Assessing Cerebral Metabolism in the Immature Rodent: From Extracts to Real-Time Assessments

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Abstract

Brain development is an energy-expensive process. Although glucose is irreplaceable, the developing brain utilizes a variety of substrates such as lactate and the ketone bodies, β -hydroxybutyrate and acetoacetate, to produce energy and synthesize the structural components necessary for cerebral maturation. When oxygen and nutrient supplies to the brain are restricted, as in neonatal hypoxia-ischemia (HI), cerebral energy metabolism undergoes alterations in substrate use to preserve the production of adenosine triphosphate. These changes have been studied by in situ biochemical methods that yielded valuable quantitative information about high-energy and glycolytic metabolites and established a temporal profile of the cerebral metabolic response to hypoxia and HI. However, these analyses relied on terminal experiments and averaging values from several animals at each time point as well as challenging requirements for accurate tissue processing. More recent

methodologies have focused on in vivo longitudinal analyses in individual animals. The emerging field of metabolomics provides a new investigative tool for studying cerebral metabolism. Magnetic resonance spectroscopy (MRS) has enabled the acquisition of a snapshot of the metabolic status of the brain as quantifiable spectra of various intracellular metabolites. Proton (^1H) MRS has been used extensively as an experimental and diagnostic tool of HI in the pursuit of markers of long-term neurodevelopmental outcomes. Still, the interpretation of the metabolite spectra acquired with ^1H MRS has proven challenging, due to discrepancies among studies, regarding calculations and timing of measurements. As a result, the predictive utility of such studies is not clear. ^{13}C MRS is methodologically more challenging, but it provides a unique window on living tissue metabolism via measurements of the incorporation of ^{13}C label from substrates into brain metabolites and the localized determination of various metabolic fluxes. The newly developed hyperpolarized ^{13}C MRS is an exciting method for assessing cerebral metabolism in vivo, that bears the advantages of conventional ^{13}C MRS but with a huge gain in signal intensity and much shorter acquisition times. The first part of this review article provides a brief description of the findings of biochemical and imaging methods over the years as

well as a discussion of their associated strengths and pitfalls. The second part summarizes the current knowledge on cerebral metabolism during development and HI brain injury.

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Introduction

The concentrations of various tissue metabolites are considered valuable indicators of both normal and abnormal conditions and have potential to be biomarkers for various pathological states. The array of metabolites that reflects the status of neural tissue is termed the “neurochemical profile” or “metabolic profile.” The metabolic profile is dynamic, varying with rapid fluxes, and is currently considered the most predictive expression of health or disease [1, 2].

The most common analytical technique deployed for metabolomic studies is nuclear magnetic resonance (NMR) spectroscopy or magnetic resonance spectroscopy (MRS), as it yields highly specific biochemical information, characteristic of specific regions of the brain and pathological states [3]. In the case of experimental cerebral ischemic injuries, MRS is especially useful since it can differentiate between the neurochemical profiles of the core lesion, the adjacent penumbra, and healthy unlesioned tissue [4]. The metabolic alterations of tissue often precede other anatomical and functional changes that can be observed with anatomical MR imaging (MRI). Thus, MRS can show disturbances even when the brain appears anatomically normal [5, 6], and the analysis of data obtained from MRS spectra has the potential to identify useful biomarkers predictive of injury or recovery.

Cerebral hypoxia-ischemia (HI), whether clinical or experimental, produces a reduction in the delivery of both glucose and oxygen to the brain to levels insufficient to meet the brain’s energetic demands, and, if prolonged, this results in energy failure and cell death [7, 8]. Restoration of cerebral blood flow upon resuscitation, while providing needed substrate and oxygen, also sets into motion a cascade of biochemical events that can prolong metabolic perturbations and further cell death, as originally described in experimental stroke models in adult animals [9]. It is now appreciated that similar events occur in newborns, with some notable differences related to the developmental status of the brain [10, 11]. Thus, it is important to study cerebral HI in developmentally relevant animal models such as the immature rodent.

The majority of experimental studies of brain metabolism in neonatal HI have utilized a combination of unilat-

eral common carotid artery ligation followed by a period of systemic hypoxia, known as the Vannucci model [12]. This model, originally developed in the immature rat and adapted for mice [13], produces a selective neuronal death or infarction in the hemisphere ipsilateral to the ligation, the severity of which ranges from little or no injury to severe injury; occlusion of the artery or hypoxia alone do not result in brain damage [14]. Systemic hypoxia, in combination with unilateral carotid artery ligation, results in a transient unilateral ischemia, that, if sustained, causes cerebral energy failure and cell death. Since its original description in 1981, the Vannucci model has been widely used and adapted to various species, and it is considered one of the most reproducible models of perinatal HI injury to date [15].

