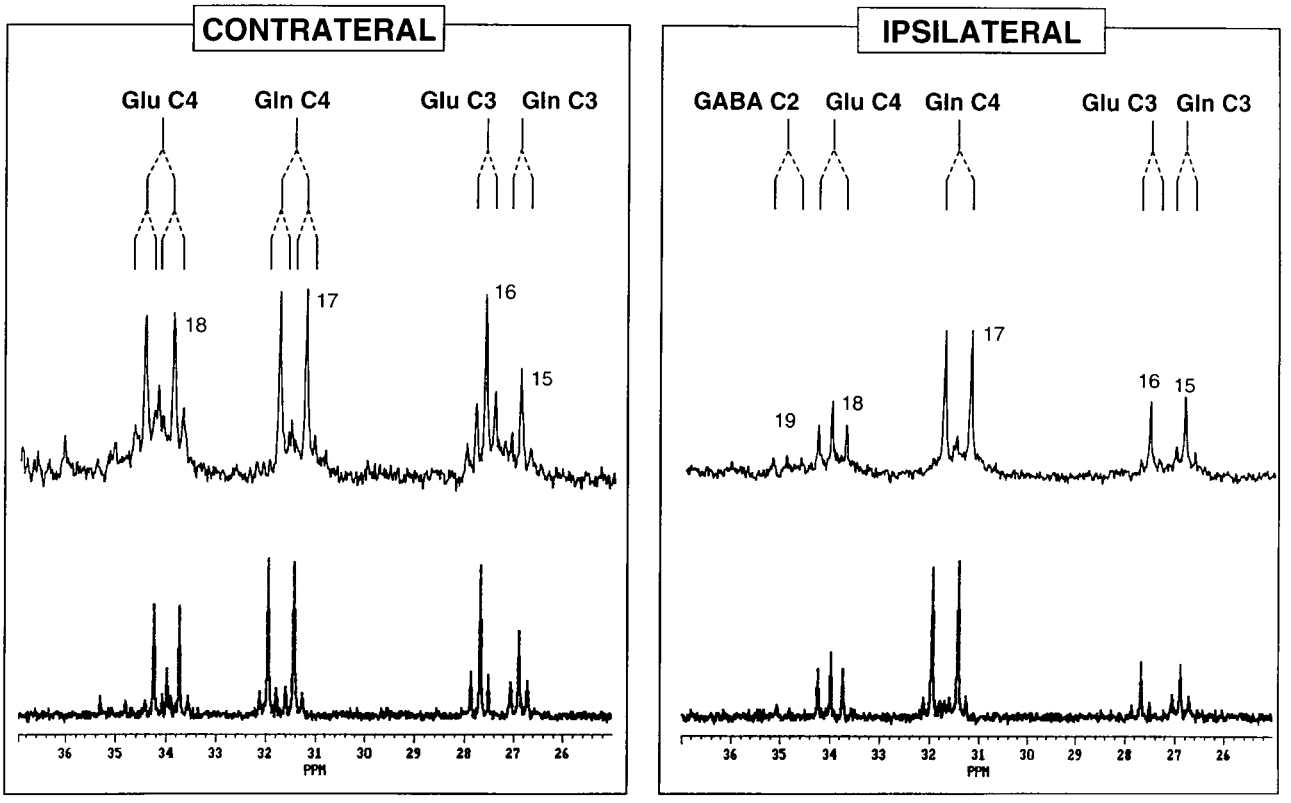

Figure 4. Representative experimental (top) and simulated (bottom) ^{13}C NMR spectra from extracts of each cerebral hemisphere 24 hours after FCI. Simulations were performed using the METASIM program configured to calculate the ^{13}C NMR spectra resulting from (1,2-$^{13}C_2$) acetate metabolism in the network described in Figure 1. Fifteen iterations were used with an artificial line broadening of 1 Hz and a simulated signal/noise ratio of 35/1. Pool size (μmol/g wet weight) and relative flux values used for the depicted simulations of ipsilateral (contralateral) hemispheres are Glu_g 0.66 (0.16); Glu_n 5.0 (5.5); Glutamine 7.5 (6.1); GABA 1.2 (1.2); flux 1 0.4 (0.45); flux 2 0.29 (0.53); flux 3 0.69 (0.98); flux 4 0.67 (0.69); flux 5 0.11(0.36); flux 6 0.30 (0.02); flux 7 0.33 (0.30); flux 8 0.24 (0.45); flux 9 0.89 (0.66); and flux 10 0.65 (0.19).

