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Imaging brain metabolism using hyperpolarized ^{13}C magnetic resonance spectroscopy

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Abstract

Aberrant metabolism is a key factor in many neurological disorders. The ability to measure such metabolic impairment could lead to improved detection of disease progression, and development and monitoring of new therapeutic approaches. Hyperpolarized ^{13}C magnetic resonance spectroscopy is a developing imaging technique which enables non-invasive measurement of enzymatic activity in real time in living organisms. Primarily applied in the fields of cancer and cardiac disease so far, this metabolic imaging method has recently been used to investigate neurological disorders. This review summarizes the preclinical research developments in this emerging field, and discusses future prospects for this exciting technology, which has the potential to change the clinical paradigm for patients with neurological disorders.

Keywords

hyperpolarized ^{13}C magnetic resonance spectroscopy; metabolic imaging; MRI; neurological disorders; preclinical models; immunometabolism; clinical translation

Hyperpolarized ^{13}C metabolic imaging of neurological disorders: a new application for a developing technology

Metabolic changes are increasingly being recognized as key players in neurological diseases, and may reveal exciting new avenues for treatment of these disorders. It is critical to develop new tools for monitoring brain metabolism through disease progression and in response to treatment, but such development remains complex due to our inability to easily sample brain tissue. New neuroimaging methods are beginning to address the crucial need for non-invasive assessment of brain metabolism.

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Hyperpolarized ^{13}C magnetic resonance spectroscopy (MRS)/spectroscopic imaging (MRSI, see Glossary) is an emerging technology which enables *in vivo* acquisition of dynamic metabolic data, providing unprecedented insights into the *in vivo* metabolic status of the organ of interest. ^{1}H and non-hyperpolarized, thermal ^{13}C MRS have both been used to acquire clinical data on steady-state human brain metabolism[1–3]. However these methods are not used widely in the clinic, and in particular, thermal ^{13}C MRS requires long scan times and infusions of ^{13}C-labeled substrate. Hyperpolarized ^{13}C MRS brings new information to the field of metabolic imaging. First published in 2003 [4], this method first requires the hyperpolarization and rapid liquid dissolution of a ^{13}C-labeled compound, typically pyruvate, a process typically carried out in an automated fashion using a hyperpolarizer. Hyperpolarization of a compound causes it to become more than ten thousand-fold more visible by MRI, due to the increased signal-to-noise ratio. Figure 1 (Key Figure) summarizes the typical steps of a hyperpolarized ^{13}C MRS/I preclinical experiment. Because the hyperpolarized state lasts only for a short time once in solution (referred to as the relaxation time, or T_1), injection into the biological system of interest (cells, animal model, human) must be rapid (10–15s in animals) and coincide with the time of data acquisition. Typical MR hardware is then used to obtain ^{13}C MR spectra (slab, 2D or 3D, single time point or dynamic), where the resonances of the injected substrate and its downstream metabolic products can be seen. Following injection of hyperpolarized [1-^{13}C]pyruvate, the most commonly used probe, the resonances of hyperpolarized [1-^{13}C]lactate, hyperpolarized [1-^{13}C]alanine, and in some cases hyperpolarized ^{13}C bicarbonate, are observed in the living brain, reflecting enzymatic conversion via lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and pyruvate dehydrogenase (PDH), respectively. Many new compounds for injection are under development, and will be discussed later. MR spectra showing area(s) under the curve(s), corresponding metabolic ratios of product:substrate (e.g. lactate:pyruvate), or kinetic pseudo-rate constants for substrate to product conversion (e.g. k_{PL}) can then be reported from hyperpolarized data.

By using preclinical models, hyperpolarized ^{13}C metabolic data can be validated either through correlation analyses with “ground truth” *ex vivo* biochemical analyses, or through comparison to more widely-applied clinical imaging techniques to assess the added value of the method. Clinically, hyperpolarized ^{13}C MRS/I can provide access to otherwise inaccessible metabolic information, given the rarity of human brain tissue biopsy sampling.

So far, hyperpolarized ^{13}C MRS/I has been primarily applied in the fields of cancer and to a lesser extent cardiovascular disease, with clinical studies underway in both areas. More recently, exciting data have been acquired in preclinical models of neurological disorders and neuroinflammation, in which metabolic changes have been observed with disease progression and following treatment. This new investigative direction of neurological disorders may improve our understanding of *in vivo* brain metabolism not only preclinically, but also in patient populations, since hyperpolarized ^{13}C MRS/I has already been translated to the clinic. In this review we summarize the current applications of hyperpolarized ^{13}C MRS/I in the non-oncological neurological field, consider current challenges, and discuss prospects for future work.

Hyperpolarized [1-^{13}C]pyruvate provides non-invasive metabolic preclinical information in the healthy and diseased brain

The conversion of pyruvate to lactate sits at a key intersection in energetic metabolism pathways, with LDH being present in most cell types across the body. This enzymatic step is affected in a multitude of diseases, and consequently often modulated in response to treatment. As a result, an ability to non-invasively visualize pyruvate to lactate conversion opens the door to vast opportunity for possible applications in disease monitoring and assessment of therapeutic response.

Understanding hyperpolarized ^{13}C MRS/I data from the healthy brain at different ages is essential if one is to investigate disease development and treatment response.

