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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, cerebral energy metabolism undergoes alterations in substrate use in order to preserve the production of ATP. These changes have been studied with *in situ* biochemical methods yielding valuable quantitative information about high energy and glycolytic metabolites and establishing a temporal profile of the cerebral metabolic response to hypoxia and hypoxia-ischemia (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 (^{1}H) 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 ^{1}H MRS has proven challenging due to discrepancies in calculations and timing of measurements among studies. As a result, the predictive utility of the results is not clear. ^{13}C-MRS is methodologically more challenging but provides a unique window on living tissue metabolism, through measurements of the incorporation of ^{13}C label from substrates into brain

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metabolites and localized determination of various metabolic fluxes. The newly developed hyperpolarized ^{13}C (HP-^{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.

Keywords

Vannucci model; neonatal hypoxia ischemia; metabolomics; magnetic resonance spectroscopy; hyperpolarized pyruvate; glucose; lactate

1. Introduction

The concentrations of various tissue metabolites are considered valuable indicators of both normal and abnormal conditions and have potential as biomarkers for various pathological states. The array of metabolites that reflect 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 that is employed for metabolomic studies is Nuclear Magnetic Resonance (NMR) spectroscopy (or Magnetic Resonance Spectroscopy – MRS) as it yields highly specific biochemical information, characteristic for particular regions of the brain and particular 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 the healthy unlesioned tissue [4]. The metabolic alterations of a tissue often precede other anatomical and functional changes that can be observed with anatomical MR (magnetic resonance) imaging (MRI). Thus, MRS can show disturbances even when the brain appears anatomically normal [5,6] and data analysis from MRS spectra has the potential to identify useful biomarkers predictive of injury or recovery.

Cerebral 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, 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 the newborn 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.

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The majority of experimental studies of brain metabolism in neonatal HI have utilized a combination of unilateral 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 its adaptations in 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 to severe, injury; occlusion of the artery or hypoxia alone do not result in brain damage [14]. The systemic hypoxia in combination with the 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 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 as glucose, namely lactate 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 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 non-invasive 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 as synthesis of old and new data will highlight the usefulness of the current metabolomic studies in the pursuit of biomarkers of injury and recovery.

2. 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 bi-directional 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

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the basement membrane [26]. From there, glucose is transported across the neural cell membranes of neurons (GLUT3) and glial cells (GLUT1), where it is phosphorylated to glucose-6-phophate (glucose-6-P) via hexokinase [25]. Glucose-6-P can be metabolized through three 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 lactate, transaminated to alanine or enter the mitochondria. In the mitochondria, it is decarboxylated by the pyruvate dehydrogenase complex (PDH) yielding the TCA cycle substrate, acetyl CoA. Acetyl CoA condenses with oxaloacetate, entering the tricarboxylic acid (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) (Figure 1). The TCA cycle provides the bulk of energy required for cerebral function. Complete oxidation of one molecule of glucose through the TCA cycle produces 30–32 ATP [28].

Glucose-6-P also serves as an essential substrate for the PPP pathway. The PPP pathway generates pentoses for nucleotide acid production, needed for DNA synthesis, and NADPH to manage oxidative stress and synthetize lipids through the regeneration of glutathione (GSH). Metabolism of glucose through the PPP pathway eventually results in fructose-6-P. Fructose-6-P will generate glyceraldehyde and consequently pyruvate. PPP followed by pyruvate carboxylation in astrocytes [29], will generate glutamate/ glutamine and GABA through backflux conversion of oxaloacetate to succinate and α-ketoglutarate (Figure 1) [30]. GSH acts as the substrate for glutathione peroxidase to reduce reactive oxygen species (ROS). Its activity is higher in the neonatal brain than in the adult 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 astrocytes. Concentrations of brain glycogen are high in the fetal rodent brain but 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 the differing metabolic rates [33].

