Altered Astrocyte–Neuronal Interactions After Hypoxia-Ischemia in the Neonatal Brain in Female and Male Rats

Tora Sund Morken, MD, PhD; Eva Brekke, MD, PhD; Asta Håberg, MD, PhD; Marius Widerøe, MD, PhD; Ann-Mari Brubakk, MD, PhD; Ursula Sonnewald, PhD

Background and Purpose—Increased susceptibility to excitotoxicity of the neonatal brain after hypoxia-ischemia (HI) may be caused by limited capacity of astrocytes for glutamate uptake, and mitochondrial failure probably plays a key role in the delayed injury cascade. Male infants have poorer outcome than females after HI, possibly linked to differential intermediary metabolism.

Methods—[1-13C]glucose and [1,2-13C]acetate were injected at zero, 6, and 48 hours after unilateral HI in 7-day-old rats. Intermediary metabolism was analyzed with magnetic resonance spectroscopy.

Results—Mitochondrial metabolism was generally reduced in the ipsilateral hemisphere for ≤6 hours after HI, whereas contralaterally, it was reduced in neurons but not in astrocytes. Transfer of glutamate from neurons to astrocytes was increased in the contralateral, but not in the ipsilateral hemisphere at 0 hour, and reduced bilaterally at 6 hours after HI. The transfer of glutamine from astrocytes to glutamatergic neurons was unaltered in both hemispheres, whereas the transfer of glutamine to GABAergic neurons was increased ipsilaterally at 0 hour. Anaplerosis (astrocytes) was decreased, whereas partial pyruvate recycling (astrocytes) was increased directly after HI. Male pups had lower astrocytic mitochondrial metabolism than females immediately after HI, whereas that of females was reduced longer and encompassed both neurons and astrocytes.

Conclusions—The prolonged depression in mitochondrial metabolism indicates that mitochondria are vulnerable targets in the delayed injury after neonatal HI. The degree of astrocytic malfunction may be a valid indicator of outcome after hypoxic/HI brain injury and may be linked to the differential outcome in males and females. (Stroke. 2014;45:2777-2785.)

Key Words: cerebral hypoxia ischemia • magnetic resonance spectroscopy • neuron glia • rats

Neonatal hypoxia-ischemia (HI) is a major public health problem, and survivors may exhibit life-long disabilities and cognitive impairments. The stop in delivery of glucose and oxygen compromises mitochondrial oxidative metabolism, causing an immediate fall in energy levels and glutamate release. Subsequent receptor overstimulation initiates an excitotoxic injury cascade, of which many downstream mediators converge on and specifically target mitochondria. On re-establishment of cerebral blood flow and oxygen delivery to the tissue, mitochondrial oxidative metabolism resumes, leading to a not only transient recovery in energy levels but also oxidative stress. Because mitochondria are vulnerable to reactive oxygen species, they are not only generators but also targets of such stress. Permanent metabolic failure of this organelle probably plays a key role in the secondary decline in energy levels and delayed cell death, which characterize neonatal HI.

In normal neurotransmission in the adult brain, astrocytes protect against excitotoxicity through uptake and recycling of extracellular glutamate via conversion to glutamine in the glutamate–glutamine cycle. Interestingly, glutamate transfer from neurons to astrocytes is low in the neonatal brain, possibly because of low expression of astrocytic glutamate transporters. It is conceivable that this reduces the capacity of uptake of pathologically increased extracellular glutamate such as after HI. In combination with an abundance of hyper-sensitive glutamate receptors, this might explain the particular vulnerability to excitotoxicity of the neonatal brain.

Mitochondrial oxidative metabolism is also intimately coupled with tricarboxylic acid (TCA) cycling and synthesis of neurotransmitters glutamate, aspartate, and GABA. Astrocytes are essential for the preservation of these neurotransmitter pools through de novo synthesis dependent on...
pyruvate carboxylation and the above-mentioned recycling of glutamate in the glutamate-glutamine cycle. The role of astrocytes, as well as mitochondrial dysfunction as essential modulators of outcome after neonatal HI, has therefore recently gained increasing interest.

In this study, we investigated the temporal trajectory of neuronal and astrocytic mitochondrial metabolic function, neurotransmitter synthesis, and transfer of substrate between neurons and astrocytes in the (GABA)-glutamate-glutamine cycle during the first 48 hours after neonatal HI in a rat model. To this end, we used magnetic resonance spectroscopy after injection of [1-13C]glucose and [1,2-13C]acetate. The largest proportion of mitochondrial glucose metabolism takes place in neurons in the neonatal and in the adult brain. Acetate has been shown to be metabolized by astrocytes and not by neurons in the adult brain, even though the mechanism for this is unknown. The same has been demonstrated in neurons, and the mechanism for this is unknown. 

Methods
Details about materials, analytical methods, animal handling, and procedures can be found in the online-only Data Supplement.

Animal Procedures and Experimental Groups
All experiments were conducted in accordance with the European Communities Council Directive of 1986 (86/609/EEC) and were approved by the Norwegian Animal Research Authority (Forskningsdyrvalget). The Vannucci model for neonatal HI was used. P7 Sprague-Dawley rats were randomly distributed to either HI (males, n=16; females, n=9) or sham-operation (males, n=9; females, n=9). Animals were injected intraperitoneally with [1-13C]glucose (564 mg/kg; 0.3 mol/L) and [1,2-13C]acetate (504 mg/kg; 0.6 mol/L) at 1 of 3 time-points: directly (0 hour) after hypoxia; n=10 (HI), n=9 (sham); 6 hours after hypoxia: n=10 (HI); and 48 hours after hypoxia: n=9 (HI), n=9 (sham). One animal in the HI group injected at 0 hour had an unsuccessful injection. Therefore, n=9 for the 13C data, and n=10 for the 1H data in this group. Pups were decapitated 30 minutes after injection as described earlier, blood was collected, and plasma samples were separated. Heads were snap-frozen in liquid N, and stored at −80°C until extraction. Plasma samples were volume corrected, and the ipsilateral and contralateral hemispheres were dissected, weighed, and extracted separately using a methanol–chloroform method.

High-Performance Liquid Chromatography
Amino acids in tissue extracts were quantified by high-performance liquid chromatography on a Hewlett Packard 100 system (Agilent Technologies, Palo Alto, CA) as earlier described.