Glucose plays a critical role in maintaining cerebral energy metabolism. Although the immature brain utilizes other substrates as well, i.e., lactate (Lac) and the ketone bodies, acetate and β -hydroxybutyrate [16, 17], under hypoxic and hypoxic-ischemic conditions, glucose is the only substrate capable of sustaining cerebral energy demands, through its capacity to be consumed by anaerobic glycolysis and the production of lactic acid and adenosine triphosphate (ATP) [18]. The obligate shift to anaerobic metabolism of glucose increases the glycolytic demand and can lead to energy failure and cell death if glucose delivery is compromised, such as in the neonatal brain [19]. Thus, studying glycolytic metabolism in the neonatal brain is of utmost importance. Past studies on neonatal rodents laid a firm foundation for the understanding of the temporal and regional changes in the metabolism of the immature brain during development and injury [20–24]. Metabolomic studies now seek to re-establish this knowledge and use it in conjunction with the advantage of fast, noninvasive detection, as a powerful and accurate diagnostic tool.

Although different animal models of cerebral ischemia can affect cerebral metabolism differently, the cerebral metabolic changes during and following HI have been most thoroughly described in the Vannucci model [12, 14]. In addition, our current metabolomic studies focus on cerebral metabolism in the neonatal rodent, subjected to this same experimental insult. The goal of this review is to highlight what these new longitudinal methods add to the picture and how best to interpret the current MRS data in the context of what has been previously described for this model. We propose that such a synthesis of old and new data will highlight the usefulness of the current metabolomic studies in search of biomarkers of injury and recovery.

Principal Metabolic Pathways of Glucose

Glucose is the principal and obligatory energy substrate for the mammalian brain. The delivery of glucose from the blood to the brain depends on transport of this nutrient across the endothelial cells of the blood-brain barrier (BBB) and the plasma membranes of the neurons and glia. This process is mediated by the glucose transporter proteins, GLUT1 and GLUT3, through facilitated diffusion, which is an energy independent, bidirectional transport of glucose across a concentration gradient [25]. Glucose in the circulation is initially transported via GLUT1, across both the luminal and abluminal membranes of the microvascular endothelial cells, where it freely diffuses through the extracellular space of the basement membrane [26]. From there, it is transported across the neural cell membranes of neurons (via GLUT3) and glial cells (via GLUT1), where it is phosphorylated to glucose-6-phosphate (glucose-6-P) via hexokinase [25]. Glucose-6-P can be metabolized by means of 3 different pathways, giving rise to a variety of substrates which can then be further metabolized: (1) glycolysis, (2) the pentose phosphate pathway (PPP), and (3) glycogenogenesis in astrocytes.

First, glucose-6-P can be metabolized via the glycolytic pathway, producing 2 molecules of pyruvate and generating ATP. The resulting pyruvate can then either be reduced to Lac, transaminated to alanine, or enter the mitochondria. In the mitochondria, it is decarboxylated by the pyruvate dehydrogenase complex, yielding the tricarboxylic acid (TCA) cycle substrate, acetyl-CoA. Acetyl-CoA condenses with oxaloacetate, entering the TCA cycle [27]. After several steps, α -ketoglutarate is formed, which can either stay in the cycle, or yield glutamate, glutamine and γ -aminobutyric acid (GABA) (Fig. 1). The TCA cycle provides the bulk of the energy required for cerebral function. Complete oxidation of 1 molecule of glucose through the TCA cycle produces 30–32 ATP [28].

Glucose-6-P also serves as an essential substrate for the PPP. The PPP generates pentoses for nucleotide acid production, needed for DNA synthesis, and also NADPH, to manage oxidative stress and synthesize lipids through the regeneration of glutathione (GSH). Metabolism of glucose via the PPP eventually results in fructose-6-P. Fructose-6-P will generate glyceraldehyde and, consequently, pyruvate. The PPP, followed by pyruvate carboxylation in astrocytes [29], will generate glutamate/ glutamine and GABA by backflux conversion of oxaloacetate to succinate and α -ketoglutarate

Fig. 1. Simplified presentation of pyruvate metabolism. Glucose is metabolized via glycolysis, leading to pyruvate which can then either be reduced to lactate, transaminated to alanine, or enter the tricarboxylic acid (TCA) cycle via pyruvate dehydrogenation (PDH) as acetyl-CoA. If glucose is metabolized through the pentose phosphate pathway (PPP), the resulting pyruvate may undergo pyruvate carboxylation (PC) in astrocytes and then enter the TCA cycle backflux from oxalo-acetate to succinate. Pyruvate is thus an intermediate, common to oxidative phosphorylation and anaerobic glycolysis. The conversion of pyruvate to lactate is bidirectional; lactate is utilized as an alternative substrate for energy production during the early postnatal period in the rodent and when the glucose supply is short, by converting back into pyruvate.

(Fig. 1) [30]. GSH acts as the substrate for GSH peroxidase to reduce reactive oxygen species. Its activity is higher in the neonatal than in the adult brain, owing to the accelerated cellular growth and proliferation which results in increased lipid consumption for the composition of cell membranes [31].

