Report – CAEN Category 1A: Visit by the Applicant to Another Laboratory

Visitor: Gustavo Ferreira, Federal University of Rio de Janeiro, Brazil
Host: Prof. Mary C. McKenna, University of Maryland, Baltimore, USA
Period of training: March 5 to May 4, 2016.

NMR spectroscopy is currently the most potent methodology to assess metabolism in specific metabolic pathways in various cell types (notably neurons and astrocytes), as well as neuron → astrocyte, and astrocyte → neuron interactions, by the labeling in individual carbon atoms of key metabolites and neurotransmitters. ^{13}C-NMR also allows the determination of the efficacy of therapeutic treatments for the prevention and/or mitigation of metabolic abnormalities in these cell types. ^{1}H-NMR can determine the concentration of metabolites that provide additional information about the structural and metabolic integrity of brain. This is widely used clinically to evaluate developing brain, and brain alterations after injury. The aim of this visit was to qualify Gustavo Ferreira for NMR studies at his lab in Brazil by providing him the background knowledge and understanding of NMR, and also providing hands-on instruction in NMR experiments and instrument use. Gustavo has been working with brain metabolism and bioenergetics in his projects, mainly using enzyme and radioactive assays, as well as polarographic experiments.

Therefore, NMR spectroscopy will provide him a powerful new tool to obtain further insights into his previous findings and the capability to expand the experimental possibilities with cutting edge NMR techniques. The training on ^{1}H and ^{13}C-NMR analysis and interpretation were carried out at the Department of Pediatrics, University of Maryland School of Medicine (MD, USA), under the supervision of Prof. Mary C. McKenna (Figure 1). The University of Maryland School of Medicine has several NMRs including a Bruker Avance III 950 MHz, which is the most powerful magnet in an Academic Institution in the United States of America (Figure 2). Dr. McKenna's group has expertise in well-established techniques for assessment of bioenergetics and neurotransmitter metabolism by NMR spectroscopy.

Figure 1. Gustavo (right) and Dr. McKenna (left) at the Department of Pediatrics, University of Maryland, Baltimore.

Figure 2. Gustavo and Dr. McKenna with Bruker 950 MHz NMR at the NMR Core Facility of the University of Maryland School of Medicine.

During the period of the visit, analysis and interpretation of both ^{1}H- and ^{13}C-NMR data were extensively performed using many labeled substrates, including [1,6-^{13}C]glucose, [1,2-^{13}C]glucose, [1,2-^{13}C]acetate and others, which allows the investigation of different metabolic pathways depending on the labeled substrate used in the experiments. The methodologies learned during training will now be used in studies at the Institute of Medical Biochemistry of the Federal University of Rio de Janeiro, where Gustavo holds a position, which already has the necessary NMR equipment for these assessments.

The experimental model chosen for this training was a modified Rice-Vannucci model of neonatal hypoxia-ischemia (HI) (Rice et al., 1981) on PN 10 Sprague-Dawley rats. Data was acquired from contralateral and ipsilateral cerebral cortex at different time points post-HI (immediately after, and 8 days after, or 28 days after HI). [1,6-^{13}C]Glucose was administered and rats were euthanized 30 minutes after injection of the ^{13}C-labelled compounds. Brains were rapidly frozen for the extraction of metabolites and the amount, pattern, and fractional enrichment of labeled metabolites were determined by ex vivo ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy as reported by Dr. McKenna's group and others (Richards et al., 2007; Morken et al., 2014).

In Brazil, mortality of children who suffered neonatal asphyxia can reach up to 30%, and varies amongst states (Brenelli-Vitali et al., 2003; Daripa et al., 2013). Therapeutic hypothermia is the current strategy of choice for prevention or mitigation of secondary energy failure in children who have suffered HI episodes. However, approximately 40% of survivor children still show impaired development after therapeutic hypothermia, indicating that the damage induced

by HI is too severe, and that different therapeutic approaches are necessary for some children (Wyatt et al., 2007; Thoresen et al., 2013; Chalak et al., 2014). Considering that the therapeutic and pharmacological interventions available today are not completely effective in improving the neurological status of children after HI (Vannucci, 1990), new strategies need to be investigated to target the different components of the brain damage due to the ischemic cascade and subsequent reperfusion (Broccard, 2006). There is a critical need to identify new therapies to protect the developing brain since acute alterations in energy metabolism and prolonged metabolic dysregulation after neonatal HI leave the brain vulnerable and unable to support many processes essential for normal development. Therapies that decrease or attenuate the secondary energy failure after HI may lead to improved outcome. Dr. McKenna and her collaborators are determining the effects of acetyl-L-carnitine and other promising neuroprotective strategies (Scafidi et al., 2010; Xu et al., 2015; Demarest et al., 2016; Waddell et al., 2016).