3. Measurement of metabolites in brain extracts

In the 1970's Oliver Lowry and colleagues 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 post-mortem changes [34]. Such studies in adult rodents utilized *in situ* funnel freezing of the brain. However, due to both the thin skull and the lower metabolic rate of the immature brain, snap-freezing of the pup, or head, in liquid N_2 proved sufficient to keep these changes to a minimum [35]. Tissue was kept at−70°C to −80°C until dissection and powdering of the brain, at −20°C. The frozen brain was then acid-extracted and the

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neutralized extracts were used for fluorometric analysis. Similar analyses were also performed on plasma and CSF samples from these animals, allowing for the calculation of blood/brain and CSF/brain ratios.

Errors inherent in this method include lower values for AMP and higher values for inorganic phosphate (P_i) when frozen tissues were acidified at temperatures below 0°C. To keep post-mortem glycolytic and energy consumption to a minimum freezing must be complete in under 60 sec, tissue must be maintained at −80 C until acid extraction, and extracts must be maintained at −20°C until analysis [34]. Limitations in this methodology include the challenge of strict adherence to the temperature requirements of brain processing, difficulty in regional measurements, and inability to correlate metabolic changes to outcome.

Despite these 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 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, lactate, pyruvate, β-hydroxybutyrate, acetoacetate, creatine and of high energy compounds, namely ATP, ADP and phosphocreatine (PCr) were measured with fluorometric enzymatic techniques [34].

Intracellular pH was also calculated through ratios of ATP/ADP and creatine/PCr. During HI the pH in the ipsilateral hemisphere decreased, whilst in the contralateral hemisphere it remained stable, despite the similarity between the increased lactate concentration in both hemispheres [22,36]. In the immature brain, it is tissue acidosis and not lactate concentrations that correlate with tissue damage, likely due to the ability of lactate 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 the 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, on 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 limitation of cellular substrate (glucose) supply to the ipsilateral hemisphere and culminates in brain damage [23]. It is important to note that the duration of hypoxia-ischemia in these early studies was 180 minutes, consistent with a severe degree of brain injury, with the increase in the mitochondrial redox state occurring at 2 to 3 hours of HI and cannot be assumed to occur following milder results.

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4. Nuclear Magnetic Resonance (NMR) techniques *in vivo*

MR techniques have provided essential contributions 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 noninvasive detection of numerous intracellular metabolites in otherwise inaccessible tissues such as the brain. It therefore is a quantitative study of a complex mixture of metabolites, visualizing multiple compounds at the same time, minus 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 enable the investigation of perinatal cerebral energy metabolism *in vivo* [37,38].

4.1 ^{1}H Magnetic Resonance Spectroscopy (^{1}H MRS)

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

The most relevant feature of the ^{1}H MRS spectra to hypoxic-ischemic injury in neonates is a variable sized double lactate peak. Lactate is the end product of anaerobic respiration and increased lactate 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 lactate maintains steady state levels, concentrations rise during hypoxia and hypoxia-ischemia [19], but are rapidly cleared on resuscitation through conversion to pyruvate as well as transport to the blood [38]. Lactate levels during the subsequent evolution of injury, depend on the severity of the insult and 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 lactate detected on ^{1}H MRS early after the insult can be used as a predictor of outcome. However, there is continued debate over the interpretation of the elevated lactate detected with ^{1}H MRS. Not all infants with abnormal neurodevelopmental scores exhibit high lactate [43]. Yet, in almost all infants with poor outcomes, lactate on ^{1}H MRS persists beyond one month after birth. While the initial lactate peak in the acute phase of HI may be due to anaerobic metabolism, persistence of elevated lactate for days to months may indicate long-term perturbation of cerebral energy metabolism [42,44]. Alternatively, high lactate 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.