The adult brain has been investigated by hyperpolarized ^{13}C MRS/I in several preclinical proof-of-concept studies. The feasibility of using both hyperpolarized [1-^{13}C]pyruvate to study brain metabolism using a variety of MR systems and radiofrequency (RF) coils has been confirmed, with conversion of hyperpolarized [1-^{13}C]pyruvate to hyperpolarized [1-^{13}C]lactate and hyperpolarized ^{13}C bicarbonate detected in mouse and rat brain via imaging at 1 or 3 Tesla [5–7]. Hyperpolarized [1-^{13}C]alanine has also been detected, although this metabolite is likely to arise from the soft tissue surrounding the healthy brain[5].

Before moving to the clinic, with the first clinical manuscript reported in 2013, hyperpolarized ^{13}C MRS/I was also shown feasible in the non-human healthy primate brain using a clinical 3 Tesla MR system and the clinical SpinLab hyperpolarizer [8]. Interestingly, values for hyperpolarized ^{13}C lactate:pyruvate were found to be similar to those seen in rodent studies.

Only one preclinical study to date has, to our knowledge, used hyperpolarized ^{13}C MRS/I to investigate brain development. This longitudinal study in mice measured cerebral conversion of hyperpolarized [1-^{13}C]pyruvate to hyperpolarized [1-^{13}C]lactate at multiple timepoints between postnatal days 18 to 60, and observed that this conversion decreased with age [9]. Total brain volume as measured by conventional MRI, however, remained largely unchanged, demonstrating the added value of metabolic imaging [9]. In the healthy aging brain, hyperpolarized ^{13}C MRS/I research remains to date very limited; there is also much opportunity for understanding age-linked neurological disorders, such as Alzheimer’s disease [10]. As an initial step into the aging field, hyperpolarized ^{13}C MRS has been used to study healthy older mice. Administration of dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor, was shown to decrease cerebral hyperpolarized [1-^{13}C]pyruvate to hyperpolarized [1-^{13}C]lactate conversion. An impairment in spatial learning was observed with DCA treatment over time, assessed using a Morris water maze [11].

Immunometabolism

Immunometabolism refers to the metabolic alterations that occur following activation of immune cells, generally in response to pathogens or noxious stimuli. Although immune cell

activation is central to many brain diseases, clinically translatable methods that enable the specific imaging of cells from the immune system and immune subtypes are still lacking. On activation, innate immune cells undergo metabolic reprogramming that give rise to pro-inflammatory or anti-inflammatory subsets [12]. Therefore, hyperpolarized ^{13}C MRS/I is a highly promising technique for non-invasively detecting and monitoring inflammatory status.

Classically activated pro-inflammatory macrophages, generally associated with tissue damage, have been shown to increase their glucose uptake, glycolysis and conversion of pyruvate into lactate [12]. *In vitro* studies conducted in mouse macrophage cells using hyperpolarized [1-^{13}C]pyruvate showed that, after activation using the toxin lipopolysaccharide (LPS), hyperpolarized ^{13}C lactate:pyruvate was significantly increased compared to non-treated macrophages [13]. This increased ratio was explained by an increase in LDH activity and gene transcription, increased cofactor nicotinamide adenine dinucleotide (NADH), and decreased expression of monocarboxylate transporters 1 and 4 (MCT1/4), resulting in an increased intracellular lactate concentration (see also Supplementary Table 1). Further, hyperpolarized [1-^{13}C]pyruvate was able to detect the effect of a nonsteroidal anti-inflammatory drug affecting LDH affinity. In line with these findings, a single intracranial injection of LPS in the mouse brain resulted in increased hyperpolarized [1-^{13}C]lactate and hyperpolarized ^{13}C lactate:pyruvate at the site of injection [14]. These changes were in alignment with increased numbers of microglia/macrophages and astrocytes.

Activation of lymphocyte T cells and the resultant altered metabolism[15] has also been detected using hyperpolarized [1-^{13}C]pyruvate; activated CD4+ T cells showed a three fold higher hyperpolarized ^{13}C lactate:pyruvate compared to non-activated cells[16].

Neurological disorders

Energetic imbalance and abnormal energy metabolism have been implicated in most neurodegenerative disorders [17, 18]. There are multiple causes of alterations in brain metabolism which may arise from direct insults to the brain tissue itself and/or as a result of systemic modifications occurring in peripheral organs. So far, only a small number of preclinical studies have used hyperpolarized ^{13}C MRS/I approaches to assess metabolic impairment in the diseased brain outside the oncological field, and these are limited to hyperpolarized [1-^{13}C]pyruvate.

In models of traumatic brain injury (TBI), hyperpolarized ^{13}C lactate:pyruvate increased at the injury site within hours and remained elevated for several weeks following impact [19, 20]. Further, hyperpolarized ^{13}C bicarbonate:lactate was decreased at early time points post-injury [20]. When investigated, these changes were found to be linked to decreased PDH activity with no changes in LDH activity. The cellular sources driving these metabolic changes remain unclear and most likely involve multiple cell types, such as dying neurons and inflammatory cells. Furthermore, interpretation of the results is challenging due to physical alterations of the brain, including blood brain barrier (BBB) disruption, bleeding and edema. However, interestingly, at subacute timepoints, microglial cells were shown to

likely be a major contributor to the increased hyperpolarized ^{13}C lactate:pyruvate, via use of a diet that specifically depletes the microglial population [19].