13C and 1H Magnetic Resonance Spectroscopy
Lyophilized samples were dissolved in 120 μL D,O containing ethylene glycol and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TSP). 1H and proton decoupled 13C magnetic resonance spectra were obtained using a 14.1T Avance III magnet (Bruker BioSpin GmbH, Rheinstetten, Germany) using a QCI CryoProbe. Total amounts of metabolites were quantified using the 1H spectra, corrected for 13C containing isotopomers. Metabolites labeled with 13C were quantified from 13C spectra and corrected for naturally abundant 13C. These 13C-labeled substances give information about the different metabolic pathways used by [1-13C]glucose (metabolized in neurons to a larger extent than in astrocytes) and [1,2-13C]acetate (probes astrocyte metabolism; Figure 1). Using this information, acetate metabolism and the amount of glucose metabolized via glycolysis, pyruvate carboxylase (in astrocytes only) and pyruvate dehydrogenase (PDH) could be approximated in addition to glutamate and glutamine transfer between neurons and astrocytes. For details on spectral acquisition, labeling patterns, and calculations of metabolic ratios see online-only Data Supplement.

Statistics
Data are presented as mean±SEM unless stated otherwise. For 2-group comparisons Student t test was used, whereas for 3-group comparisons 1-way ANOVA with post hoc Bonferroni correction was applied. A P<0.05, Bonferroni corrected, was chosen as level of significance. Statistical analyses were performed in SPSS version 19.0 (IBM, Chicago, IL).

Results
Bodyweight and weight of hemispheres were similar across experimental groups (for numbers see online-only Data Supplement). Plasma samples were analyzed using magnetic resonance spectroscopy and no differences in glucose, acetate,
Glycolysis
Immediately after HI, total lactate and \([3-^{13}C]\)lactate were increased 4- and 3-fold in the ipsilateral and contralateral hemispheres, respectively, whereas percentage enrichment was unchanged. At 6 hours, levels were normalized. Alanine and \([3-^{13}C]\)alanine were increased 7- and 5-fold in the ipsilateral and contralateral hemispheres, respectively, and were still increased at 6 hours. Percentage enrichment with \([3-^{13}C]\)alanine was decreased at both 0 and 6 hours after HI (Table I in the online-only Data Supplement).

Total Amounts and Labeling of Amino Acids
Total glutamate content after HI was unaltered versus sham at all time-points. Glutamine content was lower immediately after HI in the ipsilateral when compared with that in the contralateral hemisphere, but by 6 hours it was higher in both hemispheres when compared with sham. GABA content was elevated when compared with sham in both hemispheres at 0 hour with ipsilateral GABA levels being higher than the contralateral. In both hemispheres, GABA was normalized by 6 hours. Aspartate levels were lower than sham at 0 hours in both hemispheres and was normalized by 6 hours (Figure 2).

Labeling from \([1-^{13}C]\)glucose via PDH-producing \([4-^{13}C]\)glutamate, \([4-^{13}C]\)glutamine, \([2-^{13}C]\)GABA, and \([2-^{13}C]\)aspartate was reduced after HI at both 0 and 6 hours in the ipsilateral hemisphere versus sham.

In the contralateral hemisphere, labeling from \([1-^{13}C]\)glucose via PDH was reduced in the same metabolites except for production of \([4-^{13}C]\)glutamine, which was unaltered at 0 and reduced at 6 hours.

Labeling from \([1,2-^{13}C]\)acetate producing \([4,5-^{13}C]\)glutamate was reduced at 0 and 6 hours after HI in the ipsilateral hemisphere versus sham. Amounts of \([4,5,^{13}C]\)glutamine and \([1,2-^{13}C]\)aspartate were reduced at 0 hour and normalized by 6 hours after HI, whereas \([1,2-^{13}C]\)GABA was unaltered at all time-points after HI.

In the contralateral hemisphere, label incorporation from \([1,2-^{13}C]\)acetate into glutamate, glutamine, and GABA was unaltered at 0 hour after HI, whereas in aspartate it was lower when compared with sham.

Transfer Ratios
The transfer of glutamate from glutamatergic neurons to astrocytes was unaltered at 0 hour but was lower at 6 hours after HI in the ipsilateral hemisphere versus sham. The transfer was higher in the contralateral hemisphere than in both the ipsilateral hemisphere and in sham at 0 hour after HI and lower than sham at 6 hours after HI (Figure 3).

The transfer from astrocytes to glutamatergic neurons was unaltered in both hemispheres when compared with sham at all time-points after HI. It was higher in the ipsilateral when compared with the contralateral hemisphere at 0 hour.

The transfer of glutamine from astrocytes to GABAergic neurons was higher in the ipsilateral hemisphere when compared with that in both sham and the contralateral hemisphere at 0 hour and was normalized by 6 hours after HI. In the contralateral hemisphere, this transfer was unaltered at all time-points after HI.

Pyruvate Anaplerosis and Cataplerosis
Pyruvate carboxylation (anaplerosis) was decreased immediately after HI in both hemispheres. The conversion of oxaloacetate or malate into pyruvate represents the first step in pyruvate recycling and will be termed pyruvate cataplerosis hereafter. This was seen in the amounts of \([1,2,^{13}C]\)lactate and \([1,2-^{13}C]\)alanine, which were both elevated immediately after HI. Although there was no detectable \([2-^{13}C]\)lactate/alanine above natural abundance at any time-points (Figure 4).

Sex Differences
After HI, the amount and percentage enrichment with \([4,5,^{13}C]\)glutamate from \([1,2-^{13}C]\)acetate was lower in males than females at 0 hours after HI. Furthermore, in male HI animals amount and percentage enrichment with \([4,5,^{13}C]\)glutamine was reduced versus male shams. However, labeling from \([1,2-^{13}C]\)acetate in glutamate was still depressed in females at 6 hours after HI versus female shams, whereas that of males was normalized. The amount and percentage enrichment with \([4-^{13}C]\)glutamate/glutamine from \([1-^{13}C]\)glucose were similar between the sexes at 0 hour after HI but were lower at 6 hours in female than in male HI animals. There were no sex differences in shams or in the HI group at 48 hours after HI (Table).

