Therapeutic hypothermia and Interleukin-1 blockade as neuroprotective strategies in neonatal encephalopathy and arterial ischemic stroke

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October 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

« Loin d'épuiser une matière, on ne doit en prendre que la fleur. »

Jean De La Fontaine

"Eventually, all models are wrong, but some are useful."

George E. P. Box

TABLE OF CONTENTS

ACKNOWLEDGEMENTS / REMERCIEMENTS

My graduate studies were supported by the Brain Canada Foundation, Fonds de Recherche du Québec – Santé (FRQ-S), Research Institute of the McGill University Health Centre (RI-MUHC) – Desjardins Studentship in Child Health Research, Montreal Children Hospital Foundation – Hoppenheim Fund, Faculté de Médecine et des Sciences de la Santé de l'Université de Sherbrooke – Bourse "Tranzyme Pharma", Integrated Program in Neuroscience of McGill University. Merci à tous ces organismes subventionnaires d'avoir permis la réalisation de ce projet de recherche !

First, I would like to personally recognize and express my gratitude to my supervisor Dr. Guillaume Sébire. Je tiens à vous remercier de m'avoir soutenue, écoutée, challengée et comprise durant toutes ces belles années (de mon premier stage en fin de DUT jusqu'au doctorat). Merci de m'avoir fait confiance et de m'avoir laissée de l'autonomie dans mon projet de recherche comme dans le laboratoire. Ces expériences m'ont fait m'épanouir et évoluer tant sur le plan scientifique que personnel. Encore merci pour votre complicité, votre franchise et votre bienveillance tant appréciées au fil des années. Au plaisir de partager quelques bulles champenoises à l'avenir !

I would like to thank my advisory committee members, candidacy examiners and mentor: Dr. Jasna Kriz, Dr. Pia Wintermark, Dr. Sylvain Chemtob and Dr. Pierre Lachapelle. Merci pour vos questions pertinentes, commentaires et précieux conseils qui ont permis d'améliorer mon projet de recherche et de me faire progresser comme scientifique.

Merci aux membres du jury Dr. Pia Wintermark, Dr. Edith Hamel et Dr Gregory Lodygensky de prendre le temps d'évaluer le travail de ces quatre dernières années et de m'apporter leurs commentaires.

I am also grateful to the main collaborators of my Ph.D. project: merci à Dr. Mickaël Dinomais pour les conseils en neuroimagerie et la belle découverte des heat-maps. Un merci spécial au visiting scholar Dr. Stéphane Chabrier pour le beau partage scientifique, le respect de la langue française, les anecdotes de quand « tu étais petit » et ton humour légendaire (parce que malgré les poils de nos rats et leur agénésie carotidienne réversible, on reste écologique !). Je nous souhaite de poursuivre cette belle collaboration et complicité pendant de nombreuses années.

A special thanks to all Sébire's lab team with whom I had the chance to work, learn and laugh in French or in English since 2013. Un grand merci à Marie-Julie Allard de m'avoir tracé la voie du doctorat, pour ton soutien et tes précieux conseils durant les dernières années; ainsi qu'à Khadidja Chelabi pour le beau travail d'équipe durant les chirurgies. Taghreed Ayash and Seline Vancolen: it was a pleasure to brainstorm and work together this year. I hope to see you again apart from this COVID-19 period.

Finalement, je voudrais remercier ma famille et mes amis pour votre soutien et vos encouragements. Merci à tous les membres de ma famille qui ont contribué de près ou de loin à mon cheminement personnel et professionnel. Que ce soit à 800 ou 5000 km je trouve toujours une oreille attentive, du support moral et même un certain intérêt scientifique. Merci de pouvoir toujours compter sur vous, j'ai hâte de vous voir bientôt en personne.
Merci à mon chéri pour ton amour et ton soutien indéfectible. Merci de m'épauler au quotidien, d'appuyer mes décisions (ou pas lorsqu'elles sont mauvaises), de m'apporter du bonheur et du réconfort dans ma vie.

Un merci particulier à Annie Roy, toujours fidèle au poste malgré les hauts et les bas. Merci à Andréanne Loiselle et Élise Gullickson-Larouche pour l'amitié de longue date et le soutient pré-soutenance. Merci également à Louise Merly et Antoine Giraud pour les menus du pauvre, les discussions scientifiques (et non-scientifiques) et les moments inoubliables. Merci à mes amis d'enfance qui sont toujours là pour moi en dépit de la distance et du décalage horaire.

Did I forget someone? Maybe…I am not sure…Oh of course I did (how dare I?) Almost forgot about McPinsky. Really special thank you to you, dear drummer friend. Always there when needed. I'm pretty glad we're always stand together, in so many groups now.

ABSTRACT

Neonatal encephalopathy (NE) and neonatal arterial ischemic stroke (NAIS) are either diffuse or focal brain injuries resulting both from hypoxic-ischemic events and/or pathogen-induced inflammation. NE affects up to 1% of live births. NAIS affects 0.3% of live births. Treatment options are symptomatic in both diseases. A curative therapeutic option is only available in NE due to pure HI and consists in therapeutic hypothermia (HT). HT, however, provides limited neuroprotection and around 50% of treated neonates will experience moderate to severe neurologic disabilities. It is still unclear if HT confers beneficial effects when there is combined aggression of hypoxia-ischemia (HI) and pathogen-induced inflammation, which is one of the most common pathophysiological scenario of both NE and NAIS.

Human neuropathological studies and experimental animal models of perinatal brain injury reveal that Interleukin (IL)-1β – among other pro-inflammatory cytokines – is implicated in the pathophysiological cascade leading to brain injury. Besides, preclinical studies did not bring evidence of an effect of HT on the IL-1 cascade. Therefore, postnatal systemic administration of Interleukine-1 receptor antagonist (IL-1Ra: a natural blocker of IL-1) may be a potential therapeutic intervention of postnatal brain injury, such as NE and NAIS. However, IL-1Ra is not approved for the treatment or prevention of postnatal brain injury, and further studies are needed to determine its safety and efficacy. No studies have been conducted to assess the efficacy of IL-1Ra with other therapies, such as HT.