The third pathway of cerebral glucose utilization is the synthesis of glycogen. This process takes place almost exclusively in the astrocytes. Concentrations of brain glycogen are high in the fetal rodent brain, but they fall to near adult levels during the first week of postnatal life [32]. Brain glycogen provides an additional energy source for

astrocytes during cerebral hypoxia or HI, and the proportion of its depletion as an energy source during HI is similar in adults and neonates, if one takes into account their differing metabolic rates [33].

Measurement of Metabolites in Brain Extracts

In the 1970s, Lowry and Passonneau [34] developed a quantitative method of fluorometric analysis to study concentrations of glycolytic and high-energy phosphates in brain extracts. Preservation of cerebral metabolites is crucially dependent on near-instantaneous freezing of the brain due to rapid postmortem changes. Such studies in adult rodents utilized *in situ* funnel freezing of the brain. However, due to both the thin skull and lower metabolic rate of the immature brain, snap-freezing of the pup, or head, in liquid N₂ proved sufficient to keep these changes to a minimum [35]. The tissue was kept at -70° to -80°C until dissection and powdering of the brain at -20°C . The frozen brain was then acid-extracted, and the neutralized extracts were used for fluorometric analysis. Similar analyses were also performed on samples of plasma and cerebrospinal fluid from these animals, allowing for the calculation of the blood/brain and cerebrospinal fluid/brain ratios.

Errors inherent in this method include lower values for adenosine monophosphate and higher values for inorganic phosphate when frozen tissues are acidified at temperatures $<0^{\circ}\text{C}$. To keep postmortem glycolytic and energy consumption to a minimum, freezing must be completed in <60 s, tissue must be maintained at -80°C until acid extraction, and extracts must be maintained at -20°C until analysis [34]. Limitations of this methodology include the challenge of strict adherence to the temperature requirements of brain processing, the difficulty in obtaining regional measurements, and the inability to correlate metabolic changes to outcome. Despite such limitations, this methodology was central to establishing details of cerebral glycolytic and high-energy metabolism in the fetal and developing rodent brain and, subsequently, to describe the temporal pattern of metabolism during and following HI [20–24]. While a thorough description of these studies is beyond the scope of this review, they are briefly described below as a context for the interpretation of the more recent *in vivo* studies.

Following the description of the Vannucci model [12], the same method of extraction was used to shed light on the metabolic changes during the evolution of brain damage following HI. After exposure of rat pups to hypoxia,

concentrations of glucose, Lac, pyruvate, β -hydroxybutyrate, acetoacetate, creatine (Cr), and high-energy compounds, namely ATP, adenosine diphosphate, and phosphocreatine (PCr) were measured with fluorometric enzymatic techniques [34]. Intracellular pH was also calculated through ratios of ATP/adenosine diphosphate and Cr/PCr. During HI, the pH in the ipsilateral hemisphere decreased, whilst in the contralateral hemisphere, it remained stable despite the similarity between the increased Lac concentration in both hemispheres [22, 36]. In the immature brain, it is tissue acidosis and not Lac concentrations that correlate with tissue damage, likely due to the ability of Lac to freely enter or leave the brain via enhanced transport capacity [17, 22].

Finally, the cytoplasmic and mitochondria redox states (NAD^+/NADH) were calculated, utilizing the measured concentrations of the oxidized and reduced products of lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH) reactions in both cerebral hemispheres during and following HI. Although the cytoplasmic ratio decreased (became more reduced) in both hemispheres during HI, upon resuscitation, the redox state normalized in the contralateral hemisphere while remaining reduced ipsilaterally [22]. This was in contrast to the mitochondrial redox state, which increased (became more oxidized) in the ipsilateral hemisphere while remaining normal in the contralateral hemisphere. This paradoxical mitochondrial re-oxidation was observed to temporally parallel the depletion of high-energy phosphate reserves that results from a limited cellular substrate (glucose) supply to the ipsilateral hemisphere and culminates in brain damage [23]. It is important to note that the duration of HI in these early studies was 180 min, consistent with a severe degree of brain injury, with the increase in the mitochondrial redox state occurring at 2–3 h of HI. It cannot be assumed that this occurs following milder results.

NMR Techniques *in vivo*

MR techniques have made an essential contribution to the evolution of *in vivo* biochemistry and our knowledge of neonatal tissue metabolism. MRS is a unique experimental technique that measures temporal changes in cellular metabolite levels and offers a snapshot of the metabolic status of the tissue. In other words, it allows the non-invasive detection of numerous intracellular metabolites in otherwise inaccessible tissues such as the brain. It is therefore a quantitative study of a complex mixture of metabolites, visualizing multiple compounds at the same time, mi-

nus the chemical manipulation of the samples. The information that becomes available with MRS can then be assessed in conjunction with traditional anatomic imaging. Overall, MRS is a safe, noninvasive technique, and one of the few modalities at hand that enables the investigation of perinatal cerebral energy metabolism in vivo [37, 38].