Discussion
Glycolysis and Pyruvate Metabolism
Glucose and Lactate
It is well known that during HI, there is depletion of glucose content because of low glucose delivery and increased anaerobic glycolysis with a compensatory increase in glucose transporters in the hours after HI. Our data are in agreement with such an increase in anaerobic glycolytic activity during and immediately after HI not only in the ipsilateral but also in the contralateral hemisphere, where the elevation in lactate content immediately after HI may result from a similar increase caused by hypoxia. This elevation was also found in plasma, as expected from the whole-body hypoxia. However, at 6 hours after HI, the lactate content was no longer elevated in either hemisphere, indicating rapid normalization of glycolysis in brain and body. This is in accordance with earlier reports of neonatal HI but contrasts the prolonged lactate elevation that follows ischemia in adult brain, possibly explained by the high capability of the neonatal brain to use lactate as an energy substrate, as well as its high lactate transport capacity over the blood brain barrier.

Alanine
Alanine and \([3-^{13}C]\)alanine levels were increased in both hemispheres for ≤6 hours in agreement with earlier reports on neonatal HI and adult ischemia in rat brain. The fall in percentage \(^{13}C\)-enrichment indicates that the alanine increase occurred mainly during HI, before label was injected. The concurrent increase in valine, phenylalanine, isoleucine, leucine,
Figure 2. Total and labeled amounts of amino acids: brain extracts of P7 rats injected with [1-13C]glucose and [1,2-13C]acetate were analyzed using 1H-MRS (total amounts) and 13C-MRS (labeled amounts). Number of animals: 0-hour sham: 9; 0-hour hypoxia-ischemia (HI): 10 for 1H data, 9 for 13C data; 6-hour HI: 10; 48-hour sham: 9; 48-hour HI: 9. Results are presented as mean±SEM. Open circle, sham; black square, HI and ipsilateral hemisphere; gray triangle, HI and contralateral hemisphere. A, Total amounts of glutamate, glutamine, GABA and aspartate. B and C, Labeled amounts of the same amino acids resulting from metabolism of [1-13C]glucose or [1,2-13C]acetate, respectively. Significant differences (P<0.05) are indicated: *ipsilateral vs sham, +contralateral vs sham, §ipsilateral vs contralateral. Same symbols as groups (in brackets), comparisons between different time-points within group. MRS indicates magnetic resonance spectroscopy.
Figure 3. Transfer of glutamate/glutamine between neurons and astrocytes: brain extracts of P7 rats injected with [1-13C]glucose and [1,2-13C]acetate were analyzed using 1H-MRS (total amounts for calculation of percentage enrichment) and 13C-MRS (labeled amounts). Number of animals: 0-hour sham: 9; 0-hour hypoxia-ischemia (HI): 9; 6-hour HI: 10; 48-hour sham: 9; 48-hour HI: 9. The cartoons illustrate the principles for the equations used to calculate transfer shown in the graphs. For details, see Methods in the online-only Data Supplement. The large squares represent total amounts of glutamate/glutamine, whereas the smaller dark squares represent labeled amounts. A proportion of the unlabeled and labeled amino acids is transferred from one cell type to the other. The sizes do not reflect exact stoichiometric relations.

A, Transfer from glutamatergic neurons to astrocytes in nanomole per gram during 30 minutes. B, Transfer from astrocytes to glutamatergic neurons in nanomole per gram during 30 minutes. C, Transfer from astrocytes to GABAergic neurons in nanomole per gram during 30 minutes. Results are presented as mean±SEM. Open circle, sham; black square, HI and ipsilateral hemisphere; gray triangle, HI and contralateral hemisphere. Significant differences (P<0.05) are indicated: *ipsilateral vs sham, +contralateral vs sham, §ipsilateral vs contralateral. Same symbols as groups (in brackets), comparisons between different time-points within group. GAD indicates glutamic acid decarboxylase; GS, glutamine synthetase; MRS, magnetic resonance spectroscopy; PAG, phosphate activated glutaminase; PDH, pyruvate dehydrogenase; and TCA, tricarboxylic acid.
and lysine (results not shown), which are substrates for transamination, indicates depression in the formation of glutamate from α-ketoglutarate ≤6 hours after HI. Cataplerosis via pyruvate production from TCA cycle intermediates, which happens in astrocytes only, may also have contributed to the elevations in lactate and alanine, as discussed below.

Mitochondrial Metabolism and Transfer Between Neurons and Astrocytes

Neuronal Mitochondrial Metabolism
Neuronal mitochondrial metabolism of acetyl CoA from [1-13C]glucose was depressed in both hemispheres ≤6 hours after HI, a time-point well beyond complete reperfusion and re-establishment of oxygen delivery to the tissue. This supports that prolonged post-HI impairment of mitochondrial metabolism is caused by other damaging factors in the excito-oxidative cascade than lack of oxygen, such as oxidative stress and excitotoxicity. It should be noted that glutamatergic excitotoxicity is a major cause of neuronal death in neonatal HI.

Astrocytic Mitochondrial Metabolism
Neurons are generally assumed to be more vulnerable to HI than astrocytes. Nonetheless, astrocytic mitochondrial metabolism was clearly affected in the ipsilateral hemisphere, seen in the reduction in amounts of [4,5,6-13C]glutamate/glutamine labeled from [1,2-13C]acetate immediately after HI with full and partial recovery by 6 hours in glutamine and glutamate, respectively. This is in agreement with in vitro studies demonstrating vulnerability of astrocytes to hypoxia and oxygen glucose deprivation. In contrast, mitochondrial metabolism of [1,2-13C]acetate in the contralateral hemisphere was unaltered in the above-mentioned metabolites. Thus, astrocytic mitochondrial metabolism was better preserved in the hemisphere that was exposed to hypoxia only (contralateral), whereas neurons were severely affected after both hypoxic (contralateral) and hyperoxic (ipsilateral) HI.30,31