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Aside from lactate, there is much interest in NAA, due to its importance as a neuronal marker [48]. NAA is synthetized in neurons in a reaction catalyzed by 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 used for synthesis of fatty acids such as myelin [50,51]. Several studies have further suggested that NAA levels reflect neuronal mitochondrial function, since its levels are lowered with inhibition of mitochondrial respiration [52]. Although its absolute concentration varies from species to species, there is a steady increase in the levels of NAA from the neonatal period to adulthood. Studies in rats document a rapid increase between postnatal day 5 and 20 in the rat brain [53]. In infants, despite regional differences, ^{1}H MRS found NAA levels to be 50% of those in adults. Provided that the exponential time course of the NAA increase is extended to the *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/choline (Cho) ratios have proven to be correlated with unfavorable neurodevelopmental outcomes at 2 and 30 months of age [55,56]. Some studies have found an increased Lac and decreased Cho combination to better correlate to the outcome [57,58] while others discovered a more significant association with increased Lac and decreased NAA levels [43,59,60]. Similar to the differences in the observed lactate peaks, this divergence has again been attributed to differences in the timing of the imaging investigation with respect to the HI insult and to 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 by *in vivo* ^{1}H-MRS. Particularly, Malisza et al [61] observed substantial increase of Lac and reduction of 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 of 28 days post injury, using localized *in vivo* ^{1}H-MRS. Significant alterations in the metabolic profile of the ipsilateral hippocampus were detected at 24h post-HI and were indicative of oxidative stress such as reduced glutathione (GSH) and Tau protein, while the impaired mitochondrial phosphorylation and the consequent reliance on anaerobic metabolism were evidenced by the persistently high level of Lac and depleted PCr in the majority of HI subjects at 24 h post-HI [62].

MRS is technically demanding in the clinical setting; its success is heavily dependent on its sensitivity and the 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 low sensitivity which is 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 therefore rendering shimming (termed as the minimization of inhomogeneity B_o between different diamagnetic tissues) even more lengthy and challenging [2].

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Most MRS studies on brain tissue have used the ^{1}H nucleus due to its high natural abundance (99.99%) and its high prevalence in metabolites. In the last 15 years ^{1}H MRS spectroscopy has benefitted greatly from the application of higher 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 the 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 the low sensitivity for the detection of non-proton nuclei as they are characterized by a substantially lower gyromagnetic ratio and lower natural abundance compared to the ^{1}H nucleus. These weaknesses may be overcome in a number of ways, one of which is proton decoupling wherein ^{13}C signals become detached from their neighboring ^{1}H atoms and may appear as singlets on the spectra. Alternatively, introducing the respective label in an injected metabolic substrate that doesn't 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].

4.2 ^{13}C Magnetic Resonance Spectroscopy (^{13}C MRS)

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 different positions in the same molecule (^{13}C isotopomers). 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 through mathematical modeling [64,65].

Since glucose is the primary fuel for brain energy metabolism, most dynamic studies with ^{13}C MRS, have preferably used ^{13}C- labeled glucose for metabolic studies in the brain. Metabolism of ^{13}C glucose leads to clearly labelled patterns at the level of lactate, alanine and in metabolites produced from the first cycle of the TCA. Injection with [1,2-^{13}C] glucose to P7 rats and analysis of their extracted cerebral hemispheres with ^{1}H-^{13}C MRS was used in order to observe changes in the PPP and the activity of pyruvate carboxylase in astrocytes post-HI. In accordance with previous studies [20], glucose increased bilaterally and lactate accumulated, signifying the increased anaerobic glycolytic flux. Opposite to the researchers' expectations, PPP activity appeared reduced and they interpreted the results as mitochondria hypometabolism [41]. An alternative explanation is that during early recovery lactate 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 the relatively low sensitivity of *in vivo* NMR. Therefore, researchers must rely on detecting the ^{13}C label in larger pools of

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brain amino acids, such as glutamate or glutamine and then, indirectly calculating TCA cycle activity [66]. Thus, labelled 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 two major nuclei used for NMR studies (^{1}H 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 in order to increase 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 (minutes to hours) required for acquisition and the constant infusion of large volumes of ^{13}C-metabolites needed to get signal render ^{13}C MRS inapplicable to young animals with small body volumes and thus limit its feasibility to cultured cells and extracted tissues [68].