¹H Magnetic Resonance Spectroscopy

¹H MRS has been extensively used for >20 years in studying cerebral metabolism in rodents and newborns in pursuit of the biomarkers of long-term neurodevelopmental outcome. While there are numerous publications concerning the rat brain, there are only a few about mice [4, 39]. In human neonates, most studies with ¹H MRS have been restricted in quantifying changes in the main peaks of the ¹H MRS spectrum, which include N-acetyl-aspartate (NAA), choline (Cho), Cr, and Lac, due to their simplicity and robustness in identification [40] (Fig. 2).

The most relevant feature of the ¹H MRS spectra to hypoxic-ischemic injury in neonates is a variable-sized double Lac peak. Lac is the end product of anaerobic respiration, and increased Lac in the brain is indicative of the level of anaerobic glycolysis, where the need for oxygen exceeds supply [41]. Whilst during normal brain development cerebral Lac maintains steady state levels, concentrations rise during hypoxia and HI [19] but are rapidly cleared on resuscitation through conversion to pyruvate as well as transport to the blood [38]. Lac levels during the subsequent evolution of injury depend on the severity of the insult and the extent of cell death, from essentially normal in mild/moderate injury to elevated following severe injury [42].

Thus, it might be reasonable to assume that high Lac detected on ¹H MRS early after the insult can be used as a predictor of outcome. However, there is continued debate over the interpretation of the elevated Lac detected with ¹H MRS. Not all infants with abnormal neurodevelopmental scores exhibit high Lac [43]. Yet, in almost all infants with poor outcomes, Lac on ¹H MRS persists beyond 1 month after birth. While the initial Lac peak in the acute phase of HI may be due to anaerobic metabolism, the persistence of elevated Lac for days to months may indicate long-term perturbation of the cerebral energy metabolism [42, 44]. Alternatively, high Lac could be stemming from the breakdown of glycogen in astrocytes that have replaced the inflammatory/dead tissue at the injury site [45–47]. Explanations for differences among studies have been attributed to differences in the calculation methods or in the timing of the spectroscopic examination.

Aside from Lac, there is much interest in NAA, due to its importance as a neuronal marker [48]. NAA is synthe-

tized in neurons in a reaction catalyzed by an N-acetyltransferase-8-like protein and serves as a precursor for the synthesis of N-acetylaspartylglutamate (NAAG), a possible neurotransmitter [49]. It is typically localized in neurons and immature oligodendrocytes where it is catabolized to aspartate and acetate, the latter being used for synthesis of fatty acids such as myelin [50, 51]. Several studies have further suggested that the level of NAA reflects neuronal mitochondrial function, since it is lowered with inhibition of mitochondrial respiration [52]. Although its absolute concentration varies from species to species, there is a steady increase in the level of NAA from the neonatal period to adulthood. Studies on rats document a rapid increase between postnatal day 5 (P5) and P20 in the rat brain [53]. In infants, despite regional differences, ¹H MRS found the NAA level to be 50% of that in adults. Provided that the exponential time course of the NAA increase is extended to in utero life as well, it can be justifiably considered a biomarker of brain maturation and integrity [54].

In clinical practice, the most commonly used prognostication tools have been the ratios of metabolites detected in the spectra. Particularly, high Lac/NAA and Lac/Cho ratios have proven to be correlated with unfavorable neurodevelopmental outcomes at 2 and 30 months of age [55, 56]. Some studies found an increased Lac and decreased Cho combination to better correlate with outcome [57, 58], while others discovered a more significant association with increased Lac and decreased NAA levels [43, 59, 60]. As with the differences in the observed Lac peaks, this divergence has again been attributed to differences in the timing of the imaging investigation with respect to the HI insult and differences in the echo time (TE) used to acquire the spectra.

In addition to the clinical studies, a few papers have reported metabolite changes in rodent HI models detected by in vivo ¹H MRS. Malisza et al. [61] observed a substantial increase in Lac and reduction in NAA in the hypoxic-ischemic hemisphere in animals that developed an infarction up to 48 h after the insult. Xu et al. [62] attempted to characterize longitudinal changes of metabolites in the cortex and hippocampus of HI rats during the critical development period up to 28 days post-injury, using localized in vivo ¹H MRS. Significant alterations in the metabolic profile of the ipsilateral hippocampus were detected at 24 h post-HI, and were indicative of oxidative stress such as reduced GSH and Tau protein, while the impaired mitochondrial phosphorylation and consequent reliance on anaerobic metabolism were demonstrated by the persistently high level of Lac and depleted PCr in the majority of the HI subjects [62].

MRS is technically demanding in the clinical setting; its success is heavily dependent on its sensitivity, and results may be complicated by the use of intensive care equipment [55]. In experimental studies, in the rodent brain, and especially in mice, MRS suffers from a low sensitivity, reflected in a low signal-to-noise ratio (SNR) owing to the small size of the area of interest. A poor SNR limits the detection of metabolites at small concentrations. The inherently small size of the rodent brain is further complicated when one tries to localize measurements to functionally different cerebral areas, thereby rendering shimming (termed as the minimization of inhomogeneity B_0 between different diamagnetic tissues) even more lengthy and challenging [2].