Figure 4. Pyruvate carboxylation and pyruvate recycling in astrocytes: brain extracts of P7 rats injected with [1,2-13C]acetate were analyzed using 13C-MRS. Number of animals: 0-hour sham: 9; 0-hour hypoxia-ischemia (HI): 9; 6-hour HI: 10; 48-hour sham: 9; 48-hour HI: 9. Results are given in nmol/g of compound accumulated in the time between injection and euthanasia, which was 30 min. A, Anaplerosis is necessary for net formation of glutamate, glutamine, and GABA. The difference between [3-13C]aspartate and [2-13C]aspartate reflects pyruvate carboxylase (PC) activity. B, Pyruvate recycling is necessary for complete oxidation of glutamate, glutamine, and GABA and can be divided in a cataplerotic step and re-entry into the tricarboxylic acid (TCA) cycle. The amount of [1,2-13C]lactate/alanine reflects the cataplerotic step in astrocytes. [1,2-13C]alanine was only observed in HI animals at 0 hour (shown as bars at 0 hour), whereas [1,2-13C]lactate was present at all time-points in all groups (shown as points). For more details, see Figure 1 for labeling patterns and Methods in the online-only Data Supplement. Results are presented as mean±SEM. Open circle, sham; black square or black bar, HI and ipsilateral hemisphere; gray triangle or gray bar, HI and contralateral hemisphere. Significant differences (P<0.05) are indicated: *ipsilateral vs sham, +contralateral vs sham. Same symbols as groups (in brackets), comparisons between different time-points within group. GAD indicates glutamic acid decarboxylase; GS, glutamine synthetase; ME, malic enzyme; MRS, magnetic resonance spectroscopy; PAG, phosphate activated glutaminase; PDH, pyruvate dehydrogenase; and PEPCK, phosphoenolpyruvate carboxykinase.
HI (ipsilateral) conditions. Moreover, it has been shown in adult animals that PDH activity is severely depressed after ischemia/reperfusion injury.\textsuperscript{32,33} If this is also the case in the neonate, one could speculate that decreased PDH activity would inhibit glucose consumption but not ketone-body and \textsuperscript{1,2-13C}acetate metabolism, and this might explain some of the findings of preserved glial metabolism when compared with neuronal deficits in the present study. Furthermore, the preservation of astrocytic function and thereby the probably preserved metabolic support after hypoxia exposure may explain why the neuronal mitochondrial hypometabolism of the contralateral hemisphere that was observed in the present study usually does not produce tissue damage in this model of neonatal HI.\textsuperscript{21} Thus, the degree of astrocytic malfunction may be a determining factor for the outcome after hypoxic and HI brain injury.

Neuronal Transfer of Glutamate to Astrocytes

During HI, there is a large release of glutamate that may originate both from neurons and astrocytes, as well as reversal of glutamate transporters.\textsuperscript{34,35} In the contralateral hemisphere, glutamate transfer seemed to be increased after hypoxia, indicating that the astrocytes were able to increase their uptake of glutamate in this phase and thus counteracted excitotoxicity. However, in the ipsilateral hemisphere, no such increase occurred. Both glutamate uptake and glutamine synthesis are energy-demanding processes, and increased transfer of glutamate in the contralateral hemisphere is, therefore, in agreement with better astrocytic function when compared with the ipsilateral hemisphere.

A decrease in transfer of glutamate from neurons to astrocytes was observed 6 hours after HI in both hemispheres, despite unaltered glutamate content. This may be because of reduced release of glutamate from neurons as a compensatory mechanism after the preceding large release.\textsuperscript{34}

As reported earlier,\textsuperscript{36,37} the total glutamine content was increased (≈150\%) in the brain at 6 hours after HI. In adult brain, it has been suggested that this increase stems from conversion of glutamate released by neurons after ischemia.\textsuperscript{24} However, our data indicate decreased transfer of glutamate from neurons to astrocytes, and when considering the large magnitude of the elevation (>1000 nmol/g) and that the measured glutamine is unlabeled, it is unlikely that it is derived from the glutamate released from neurons, which is \textsuperscript{13C} labeled. Rather, the elevated brain glutamine probably originated from the circulation because hypoxia also leads to liver malfunction and systemic rise in ammonia.\textsuperscript{38,39}

Transfer of Glutamine From Astrocytes to Glutamatergic Neurons

The transfer of glutamine from astrocytes to glutamatergic neurons was similar to sham at all time-points after HI in both hemispheres, indicating that even though the ipsilateral mitochondrial metabolism in astrocytes was reduced, the relative contribution of astrocytic glutamine to the neuronal glutamate pool was unaltered.

GABAergic Neurons and Transfer of Glutamine From Astrocytes

Total GABA was elevated immediately after HI in both hemispheres, but most pronounced in the ipsilateral, in agreement with earlier reports.\textsuperscript{14,36} Because GABA has excitatory properties in the neonatal brain,\textsuperscript{40} this may contribute to excitotoxicity. The elevation was caused by reduced degradation of GABA, and not increased synthesis, because there was no increase in \textsuperscript{[13C]}GABA. The first step of GABA catabolism is transamination leading to the formation of glutamate. The accumulation of GABA because of reduced degradation is, therefore, in agreement with the earlier mentioned elevations in other transamination substrates (alanine and the branched chain amino acids) and lends further support to a decrease in the formation of glutamate from α-ketoglutarate.

Interestingly, there was no detectable labeling of GABA from \textsuperscript{[1-13C]}glucose immediately after HI, and low labeling at 6 hours, suggesting severe impairment of mitochondrial metabolism after the preceding large release.\textsuperscript{34} However, our data indicate decreased transfer of glutamate from neurons to astrocytes, and when considering the large magnitude of the elevation (>1000 nmol/g) and that the measured glutamine is unlabeled, it is unlikely that it is derived from the glutamate released from neurons, which is \textsuperscript{13C} labeled. Rather, the elevated brain glutamine probably originated from the circulation because hypoxia also leads to liver malfunction and systemic rise in ammonia.\textsuperscript{38,39}

### Table. | Sex Differences in Glutamate and Glutamine Amounts (nmol/g Brain Tissue) and % in Response to HI |
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<td>Female</td>
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<td>Sham</td>
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<tr>
<td>[4,5-13C]glutamate</td>
<td>190±8</td>
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<tr>
<td>[4,5-13C]glutamine</td>
<td>186±22</td>
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<tr>
<td>% [4,5-13C]glutamate</td>
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<tr>
<td>% [4,5-13C]glutamine</td>
<td>8.1±0.7</td>
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<tr>
<td>[4-13C]glutamate</td>
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<td>[4-13C]glutamine</td>
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<td>% [4-13C]glutamate</td>
<td>1.9±0.4</td>
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<td>% [4-13C]glutamine</td>
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Brain extracts of P7 rats injected with \textsuperscript{[1-13C]}glucose and \textsuperscript{[1,2-13C]}acetate were analyzed using \textsuperscript{1H} -MRS (total amounts for calculation of percentage enrichment) and \textsuperscript{13C}-MRS (labeled amounts). For more details see online-only Data Supplement. Results are presented nmol/g brain tissue or % of total as mean±SEM. HI indicates hypoxia-ischemia; nd, not detectable; and MRS, magnetic resonance spectroscopy.