In this thesis, I hypothesized that HT alone or in combination with IL-1Ra would provide neuroprotective effects in a rat model of both NE and NAIS resulting from inflammatory-sensitized-HI. We further hypothesized that IL-1Ra will provide an added value to HT in alleviating brain injuries and improving motor behavioural outcomes. I aimed to investigate (1)

the safety of HT treatment in combination with IL-1Ra and (2) the efficacy of HT combined or not with IL-1Ra in terms of mortality, brain injuries, and short and long-term motor behaviour in our model of NE resulting from inflammatory-sensitized HI. This thesis shows for the first time that (*i*) HT has an impact on the pharmacodynamic parameters of IL-1Ra; revealing a future need to refine the dose of IL-1Ra used in combination to HT; (*ii*) this combined therapy is able to reduce the mortality rate and improve short-term motor behaviour; and (*iii*) HT alone reduces the occurrence and the volume of brain infarct, improves brain metabolic activity, and alleviates the long-term motor outcomes of inflammatory plus HI- induced brain injuries.

This thesis reveals the importance of studying the influence of HT on the distribution, metabolism, elimination and clinical efficacy of drugs in order to avoid toxicity or ineffectiveness. Current and future studies investigating the beneficial effect of HT in other neurological pathologies, such as stroke, subarachnoid hemorrhage, traumatic brain injury, and spinal cord injury, should take into account this particular effect. In addition, we observed a potential combined effect of IL-1Ra plus HT that needs further preclinical evaluation before moving to human clinical therapeutic trials. Our results provide evidence of the neuroprotective effect of HT in NE and NAIS resulting from inflammatory-sensitized HI. This thesis opens new research avenues on the potential benefit of HT in other postnatal brain injury sharing common determinants with NE, such as NAIS. This could pave the way towards further clinical evaluation of the benefit of HT in NAIS.

RÉSUMÉ

L'encéphalopathie néonatale (EN) et l'infarctus cérébral artériel néonatal (ICAN) sont responsables de lésions cérébrales diffuses ou focales résultant à la fois d'événements hypoxiques-ischémiques (HI) et/ou d'une inflammation induite par des agents pathogènes. L'EN affecte jusqu'à 1% des naissances vivantes. L'ICAN affecte 0,3% des naissances vivantes. Dans les deux pathologies, les options de traitement sont symptomatiques. Une option thérapeutique curative n'est disponible que chez l'EN résultant d'une pure HI et consiste en une hypothermie thérapeutique (HT). Néanmoins, l'HT n'offre pas une neuroprotection complète et environ 50% des nouveau-nés traités souffriront de troubles neurologiques modérés à sévères. Le bénéfice de l'HT est toujours incertain en cas d'agression combinée par l'HI et l'inflammation induite par un agent pathogène; qui est l'un des scénarios physiopathologiques les plus courants dans l'EN/ICAN. Les études neuropathologiques humaines et les modèles animaux de lésions cérébrales périnatales révèlent que l'interleukine (IL)-1β – parmi d'autres cytokines pro-inflammatoires – est impliquée dans la cascade physiopathologique conduisant aux lésions cérébrales. De plus, les études précliniques n'ont pas apporté la preuve concrète d'un effet de l'HT sur la cascade de l'IL-1. Par conséquent, l'administration systémique postnatale d'un antagoniste du récepteur de l'IL-1 (l'IL-1Ra : bloquant naturel de l'IL-1) pourrait être une intervention thérapeutique potentielle des lésions cérébrales induites dans l'EN/ICAN. Cependant, l'IL-1Ra n'est pas approuvé pour le traitement ou la prévention des lésions cérébrales postnatales. D'autres études sont nécessaires pour en déterminer sa sécurité d'administration et son efficacité. Aucune étude n'a été menée pour évaluer l'efficacité de l'IL-1Ra en combinaison avec d'autres thérapies comme l'HT.

Dans cette thèse, j'ai émis l'hypothèse que l'HT seule ou en combinaison avec l'IL-1Ra a des effets neuroprotecteurs dans un modèle murin d'EN/ICAN résultant de l'HI sensibilisée par

l'inflammation. Nous avons également émis l'hypothèse que l'IL-1Ra apportait une valeur ajoutée à l'HT dans l'atténuation des lésions cérébrales et l'amélioration du comportement moteur. Les objectifs étaient d'étudier (1) l'innocuité de l'HT combinée à l'IL-1Ra et (2) l'efficacité de l'HT combinée ou non à l'IL-1Ra en termes de mortalité, de lésions cérébrales et de comportements moteurs à court et à long-terme dans notre modèle d'EN/ICAN qui résulte de l'HI et de l'inflammation. Cette thèse montre pour la première fois que (*i*) l'HT a un impact sur les paramètres pharmacodynamiques de l'IL-1Ra, (*ii*) la thérapie combinée est capable de réduire le taux de mortalité et d'améliorer le comportement moteur à court-terme; et (*iii*) l'HT seule réduit l'apparition et le volume des infarctus cérébraux, améliore l'activité métabolique cérébrale et le comportement moteur à long-terme suite aux lésions cérébrales induites par l'inflammation et l'HI. Cette thèse révèle l'importance d'étudier l'impact de l'HT sur la distribution, le métabolisme, l'élimination et l'efficacité clinique des médicaments afin d'en éviter la toxicité ou l'inefficacité. Les études actuelles et futures examinant les effets bénéfiques de l'HT dans d'autres pathologies comme l'accident vasculaire cérébral, l'hémorragie sous-arachnoïdienne, les traumatismes crâniens et les lésions de la moelle épinière, devraient prendre en compte cet effet particulier de l'HT. De plus nous avons observé un effet combiné potentiel de l'IL-1Ra à l'HT nécessitant une évaluation préclinique supplémentaire avant de passer aux essais thérapeutiques cliniques chez l'humain. Nos résultats fournissent des preuves solides de l'effet neuroprotecteur de l'HT dans l'EN/ICAN résultant de l'HI sensibilisée à l'inflammation. Ces travaux pourraient ouvrir la voie à une évaluation clinique plus approfondie du bénéfice de l'HT dans l'ICAN.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BBB	Blood brain barrier
Ca2+	Calcium
CINC	Cytokine-induced neutrophil chemoattractant
CNS	Central nervous system
CP	Cerebral palsy
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular pattern
Drp	Dynamin-related protein
EEG	Electroencephalography
EPO	Erythropoietin
FADD	Fas-associated protein with death domain
GBS	Group B Streptococcus
HI	Hypoxia-ischemia
HIE	Hypoxic-ischemic encephalopathy
HMGB	High-mobility group box
HT	Hypothermia
hr	Human recombinant
ICAM	Intercellular adhesion molecule
IL	Interleukin
IL-1Ra	Interleukin -1 receptor antagonist
IL-1RAcP	Adapter protein of the IL-1β receptor
LPS	Lipopolysaccharide of E. coli
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MCP	Monocyte chemoattractant protein
MLKL	Mixed lineage kinase domain-like protein
MMP	Matrix metallopeptidase
MRI	Magnetic resonnance imaging
MyD	Myeloid differentiation primary response gene
NAIS	Neonatal arterial ischemic stroke
NE	Neonatal encephalopathy
NFkB	Nuclear factor
NIRS	Near-infrared spectroscopy
NLR	Nod-like receptor
NMDA	N-methyl-D-aspartate
OR	Odds ratio