and pool size measurements presented in Figure 5 represent the weighted averages of the value of each parameter over the complete ipsilateral or contralateral hemispheres.

Discussion

Cerebral ischemia is the consequence of the reduction in blood flow below a critical threshold and the concomitant limitation in the supplies of primary cerebral substrates like glucose and oxygen. Under these limiting circumstances the ability of neurons and glial cells to obtain energy from substrates other than glucose contributes importantly to cellular survival. The amino acids glutamate, GABA, and glutamine are available as substrates in the extracellular fluid, and may be used as alternative fuels by astrocytes or neurons under conditions of glucose deprivation. Although it is known that these amino acids are used as substrates by enriched cultures of neurons or glial cells,[31] evidence on the physiological competence of this protective mechanism during glucose deprivation has been missing in the adult brain in vivo.

Metabolic utilization of glutamate, glutamine, and GABA as fuel alternatives or complementary to glucose is determined by their relative availability in the extracellular fluid as well as by the kinetics of their corresponding transport systems. The extracellular concentration of these amino acids in normoxic and ischemic brain tissues[32,33] and the kinetics of their uptake by cultures of cortical neurons or astrocytes have been addressed previously.[34–38] These studies allow us to calculate values for the availability of different substrates for the neuronal and glial metabolisms using in vitro experiments (Table 2) and to compare the results with those found in the present study for the in vivo brain (Figure 6).

Glucose is the main substrate for the adult brain under physiological conditions. The rates of glucose utilization as measured by the deoxyglucose method are approximately, 3.7 and 2.1 nmol glucose · min^{-1} · mg $protein^{-1}$ in cultivated astrocytes and cortical neurons, respectively.[39] Glutamate, GABA and glutamine are known to be transported to astrocytes and neurons by cell specific transporters.[34–38] Given physiological extracellular concentrations of glutamate, GABA and glutamine of approximately 1, 3 and 94 μmol/L respectively,[32,33] the amounts of glutamate and GABA transported to the astrocyte can be calculated as 0.07 and 0.0042 nmol · min^{-1} · mg $protein^{-1}$, respectively, and the amount calculated for glutamine transported to the neuron is 0.85 nmol · min^{-1} · mg $protein^{-1}$. On the other hand, tricarboxylic acid cycle rates of approximately 13 and 3 nmol min^{-1} · mg $protein^{-1}$ have been proposed for the neuronal and glial compartments in vivo.[10] Thus, under normoxic conditions complete oxidation of glutamate in the tricarboxylic acid cycle of astrocytes would represent at most 2% of their tricarboxylic cycle flux, whereas complete oxidation of glu-