4.3 Hyperpolarized ^{13}C Magnetic Resonance Spectroscopy (HP-^{13}C MRS)

The latest evolution in MRS imaging has been the use of hyperpolarized ^{13}C-labeled substrates (HP-^{13}C MRS). Dynamic nuclear polarization (DNP) can achieve 10,000-fold enhancement of the ^{13}C signals of an introduced substrate and its subsequent metabolic products. The gain in signal intensity has irreversibly altered the clinical potential of the ^{13}C labeled substrates. By applying DNP to endogenous, non-toxic, non-radioactive substances (pyruvate, urea) and administering them to a subject, HP-^{13}C MRS can monitor the administered compound's fluxes through key biochemical pathways, such as glycolysis. Contrary to ^{1}H MRS, which focuses on metabolite levels, with HP-^{13}C MRS, researchers can now track metabolic activity in real-time occurring at the 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 microwave heating. Hyperpolarized 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 hyperpolarized solid substance from a very low temperature inside the polarizer to an injectable solution, close to body temperature, and 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 hyperpolarized state [71].

One of the first applications of this technology has been the evaluation of the conversion of hyperpolarized ^{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 non-toxic, pyruvate is found at the junction of anaerobic glycolysis and oxidative phosphorylation (Figure 1). Depending on the intracellular energy state of the tissue, pyruvate is converted to a different degree to lactate, 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 (~3 s) allows for multiple acquisitions while the intravenously injected hyperpolarized [1-^{13}C] pyruvate converts rapidly into lactate in the brain. Dynamic data from the average peak height for pyruvate and lactate at each time point can be measured and an individual curve for the build-up and decay of pyruvate and lactate signals can be obtained (Figure 2). The normalized lactate level and the pyruvate to lactate 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 lactate and the more lactate is produced within a certain time period [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 lactate as fuel more efficiently than the adult. In the setting of HI, a potentially increased k_{pl} could indicate the urgent need for substrate in the absence of glucose.

The conversion of pyruvate to lactate reflects the activity of lactate dehydrogenase [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 hyperpolarized pyruvate on C-2 instead of C-1 ([2-^{13}C] pyruvate) permits the investigation of additional metabolic pathways such as the formation of glutamate [75]. No studies with hyperpolarized [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 in investigating the metabolic profile changes during HI.

The translation of the hyperpolarized 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 blood brain barrier. 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 data sets 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 a 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 normal development of the rodent brain as well as during HI brain damage as is manifested in the Vannucci model.

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5. 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 alternate cerebral fuels to supplement glucose in fulfilling the brain's energy needs during periods of starvation, during suckling, and hypoglycemia [77]. The alternate substrates lactate and the ketone bodies, β-hydroxybutyrate and acetoacetate, can assist glucose for energy production in a well oxygenated state [78].

Normal brain development depends upon a metabolic system whose role is to keep pace with the escalating oxygen substrate demands that accompany the rapid increase in brain mass and the accelerated electrochemical maturation that characterizes synaptogenesis [79]. Under normal physiological conditions, oxygen and substrate delivery to the developing brain has been considered adequate to support cerebral energy metabolism as well as maturation. The immature animal brain has substantially lower metabolic demands (1/10 of the adult 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, they can suffer an extensive injury due to unique vulnerabilities [80].

The low cerebral metabolic rate is reflected in low rates cerebral glucose utilization (CGU). While blood glucose levels are comparable between the early postnatal period and adulthood, the expression of the glucose transporter proteins, GLUT1 in the blood brain barrier 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 increased biochemical activity after the second postnatal week. A concomitant increase in enzymes of both the glycolytic and tricarboxylic acid pathways is fueled by the increased availability of glucose during this period of functional maturation with 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, such as in increased glycolytic demand or hypoglycemia. The transport of the alternate 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 blood-brain barrier and in glial cells [83]. MCT2 is the primary neuronal isoform although evidence of 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 two weeks of life ketone bodies can provide up to 60% of energetic fuel for the brain [89] and 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 are 25 times the levels of the adult [84]. This upregulation is more

conspicuous in suckling rats than adults receiving a ketogenic diet, so it may be developmental along with dietary [17,86].