As mentioned above, metabolism via cell specific metabolic pathways in astrocytes and neurons can be determined and evaluated depending on the labeled precursor used in the experiments. Figure 3 shows the different peaks identified using [1,6-^{13}C]glucose as a precursor, as well as the metabolic pathways involved in such labeling.

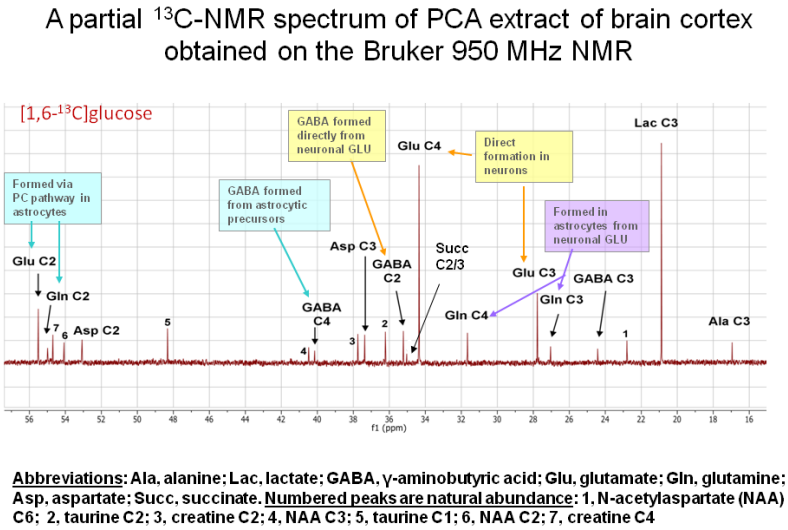

Figure 3. ^{13}C-NMR labeling pattern of metabolites labeled subsequent to the metabolism of [1,6-^{13}C]glucose in brain. Pathways are color coded to facilitate the identification.

Below is shown a typical ^{13}C-NMR spectrum of compounds labeled from the metabolism of [1,2-13C]glucose in brain from control and HI animals (Figure 4):