Most MRS studies on brain tissue have used the ^1H nucleus due to its high natural abundance (99.99%) and its high prevalence in metabolites. In the last 15 years, ^1H MRS has benefitted greatly from the application of more highly magnetic fields. To achieve high spectral quality in the small-sized mouse brain, efficient second-order shimming through strong enough coils and high pulse sequence performance are required [39].

Technological developments and refinements have also enabled the use of heteronuclear MRS (x-nuclei MRS) in humans and small animals. The term “heteronuclear” refers to detection of the resonances of nuclei other than protons such as ^{17}O , ^{31}P , and ^{13}C . These methods are still challenging due to their low sensitivity for the detection of nonproton nuclei as they are characterized by a substantially lower gyromagnetic ratio and lower natural abundance compared to the ^1H nucleus. Such weaknesses may be overcome in a number of ways, one of which is proton decoupling, whereby ^{13}C signals become detached from their neighboring ^1H atoms and may appear as singlets on the spectra. Alternatively, introducing the respective label in an injected metabolic substrate that does not affect its biochemistry, such as replacing the ^{12}C isotope with ^{13}C at a specific carbon, can singularly increase its detection over background signals [63].

^{13}C Magnetic Resonance Spectroscopy

The properties of the ^{13}C nucleus can be used to study glucose metabolism in neurons and glia, as well as neurotransmitter cycling. ^{13}C MRS is a unique type of spectroscopy which allows the investigation of the “backbone” of organic compounds that can incorporate carbon after the application of ^{13}C -labeled glucose or equivalent substrates. The high chemical specificity of the ^{13}C nucleus allows its detection within different molecules as well as at different positions in the same molecule (^{13}C isotopomers). In this way, it is possible to follow the fate of the

^{13}C label to investigate enzyme activities and changes in metabolites that participate in pathways fueled by glucose. In certain cases, the metabolic flux can also be indirectly estimated by mathematical modeling [64, 65].

Since glucose is the primary fuel for brain energy metabolism, most dynamic studies with ^{13}C MRS prefer to use ^{13}C -labeled glucose for metabolic studies in the brain. The metabolism of ^{13}C glucose leads to clearly labeled patterns at the level of Lac and alanine, and in metabolites produced from the first cycle of the TCA. Injection of (1,2- ^{13}C) glucose in P7 rats and the analysis of their extracted cerebral hemispheres with ^1H and ^{13}C MRS was used to observe changes in the PPP and the activity of pyruvate carboxylase in astrocytes post-HI [41]. In accordance with previous studies [20], glucose increased bilaterally and Lac accumulated, signifying the increased anaerobic glycolytic flux. Contrary to the researchers’ expectations, the PPP activity appeared to be reduced, and they interpreted the results as mitochondria hypometabolism. An alternative explanation is that, during early recovery, Lac is the principal substrate for the TCA cycle [22], thus decreasing the appearance of the label derived from the injected glucose in glutamate.

Unfortunately, most of the molecules that are labeled during a ^{13}C glucose infusion cannot be detected by NMR due to their low concentration and to the relatively low sensitivity of in vivo NMR. Therefore, researchers must rely on detecting the ^{13}C label in larger pools of brain amino acids, such as glutamate or glutamine, and then indirectly calculating the TCA cycle activity [66]. Thus, labeled glucose does not provide complete information on energy metabolism, especially in the neonatal period, and even more so in conditions such as HI where the brain also utilizes other substrates.

Despite the wealth of highly specific information that can be acquired on metabolites and metabolic rates, ^{13}C MRS is a very challenging modality, perhaps even more so than the other 2 major nuclei used for NMR studies (^1H and ^{31}P). The difficulties that arise are mainly technical issues related to the ^{13}C nucleus. ^{13}C has a natural abundance of approximately 1% in living organisms. While this greatly enhances the specificity of detection, the administration of exogenous ^{13}C -labeled precursors becomes mandatory to increase the sensitivity. Nonetheless, even if the problem of low sensitivity is somewhat alleviated by the ^{13}C -enriched label, the use of the label itself adds to the barrier, since it compounds the cost and complexity of the experimental procedure [67]. Lastly, the long scan times (from minutes to hours) required for acquisition and the constant infusion of large volumes of ^{13}C metabolites that are needed to get a signal render ^{13}C