\textsuperscript{*}P<0.05 vs sham; †P<0.05 male vs female; ‡P≤0.001 vs sham.
metabolism in the GABAergic neurons. In contrast, the amount of GABA labeled from [1,2-13C]acetate was preserved at all time-points, and the transfer of glutamine from astrocytes to GABAergic neurons was in fact increased immediately after HI. This indicates that astrocytes supplied GABAergic neurons with metabolic support in the form of glutamine in the acute phase after HI, and that GABAergic neurons were capable of synthesizing GABA from this glutamine via glutamate, even though mitochondrial metabolism was depressed at this time-point.

Pyruvate Carboxylation in Astrocytes (Anaplerosis)
Anaplerosis via carboxylation of pyruvate was decreased in both hemispheres after HI seen in the labeling in aspartate and in total aspartate, in accordance with earlier reports. However, in agreement with our earlier findings, the carboxylation of pyruvate relative to dehydrogenation (pyruvate carboxylase/PDH ratio) in the present study was similar to sham (data not shown), indicating a relative preservation of anaplerosis after HI. This contrasts adult brain ischemia where there is a larger decrease in carboxylation than dehydrogenation of pyruvate. In combination with the preserved or increased transfer of glutamine from astrocytes to neurons, such a relative preservation of anaplerosis may have both beneficial (supply of substrate for TCA cycling) and harmful effects (supply of de novo neurotransmitters to compromised neurons in a period of ongoing excitotoxicity).

Pyruvate Recycling (Cataplerosis)
[1,2-13C]lactate/alanine produced from [1,2-13C]acetate was elevated after HI, whereas there was no detectable [2-13C]lactate/alanine produced from [1-13C]glucose, indicating increase in the cataplerotic step of pyruvate recycling in astrocytes but not in neurons. When malic enzyme is involved, this cataplerotic step generates the reducing agent NADPH, while re-entry of pyruvate into the TCA cycle, representing complete pyruvate recycling and thus complete oxidation of substrates, such as glutamate, may potentially be a source of ATP. Both mechanisms could be protective after HI, and pyruvate recycling is elevated after adult brain ischemia. Interestingly, a neuroprotective effect of pyruvate supplementation after neonatal HI has been reported. However, although the cataplerotic step was clearly increased in the present study, no increase in re-entry of pyruvate into the TCA cycle was detected in [13C] glutamate.

Sex Differences
It is well known that male neonates have a poorer neurodevelopmental outcome after brain injury when compared with females. We observed differences in metabolism between sexes, indicating that the neonatal male brain has a larger immediate depression in astrocytic function after HI but recovers more rapidly. In comparison, the female brain has a less severe immediate but more prolonged depression that encompasses both neurons and astrocytes. It is conceivable that these differences in mitochondrial metabolism may be connected to the identified sex-linked differential apoptotic pathways in the delayed injury cascade after HI or increased susceptibility to oxidative stress in astrocytes from male brains.

Clinical Implications
In the present study, prolonged and pronounced posts ischemic mitochondrial defects, which have been suspected for decades, were demonstrated. These findings point to the mitochondrion not only as a vulnerable organelle in the delayed injury cascade after HI but also as a potential target for therapeutic agents. Furthermore, striking differences in astrocytic function between the contralateral and ipsilateral hemisphere were found, whereas neurons were similarly depressed in both hemispheres. In this model of HI, the contralateral hemisphere is apparently unaffected, and a preserved astrocytic mitochondrial metabolism and increased uptake of glutamate after HI may be the key factors that protect this hemisphere from tissue damage, even when neuronal mitochondrial function is severely compromised. Whether improving astrocytic post-HI metabolic function may represent a future treatment should be investigated. Also, our findings of more severely impaired astrocyte metabolism in males may be connected to the poorer neurodevelopmental outcome in male infants and emphasize the need to study both sexes separately after neonatal HI. Finally, the presence of pyruvate cataplerosis implies that pyruvate recycling may be present in the neonatal brain after HI, which could potentially be a neuroprotective measure that may be exploited in future after HI treatment.

Conclusions
The pronounced and prolonged depression in mitochondrial metabolism agrees with emerging evidence of mitochondria as vulnerable targets in the delayed injury cascade after neonatal HI. Furthermore, the degree of astrocytic malfunction may be a valid indicator of outcome after hypoxic and HI brain injury in neonates. Our findings point to mitochondria, pyruvate recycling, and in particular astrocytes as potential therapeutic targets.

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Disclosures
None.

References


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Supplemental Methods and Materials

Materials

[1,13C]Glucose and [1,2,13C]acetate (99% 13C enriched) were from Cambridge Isotope Laboratories (Woburn, MA), D2O (99.9%) from CDN Isotopes (Pointe-Claire, Canada), ethylene glycol from Acros Organics (Geel, Belgium) and 3-(trimethylsilyl)-1-propane-sulfonic acid (TSP) from Sigma-Aldrich (St. Louis, MA). All other chemicals were of the purest grade available from regular commercial sources.

Animals

Four Sprague-Dawley rats (Scanbur AS, Norway), mated at the Comparative Medicine Core Facility at the Norwegian University of Science and Technology (Trondheim, Norway), gave birth to 48 pups. Dams with litter were kept on a 12:12 hours light:dark cycle with food and water ad libitum. At P7 sexed pups with average bodyweight of 12.8 ± 1.0 g (mean ± 95% CI) were randomly distributed so that each experimental group contained 2-3 pups from each litter.

Hypoxia-ischemia

P7 rats were anaesthetized with isoflurane (4% induction, 2% maintenance in O2; Baxter, Allerød, Denmark) and placed on a water-heated thermal pad (Gaymar Industries Ltd., Orchard Park, NY). The right common carotid artery was identified, thermo-cauterized and severed. In sham-operated littersmates the carotid artery was identified under anesthesia, but not severed. The surgical procedure including induction of and recovery from anesthesia took <10 minutes. Pups were returned to their dam for recovery and feeding for a minimum of two hours and a maximum of three hours. Thereafter pups were put in a fiberglass box inside an incubator where temperature and oxygen concentrations were constantly monitored. Temperature was kept at 36 ± 0.5 ºC. The box was flushed with pre-heated humidified 8% O2 in 92% N2 until oxygen concentration in the box was 8% after which the flow was kept at 5L/min keeping the oxygen concentration at 8% for 90 min. During hypoxia sham-operated littersmates were kept separate from the dam in a heated atmosphere (36 ± 0.5 ºC).