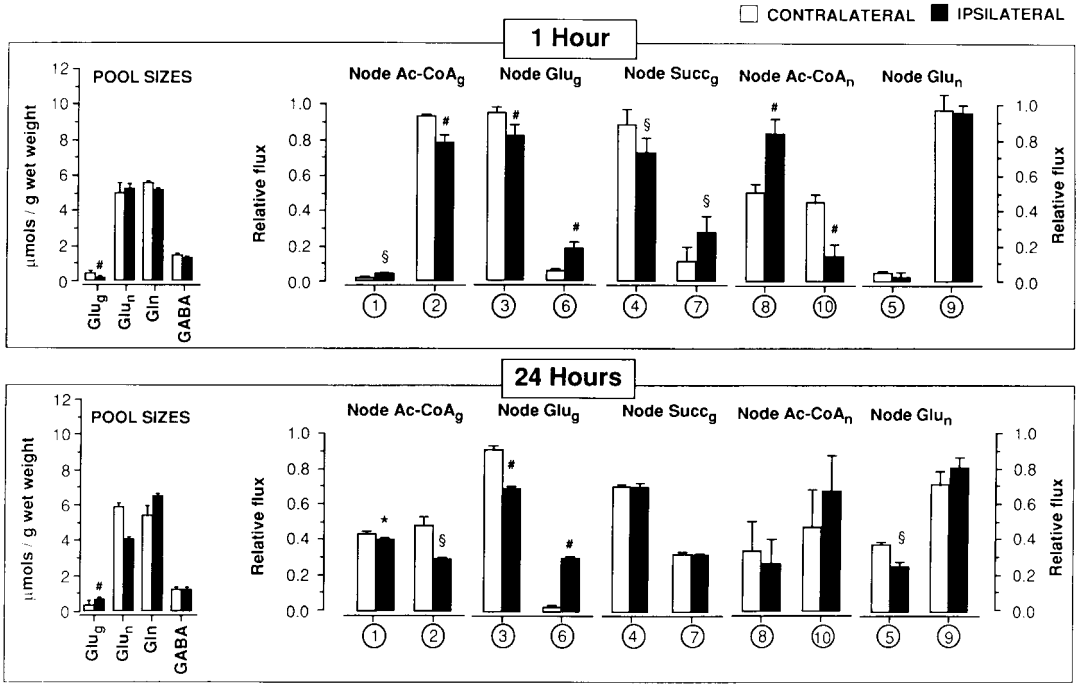

Figure 5. Pool sizes and relative flux values obtained from computer-assisted interpretations of ^{13}C NMR spectra from extracts of both hemispheres of rat brain infused with (1,2-$^{13}C_2$) acetate, 1 hour and 24 hours after FCI. Values were optimized iteratively using METASIM as indicated in "Materials and Methods" (c.f. References 14 and 15) and illustrated in Figure 4. Results represent the mean±SD of the fittings performed on four different ^{13}C NMR spectra of extracts from the ipsilateral (dark bars) and contralateral (light bars) hemispheres of the 1-hour and 24-hour groups, respectively. Circled numbers and node names refer to the nomenclature of Figure 1. Subscripts "g" or "n" refer to the glial or neuronal glutamate pools. Relative flux refers to the probability (P_i) of occurrence of a flux ($0 < P_i < 1$), as compared with the added probability of occurrence of all fluxes converging on the same node taken arbitrarily as one ($\Sigma P_i = 1$). *$P<0.05$, §$P<0.01$, #$P<0.001$.

tamine would contribute at most 6% of the tricarboxylic acid cycle flux in neurons. These results match well with those obtained in the present study, indicating that the contribution of glutamate oxidation to the energetics of the astrocytes (or glutamine to that of neurons) is minor under physiological conditions. This situation is observed in the contralateral hemisphere of the 1-hour group.

Under ischemic conditions the availability of extracellular substrates for neurons and astrocytes undergoes a drastic change. Glucose deprivation and hypoxia cause the extracellular levels of glutamate, glutamine, and GABA to rise to approximately 200, 250, and 20 μmol/L, respectively.[32,33] These concentrations saturate the corresponding amino acid transport systems of neurons or astrocytes, transiently increasing the metabolic availability of glutamine or glutamate and GABA, in the respective intracellular environments (Table 2). The calculated rates of amino acid transport under ischemic conditions represent an important increase in the intracellular metabolic availability of glutamate and GABA or of glutamine for oxidation. The results obtained in the

TABLE 2. Calculated Availability of Glutamate, Glutamine, and GABA as Oxidative Substrates in Cultivated Neurons and Glial Cells Under Normoxic or Ischemic Conditions