Fig. 2. Examples of representative spectra from individual voxels of the ischemic/hypoxic-ischemic murine brain acquired with ^1H MRS (**a**) and $\text{HP-}^{13}\text{C}$ MRS (**b**). ^1H MRS spectra allow access to the steady state of a collection of metabolites using their specific protons while $\text{HP-}^{13}\text{C}$ MRS shows the dynamic changes of a single, labeled substrate during its infusion and metabolism in a living organism. The metabolic data that can be acquired with these 2 methods are different in nature but complement each other. **a** ^1H MRS spectra of the nonischemic contralateral striatum (left) and of the ipsilateral to the brain injury striatum (right) of an adult mouse (the MCAO model) taken at 24 h after reperfusion. Lactate is the only significantly increased metabolite, while most of the rest of the metabolite concentrations are decreased on the ipsilateral side. **b** Spectra acquired from single voxels positioned on the contralateral (left) and ipsilateral (right) hemispheres with a dual-

shifted $^1\text{H}/^{13}\text{C}$ coil during intravenous injection of hyperpolarized pyruvate to a postnatal day 10 (P10) mouse 4–6 h after HI. The pyruvate and lactate signals at a single time point during the acquisition are represented by 2 peaks at their respective frequencies. The low pyruvate signal ipsilateral to the injury reflects the impeded perfusion and edema, which result in reduced delivery of the label to the area. Furthermore, a higher pyruvate to lactate conversion rate is expected to relate to a higher reliance on anaerobic metabolism. Arrows indicate changes relative to the healthy contralateral side. Metabolites in the spectra are assigned as follows: Ala, alanine; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamate; GSH, glutathione; Ins, myoinositol; NAA, N-acetyl-aspartate; Tau, taurine; tCho, total choline; tCr, total creatine; Lac, lactate; Pyr, pyruvate.

MRS inapplicable to young animals with small body volumes, thus limiting its feasibility for use in cultured cells and extracted tissues [68].

HP ^{13}C Magnetic Resonance Spectroscopy

The latest evolution in MRS has been the use of hyperpolarized ^{13}C ($\text{HP-}^{13}\text{C}$)-labeled substrates. Dynamic nuclear polarization (DNP) can achieve a 10,000-fold enhancement of the ^{13}C signals of an introduced substrate and its subsequent metabolic products. This gain in signal intensity has irreversibly altered the clinical potential of the ^{13}C -labeled substrates. By applying DNP to endogenous, non-

toxic, nonradioactive substances (pyruvate and urea) and administering these to a subject, $\text{HP-}^{13}\text{C}$ MRS can monitor the administered compound's fluxes via key biochemical pathways, such as glycolysis. Contrary to ^1H MRS, which focuses on metabolite levels, with $\text{HP-}^{13}\text{C}$ MRS, researchers can now track metabolic activity in real time, i.e., occurring at the actual moment of the acquisition [69].

DNP is based on polarizing the nuclear spins of a molecule in the solid state. It requires the presence of unpaired electrons, such as organic free radicals. The high electron polarization spin is then transferred to the nuclear spins of the sample (the ^{13}C -labeled substrate) by mi-

crowave heating. The HP state is acquired inside a DNP polarizer machine at cryogenic temperatures (0–2 K). However, the nuclear spins return to thermal equilibrium in a very short time once exposed to room temperature. Thus, the main challenge that must be overcome is the transition of the HP solid substance from a very low temperature inside the polarizer to an injectable solution close to body temperature, and then the transfer to the target organ without significant loss of polarization [70]. For this reason, the ^{13}C nucleus is ideal as its longitudinal relaxation time T_1 is sufficiently longer than the time required for the imaging acquisition, so that it can be distributed to the brain while retaining its HP state [71].

One of the first applications of this technology has been the evaluation of the conversion of HP- ^{13}C -labeled pyruvate to ^{13}C lactate. The choice of pyruvate as the injected labeled substrate is supported by the fact that, aside from being an endogenous substance and thus nontoxic, pyruvate is found at the junction of anaerobic glycolysis and oxidative phosphorylation (Fig. 1). Depending on the intracellular energy state of the tissue, pyruvate is converted to a different degree to Lac, alanine, and carbon dioxide [69].

Chen et al. [72] applied this method to the immature murine brain to investigate the metabolic changes of glucose utilization during development. The fast acquisition time (approx. 3 s) allows for multiple acquisitions while the intravenously injected HP (1- ^{13}C) pyruvate converts rapidly into Lac in the brain. Dynamic data from the average peak height for pyruvate and Lac at each time point can be measured, and an individual curve for the build-up and decay of pyruvate and Lac signals can be obtained (Fig. 2). The normalized Lac level and the pyruvate to Lac conversion rate (k_{pl}) were calculated as parameters of metabolic rate in maturation and both appeared to decrease linearly with increasing age. The higher the k_{pl} , the faster the pyruvate bolus turns into Lac and the more Lac is produced within a certain period of time [72]. Thus, conversion rates can reveal important information about the glycolytic flux and the metabolic needs of the brain; k_{pl} is higher in the suckling P18 mouse, when the developing brain can transport and utilize Lac as fuel more efficiently than the adult brain can. In the setting of HI, a potentially increased k_{pl} could indicate the urgent need for a substrate in the absence of glucose.