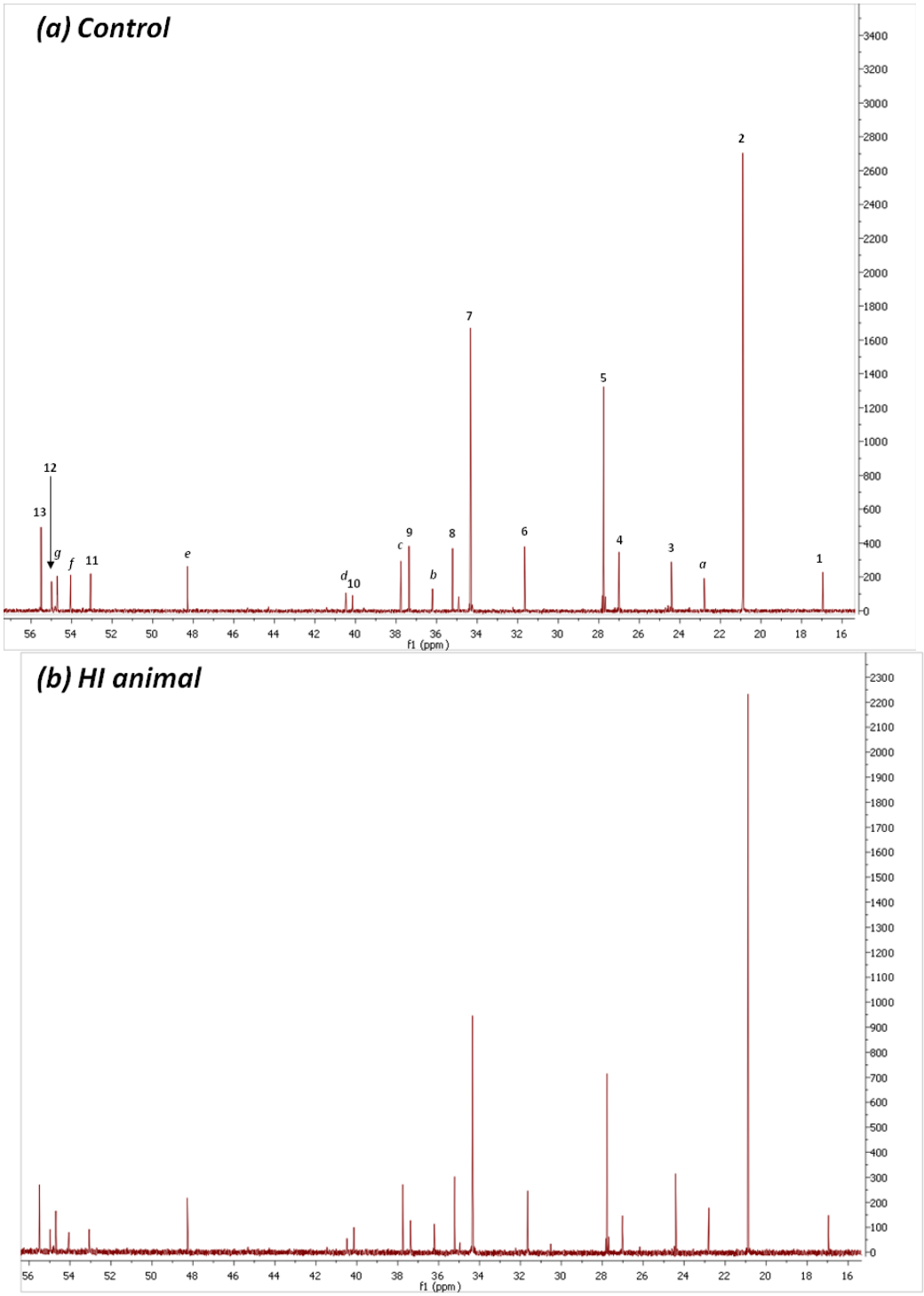

Figure 4. Representative spectra of perchloric acid extracts of brain from control (a) and HI animals (b) who were injected with [1,6-^{13}C]glucose at 28 days after HI. Peaks of interest from metabolism of labeled glucose are numbered in the upper chart. Peaks from upper and lower charts are lined up to facilitate the comparison. 1: alanine C3; 2: lactate C3; 3: GABA C3; 4: glutamine (Gln) C3; 5: glutamate (Glu) C3; 6: Gln C4; 7: Glu C4; 8: GABA C2; aspartate (Asp) C3; 10: GABA C4; 11: Asp C2; 12: Gln C2; 13: Glu C2; Peaks representing natural abundance are labeled with letters in the same chart. a: N-acetylaspartate (NAA) C6; b: taurine C2; c: creatine C2; d: NAA C3; e: taurine C1; f: NAA C2; g: creatine C4.

In the figure above it can be noted that metabolism was markedly shifted toward lactate formation in HI animals. It is also observed that overall metabolism is decreased (note the lower amount of glutamine labeling in both C4 and C3 from its immediate precursors C4 and C3 labeled glutamate, whilst GABA C2 and C3, that comes from the same glutamate precursors, appears to be increased). Figure 5 shows a representative spectrum of ^{1}H-NMR spectroscopy in cerebral cortex from rat pups. many metabolites can be identified and quantified using this technique, namely lactate (Lac), alanine (Ala), GABA, N-acetylaspartate (NAA), glutamate (Glu), glutamine (Gln), succinate (Suc), aspartate (Asp), creatine (Cr), choline-containing compounds (Cho), taurine (Tau) and myo-inositol (Myo-Ins).