Substrate Availability (v)/Condition	Neuron, nmol · min^{-1} · mg protein^{-1}		Extracellular Fluid Concentration EFC μmol/L		Astrocyte, nmol · min^{-1} · mg protein^{-1}	
	Normoxia	Ischemia	Normoxia	Ischemia	Normoxia	Ischemia
Glutamate	0.9[a]	20.5[a]	1[b,c]	200[b,c]	0.07[d]	4.2[d]
Glutamine	0.85[e]	2.17[e]	94[b,c]	250[b,c]	1.4[f]	3.5[f]
GABA	0.09[g]	0.15[g]	3[b,c]	20[b,c]	0.0042[h]	0.0083[h]
Tricarboxylic acid cycle rate[i]	13[j]	NA	...	...	3[j]	NA

Intracellular substrate availability (v) was calculated using the expression $v = V_{max} \cdot EFC/(K_m + EFC)$ where K_m (μmol/L) and V_{max} (nmols · min^{-1} · mg protein^{-1}) refer to the kinetic constants of the transport system in enriched cultures of neurons or glial cells and EFC is the concentration of the substrate (μmol/L) in the interstitial fluid of the brain. V_{max}/K_m or EFC values used to calculate v were [a]23/24 (34), [b](32), [c](33), [d]6.1/91 (35), [3]28.2/3000 (36), [f]50.2/3300 (36), [g]0.171/2.8 (37), [h]0.01/4.1 (37), [i]nmol acetyl-CoA · min^{-1} · mg protein^{-1}, [j](10). Kinetic constants and EFC values were taken from the references indicated in parentheses. A value of 100 mg protein · g^{-1} wet weight was used to transform measurements carried out in the whole brain (μmol^{-1} · min^{-1} · g wet weight) into nmol · min^{-1} · mg protein^{-1}; NA, not applicable.

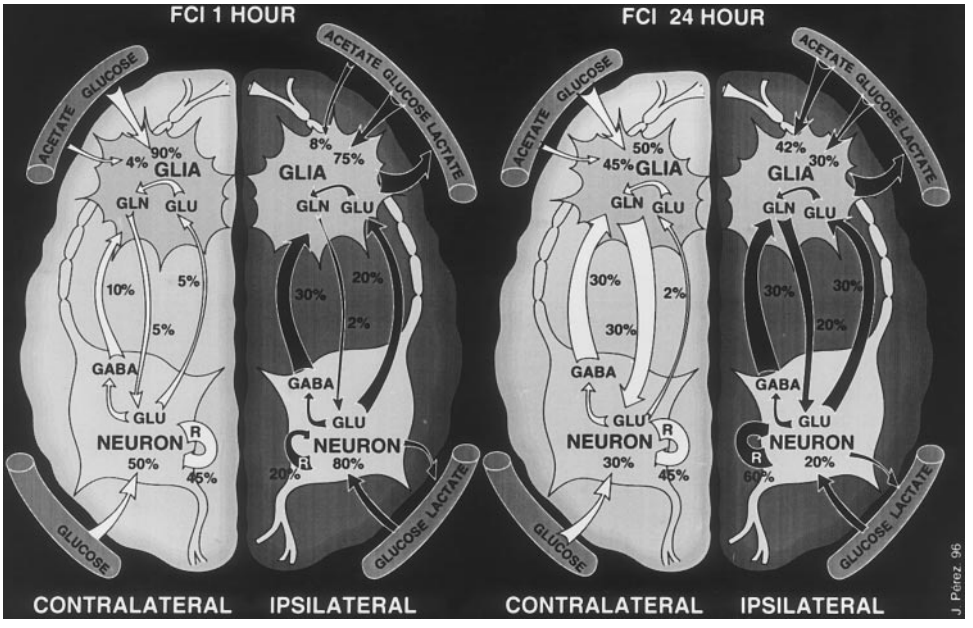

Figure 6. Summary of neuronal-glial interactions in both hemispheres of rat brain infused with (1,2-$^{13}C_2$) acetate, 1 and 24 hours after FCI. Primary cerebral substrates and metabolic end products in plasma are shown schematically in the blood vessels located outside the hemispheres. Exchanges of glutamate, glutamine, and GABA between the tricarboxylic acid cycles of neurons and glial cells are shown by arrows inside the hemispheres. The relative size of the arrows indicates approximately the relative contributions of the fluxes. Black arrows indicate ipsilateral fluxes; white arrows, contralateral fluxes. Numbers indicate the contribution (%) of the corresponding flux, taken from Figure 5.