The transition from lower CGU in the suckling animal to the high CGU in the adult is also mediated through changes in the expression of enzymes for substrate use in the brain. The development of enzymes associated with the complete aerobic utilization of glucose as well as glycolysis, such as hexokinase, citrate synthase, lactate dehydrogenase and pyruvate carboxylase has been shown to have absolute correlation with the onset of full neurological competence in the rodent brain. The activity of these enzymes increases several-fold until days 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 occurs during the early suckling period [93].

6. Cerebral metabolism in hypoxic ischemic injury

During experimental ischemia in adult models, rates of CGU are nearly triple 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 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 depletion of 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 lactate has been shown in the neonate immediately after the insult [19,95]. Brekke et al [41] reconfirmed through their results that during the hypoxic ischemic episode, there is a dramatic shift towards the anaerobic utilization of glucose with approximately three-fold increase in the production of lactate and rapid depletion of brain glucose. The lactate accumulation that is 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 for adults, lactate is not deleterious to the neonatal brain and does not contribute to the decrease of the pH and the subsequent intracellular acidosis that develops during cerebral HI, until a certain threshold is passed [36].

However, within four hours after the insult, brain lactate levels normalize [20,22]. Upon reperfusion and reoxygenation, the accumulated lactate is rapidly metabolized into pyruvate, providing 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 lactate and ketone bodies across the blood brain barrier through the MCTs. In fact, any condition that results in elevated lactate levels systemically, promotes its

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uptake and utilization by the immature brain, thus sparing the consumption of glucose [17]. Indeed, contrary to what happens in adult cerebral ischemia, the PDH 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 lactate, 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 lactate which also explains the concurrent elevation in pyruvate. The elevated pyruvate in turn, inhibits glycolysis [20]. Lactate 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.

The study of metabolomic biomarkers for HI in the immediate neonatal period is not a trivial task and requires very specific considerations, unique to this disease, and population. Essentially, in order to truly understand its pathophysiological processes and find solutions, researchers and clinicians should employ more of a holistic, instead of reductionist, approach, which has traditionally focused on small numbers or individual metabolites in isolation. Metabolomics can aid in this way, 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 of metabolites, which in turn, reflect 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 do exist between normal and hypoxic-ischemic conditions. These different MRS techniques can be combined to assess different sources of information: ^{1}H 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 on real-time metabolic conversions.