P	Postnatal day
PAMP	Pathogen-associated molecular pattern
PET	Positron emission topography
PGE2	Prostaglandin E2
R	Receptor
RIP	Receptor interacting-protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEP	somatosensory evoked potential
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated death domain
VCAM	Vascular cell adhesion molecule

LIST OF FIGURES

CHAPTER I:

CHAPTER II:

CHAPTER III:

CHAPTER IV:

CHAPTER V:

LIST OF TABLES

CHAPTER I:

PREFACE

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Neonatal encephalopathy (NE) affects around 2 to 6 per 1000 term newborns and is the second most common cause of childhood disabilities. Despite the use of therapeutic hypothermia (HT) as a standard of care, the incidence of NE and its devastating outcomes remain a major issue for the patient, its family as well as the society. Ongoing research surrounding add-on neuroprotective strategies against NE is important as HT effects are limited, leaving 50% of treated patient with neurological sequelae. NE is mainly due to the compounding effects of hypoxia-ischemia (HI) and perinatal inflammation resulting from placental infections or neonatal sepsis. The combination of infection/inflammation plus HI is frequent, however, it is unclear if HT is effective in this context. This thesis explores for the first time the safety and efficacy of HT combined with interleukin-1 (IL-1) blockade – using IL-1 receptor antagonist (IL-1Ra) – in order to prevent NE resulting from inflammatory-sensitized HI. The main contributions of this thesis include the demonstration of (*i*) the effect of HT on IL-1Ra pharmacodynamic parameters, (*ii*) the benefit of IL-1Ra combined to HT in terms of mortality and motor behavioural improvement, and (*iii*) the major neuroprotective effects of HT in our model of inflammation plus HI.

Chapter I introduces NE, inflammatory pathways at plays in brain damage, IL-1Ra and its effect in postnatal brain injury, HT and its limitations. **Chapter I** also presents the rationale of the studies included, the general hypothesis and aims of this thesis.

Chapter II (**manuscript 1**) investigates the safety and efficacy of HT in combination with IL-1Ra. We showed that HT alters pharmacodynamic parameters of IL-1Ra leading to a paradoxical

upregulation of the immune system and lack of effectiveness of IL-1Ra plus HT-treated rats in terms of histological measures of brain injury.

Chapter III (**manuscript 2**) compares the effect of sole HT versus IL-1Ra plus HT in terms of mortality and short-term motor behaviour after LPS+HI exposure. We showed a beneficial effect of the combined therapy in these parameters in our model of inflammatory-sensitized NE.

Chapter IV (**manuscript 3**) unravels the neuroprotective effects of HT in our model of NE resulting from inflammation plus HI. Using advanced imaging techniques, we showed a major impact of HT on the occurrence, and volume, of brain infarct measured by magnetic resonance imaging (MRI). Besides, HT enhanced brain metabolic activity measured by positron emission tomography (PET)-Scan and improved long-term motor behaviour.

Finally, **chapter V** summarizes, discusses and concludes on the findings included in this thesis. It also proposes future directions for this research project.

CONTRIBUTION OF AUTHORS

Doctoral studies of M. Chevin led to four articles as first author: three original articles and a review article (see appendix #2).

1. Manuscript 1 (Chapter II)

Chevin M, Guiraut C, Sébire G. Effect of hypothermia on interleukin-1 receptor antagonist pharmacodynamics in inflammatory-sensitized hypoxic-ischemic encephalopathy of term newborns. (2018) *J Neuroinflammation* Jul 30; 15(1):214. PMID: 30060742.

MC and GS created the study design (animal model, experimental planning). MC and CG carried out the experiments. MC obtained the results included in this manuscript, performed the statistical analyses, created the figures, and wrote the first draft of the manuscript. MC and GS wrote and revised the manuscript and participated in its edition during the reviewing process. CG provided a critical review of the manuscript. All the work was conducted under the supervision of GS.

2. Manuscript 2 (Chapter III)

Chevin M, Chelabi K, Chabrier S, Sébire G. Added value of interleukin-1 blockade to hypothermia in the treatment of neonatal encephalopathy. (2020) *Am J Obstet Gynecol*. Sep;223(3):458-460. PMID: 32184151.

MC and GS conceived and designed the work. MC and KC performed the experiments. MC analyzed and interpreted the data. MC, SC and G.S. wrote the paper. SC provided a critical review of the manuscript. All the work was conducted under the supervision of GS.

3. Manuscript 3 (Chapter IV)

Chevin M, Chabrier S, Dinomais M, Bedell BJ, Sébire G. Benefits of hypothermia in neonatal arterial ischemic strokes: a preclinical study. (2020) *Int J Dev Neurosci.* Jun;80(4):257-266. PMID: 32115740.

MC, SC, and GS initially planned the study (animal model development, ethical approval, experiments). MC and SC performed the experiments. MD and BJB performed the imaging analyses and interpretation of data. MC obtained the results included in this manuscript, performed the statistical analysis, and created the figures. MC and SC drafted the manuscript. MD and BJB provided a critical review of the manuscript. All the work was conducted under the supervision of GS.