Further evidence that pro-inflammatory macrophages can be responsible for the observed increased hyperpolarized ^{13}C lactate:pyruvate was provided from two mouse models of multiple sclerosis (MS) [21, 22]. MS is an autoimmune disease that leads to destruction of myelin sheaths and results in motor and cognitive deficits [23]. Increased hyperpolarized ^{13}C lactate:pyruvate was seen in brain lesions, where high levels of macrophages were detected. These changes were linked to macrophages upregulating the enzyme PDK1, leading to inhibition of PDH activity and increased flux towards lactate production. This increase was not detected in transgenic mice that do not elicit an immune response due to their lack of the fractalkine receptor (CX3CR1) [24].

Hyperpolarized [1-^{13}C]pyruvate was successfully used to identify metabolic alterations in a model of ischemic stroke induced by intracranial injections of the vasoconstrictor endothelin-1 [25]. Hyperpolarized [1-^{13}C]pyruvate and hyperpolarized [1-^{13}C]lactate, but not hyperpolarized ^{13}C lactate:pyruvate, were increased in the stroke region (penumbra) compared to the contralateral hemisphere. Although no *ex vivo* validations were performed, these findings indicate an increased delivery of hyperpolarized [1-^{13}C]pyruvate. This increased delivery is possibly linked to increased blood flow/perfusion or uptake across the BBB, as well as an increased conversion into lactate, which the authors suggest indicates an increased LDH enzymatic activity.

Increases in hyperpolarized ^{13}C lactate:pyruvate were described in the brains of non-diabetic mice fed a high fat diet for a 24-week period, and were correlated with decreased PDH activity, but not LDH activity in the cortex and striatum [26]. Importantly, this study is the first to report a significant correlation between the hyperpolarized ^{13}C lactate:pyruvate and cognitive deficits, evaluated in this case via a Morris Water Maze behavioral assessment. Interestingly, in a follow up study of diabetic mice fed a high fat diet for a 24-week period, hyperpolarized ^{13}C lactate:total ^{13}C was increased in the hippocampus [27]. *Ex vivo* analyses of tissue revealed increased lactate levels that were not explained by modulations of LDH, but were correlated with the phosphorylated adenosine triphosphate (ATP) citrate lyase protein, highlighting a metabolic adaptation in the presence of limited glucose availability.

Following acute liver failure, ^{13}C MRS/I of hyperpolarized [1-^{13}C]pyruvate revealed increased brain hyperpolarized ^{13}C lactate:pyruvate and hyperpolarized ^{13}C alanine:pyruvate before the appearance of clinical symptoms, and further increases of these ratios with disease progression [28]. In this case, it is not determined whether [1-^{13}C]alanine is indeed being visualized in the brain or in the surrounding tissue. Although lacking validation through molecular *ex vivo* analyses of tissue, these findings shed light on the pathogenesis of cerebral edema in acute liver injury, and suggest that lactate produced from anaerobic metabolism is a crucial factor in the development of brain edema.

Developing new hyperpolarized probes to target the full breadth of metabolic changes occurring in neurological disorders

The broad applications of hyperpolarized [1-^{13}C]pyruvate has been discussed above, but there are many additional novel hyperpolarized probes in development. Such probes could provide valuable information on the changing metabolism in disease and enable early assessment of treatment response. It is important to note that, while many metabolic pathways are of potential interest in neurological disorders, not all metabolites can be hyperpolarized. For success as a hyperpolarized probe, the metabolite of interest should have the following physical properties: (1) can be labelled with ^{13}C at the position of interest (which is often costly), (2) either be a liquid at room temperature, or dissolve in a safe solvent at high concentration (at least 1M), (3) form a glass when frozen quickly. From an MR perspective, both substrate and products of interest need to have distinct MR frequencies, and relaxation times long enough to be visualized during a MR experiment. Finally, from a biological perspective, the metabolite of interest should be rapidly transported to the organ of interest (and if necessary through the cell membrane), be metabolized rapidly, and be physiologically safe in the 1–200mM range. Despite these steep requirements, many hyperpolarized probes in early development have so far been demonstrated feasible for application in healthy animals (Figure 2). Hyperpolarized [2-^{13}C]pyruvate differs from the widely used [1-^{13}C]pyruvate only by the position of the ^{13}C label; protocols for its use are identical. From a metabolic perspective, however, hyperpolarized [2-^{13}C]pyruvate enables investigation of tricarboxylic acid (TCA) cycle metabolism, as the ^{13}C label in the C2 position results in the MR-visible products of not only [2-^{13}C]lactate, but also [5-^{13}C]glutamate and [1-^{13}C]citrate [29]. It must be cautioned that detection of metabolites besides [2-^{13}C]lactate is not a given, and may be dependent on multiple biological and technical factors [30]. Data acquired in the rat brain following injection of hyperpolarized [1-^{13}C]ethyl pyruvate, a lipophilic probe used for its ability to more easily cross the BBB, showed a comparable average lactate level to that observed following hyperpolarized [1-^{13}C]pyruvate injection [31].

Imaging metabolism of hyperpolarized [2,3,4,6,6-$^{2}H_5$, 3,4-$^{13}C_2$]glucose has been shown feasible in the healthy mouse brain [32]. Through detecting hyperpolarized [1-^{13}C]pyruvate and hyperpolarized [1-^{13}C]lactate shortly after injection, this probe is a potentially useful complement to fluorodeoxyglucose (FDG)-positron emission tomography (PET), informing on metabolism rather than uptake.