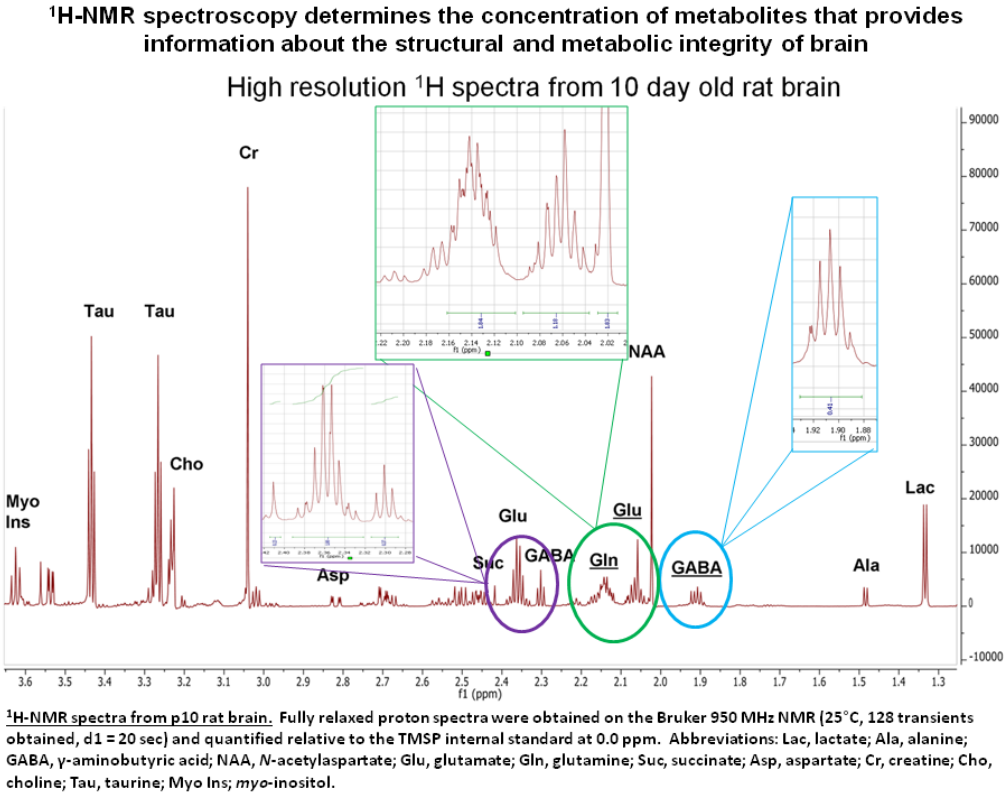

Figure 5. Representative spectrum of ^{1}H-NMR spectroscopy in cerebral cortex from rat pups. As depicted in the figure, many metabolites can be identified and quantified using this technique, namely lactate (Lac), alanine (Ala), GABA, N-acetylaspartate (NAA), glutamate (Glu), glutamine (Gln), succinate (Suc), aspartate (Asp), creatine (Cr), choline-containing compounds (Cho), taurine (Tau) and myo-inositol (Myo-Ins).

Spectra from animals injected with [1,2-^{13}C]glucose were also analyzed, the only precursor that enables the determination of metabolism via the pentose phosphate pathway. When [1,2-^{13}C]glucose is metabolized via glycolysis all labeled carbons are kept; whilst when it is metabolized by pentose phosphate pathway one labeled carbon is lost as $^{13}CO_2$, thus allowing

us to distinguish and compare the relative metabolism of glucose through glycolysis or pentose phosphate pathway under different experimental conditions. Another precursor often used is acetate which is taken up almost exclusively by astrocytes. Thus by using ^{13}C-acetate as a precursor in living animals it is possible to determine metabolism in the TCA cycle in astrocytes, and also to determine metabolite trafficking between astrocytes and neurons and synthesis of glutamate and GABA from glial precursors.

Due to the short-term visit and limitations with IACUC regulations and trainings, Gustavo was not able to be trained on the neonatal HI model during his initial visit. Instead, frozen tissues from previous experiments carried out at Dr. McKenna's lab were used in this training. Gustavo prepared tutorial files to be used for NMR spectroscopy training of post-graduate students in Brazil. During the period of his initial visit Gustavo and Dr. McKenna wrote and submitted a book chapter together entitled *"Enzyme complexes important for the glutamate-glutamine cycle"*, in *"Advances in Neurobiology" (in press)*, which was only possible due to this award kindly provided by ISN. In addition, since the initial collaboration between Gustavo and Dr. McKenna's lab was considered very fruitful for both the visitor and the host, Gustavo was invited to return to Dr. McKenna's lab for a second visit, which allowed Gustavo to expand his hands-on experience with NMR spectroscopy and learn the rat pup model of neonatal hypoxia-ischemia and hypothermia, as well as the tissue extraction for NMR analysis.

The strategies learned for studying brain metabolism can be readily implemented in Gustavo's laboratory in Brazil and have broad applicability for determining brain alterations and efficacy of neuroprotection in other conditions leading to developmental brain injury. The knowledge and expertise gained during this overseas training will be applied in the future in teaching activities in undergraduate and postgraduate studies in biochemistry and neuroscience, applying the knowledge to improve the mentoring and training of students and researchers in Brazil, as well as in future research projects in the fields of biochemistry and neurosciences of Gustavo's group.

Acknowledgements

Gustavo and Dr. McKenna acknowledge this ISN initiative at such a critical stage of career development.

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