MR spectroscopy

MR spectroscopy was performed on a 14.1T Ultrashielded Plus Avance III magnet (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 600 MHz (for 1H) using a QCI CryoProbeTM. 1H-MR spectra were accumulated with a pulse angle of 90°, 2.7s acquisition time and 10s relaxation delay. The number of scans was 128. Proton decoupled 13C MR spectra were obtained using an acquisition time of 1.7s, 0.5s relaxation delay and a 30° pulse angle. Scans were accumulated at 30 kHz spectral width with 98 K data points. The number of scans was typically 11000. Relevant peaks in the spectra were identified and integrated using TopSpinTM 3.0 software (Bruker BioSpin GmbH, Rheinstetten, Germany). Concentrations of metabolites were calculated from the integrals of the peaks using TSP (1H-MR spectra) or ethylene glycol (13C-MR spectra) as internal quantification standards. Concentrations from the 1H-MR spectra were corrected for the number of protons that constituted the peak. Correction factors for incomplete relaxation and nuclear Overhauser effects in the 13C spectra were applied to all spectra. The integrals of singlets from the 13C MR spectra were corrected for naturally abundant 13C.
Percent excess $^{13}$C enrichment was calculated after subtracting natural abundance where appropriate, and is in the following referred to as % enrichment.

Glucose percent enrichment was calculated from the $^1$H-MR spectra using the α-glucose satellites, representing protons on the $^{13}$C labeled glucose in the α-C1 position, over the sum of unlabeled α-glucose and its satellites.

**Metabolic model**
We used $^1$H and $^{13}$C MR spectroscopy after injection of [1-$^{13}$C]glucose and [1,2-$^{13}$C]acetate to study metabolism. Even though neonates metabolize ketone bodies to a larger extent than adults, glucose still contributes more than half of the overall energy. However, at the acetyl CoA stage, the largest proportion is metabolized in the neuronal TCA cycle. It has been calculated that at least 66% of the glucose at the level of acetyl CoA is metabolized in neurons also in the neonatal brain. Therefore, the labeling originating from glucose in TCA cycle intermediates as well as in glutamate and GABA will mostly reflect neuronal metabolism.

Hassel et al have shown that approximately 40% of glutamine is labelled from neuronal glutamate derived from [1-$^{13}$C]glucose. Also, pyruvate carboxylase is only present in astrocytes. Therefore, any labeling originating from pyruvate carboxylation must have taken place in astrocytes.

Acetate, in the adult, has been shown to be metabolized by astrocytes and not by neurons, even though the mechanism for this is unknown. We have demonstrated that the same is the case in the neonatal brain. Thus, acetate can be used to probe astrocytic metabolism.

Moreover, the major glutamate pool is localized in glutamatergic neurons. Therefore, the only way of getting substantial labeling from acetate in glutamate is through metabolism of acetate in astrocytes leading to labeling in glutamine which is later transferred to neurons and converted into glutamate.

Thus, analysis of labeling patterns following injection of [1-$^{13}$C]glucose and [1,2-$^{13}$C]acetate with particular emphasis on labeling patterns in glutamate and glutamine provides information of differential metabolism in neurons, and astrocytes, and the transfer of glutamate from neurons to astrocytes as well as transfer of glutamate from astrocytes to neurons.

**Labeling from [1-$^{13}$C]glucose and [1,2-$^{13}$C]acetate**
Labeling patterns are shown in Figure 1 in the main article. For additional figures illustrating this see Morken et al and Melø et al.

**Labeling patterns after injection of [1-$^{13}$C]glucose**
When metabolized via glycolysis, [1-$^{13}$C]glucose will yield one molecule of unlabeled pyruvate (not discussed further) and one molecule of [3-$^{13}$C]pyruvate. If the pyruvate is metabolized via pyruvate dehydrogenase (PDH) it leads to [2-$^{13}$C]acetyl CoA which can condense with oxaloacetate to form [2-$^{13}$C]citrate. After conversion to cis-aconitate and isocitrate, α-[4-$^{13}$C]ketoglutarate will be formed which can give rise to [4-$^{13}$C]glutamate, [4-$^{13}$C]glutamine and [2-$^{13}$C]GABA (from [4-$^{13}$C]glutamate). If α-[4-$^{13}$C]ketoglutarate stays
in the TCA cycle, the $^{13}$C label will be scrambled in the symmetrical molecule succinate to yield equal amounts of $[2^{-13}C]$ or $[3^{-13}C]$succinate, fumarate, malate, oxaloacetate and aspartate. $[2^{-13}C]$ or $[3^{-13}C]$oxaloacetate can condense with acetyl CoA and yield, $[2^{-13}C]$ or $[3^{-13}C]$glutamate/glutamine and $[3^{-13}C]$GABA from the 2nd turn of the TCA cycle if the acetyl-CoA is unlabeled. If labeled, the molecules will in addition be labeled in the positions mentioned above from the 1st turn of the TCA cycle ($[2,4^{-13}C]$glutamate/glutamine or $[3,4^{-13}C]$glutamate/glutamine and $[2,3^{-13}C]$GABA or $[2,4^{-13}C]$GABA). Glycolysis followed by pyruvate carboxylation via PC will generate label in $[3^{-13}C]$oxaloacetate which can be transaminated to $[3^{-13}C]$aspartate. If the $[3^{-13}C]$oxaloacetate condenses with unlabeled acetyl CoA it leads to formation of $[4^{-13}C]$citrate and thereafter $[2^{-13}C]$glutamate/glutamine followed by equal amounts of $[1^{-13}C]$- and $[4^{-13}C]$succinate, fumarate, malate, oxaloacetate and aspartate. However, if $[3^{-13}C]$oxaloacetate equilibrates to succinate (backflux$^{15}$) it leads to equal amounts of $[3^{-13}C]$- and $[2^{-13}C]$succinate, fumarate, malate, oxaloacetate and aspartate.

**Labeling patterns after injection of $[1,2^{-13}C]$acetate (Figure 1 in main article)**

$[1,2^{-13}C]$acetate will enter the TCA cycle via $[1,2^{-13}C]$acetyl CoA and yield $[4,5^{-13}C]$glutamate/glutamine and $[1,2^{-13}C]$GABA. If the label remains in the TCA cycle for a 2nd turn, the product will be equal amounts of $[1,2^{-13}C]$- and $[3^{-13}C]$glutamate/glutamine and $[3^{-13}C]$- and $[4^{-13}C]$GABA.