Hyperpolarized [1-^{13}C]lactate has itself been used as a probe; increased hyperpolarized [1-^{13}C]pyruvate:lactate was observed in the brain shortly after reperfusion of a middle cerebral artery occlusion mouse model of stroke. No lesion was detected using conventional MRI [33]. Interestingly, therapeutic strategies have identified lactate as a possible neuroprotective agent to limit cerebral damage after stroke, and thus hyperpolarized [1-^{13}C]lactate could be an effective theranostic agent, although it must be noted that use of hyperpolarized [1-^{13}C]lactate may be BBB limited [34].

Widening the scope further, hyperpolarized 2-keto[1-^{13}C]isocaproate (KIC) conversion to hyperpolarized [1-^{13}C]leucine by branched-chain amino acid aminotransferase (BCAT)

enzyme has been imaged in the healthy rat brain [35], which could be informative with regards to the metabolism of glutamate – a key metabolite in astroglia, microglia and neurons.

Hyperpolarized [1-^{13}C]glutamate and hyperpolarized [1-^{13}C] acetate could provide valuable *in vivo* information. However, they currently face the same challenge: the resonance frequency of the product is very close to that of the injected substrate, making data analysis complex. If this could be overcome, both probes could be usefully applied to the study of brain metabolism. Hyperpolarized [1-^{13}C]glutamate has been shown to provide information on glutamate to glutamine conversion in the healthy rat brain, although it required BBB opening [36]. This probe is potentially useful for studying chronic excitotoxicity, a symptom associated with many neurodegenerative disorders [37], but probe administration may have unwanted side effects [38, 39]. Brain cells such as astrocytes also metabolize acetate as fuel, and reactive astrocytes are major players in multiple neurodegenerative disorders. Thus, it is promising to note that injection of hyperpolarized [1-^{13}C]acetate *in vivo* resulted in observation of the TCA cycle intermediate α-ketoglutarate in the rat brain[40].

Oxidative stress is a key player in neuroinflammatory disorders, so a non-invasive *in vivo* measure of it in the brain would be incredibly valuable. Hyperpolarized [1-^{13}C]dehydroascorbic acid (DHA) has been shown to cross the BBB and be converted to hyperpolarized [1-^{13}C]vitamin C alongside oxidation/reduction of glutathione (GSH). As such, the conversion of hyperpolarized [1-^{13}C]DHA to hyperpolarized [1-^{13}C]vitamin C could be used as a marker of oxidative stress [41, 42]. In the rat brain, production of hyperpolarized [1-^{13}C]vitamin C from hyperpolarized [1-^{13}C]DHA was shown to be significantly reduced after treatment with diethyl maleate, a GSH depleting agent [43]. Unfortunately, administration of this hyperpolarized probe causes respiratory and cardiac distress in animal models [44], and developments are thus required before this probe can progress to the clinic.

In looking to image the transient but vital oxidative status of the brain, hyperpolarized [1-^{13}C]acetoacetate must also be considered. Hyperpolarized [1-^{13}C]acetoacetate to hyperpolarized [1-^{13}C]beta-hydroxybutyrate (BHB) conversion is dependent on, and therefore can be a biomarker for, NAD^+/NADH ratio – a proxy for the redox status of the cells. Detection of this conversion was recently shown feasible in the mouse brain [45]. Finally, conversion of hyperpolarized γ-glutamyl-[1-^{13}C]glycine to [1-^{13}C]glycine via γ-glutamyl-transferase (GGT) has been observed in healthy brain tissue [46]. GGT is responsible for maintaining cysteine levels for GSH production, and thus changes in hyperpolarized γ-glutamyl-[1-^{13}C]glycine metabolism may be informative in times of oxidative stress and GSH depletion.

Anti-inflammatory macrophages, associated with repair functions, are characterized by their ability to upregulate the enzyme arginase, which converts arginine into urea and ornithine, the latter being a substrate for synthesis of polyamine and collagen. Hyperpolarized [6-^{13}C]arginine detected arginase activity in an *in vitro* study using primary mouse myeloid-derived suppressor cells (MDSCs) [47]. Hyperpolarized ^{13}C urea:arginine was significantly increased in MDSCs compared to control bone marrow cells. Despite the fact that *in vivo*

translation of hyperpolarized [6-^{13}C]arginine is challenged by a relatively short T_1, new arginine preparation formulations and labeling strategies (e.g. [6-^{13}C,$^{15}N_3$]arginine) have demonstrated improved arginine imaging, as exemplified in the rodent liver [48, 49].

Translation to the clinic: Hyperpolarized ^{13}C pyruvate studies and clinical trials investigating human brain metabolism

The first-in-human study using hyperpolarized ^{13}C MRS/I, published in 2013, was conducted in patients with prostate cancer and proved the feasibility and safety of using hyperpolarized [1-^{13}C]pyruvate in the clinical setting [50]. A few years later, in 2018, the first hyperpolarized ^{13}C MRS/I studies in the human brain were performed in patients with glioma [51–55]. In addition to essential information on tumor metabolism (not discussed here [56]), these early studies provided the first hyperpolarized ^{13}C data on the non-tumor brain tissue. In this normal-appearing tissue, hyperpolarized [1-^{13}C]pyruvate was transported across the BBB and converted to the metabolic products hyperpolarized [1-^{13}C]lactate and hyperpolarized ^{13}C bicarbonate. Most studies agreed that hyperpolarized [1-^{13}C]pyruvate signal is highest in the vascular compartment, with the strongest signal intensity occurring in the superior sagittal sinus and transverse sinuses. Hyperpolarized [1-^{13}C]lactate production was observed throughout the brain [53, 55], and appeared significantly higher in cortical/juxtacortical regions than in white matter tracts [51, 53]. Hyperpolarized ^{13}C bicarbonate detection was more challenging [51], but showed higher signal in gray matter than in white matter [53]. Subsequent studies have focused on improving acquisition sequences [53], hardware configurations [54] and modelling of the kinetics of enzymatic pyruvate metabolism, to extract pseudorate constants for conversion of hyperpolarized [1-^{13}C]pyruvate to lactate (k_{PL}) and bicarbonate (k_{PB}) [55].