CHAPTER I: INTRODUCTION

REVIEW OF THE RELEVANT LITERATURE

The literature review will focus on: (1) the description of two human conditions of the term newborns leading to brain injury, their similarity in terms of causes and risk factors, (2) preclinical models used to mimic these pathologies, (3) mechanisms at play in brain injuries with a focus on the interleukin-1 (IL-1)β, and (4) therapeutic hypothermia (HT) and its limitations to prevent brain injuries.

1. Brain injury in the term newborn

The most common disorders leading to brain injury in term newborns are neonatal encephalopathy (NE) and neonatal arterial ischemic stroke (NAIS) (Austin, 2019). NE is the leading cause of acquired brain injury and neurodisability, with an incidence of 2 to 6 per 1000 live births (Kurinczuk et al., 2010). With a birth prevalence of 1-3000 to 1-6000, NAIS is the second most common cause of neonatal seizures and the most common cause of childhood hemiplegia (Dunbar and Kirton, 2018; Fluss et al., 2019; Kirton and Deveber, 2013).

1.1. Neonatal encephalopathy

NE is a syndrome characterized by clinical abnormalities of the central nervous system that occur in the term newborn (born after the 37th week of gestation), *i.e.* between birth and the 28th day of life (Ferriero and Koch, 1985). Patients with NE present a reduced level of consciousness up to coma, epileptic seizures, feeding and respiratory difficulties, low tone and abnormal archaic reflexes (Wu et al., 2004)(**Table 1**). The causes of NE are multiple. They can be combined and are not always clearly identified. In fact, the mechanisms and precise causes of NE remain unclear in a large proportion of patients (Wu et al., 2004). Nevertheless, efforts to clarify the

pathophysiological mechanisms leading to NE led to the establishment of diagnostic criteria defining a subcategory that is hypoxic-ischemic encephalopathy (HIE). HIE – for which the cause is considered to be a limitation of oxygen and blood flow near the time of birth – accounts only for 10% to 30% of NE (Kurinczuk et al., 2010; Wachtel et al., 2019). The diagnostic criteria for HIE are a low APGAR score at 10 minutes of life, metabolic acidosis at birth, as well as the need to be maintained ventilated beyond 10 minutes of life. These events demonstrate the presence of an episode of HI without ruling out other associated cause(s), possibly preceding the HI. Acute HIE around the time of birth remains a major cause of death and life-long disabilities, including motor and non-motor impairments (*e.g.* cognitive dysfunction, behavioural and educational difficulties) (Azzopardi et al., 2014; Lemyre and Chau, 2018; Pin et al., 2009). Cerebral palsy (CP) – which is defined by permanent disorders of the development of movement and posture – occurs in more than 20% of term infants with an history of NE (Battin and Sadler, 2018; Colver et al., 2014; McIntyre et al., 2013).

Since 2010, HT has been the mandatory standard of care for HIE in neonates, which could improve their neurodevelopmental outcomes (Edwards et al., 2010; Hoehn et al., 2008), see paragraph 4.

Category	Moderate encephalopathy	Severe encephalopathy
1. **Level of consciousness**	Lethargy	Stupor/coma
2. **Spontaneous activity**	Decreased activity	No activity
3. **Posture**	Distal flexion, full extension	Decerebrate (arms extended and internally rotated, legs extended with feet in forced plantar flexion)
4. **Tone**	Hypotonia (focal, general)	Flaccid
5. **Primitive reflexes**		
Suck	Weak	Absent
Moro	Incomplete	Absent
6. **Autonomic system**		
Pupils	Constricted	Skew deviation/dilated/nonreactive to light
Heart rate (HR)	Bradycardia	Variable HR
Respirations	Periodic breathing	Apnea

Table 1. Criteria defining moderate or severe encephalopathy. HIE is classified according to its severity: mild, moderate or severe depending on the level of consciousness of the child, his tone and reflexes, the presence of epileptic seizures and duration of symptoms for 7 days after birth. Figure reproduced with permission from (Lemyre and Chau, 2018), copyright from Creative Commons Attribution License (CC BY).

1.2. Neonatal arterial ischemic stroke

NAIS is one of the commonest forms of pediatric stroke (Dunbar and Kirton, 2018; Fluss et al., 2019). NAIS results from the occlusion of one or several major cerebral arteries likely due to an embolus from placental origin leading to a permanent brain infarct (detailed explanation about the

physiopathological hypothesis are presented in the review article (Giraud et al., 2017) (see appendix #2). This condition is characterized by repeated focal seizures that occur in term / near-term newborn between birth to 28 days of life. For less than 30% of them, they also present at birth an altered level of consciousness, abnormal tone and feeding, and/or respiratory (Fluss et al., 2019; Kirton et al., 2011). NAIS cause a heavy burden of life-long motor, cognitive, and/or behavioural disabilities. One third of affected newborns will have CP, one quarter will have epilepsy, and half of them will suffer from behavioural and learning difficulties (Fluss et al., 2019). The pathophysiology of NAIS remains largely unknown; hence, there is no evidence-based preventive or curative neuroprotective strategy available for patients affected by NAIS.

1.3. Brain regions mainly affected by HIE and NAIS

Term Newborns suffering from HIE have widespread brain injuries. There are three main patterns of damage that may be combined: (1) impairment of the neocortex and adjacent subcortical regions affecting mainly paracentral frontoparietal regions; (2) involvement of basal grey nuclei including lenticular nuclei and thalami (especially ventrolateral nuclei); (3) the so-called watershed which affects frontal or parieto-occipital junctions at the border of the main cerebral arterial territories (**Figure 1**) (Rutherford et al., 2010; Sorokan et al., 2018; Yager and Ashwal, 2009).