The conversion of pyruvate to Lac reflects the activity of LDH [73]. When using HP (1- ^{13}C) pyruvate, the labeled carbon from pyruvate is eliminated as carbon dioxide in exchange with bicarbonate during the conversion of pyruvate to acetyl-CoA. In theory, the time course of the labeled bicarbonate reflects the activity of the TCA cycle since almost all acetyl-CoA enters the TCA cycle

[74]. However, the downstream metabolic fate of acetyl-CoA cannot be directly observed. Labeling HP pyruvate on C-2 instead of on C-1, i.e., (2- ^{13}C) pyruvate, permits the investigation of additional metabolic pathways such as the formation of glutamate [75]. No studies using HP-(2- ^{13}C) pyruvate have been conducted on the immature rodent brain thus far. Still, direct measurements of oxidative phosphorylation and glutamate could prove valuable for investigating the metabolic profile changes during HI.

The translation of the signal acquired with HP- ^{13}C MRS into cerebral metabolic rates is still not completely straightforward. The observed in vivo signals are influenced by the influx of metabolites produced systemically, the cerebral blood volume, and the rate of transport across the BBB. So far, these problems have only been addressed in vitro by performing HP- ^{13}C MRS on perfused brain slices [76].

The interpretation of large datasets that have become available from such diverse enzymatic and imaging techniques can be quite challenging. Knowledge of the metabolic processes that take place in the neonatal brain and how they are affected by maturation and injury is, without doubt, essential to develop a baseline for the assessment of the different metabolite and parameter values. In the following sections, we provide a synopsis of the results published thus far on cerebral metabolism during the normal development of the rodent brain as well as during HI brain damage, as is manifested in the Vannucci model.

Cerebral Metabolism in Normal Development

The brain requires a continual supply of substrate and oxygen. The prevailing dogma is that the principal substrate that supports brain metabolism throughout all phases of development is glucose. However, neonates and, to a lesser degree, adults can utilize alternative cerebral fuels to supplement glucose in fulfilling the brain's energy needs during periods of starvation, suckling, and hypoglycemia [77]. The alternative substrates Lac and the ketone bodies (β -hydroxybutyrate and acetoacetate) can assist glucose to produce energy in a well-oxygenated state [78].

Normal brain development depends upon a metabolic system that must keep pace with the escalating oxygen substrate demands which accompany the rapid increase in brain mass and the accelerated electrochemical maturation that characterizes synaptogenesis [79]. Under normal physiological conditions, the delivery of oxygen and substrate to the developing brain is considered adequate to support cerebral energy metabolism as well as maturation. The immature animal brain has substantially lower

metabolic demands (one-tenth of that of the adult brain, in the first postnatal week), allowing it to survive prolonged periods of hypoxia and creating the belief that the immature brain is “resistant” to hypoxia and HI [32]. However, it is now clear that although the immature animal can survive prolonged hypoxia, it can suffer extensive injury due to its unique vulnerabilities [80].

The low cerebral metabolic rate is reflected in a low rate of cerebral glucose utilization (CGU). Blood glucose levels in the early postnatal period and adulthood are comparable, but the expression of the glucose transporter proteins, GLUT1 in the BBB and glia and GLUT3 in neurons, is also low in the neonatal rodent brain and increases developmentally in concert with increases in CGU, coincident with synaptogenesis and an increased biochemical activity after the second postnatal week. A concomitant increase in the enzymes of the glycolytic and TCA pathways is fueled by the increased availability of glucose during this period of functional maturation, with a higher expression of GLUT3 [81, 82]. Thus, during normal development, the capacity of the glucose transporter system is sufficient to fuel the glycolytic demands of the immature brain. However, CGU becomes transport-limited when the capacity of this system is stressed, e.g., with increased glycolytic demand or hypoglycemia. The transport of the alternative substrates across the BBB and into the neural cells is mediated by a different transporter protein family, the monocarboxylate transporters (MCT). In the rodent and human brain, the isoform MCT1 is widely expressed in the vascular endothelial cells of the BBB and in glial cells [83]. MCT2 is the primary neuronal isoform, although evidence of the expression of MCT1 has also been found in the neuropil and neurons [84].

Rodent breast milk, and to a lesser extent human breast milk, is characterized by a high fat content which supports ketogenesis and increased levels of circulating ketone bodies [85–87]. With the onset of suckling, circulating levels of β -hydroxybutyrate increase nearly 4-fold within the first 24 h [88]. During the first 2 weeks of life, ketone bodies can provide up to 60% of the energetic fuel for the brain [89], and they account for at least 30% of the total energy metabolic balance [90]. Simultaneously, levels of the MCT1 transporter increase steadily in the BBB and at P17, they are 25 times the levels of the adult [84]. This upregulation is more conspicuous in suckling rats than in adults receiving a ketogenic diet, so it may be developmental as well as dietary [17, 86].