As all these studies were performed in glioma patients who had undergone multiple treatments, one may wonder if the normal-appearing brain tissue might exhibit an altered metabolism. However, interestingly, similar findings were recently shown in the brains of healthy volunteers. The first studies in human volunteers have showed that hyperpolarized [1-^{13}C]pyruvate, lactate and bicarbonate, and hyperpolarized ^{13}C lactate:pyruvate were significantly higher in gray matter compared to white matter. Further, no significant difference was detected in k_{PL}, k, or hyperpolarized ^{13}C bicarbonate:lactate between the two tissue types [57, 58]. In an elegant study, in fact the largest study of hyperpolarized ^{13}C MRS in the healthy human brain thus far, with a sample size of 14 subjects, it was reported that following injection of hyperpolarized [1-^{13}C]pyruvate [58], lactate topography of the brain was maintained across individuals, suggesting consistent region-specific lactate biology.

Feasibility of clinical injections of hyperpolarized [2-^{13}C]pyruvate has also been confirmed, showing the non-invasive detection of hyperpolarized [2-^{13}C]lactate and hyperpolarized [5-^{13}C]glutamate in the brains of healthy volunteers [59]. This detection of TCA-cycle-mediated glutamate production in the human brain mimics preclinical observations to some extent, although detection of other metabolites (e.g. citrate) was unconfirmed.

In a small but innovative ongoing early phase 1 clinical trial (interventional, non-randomized, NCT03502967)[I] [60], brain metabolism of both hyperpolarized [1-^{13}C] and

hyperpolarized [2-^{13}C]pyruvate are being measured after TBI (n=5), for comparison to healthy controls (n=3). This study is an exciting first step into imaging neurological, non-oncological disease using hyperpolarized ^{13}C MRS/I, using the most established probes. It will complement the ongoing preclinical studies, and hopefully highlight areas of improvement to ensure future preclinical studies are most relevant. Recent TBI research has highlighted the complex nuances of the disease, which cannot be observed by conventional imaging methods. Imaging metabolism using hyperpolarized [1-^{13}C]pyruvate in these cases may well be able to shine new light on early damage after injury,.

In the wake of the TBI clinical trial, further applications will likely grow out of those other areas investigated preclinically – namely MS and cognitive impairment. In MS, in addition to well established MRI protocols, hyperpolarized [1-^{13}C]pyruvate imaging could provide valuable data on metabolic alterations both in lesioned tissue as well as in normal-appearing brain matter. Given the potential of [1-^{13}C]pyruvate to detect inflammatory processes, this could represent a valuable tool to monitor the efficacy of immune-modulatory treatments in MS and help refine therapeutic regimens. In the field of dementia and Alzheimer's disease, there have been some clinical trial disappointments following targeting of ß-amyloid, and the presence of inflammation in the brain in Alzheimer's disease is increasingly being investigated [61, 62]. Given the early preclinical data demonstrating an ability to visualize inflammation using hyperpolarized [1-^{13}C]pyruvate (discussed earlier), metabolic imaging could be an exciting new source of information for diagnosis and treatment monitoring once translated to the clinic.

Current technical and biological challenges for preclinical and clinical metabolic imaging, and ongoing development of solutions

Improvements in hyperpolarization coupled with development of metabolic probes with a longer relaxation time are essential if the potential of preclinical research (and clinical translation) is to be fully realized.

Technical advancements for increased polarization levels are underway [63, 64]. These, while improving the data achieved using hyperpolarized [1-^{13}C]pyruvate, will also provide greater capacity for new probes. The rapid loss of hyperpolarization through T_1 relaxation following probe dissolution remains one of the largest challenges for the field. From a technical standpoint, dedicated rapid acquisition schemes are required to enable rapid data acquisition and use the limited hyperpolarized signal as efficiently as possible. While most preclinical studies are performed at higher field strength (3 Tesla to 14.1 Tesla), hyperpolarized ^{13}C MRS/I does in fact benefit from lower field strength due to the increased relaxation times of most probes at lower field, showing optimal results on clinical MR systems (1.5 Tesla to 3 Tesla) or even below [5]. Following data acquisition, developments in data processing could also improve data quality, exemplified by recent development of a denoising algorithm, providing a possible increase in signal-to-noise ratios of about an order of magnitude [65].

Resources
I This study is registered with ClinicalTrials.gov.