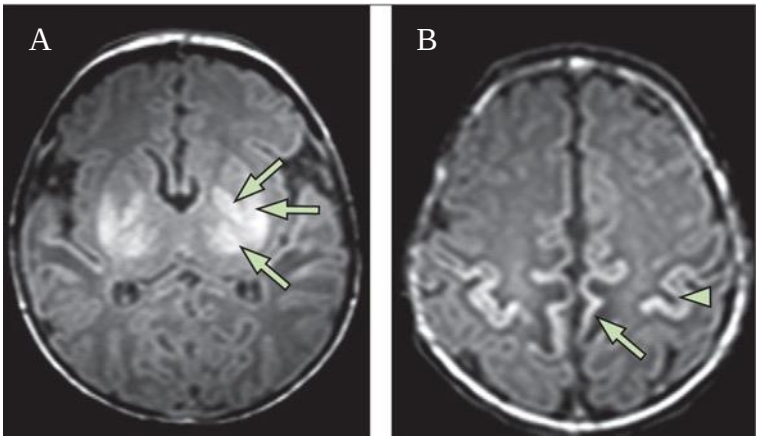

Figure 1. Magnetic resonance imaging (MRI) aspects from brain of term newborns suffering from HIE. T1-weighted MRI images in the axial plane of neonatal brain with (A) moderate basal ganglia and thalamic lesions, and (B) cortical lesions. Figure adapted with permission from (Rutherford et al., 2010), copyright from Creative Commons Attribution License (CC BY).

MRI is the modality to make the distinction between HIE and NAIS, makes the diagnosis and provides supplementary information about the prognosis. In case of NAIS, focal ischemic infarct in the anatomical territory of a brain artery will be visible (see **Figure 2** for an example). Lesions are more commonly found in the left side of the anterior (carotidian) brain circulation (Fluss et al., 2016; Kirton et al., 2011; Sorokan et al., 2018).

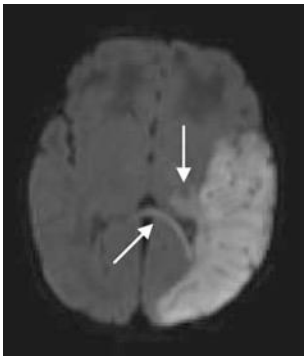

Figure 2. Example of NAIS imaging. A median cerebral artery infarct was found in diffusion weighted MRI from a neonate presenting hypotonia and focal seizures. Figure reproduced with permission from (Fluss et al., 2019).

1.4. Causes and risk factors

The majority of NE cases are associated with antepartum aggressions (prior to delivery) (Badawi et al., 1998a). Retrospective epidemiological studies have identified conditions occurring during

pregnancy as a risk factor for NE. Among the major risk factors identified are perinatal bacterial infections (mainly due to *group B streptococcus* and *Escherichia coli*), maternal fever, maternal history of epileptic seizures without fever or other neurological disorders, thyroid disorders or treatments for infertility (Badawi et al., 1998a; Speer and Hankins, 2003). In addition, pre-eclampsia (*i.e.* hypertension and end-stage renal disease), chorioamnionitis (infection/inflammation of the placenta, membranes and amniotic fluid), placental thrombosis and infarction, maternal hemorrhage following placental abruption, umbilical cord prolapse, uterine rupture, prolonged rupture of membranes, or post-term birth (> 41 weeks of gestation) are also risk factors for NE (Mcdonald, 2004; Redline and ORiordan, 2000; Redline, 2005; Redline et al., 2007). These factors have - or are associated with - perinatal infection/inflammation and/or decreased blood flow or oxygen delivery to the fetal brain, resulting in most cases from NE to HI aggression, either pure or combined with infectious and inflammatory factors (Badawi et al., 1998a; Speer and Hankins, 2003).

Intrapartum insults (during delivery) are major risk factors of NE and subsequent CP but are reported in only 8 to 10% of NE cases. These are mainly acute complications of childbirth: uterine rupture, placental abruption, cord compression or maternal bleeding (Badawi et al., 1998b; Martinez-Biarge et al., 2013).

Despite the fact that HIE and NAIS are two distinct pathologies, current evidence suggests that they shared similar determinants. Indeed, *peripartum* HI is known as a risk factor for NAIS (Fluss et al., 2019; Giraud et al., 2017; Martinez-Biarge et al., 2016). Besides, recent studies emphasize the role of perinatal infection/inflammation in NAIS (Giraud et al., 2017; Sorg et al., 2019) (see

also paragraph 1.5). Sorg and colleagues found that exposure to chorioamnionitis increased by a factor of 10 the risk of NAIS (Sorg et al., 2019). Co-occurrence of HIE and NAIS has been describe in the literature (Adami et al., 2016; Michoulas et al., 2011; Ramaswamy et al., 2004). Besides, it can be challenging to differentiate neonates having NAIS from HIE given overlapping clinical symptoms (Adami et al., 2016).

1.5. Role of infection/inflammation in HIE and NAIS

Infectious/inflammatory events exert their harmful effects most often before birth (*e.g.*, chorioamnionitis) or sometimes after (neonatal infections), and increases the risk of perinatal asphyxia and cardiac depression with subsequent brain HI (Lieberman et al., 2000). An interaction between inflammatory events (such as a chorioamnionitis) – an antepartum inflammatory episode – and an intrapartum HI has been identified as a major cause of brain damage in neonates with NE (Aly et al., 2006; Blume et al., 2008; Wu et al., 2003). For instance, it has been shown that the risk of neurological impairment, such as CP, is increasing as a function of the number and severity of placental lesions (Redline and ORiordan, 2000). In line with this result, a meta-analysis indicated a positive association between CP and chorioamnionitis: term newborns were found to have higher risk of 4.7 for CP when signs of clinical chorioamnionitis were presents (Wu and Colford, 2000). A recent multivariable analysis included 32,326 infants with detailed information about the placental pathology and neurodevelopment (Chen et al., 2020). Placental inflammatory pathology was significantly associated with neurodevelopmental outcomes at 8 months, 4 and 7 years. Placental inflammation was associated with CP in offspring up to four years of age (odds ratio (OR) of 8 for histological chorioamnionitis and perinatal CP, whatever the level of severity of chorioamnionitis) (Chen et al., 2020). Moreover, CP patients were found to have higher

concentration of pro-inflammatory cytokines within the blood, including IL-1, IL-6, IL-8, TNF-α, and others, as compared with control patients (Nelson et al., 1998). Several review articles emphasize the role of infection/inflammation in neonatal brain injury (Fleiss et al., 2015; Girard et al., 2009; Giraud et al., 2017; Hagberg et al., 2015; Nelson and Penn, 2015; Nelson and Willoughby, 2000).

Perinatal infection/inflammation has recently emerged as one of the most significant risk factors of NAIS (Giraud et al., 2017; Sorg et al., 2019). A recent review article confirmed the role of chorioamnionitis in increasing the risk for NAIS (with an OR up to 10) (Chabrier and Sébire, 2020; Sorg et al., 2019)

This combination of inflammation plus HI, which is the most frequent and aggressive sequence for the brain, has been applied to our experimental model of neonatal brain injuries.