present study (Figure 6) confirm this prediction showing increased oxidations of (^{13}C)* glutamate and (^{13}C) GABA in the glial tricarboxylic acid cycle and (^{13}C) glutamine in the neuronal tricarboxylic acid cycle of the adult brain in vivo, respectively. The detection of increased glutamate and GABA oxidation in the glial compartment agrees with previous observations in astrocyte cultures[30] and shows the important role of glial metabolism in regulating the extracellular concentrations of these amino acids in the ischemic brain tissue in situ.

Notably, an increased contribution of the previously described pyruvate recycling system[13] is observed in ipsilateral hemisphere of the 24-hour group as compared with the 1-hour group. Neuroprotective actions of this pathway may include providing the neurons with (1) an increased NADPH generation through mitochondrial and synaptosomal malic enzymes[40] and (2) a mechanism to generate acetyl-CoA from glial glutamine under conditions of reduced glucose oxidation.[14] Indeed, our current results show that the relative contribution of the pyruvate recycling system to the neuronal cycle increases in the ipsilateral hemisphere during the late phases of ischemic insult when glucose oxidation is reduced and neuronal glutamine oxidation is increased. Interestingly, recently bicarbonate has been shown to modulate glutamine synthesis in cultivated astrocytes.[41] Thus, a reduction in tissue bicarbonate caused by ischemic acidosis could contribute to the reduced neuronal glutamine oxidation and pyruvate recycling observed in the 1-hour group as compared with the 24-hour group. An alternative mechanism in which an astrocytic pyruvate recycling would provide the neurons with recycled lactate, rather than with glutamine, has been proposed, using astrocyte cultures prepared from neonatal brain tissues.[42,43] Recently, enzymes of this pathway have been shown to experience important changes in activity and cellular localization during ontogenic development, and malic enzyme and phosphoenolpyruvatecarboxykinase activities have been reported in neuronal mitochondria and synaptosomes prepared from adult brain tissues.[40,44] It should also be mentioned here that the use of lower doses of acetate could decrease the reported contributions of recycling.

In summary, our current results are consistent with important time-dependent changes in substrate selection during the development of ischemic brain damage. Glutamate and GABA or glutamine appear to be efficiently oxidized as alternative substrates to glucose by the glial or neuronal compartments of the adult brain in situ. Investigation of the effects of ischemic damage in the different perfusion zones surrounding the necrotic foci constitutes an attractive topic for future research in this field.

*We use the term (^{13}C) X to denote the total ^{13}C accumulated in metabolite X during the infusion of a ^{13}C-labeled substrate.

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Editorial Comment

In the mature brain, glucose is the predominant energy substrate with small contributions from plasma-derived lactate, β-hydroxybutyrate, and acetoacetate. In addition, components of the amino acid pool can exchange with intermediates of the tricarboxylic acid cycle (TCA) and thereby provide alternative energy substrates when glycolysis is reduced. During prolonged focal cerebral ischemia, glucose availability and acidosis may limit the activity of glycolytic and other energy-associated enzymes. Whether amino acids such as glutamate and glutamine, which are normally thought to cycle between neuronal and astrocytic compartments, undergo increased

oxidation as alternative energy substrates during ischemia in vivo is not well-established.