In terms of biological interpretation, one major area of discussion, briefly mentioned earlier, is to what extent hyperpolarized [1-^{13}C]pyruvate crosses the BBB within the time-frame of the hyperpolarized experiment [66]. Alternatives such as a more lipophilic version of pyruvate have been explored to increased BBB crossing [31]. Use of probes other than pyruvate may also be limited in their crossing of the BBB, such as lactate [34]. In combination with all the metabolic probes in development, co-injection with metabolically inert hyperpolarized probes such as [^{13}C]urea [67], [^{13}C]hydroxymethyl cyclopropane, or [^{13}C]t-butanol [68] can ensure data is not skewed by changes in perfusion. To enable investigation of brain cellular metabolism without, for example, the concerns over BBB transport, live excised rat brain slices can be studied using hyperpolarized ^{13}C MRS in a bioreactor system to maintain cellular viability [69]. Once issues with tissue slice motion during hyperpolarized injection are resolved, this technique may prove useful in future studies of the diseased brain.

Variations in the amount of metabolic product seen may be anesthesia-[70, 71], species-[72], or strain-[34] dependent. The majority of preclinical hyperpolarized ^{13}C MRS/I studies have been performed with the animals under anesthesia. Metabolic data were successfully acquired from the liver following injection of hyperpolarized [6-^{13}C, $^{15}N_3$]arginine in an awake, restrained mouse, in response to reported toxicity of this probe in isoflurane-anesthetized mice[48]. No such toxicity was seen in the awake animal. In preclinical studies, imaging awake animals require extensive training of the animals, and it would be necessary to ensure that stress does not interfere with the study goals. However, if testing in awake animals could become the standard protocol, it would be incredibly valuable in eliminating the confounder of type and depth of anesthetic, and enhance the relevance to human studies.

One particularly interesting question to answer is to what extent different cell types contribute to the observed *in vivo* signal, for example dominance of signal emanating from neurons, microglia, or astrocytes. Future work can begin to address this question, exploiting the flexibility of the hyperpolarized technique to use cell populations both in slurries [73] and bioreactor systems [74]. Developments in these systems, such as the use of micromagnetic resonance spectrometer [75] or co-culturing of multiple cell populations, will help decipher weights of contribution. Additionally, *ex vivo* biochemical analyses, the use of appropriate knock-out/knock-in mouse models, and specific metabolic pathway inhibitors could also help address these questions [21].

Clinically, there are also both technical and biological considerations to overcome before the hyperpolarized ^{13}C MRS/I technology becomes standard clinical practice in neurological disorders, as discussed above. The clinical SpinLab polarizers are available at some cost, and dedicated specific pulse sequences are required. As with any new injectable, approval of a investigational new drug application (IND) from the Food and Drug Administration (FDA) (or equivalent) is required, and concentrations of new probes have to be tested for toxicity and good quality data acquisition. It would be most valuable if data is comparable across clinical sites, although this is no small task as standardization of data acquisition and processing will be needed. A full analysis of healthy brain metabolism using several hyperpolarized probes across multiple countries and therefore multiple volunteer

populations would be optimal, to provide a diverse baseline dataset against which to assess disease states.

A detailed understanding of the biological meaning of clinical hyperpolarized data would be incredibly valuable, but is challenging given the limited ability to biopsy the imaged human tissue. Despite this, use of hyperpolarized pyruvate remains clinically valuable if it can provide distinctions between healthy, diseased, and treated brain tissue. In the short-term, hyperpolarized [1-^{13}C]pyruvate can feasibly be used in clinical studies of neurological disorders as it is patient-safe, and the hardware and data analysis are in place for brain studies in an increasing number of locations.

Concluding remarks and future perspectives

Hyperpolarized [1-^{13}C]pyruvate has been recently applied, and shown to provide informative data, in multiple rodent models of neurological diseases and neuroinflammation. Further, there are many hyperpolarized probes for *in vivo* enzymatic assessment of several metabolic pathways which have been developed and applied in the healthy brain. Moving to models of neurological disease is thus a logical and feasible next step for such probes. Although challenges exist for hyperpolarized technology in the field of brain diseases (see Outstanding Questions), clinical translation is underway in TBI patients and healthy volunteers and will likely follow in other patient populations in the upcoming years. Thus, it seems appropriate at this point to focus resources on improving hardware, acquisition capacity, processing pipelines and hyperpolarized probes; efforts which will hopefully allow investigation of multiple metabolic pathways within the human brain in health and disease. An optimized methodology could improve longitudinal monitoring of response to therapy. In combination with other imaging modalities, the hyperpolarized ^{13}C technology provides an exciting opportunity to enhance our understanding of *in vivo* brain metabolism, and in the future, to impact clinical care in a range of neurological disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

MRI: Magnetic Resonance Imaging

Typically used for visualizing and distinguishing between areas of varying physical properties of water molecules (via exploitation of the high MR visibility of protons). MRI is widely used in the clinic and can provide information on brain structure, for example, and presence of lesions such as tumors or edema.

MRS: Magnetic Resonance Spectroscopy

This technique enables visualization of steady-state levels of brain metabolites. Data are in the form of spectra. Proton spectroscopy enables detection of several metabolites with a sensitivity in the millimolar range (e.g. choline, N-acetyl aspartate, creatine). Carbon spectroscopy is also viable but due to the low natural abundance of the MR-detectable carbon-13 isotope, long acquisition times or infusion of carbon-13 labelled compounds is necessary.

MRSI: Magnetic Resonance Spectroscopic Imaging

Spatially-localized spectra can be produced from multiple parts of the brain at once, to provide spatial information on steady-state metabolite levels.