**Pyruvate cataplerosis**

The process by which oxaloacetate or malate is converted to pyruvate and subsequently acetyl CoA which can re-enter the TCA cycle is termed pyruvate recycling.$^{16}$ The conversion of oxaloacetate or malate into pyruvate represents the first step in pyruvate recycling and will in the following be termed pyruvate cataplerosis. Both glucose metabolism via PDH and PC as well as acetate metabolism can lead to $[1^{-13}C]$-, $[2^{-13}C]$- and $[3^{-13}C]$oxaloacetate (mentioned above or through subsequent cycling) and consequently $[1^{-13}C]$-, $[2^{-13}C]$- and $[3^{-13}C]$pyruvate through pyruvate recycling. However, from the first turn of the TCA cycle, metabolism of $[1^{-13}C]$glucose via PDH will lead to equal labeling of $[2^{-13}C]$- and $[3^{-15}C]$pyruvate through pyruvate cataplerosis. Thus, the amount of $[2^{-13}C]$lactate and $[2^{-13}C]$alanine should reflect neuronal pyruvate recycling from $[1^{-15}C]$glucose metabolism. Following injection of $[1,2^{-13}C]$acetate, the first step in such pyruvate recycling will be seen as $[1,2^{-13}C]$lactate/alanine (the result of conversion of $[1,2^{-13}C]$oxaloacetate/malate to $[1,2^{-13}C]$pyruvate) and $[3^{-13}C]$lactate/alanine. Thus, the amount of $[1,2^{-13}C]$lactate and $[1,2^{-13}C]$alanine should reflect astrocytic pyruvate recycling from $[1,2^{-13}C]$acetate metabolism.

**Metabolic ratios (see sections above for explanation of labeling patterns)**

**Transfer of glutamate from neurons to astrocytes (Figure 3A in main article)**

In the adult$^4$ as well as in the neonatal brain,$^5$ at least 66 % of the glucose at the level of acetyl CoA is metabolized via PDH in neurons. Furthermore, the major glutamate pool is localized in glutamatergic neurons$^13$ and we can therefore assume that $[4^{-13}C]$glutamate resulting from PDH metabolism of $[3^{-13}C]$pyruvate from glycolysis of $[1^{-15}C]$glucose is mainly labeled in neurons. Glutamate is released by neurons and taken up by astrocytes to end neurotransmission. $[4^{-13}C]$Glutamate will give rise to $[4^{-13}C]$glutamine which consequently represents glutamine synthesized from glutamate that has been transferred from the neuronal compartment. The amount of transfer of glutamate from neurons to astrocytes during the time from injection to decapitation can be expressed as:
Transfer of glutamine from astrocytes to neurons (Figure 3B and C in the main article)

Acetate is predominantly metabolized in astrocytes.\textsuperscript{17} Metabolism of [1,2-\textsuperscript{13}C]acetate leads to labeling of [4,5-\textsuperscript{13}C]glutamate. [4,5-\textsuperscript{13}C]glutamate in astrocytes is rapidly converted to [4,5-\textsuperscript{13}C]glutamine via glutamine synthetase (GS) which is exclusively localized in astrocytes.\textsuperscript{18} GS activity is high in the neonatal brain.\textsuperscript{19} [4,5-\textsuperscript{13}C]Glutamine is then sent to neurons to be converted to [4,5-\textsuperscript{13}C]glutamate. Because most of the glutamate in the brain is localized in the neuronal compartment,\textsuperscript{13} formation of most [4,5-\textsuperscript{13}C]glutamate happens when [4,5-\textsuperscript{13}C]glutamine is transferred from the astrocytic to the neuronal compartment. Therefore, the ratio between [4,5-\textsuperscript{13}C]glutamate and % enrichment in [4,5-\textsuperscript{13}C]glutamine will reflect the amount of glutamine transferred from astrocytes to neurons during the 30 minutes between injection and decapitation and can be expressed in the following equation:

\begin{equation}
\frac{[4,5-\textsuperscript{13}C]\text{glutamate}}{[4,5-\textsuperscript{13}C]\text{glutamine}} / \% \text{ enrichment}
\end{equation}

Similarly, the transfer of glutamine from astrocytes to GABAergic neurons can be expressed as:

\begin{equation}
[4,5-\textsuperscript{13}C]\text{GABA} / \% \text{ enrichment}
\end{equation}

\begin{equation}
[1,2-\textsuperscript{13}C]\text{GABA} / [4,5-\textsuperscript{13}C]\text{glutamine}
\end{equation}

\textit{Eq. 1-3} are estimates of the amount of substrate (glutamate or glutamine) in nmol/g transferred between astrocytes and neurons during the time from the ip. bolus injection of labeled substrate and arrest of metabolism by euthanization (30 minutes). We have earlier reported that there is uptake and stable availability of [1-\textsuperscript{13}C]glucose and [1,2-\textsuperscript{13}C]acetate between five to 45 minutes after injection and hence abundantly labeled glucose and acetate at 30 minutes.\textsuperscript{12}

Pyruvate carboxylation (anaplerosis) (Figure 4 in main article)

Carboxylation of [3-\textsuperscript{13}C]pyruvate will label [3-\textsuperscript{13}C]oxaloacetate which can be transaminated to [3-\textsuperscript{13}C]aspartate. [3-\textsuperscript{13}C]aspartate will also arise from pyruvate dehydrogenation of [3-\textsuperscript{13}C]pyruvate via PDH in 1\textsuperscript{st} full turn of the TCA cycle together with an equal amount of [2-\textsuperscript{13}C]aspartate because of scrambling of label in the formation of the symmetrical molecule succinate. To calculate the amount of [3-\textsuperscript{13}C]aspartate arising from pyruvate carboxylation it is necessary to subtract [2-\textsuperscript{13}C]aspartate from [3-\textsuperscript{13}C]aspartate. This can be expressed in the following equation:

\begin{equation}
PC = [3-\textsuperscript{13}C]\text{aspartate} - [2-\textsuperscript{13}C]\text{aspartate}
\end{equation}

The same principle can be used to compare PC activity with PDH activity in the following equation:

\begin{equation}
PC/PDH = (([3-\textsuperscript{13}C]\text{aspartate} - [2-\textsuperscript{13}C]\text{aspartate}) / [2-\textsuperscript{13}C]\text{aspartate}) \times 2
\end{equation}
However, it should be noted that in the case of substantial backflux, leading to the formation of [2-$^{13}$C]aspartate in addition to [3-$^{13}$C]aspartate following pyruvate carboxylation, these equations may underestimate PC activity.
Supplemental Results

Body and brain weight

Bodyweight was similar across experiment groups (mean ± 95% CI, grams): HI: 14.0 ± 2.3 (zero hours), 12.7 ± 2.1 (six hours); 12.4 ± 2.3 (48 hours, weight at post natal day 7); 16.6 ± 4.1 (48 hours, weight at post natal day 9), sham: 12.8 ± 2.4 (zero hours), 12.0 ± 2.3 (48 hours, weight at post natal day 7); 14.8 ± 4.5 (48 hours, weight at post natal day 9).