In the study of Pascual et al, the authors use an innovative approach of carbon-13 NMR spectroscopy to track changes of carbon-13 incorporation into key energy substrates and to allow computer simulation of metabolic pathways that can predict changes in glutamate, glutamine, and GABA oxidation during focal ischemia in the rat. Ischemia was induced by permanent middle cerebral artery occlusion plus bilateral carotid occlusion for 90 minutes. The critical feature of the experiment is the use of carbon-13 in both the one and two carbon positions of acetate. When these two adjacent carbons are incorporated into the TCA cycle, they provide both singlet and multiplet signatures on the NMR spectra for glutamate, glutamine, GABA, and lactate carbons. Labeled carbon positions depend on the number of turns of the TCA cycle and on a pyruvate cycling pathway. By assuming two metabolic compartment pools based in neurons and glia, the authors conclude (1) that oxidation of neuronally derived glutamate and GABA increases in the glial compartment after 90 minutes of focal ischemia and (2) that oxidation of glial-derived glutamine increases in the neuronal compartment in conjunction with an increase in activity of the pyruvate recycling pathway after 24 hours of focal ischemia. The latter effect of increased glutamine oxidation also occurred in cortex contralateral to the middle cerebral artery occlusion on the day after bilateral carotid occlusion. These results are consistent with the notions (1) that glia increase their utilization of carbon skeletons derived from amino acids for energy metabolism during the early period of focal ischemia and (2) that neurons outside the ischemic core and surviving the moderate ischemia associated with bilateral carotid occlusion increase their utilization of carbon skeletons derived from amino acids for energy metabolism 1 day after the insult.

In performing the computer simulation of the metabolic fluxes, the authors used the most simplified model consistent with the information content of their data. Reducing the model to the simplest elements is often necessary for computer iterations to converge on a solution. However, when applying these models to the in vivo situation with multiple neuronal and glial subtypes and subcellular compartments, some of which will be selectively altered by the ischemic insult, one needs to critically assess the simplifying assumptions of the model.

The main assumptions of the model are that (1) all of the labeled acetate is metabolized in the glial compartment, (2) all of the glutamine is synthesized in the glial compartment, and (3) all of the pyruvate recycling occurs in the neuronal compartment. These assumptions, which are based on reports from the literature and previous work from this laboratory, are reasonable first approximations. However, future studies need to be directed at validating these assumptions during ischemia and at further refining the model to include additional pathways.

For example, blood-brain barrier injury during ischemia may increase intracellular availability of ^{13}C-acetate relative to glucose, particularly in neurons if ^{13}C-acetate can more readily diffuse past astrocyte foot processes. Because ^{13}C-NMR is relatively insensitive, a 1.5 millimolar plasma concentration of labeled acetate was used rather than a tracer concentration. Thus, it is possible that substantial changes in intracellular acetate availability during ischemia could occur and influence the rate of glycolysis. The increased signal intensity across all peaks of the 24-hour spectra might be due to increased acetate transport across the barrier.

Another consideration is the gliosis and increased glial fibrillary acidic protein–staining typically seen 1 day after ischemia. Such phenotypic changes in astrocytes may be accompanied by an altered expression of metabolic enzymes. Thus, pyruvate recycling enzymes present in neonatal glial cultures could become prominent in postischemic astrocytes in vivo and modify the assumption of selective neuronal localization. Because the observed changes in the ratio of singlet-to-doublet resonances in C4 glutamate are presumed to reflect changes in pyruvate recycling activity, changes in cell localization of this pathway during ischemia will have a considerable impact on the data interpretation. Moreover, an increased fraction of the glutamine synthesized in glia may be metabolized by glutaminase in glial mitochondria rather than in neuronal mitochondria when neurons are selectively injured. Finally, the model does not consider the potential transport of carbon between astrocytes and neurons in the form of lactate, which is known to increase during ischemia.

This study breaks new ground in demonstrating increased use of alternative energy substrates during ischemia. However, further work is needed to validate the assumptions of the model in postischemic tissue, to expand the number of metabolic compartments and pathways, and to use other carbon-labeled substrates such as ^{13}C-glucose to complement the information derived from $^{13}C_2$-acetate.

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