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Outstanding Questions

- Hyperpolarized ^{13}C MRS/I of [1-^{13}C]pyruvate can detect metabolic impairment in several preclinical models of neurological disorders. Can this metabolic imaging technique improve diagnosis and evaluation of prognosis in patients with brain diseases?
- Modulations in immune cell metabolism have been detected by hyperpolarized ^{13}C MRS/I of [1-^{13}C]pyruvate in *ex vivo* and *in vivo* preclinical models. Can this method be used to detect neuroinflammation in patients?
- Can more sensitive acquisition and processing pipelines be developed to enable higher MR signal and better spatial resolution ?
- Can we establish a metabolic baseline dataset for healthy brain that is consistent across sites for disease state comparison?
- Initial preclinical studies showed that hyperpolarized [1-^{13}C]pyruvate to [1-^{13}C]lactate conversion is sensitive to metabolic response to therapy in several models of neurological diseases. Can hyperpolarized ^{13}C MRS/I be used to monitor drug efficacy and response to therapy in neurological disorders clinically, and are changes in these parameters observed before changes in typical patient symptoms?
- Several cerebral metabolic pathways have been shown to be accessible with hyperpolarized probes. Can hyperpolarized ^{13}C MRS/I be used to obtain a fuller picture of metabolic changes in neurological disorders using multiple, translatable hyperpolarized probes?

Highlights

- Hyperpolarized ^{13}C magnetic resonance spectroscopy is an emerging metabolic imaging technique that provides *in vivo* assessment of enzymatic fluxes after injection of hyperpolarized probes (e.g. [1-^{13}C]pyruvate)
- This technology provides valuable diagnostic and treatment monitoring information in the oncologic and cardiovascular fields, where measurement of hyperpolarized pyruvate-to-lactate conversion has been translated to the clinic
- The technique has recently been applied preclinically in several non-oncological neurological disorders, successfully detecting metabolic impairment
- Valuable metabolic data assessing hyperpolarized pyruvate-to-lactate conversion have been acquired in animal models of multiple sclerosis, traumatic brain injury, neuroinflammation, cognitive impairment, and stroke
- Clinical trials on healthy volunteers and traumatic brain injury patients are currently underway

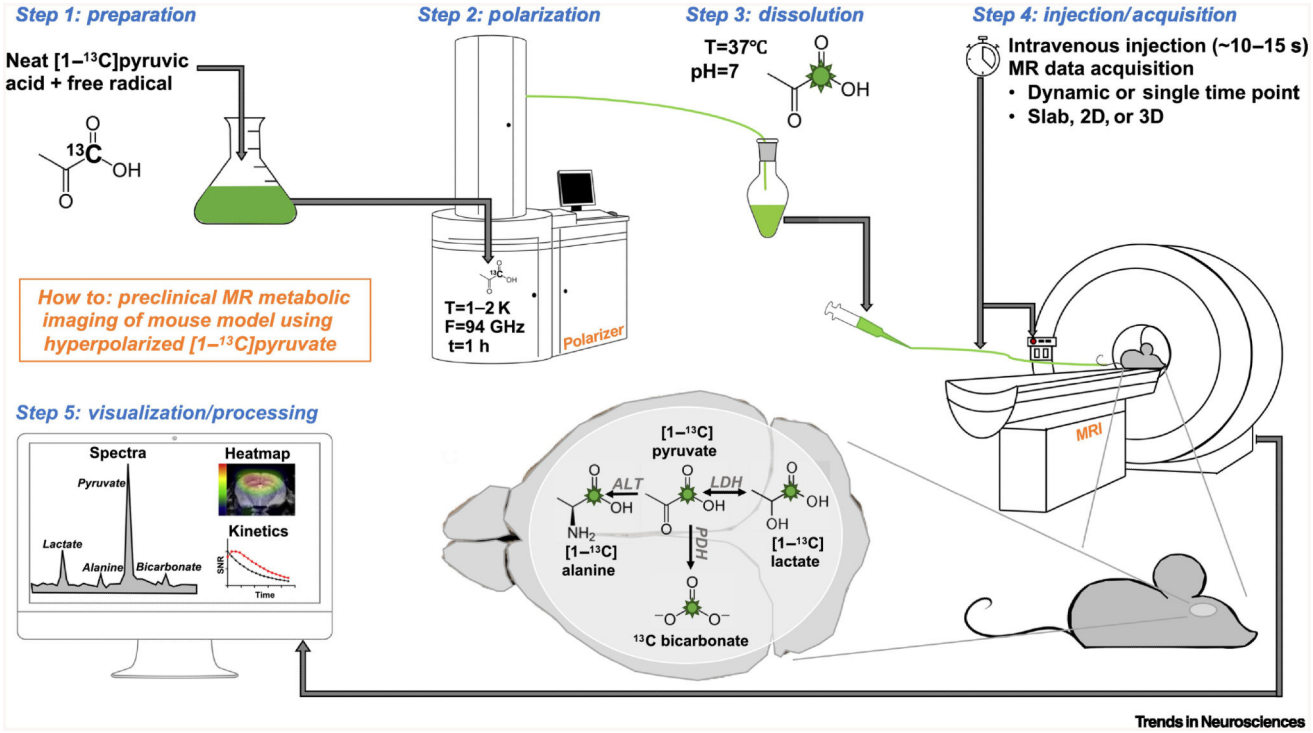

Figure 1 (Key Figure). Typical steps of preclinical MR metabolic brain imaging of a rodent model using hyperpolarized [1-^{13}C]pyruvate.