2. Preclinical model of HI and/or inflammatory-sensitized HI

2.1. Rationale of the inflammatory-sensitized HI model

The first rat model of HIE was developed by the Rice-Vannucci group in 1981 (Rice et al., 1981). The authors used pups on postnatal day 7 (P7) on which they performed ischemia (permanent ligation of the left common carotid artery) and hypoxia (3.5 h, at 8% O2) (Rice et al., 1981). However, P7 rat pups correspond in term of neuronal maturation, to the level of development of a premature newborn (32-36 weeks of gestation, Figure 3) (Patel et al., 2014). Hence, P7 pups are slightly immature in terms of brain development to appropriately reflect the stage of neuronal development of the term newborn (Sarkar et al., 2019; Yager and Ashwal, 2009) (**Figure 3**). Significant differences between P7 and P12 rat pups are found in (*i*) the pattern of brain injuries following HI insults, (*ii*) the electrical brain activity, and (*iii*) the myelination process. Briefly,

premature HI insults result in periventricular leukomalacia or diffuse white mater injury due to the vulnerability of the oligodendrocytes at this developmental stage (Patel et al., 2014; Rumajogee et al., 2016; Sarkar et al., 2019). On the other hand, term HI injuries will affect mainly grey structures, including deep grey nuclei (thalamus, basal ganglia and caudate-putamen) and cerebral cortex, with little or only secondary (Wallerian degeneration) involvement of white matter tracts (Patel et al., 2014; Towfighi et al., 1997). Besides, it has been described that the levels of cerebral activity using electroencephalography and myelination of P12 pups were similar to term brain newborns (Patel et al., 2014).

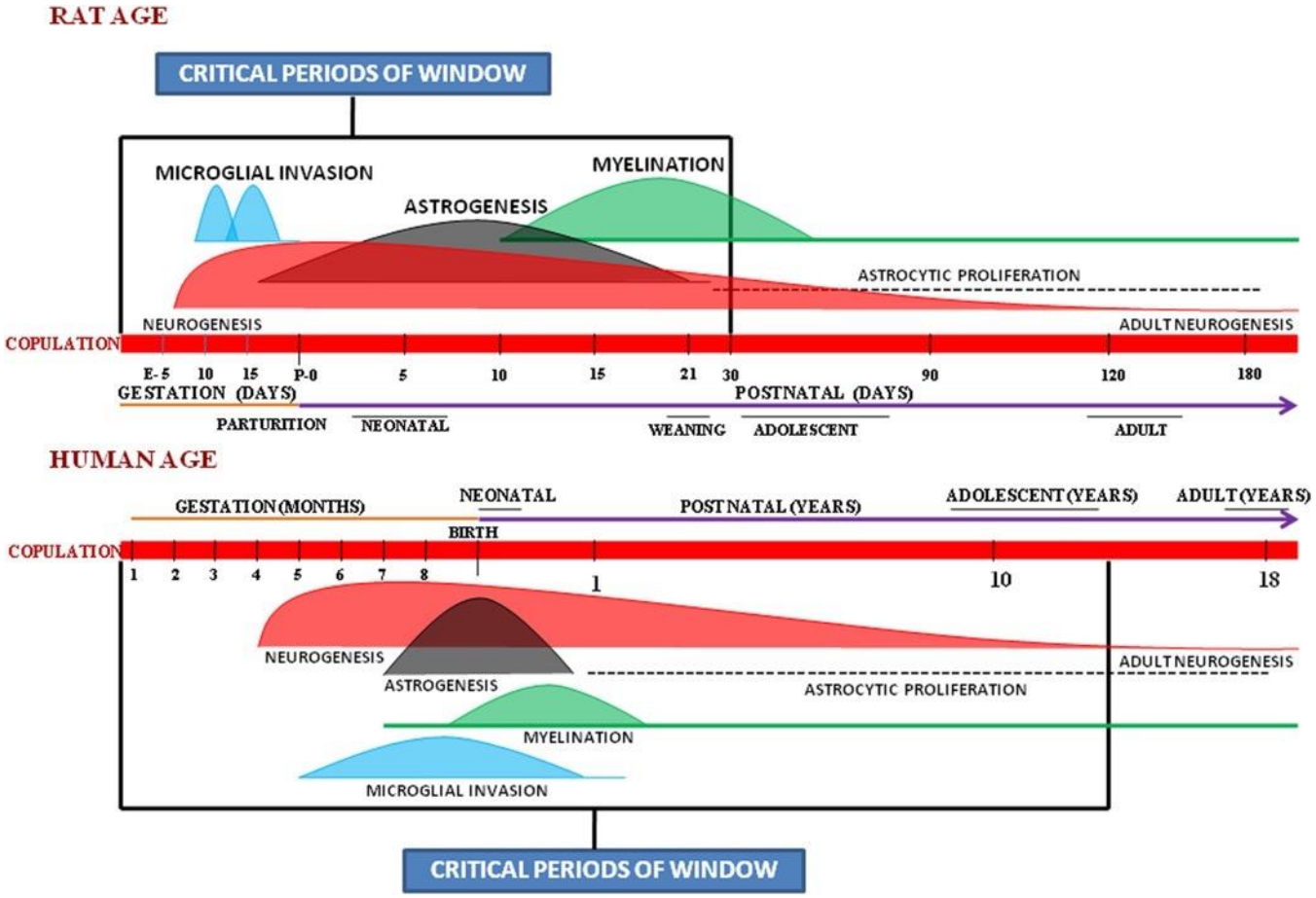

Figure 3. Summary of the time course of brain processes during development in humans and rats. Timeline of brain development starting with neurogenesis, followed by microglial invasion, astrogenesis, myelination, astrocytic proliferation and adult neurogenesis in both rat and human brains. Note that the myelination process started at P10 in rats. Figure reproduced with permission from (Sarkar et al., 2019).

Recently, the Vannucci's group and others have used P10-12 pups; which is therefore more appropriate from a translational point of view for the study of the pathophysiology of brain injuries in term human newborn (Brochu et al., 2011; Patel et al., 2015, 2014).