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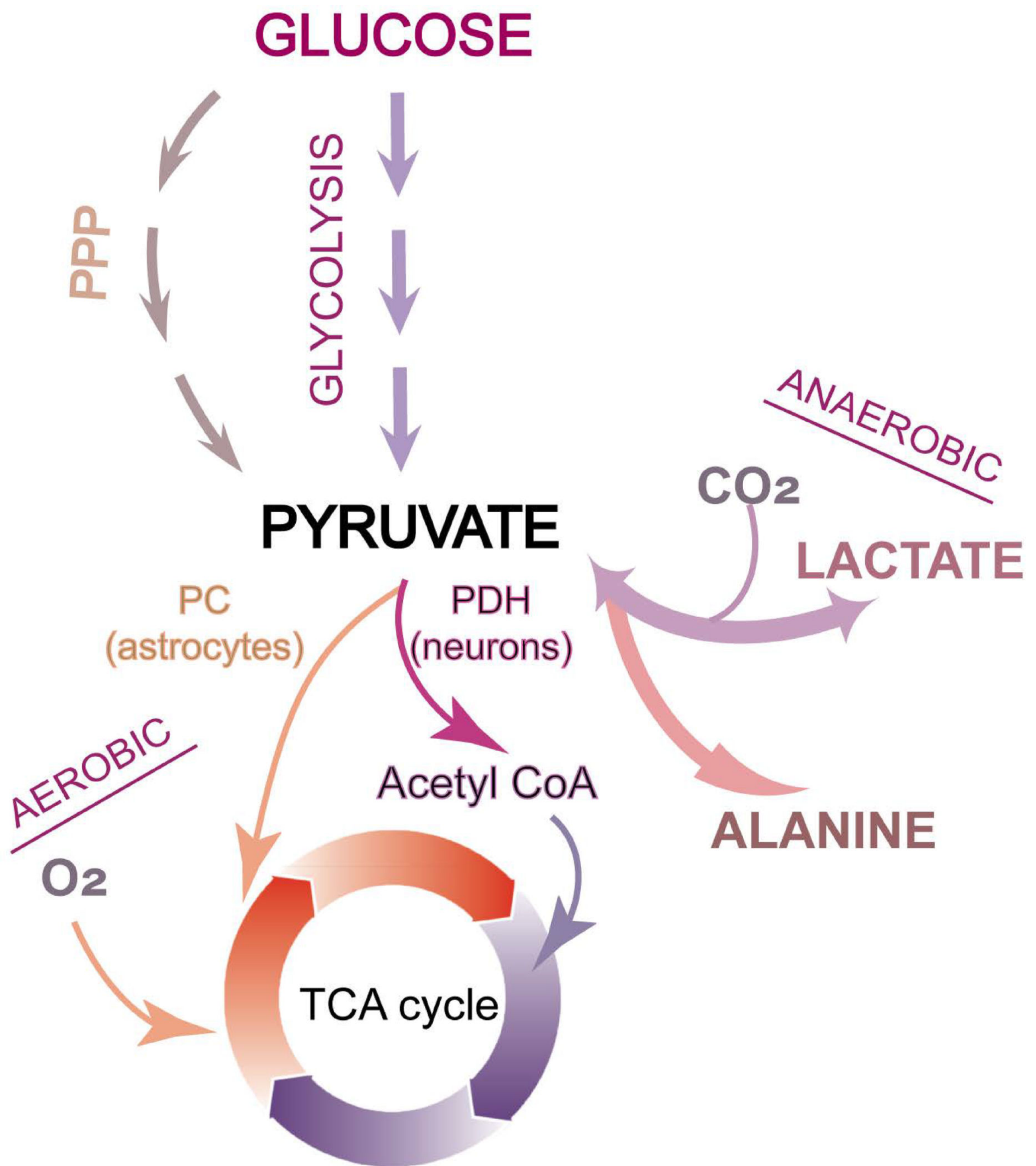

Figure 1.

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 TCA cycle via pyruvate dehydrogenation (PDH) as acetyl CoA. If glucose is metabolized through the PPP, the resulting pyruvate may undergo pyruvate carboxylation (PC) in astrocytes and then enter the TCA cycle backflux from oxaloacetate to succinate. Pyruvate is thus, an intermediate common between 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 glucose supply is short, by converting back into pyruvate.