Step 1: Neat [1-^{13}C]pyruvic acid mixed with a free radical is prepared at a high concentration (typically 14 M). Step 2: A small quantity of the preparation (5–150 uL) is placed into a hyperpolarizer for approximately 1 hour, where the sample sits at low temperature (T=1–2 K) whilst being irradiated with microwaves (F=~94 GHz). Step 3: When hyperpolarization is complete, the sample is very rapidly dissolved in a neutralizing buffer (within approximately 10s), which produces a pH 7, 37˚C solution of [1-^{13}C]pyruvate in the range of 5–100 mM. Step 4: This buffered solution is then immediately injected into the experimental subject placed inside a magnetic resonance imaging (MRI) system, over a short period of time (~15 sec). In the illustration, the injection is into a rodent model, via a tail-vein catheter. Acquisition of MR data (slab, 2D or 3D, dynamic or single timepoint acquisition) is timed with the injection of the hyperpolarized solution.

Zoomed-in brain inset: In the rodent brain, conversion of hyperpolarized [1-^{13}C]pyruvate conversion to hyperpolarized [1-^{13}C]lactate, hyperpolarized [1-^{13}C]alanine and hyperpolarized [^{13}C]bicarbonate occurs within the time frame of the MR acquisition. These conversions are via lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and pyruvate dehydrogenase (PDH) respectively. Step 5: After data processing, MR data reflecting the *in vivo* metabolism of the injected hyperpolarized compound to its downstream metabolites can be visualized, for instance by displaying spectra, heatmaps or kinetic analyses of signal-to-noise ratio (SNR) versus time.

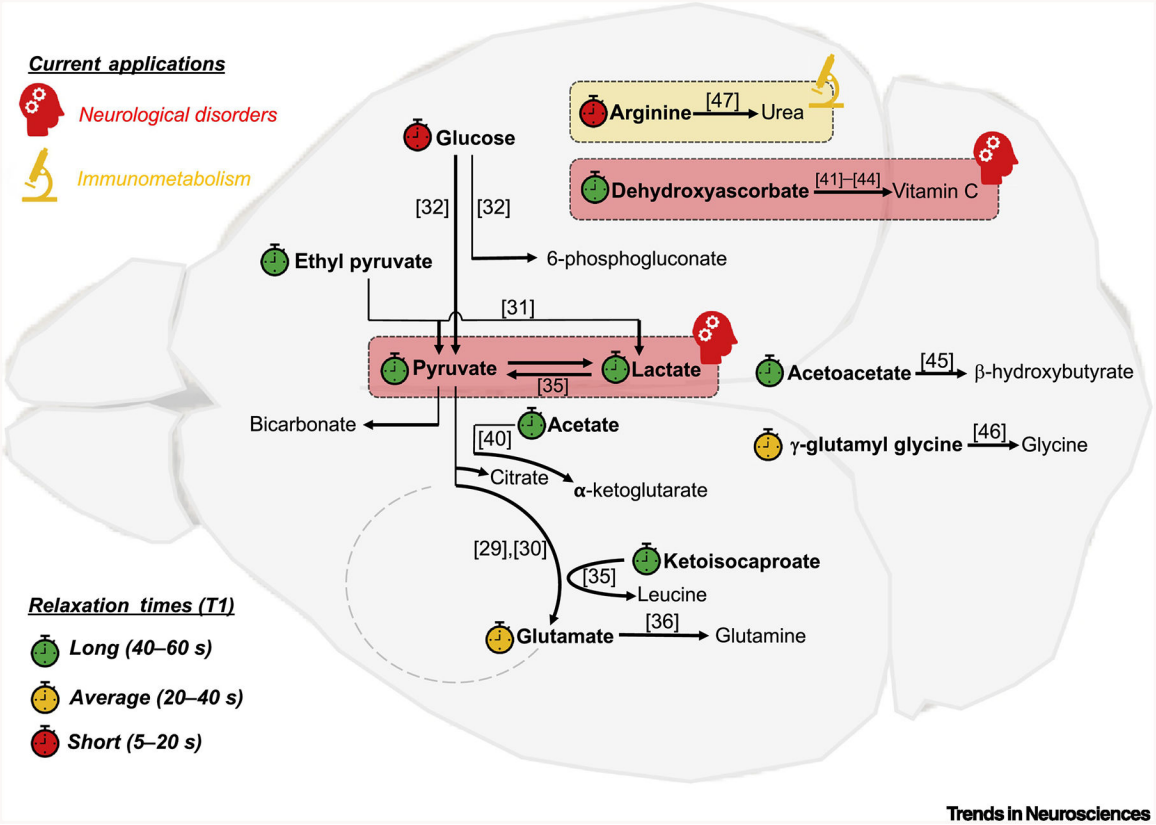

Figure 2. Overview of metabolic reactions currently investigated using hyperpolarized probes.

Hyperpolarized probes (bold) and corresponding hyperpolarized downstream product(s) currently investigated in (1) the healthy brain (all items in the schematic except arginine); (2) the field of immunometabolism (yellow box) and (3) preclinical models of neurological disorders (red boxes). References to the corresponding study/studies are noted in grey above the reaction arrows. The relaxation times (T_1) of the hyperpolarized probes are ranked as long (40–60sec, green), average (20–40sec, orange) and short (5–20sec, red), from data at the clinical magnetic field strength of 3 Tesla (extrapolated from data at other field strengths for glucose, glutamate, acetate and ketoisocaproate).