The vast majority of NE studies used HI insult only. However, HI represents only 10-30% of NE cases (Kurinczuk et al., 2010) and infection/inflammation has been identified as a risk factor for brain damage in neonates with NE (see paragraph 1.5). This is the reason why another group started to develop a novel double-hit rat model at P7 using an intraperitoneal injection of lipopolysaccharide (LPS) of *Escherichia coli* followed by HI as performed in the Rice-Vannucci model (Eklind et al., 2001). Even if this study was mainly descriptive (appearance and extent of damage) and using rat pups at a preterm level of brain development, it demonstrated for the first time the relevance to combine an inflammatory insult with a HI since this combination significantly increases the extent of injury (Eklind et al., 2001). Other preclinical studies later confirmed the synergistic effect of the LPS+HI double hit model (**Figure 4**) (Brochu et al., 2011; Coumans et al., 2003; Larouche et al., 2005). In 2004, a first behavioural study on the double hit rat model at P7 demonstrated an increased cognitive impairment in the LPS+HI group as compared to HI only (Ikeda et al., 2004). In the past, our group studied the neuroinflammatory response in

very preterm (P1) *versus* term (P12) rat pups after LPS, HI or LPS+HI exposure. The maximal inflammatory response was shown in term-like brain after the exposition of LPS+HI (Brochu et al., 2011). Our group and others further characterized the effect of LPS+HI on inflammatory processes leading to brain damage (Bonestroo et al., 2015; Savard et al., 2015, 2013).

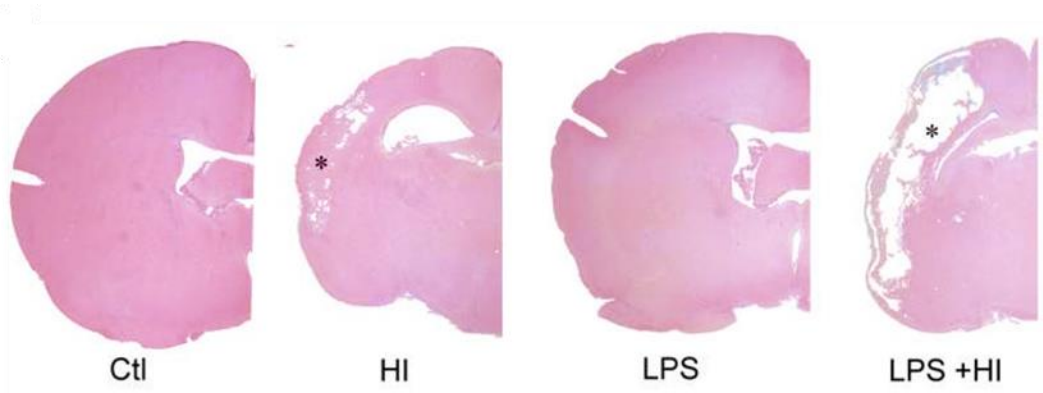

Figure 4. Synergistic effect of LPS and HI in a term (P12) rat model. Sole HI (Rice-Vannucci model) causes macroscopic lesions in the cerebral cortex, as compared to control (the stars correspond to the total loss of tissue). Exposure to LPS does not induce macroscopic brain damage. Exposure to LPS plus HI increases brain damage compared to sole HI. The inflammation caused by the presence of LPS potentiates the inflammatory response and the brain injury caused by HI insult. Figure reproduced with permission from (Brochu et al., 2011), copyright from Creative Commons Attribution License (CC BY).

2.2. Rationale for the LPS+HI model as an NAIS model

We already discussed earlier in this thesis introduction how inflammatory-induced NE and NAIS could be two pathologies that can coexists in clinic, and sometimes hardly distinguishable (Adami et al., 2016; Michoulas et al., 2011; Ramaswamy et al., 2004). Both pathologies shared similar risk

factors (see paragraph 1.5.), as well as induced comparable long-term consequences for infants; i.e. CP, epilepsy, and cognitive and behavioural impairments (see **Table 2.**) (Fluss et al., 2019; Giraud et al., 2017; McIntyre et al., 2013). MRI images of the brain is actually the main tool used to discriminate NE and NAIS (see paragraph 1.3.): with diffuse injuries observable in NE, and focal injuries in an arterial territory in NAIS (Fluss et al., 2019; Rutherford et al., 2010). Besides, in this model, the occlusion of the common carotid artery leading to focal brain injury is one of the key feature responsible for NAIS in human (see **Table 2.**) (Fluss et al., 2016; Suppiej et al., 2019).

	Neonatal encephalopathy (NE)	Neonatal arterial ischemic stroke (NAIS)
Prevalence	2/1000 term births	1/3000 to 1/6000 term births
Mortality	Around 30%	2%
Initial symptoms	Abnormal consciousness level, seizures, abnormal tonus and reflexes	Focal seizures
Long-term consequences	Cerebral palsy (20%), epilepsy (15-20%), cognitive and behavioral impairments	Cerebral palsy (33%), epilepsy (15%), cognitive and behavioral impairments
Risk factors	Hypoxia + Ischemia (HI, 10-20%) Perinatal infection-inflammation (chorioamnionitis: OR=8)	*Peripartum* hypoxia Occlusion of the cervico-encephalic artery Infection-inflammation (chorioamnionitis: OR=10)
Brain injuries (MRI)	permanent non-progressive mainly in the cortex or the basal ganglia	permanent non-progressive mainly in the area of the affected artery (middle cerebral artery, carotid artery)

Table 2. Comparison of the main clinical characteristics between NE and NAIS. This table was realized based on a literature review of several clinical studies conducted on human cases of HIE and inflammatory-sensitized NE, as well as NAIS (Aly et al., 2006; Azzopardi et al., 2014; Blume et al., 2008; Chabrier and Sébire, 2020; Chen et al., 2020; Fluss et al., 2019, 2016; Giraud et al., 2017; Mcdonald, 2004; McIntyre et al., 2013; Nelson, 2009; Redline and ORiordan, 2000; Redline, 2005; Rutherford et al., 2010; Sorg et al., 2019; Suppiej et al., 2019).

This is why, to our opinion and based on these knowledges, we think that the combination of inflammation plus HI is relevant to both NE and NAIS.