Furthermore, there were no differences in brain hemisphere weights (mean ± 95% CI, milligram): HI ipsilateral: 287.1 ± 24 (zero hours); 279.0 ± 19 (six hours); 303.7 ± 23 (48 hours); HI contralateral: 280.0 ± 19 (zero hours); 276.7 ± 16 (six hours); 318.9 ± 27 (48 hours); sham: 272.9 ± 15 (zero hours); 311.0 ± 31 (48 hours). Significant brain edema with increased water content induced by HI could be a possible confounder for the results, since measurements were corrected for brain weight. However, since brain weights were similar across experiment groups increased brain water is unlikely.
Figure I: Typical $^{13}$C NMR spectra of brain extracts of a sham-operated animal (top) and HI animals (four remaining: from the top: ipsilateral hemisphere injected at zero hours; contralateral hemisphere injected at zero hours; ipsilateral hemisphere injected at six hours; contralateral hemisphere injected at six hours). Only ppm (parts per million) values from 15-72 are shown. The most central peaks for the results/discussion are marked by the panels A-H, and are shown in greater detail to the right. The relative increase in the vertical axis is given at the bottom of the figure. Some additional peaks are marked in the spectrum from the sham-operated animal. These are given in the top left of the figure. Abbreviations: HI, hypoxic-ischemia.
Table I. Total amounts in nmol/g brain tissue, amounts of $^{13}$C-labelled isotopomers in nmol/g brain tissue and % enrichment of glucose, lactate, alanine and acetate labeled from [1-$^{13}$C]glucose and [1,2-$^{13}$C]acetate

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<th>Sham 0 h n=9</th>
<th>Sham 48 h n=9</th>
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<th>HI ipsi 6h n=10</th>
<th>HI ipsi 48 h n=10</th>
<th>HI contra 0 h n=10</th>
<th>HI contra 6h n=10</th>
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<tr>
<td>total</td>
<td>2343 ± 382</td>
<td>2342 ± 267</td>
<td>1746 ± 242 *</td>
<td>2980 ± 281 †</td>
<td>2285 ± 208 †</td>
<td>2755 ± 339</td>
<td>2641 ± 223 †</td>
<td>2348 ± 301 †</td>
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<td>[1-$^{13}$C]</td>
<td>645 ± 85</td>
<td>568 ± 91</td>
<td>478 ± 96 * †</td>
<td>682 ± 138</td>
<td>558 ± 107</td>
<td>801 ± 74</td>
<td>608 ± 116</td>
<td>542 ± 121</td>
</tr>
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<td>[%]</td>
<td>37 ± 3</td>
<td>36 ± 3</td>
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<td>32 ± 5 ↓</td>
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<td>2181 ± 246</td>
<td>6139 ± 1970 †</td>
<td>1866 ± 253 ↓</td>
<td>2176 ± 309 ↓</td>
<td>4681 ± 1307 †</td>
<td>1662 ± 193,3 ↓</td>
<td>2285 ± 288 ↓</td>
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<td>[3-$^{13}$C]</td>
<td>88 ± 22</td>
<td>112 ± 17</td>
<td>207 ± 32 * †</td>
<td>84 ± 19 ↓</td>
<td>114 ± 13 ↓</td>
<td>155 ± 14 †</td>
<td>79 ± 14 ↓</td>
<td>115 ↓</td>
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<tr>
<td>[%]</td>
<td>4.3 ± 0.8</td>
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<td>4.1 ± 0.6</td>
<td>3.7 ± 0.7</td>
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<td>total</td>
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<td>582 ± 58</td>
<td>2921 ± 234 * †</td>
<td>697 ± 100 †</td>
<td>625 ± 51 ↓</td>
<td>2071 ± 225 †</td>
<td>685 ± 112 ↓</td>
<td>686 ± 534 ↓</td>
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<tr>
<td>[3-$^{13}$C]</td>
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<td>16 ± 3</td>
<td>31 ± 8 ↑</td>
<td>12 ± 3 ↓</td>
<td>18 ± 3 ↓</td>
<td>19 ± 7</td>
<td>12 ± 3</td>
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<td>[%]</td>
<td>9.7 ± 1.0</td>
<td>8.5 ± 0.5</td>
<td>4.1 ± 0.3 ↑</td>
<td>7.2 ± 1.1 * ↑</td>
<td>8.3 ± 0.3 ↑</td>
<td>4.0 ± 0.6 ↑</td>
<td>7.4 ± 1.1 ↑</td>
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<tr>
<td>total</td>
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<td>1483 ± 193</td>
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<td>2021 ± 383</td>
<td>1671 ± 373</td>
<td>1833 ± 187</td>
<td>1918 ± 398</td>
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<td>[1,2-$^{13}$C]</td>
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<td>843 ± 167</td>
<td>1117 ± 123</td>
<td>1247 ± 320</td>
<td>996 ± 304</td>
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<td>71 ± 7</td>
<td>68 ± 8</td>
<td>75 ± 1</td>
<td>72 ± 7</td>
<td>68 ± 8</td>
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Brain extracts of P7 rats injected with [1-$^{13}$C]glucose and [1,2-$^{13}$C]acetate were analyzed using $^1$H-MRS (total amounts) and $^{13}$C-MRS (labeled amounts). At zero hours, one animal from the HI group had to be excluded due to incorrect injection. Therefore, n = 9 for the $^{13}$C data, and n = 10 for the $^1$H data. For more details and description of Eq.1 (anaplerosis: [3-$^{13}$C]aspartate → [2-$^{13}$C]aspartate) see Supplemental Materials. Results are presented as mean ± 95% CI. * $p$< 0.05 ipsilateral vs. contralateral hemisphere; † $p$< 0.05 vs. sham; ↓ $p$< 0.05 lower vs. 0 hours; ↑ $p$< 0.05 higher vs. 0 hours.
Supplemental References