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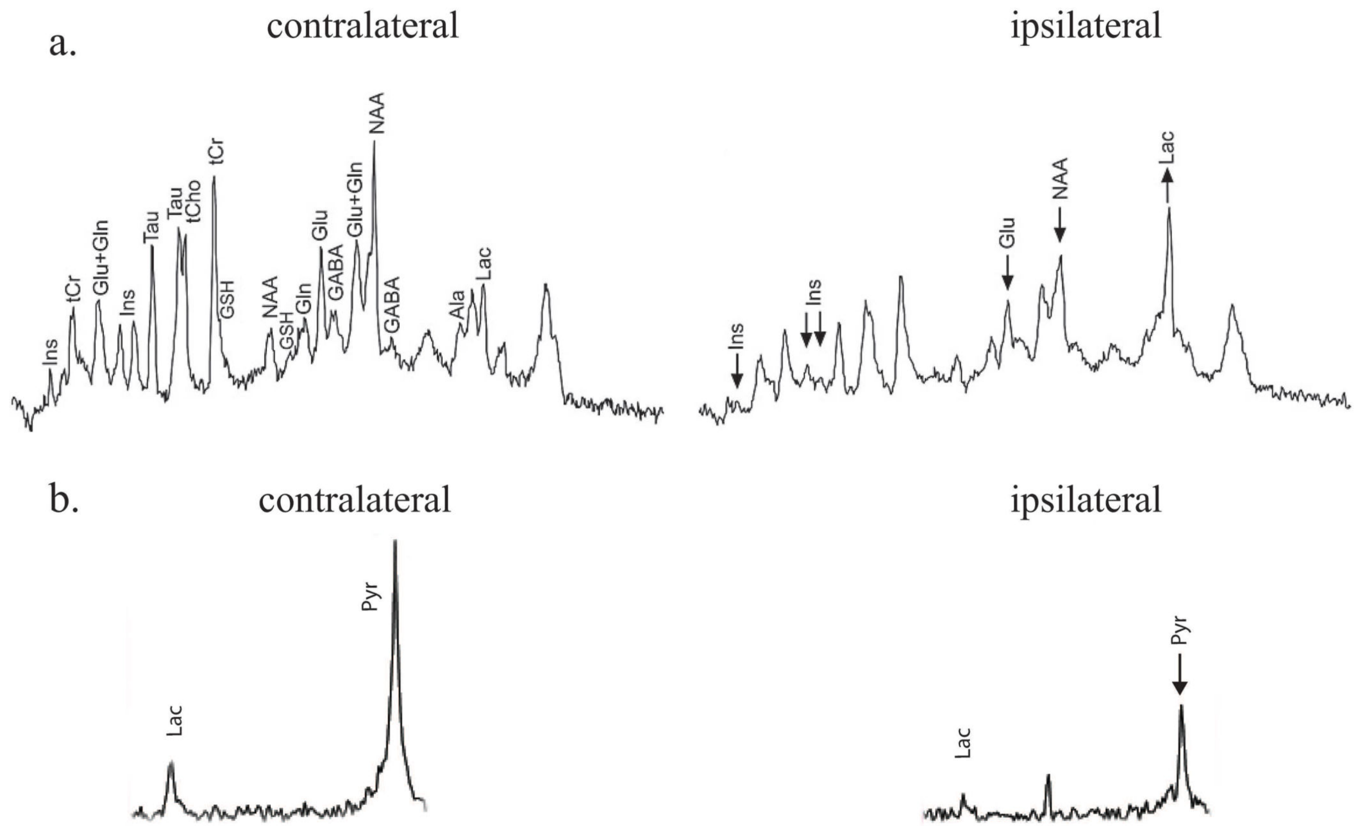

Figure 2.

Examples of representative spectra from individual voxels of the ischemic / hypoxic-ischemic murine brain acquired with **a)** ^{1}H MRS and **b)** HP-^{13}C MRS. ^{1}H MRS spectra allow access to the steady state of a collection of metabolites using their specific protons while HP-^{13}C MRS 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 the two methods are different in nature but complementary to each other. **a)** ^{1}H MRS spectra of the non-ischemic contralateral striatum (left) and of the ipsilateral to the brain injury striatum (right) of an adult mouse (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 in the ipsilateral side. **b)** Spectra acquired from single voxels positioned on the contralateral (left) and ipsilateral (right) hemispheres with a dual shifted ^{1}H-^{13}C coil during the iv injection of hyperpolarized pyruvate to a P10 mouse at 4–6 h after HI. The pyruvate and lactate signals at a single time point during the acquisition are represented by two peaks at their respective frequencies. The low pyruvate signal on the ipsilateral to the injury side reflects the impeded perfusion and edema which results 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-acetylaspartate; Tau, taurine; tCho, total choline; tCr, total creatine; Lac, lactate; Pyr, pyruvate.

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