The transition from the lower CGU in the suckling animal to the high CGU in the adult is also mediated by changes in the expression of enzymes for substrate use in the brain. The development of enzymes associated with

the complete aerobic utilization of glucose and also glycolysis, such as hexokinase, citrate synthase, LDH, and pyruvate carboxylase, has been shown to have an absolute correlation with the onset of full neurological competence in the rodent brain. The activity of these enzymes increases several-fold until P10–P15, in coordination with the developmental switch from ketone body to glucose metabolism [17, 91, 92]. Fatty acids do represent a large percentage of a newborn’s energy source, and their homogenous uptake by the brain coincides with the rapid cell proliferation and cholesterol synthesis for brain growth that occur during the early suckling period [93].

Cerebral Metabolism in Hypoxic-Ischemic Injury

During experimental ischemia in adult models, rates of CGU are nearly tripled, reflecting the increased glycolytic demand of anaerobic metabolism, fueled by a steady supply of glucose [94]. The cerebral metabolic changes observed in the neonatal brain are distinct from in the adult, due to the unique features discussed above. CGU in the immature brain is transport-limited, such that the delivery of glucose to the brain via GLUT1 cannot keep up with the increased demand due to the switch to anaerobic glycolysis. The reduction of blood flow in the hemisphere ipsilateral to the carotid ligation in the animal model further limits glucose delivery and brain glucose in this hemisphere falls precipitously, resulting in the depletion of the high-energy compounds, ATP and PCr, culminating in the characteristic energy failure and cell death. Although glucose is also reduced in the contralateral hemisphere, the maintenance of blood flow is sufficient to meet the glycolytic demand and there is no energy failure or cell death [20].

Contrary to findings for cerebral ischemia in the adult, no sustained elevation of Lac has been shown in the neonate immediately after the insult [19, 95]. Brekke et al. [41] reconfirmed by their results that, during the hypoxic ischemic episode, there is a dramatic shift towards the anaerobic utilization of glucose with an approximately 3-fold increase in the production of Lac and a rapid depletion of brain glucose. The Lac accumulation observed in the Vannucci model during the course of HI is identical in both hemispheres, despite the fact that brain injury is unilateral [22, 95]. This emphasizes the fact that, unlike in adults, Lac is not deleterious to the neonatal brain and does not contribute to the decrease of pH, and the subsequent intracellular acidosis that develops during cerebral HI, until a certain threshold is passed [36].

However, within 4 h after the insult, brain Lac levels normalize [20, 22]. Upon reperfusion and reoxygenation, the accumulated Lac is rapidly metabolized into pyruvate, providing a substrate for the TCA cycle, in addition to its rapid clearance across the BBB. Validation for these speculations can be found in the marked ability of the immature brain to metabolize and transfer Lac and ketone bodies across the BBB via the MCTs. In fact, any condition that results in elevated Lac levels systemically promotes its uptake and utilization by the immature brain, thus sparing the consumption of glucose [17]. Indeed, contrary to what happens in adult cerebral ischemia, the pyruvate dehydrogenase complex (responsible for turning pyruvate into acetyl-CoA) appears to remain active, which may also account for the rapid restoration of the TCA cycle during early recovery. Occurring in parallel with the decline of Lac is the recovery of tissue concentrations of glucose and pyruvate which initially exceed their control values. The early elevation of glucose levels reflects the glucose-sparing effect of preferential use of Lac, which also explains the concurrent elevation in pyruvate. The elevated pyruvate, in turn, inhibits glycolysis [20]. Lac levels have been shown to increase during the chronic phase of ongoing cell death and infarction in the rat [62] as well as in some human neonates [42]. The precise explanation for this continues to be debatable as discussed above.

Conclusion

This review covers the wide and complicated subject of cerebral metabolism in the immature rodent brain during physiologic and hypoxic-ischemic conditions from a metabolomics perspective. The emergence of metabolomics, as a systems biology approach, has made possible the measurement of multiple metabolites directly from complex biological systems and the creation of a phenotypic “snapshot” of a cell, tissue, or organism.

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The study of metabolomic biomarkers for HI in the immediate neonatal period is not a trivial task and requires very specific considerations that are unique to this disease and population. Essentially, in order to truly understand its pathophysiological processes and find solutions, researchers and clinicians should employ a holistic approach, instead of a reductionist approach which traditionally focuses on small numbers or individual metabolites in isolation. A metabolomics approach can be an aid, since it provides a comprehensive panel of all metabolites present in a biological system, can investigate all variations of concentrations and fluxes, and is more contextual by tracking the rapid changes in metabolites, which, in turn, reflects the status of the surrounding tissue.

In the immediate future, metabolomics will be able to assist in the prediction of mortality as well as the validation of treatments like therapeutic hypothermia. Although the predictive value of MRS imaging as the banner of the metabolomics field has not yet been fully established, it is clear that differences in metabolite ratios under normal and hypoxic-ischemic conditions do exist. The different MRS techniques can be combined to assess different sources of information: ^1H MRS can measure the steady state of metabolites using specific proton resonances, ^{13}C MRS can provide insight into the fate of the applied label and its intermediate metabolic products under steady-state conditions, and HP- ^{13}C MRS delivers information about real-time metabolic conversions.

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Disclosure Statement

The authors declare they have no conflicts of interest.

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