3. Mechanisms of brain injury induced by HI ± inflammation.

This paragraph focuses on the genesis of brain injury in term newborns. Most of the mechanisms detailed below have been described in pure or inflammatory-sensitized HI but can also be applied to NAIS.

3.1. Phases of brain injury

Brain damage resulting from pure HI origin - or HI combined to infection/inflammation - is caused by two consecutive energy failures. The first phase of injury occurs between 0 and 6 h after the exposition to HI alone or infection/inflammation plus HI. This primary energy failure leads to primary neuronal death, including unprogrammed cell death (*i.e.* excitatory cell death and necrosis, see paragraph 3.2.1) and programmed necrosis (so-called necroptosis, paragraph 3.2.2) followed by a first release of inflammatory molecules, including damage-associated molecular pattern molecules (DAMPs), cytokines, chemokines, and other (Davidson et al., 2015; Giraud et al., 2017) (**Figure 5**). After a latent phase corresponding to cerebral re-oxygenation, the energy metabolism will stabilize, before deteriorating again. The second energy failure occurs from 24 to 72 hours after the first one and is characterized by neuronal damage caused by mitochondrial dysfunction and the initiation of the apoptotic cascade (Ma et al., 2012) (**Figure 5** and paragraph 3.2.3). During this second phase, there is also release of inflammatory mediators amplifying brain damage (Khwaja and Volpe, 2008; Leviton et al., 2005).

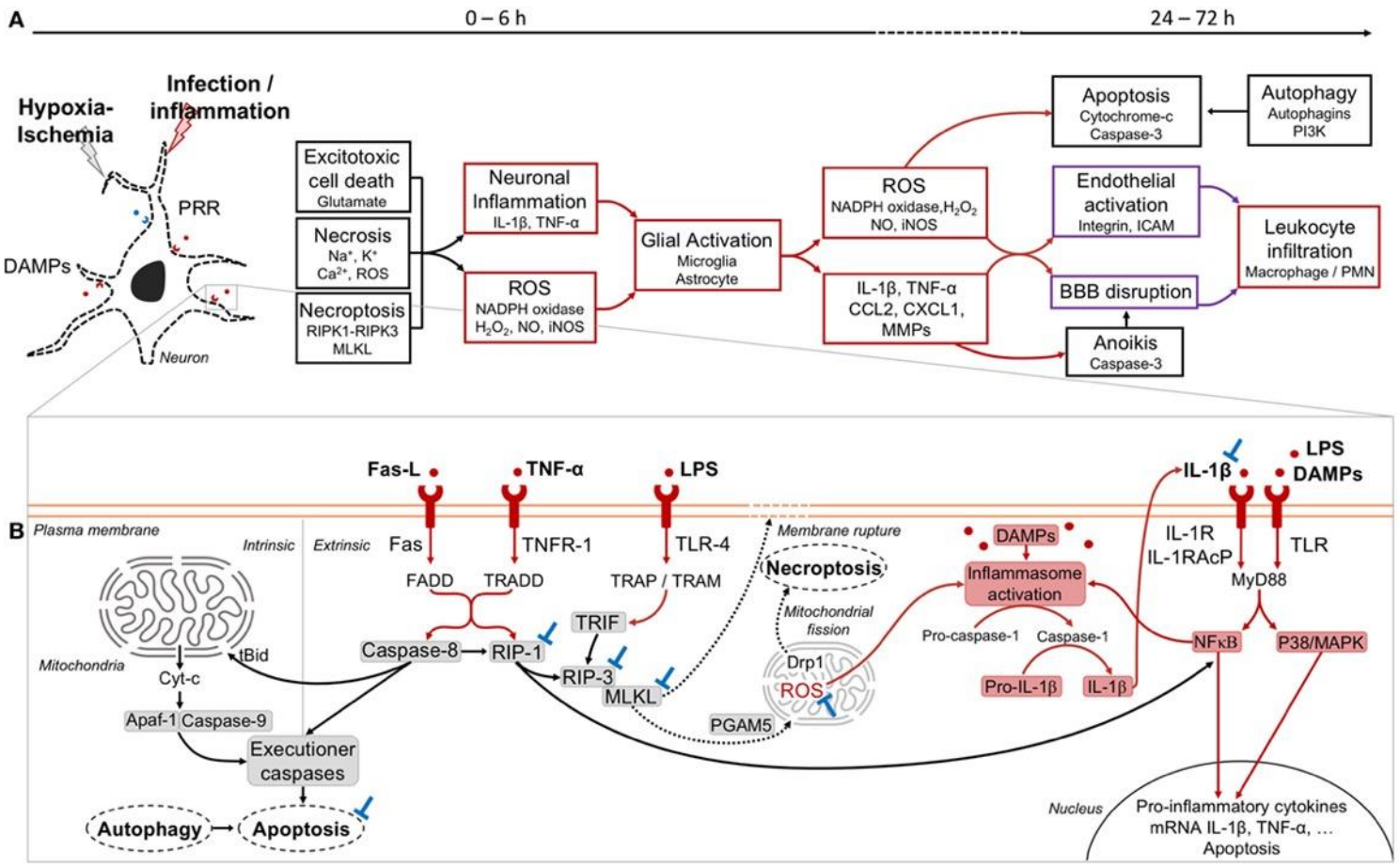

Figure 5. Phases of injury following HI ± inflammation and mechanistic pathways leading to neuronal death. Figure reproduced with permission from (Giraud et al., 2017), copyright from Creative Commons Attribution License (CC BY).

A phase of repair and reorganization will follow the massive destruction of neurons during the second phase of injury. During this third phase, new cells are created, and neural circuits are regenerated. However, the inflammation resulting from the HI still persists and the resulting apoptosis disrupts the production of new cells and neuronal survival for several months. Indeed, it was shown by Winerdal and colleagues that inflammation can persist up to three months after NE within the periphery and brain of neonates (Winerdal et al., 2012). The precise mechanisms that

induce the prolongation of the assault are not all known at present (Davidson et al., 2015; Fleiss and Gressens, 2012).

3.2. Cell death pathways at play in HIE and NAIS

3.2.1. Unprogrammed cell death

In physiological conditions, glutamate mediates excitatory synaptic transmission via the activation of glutamate receptors, including N-methyl-D-aspartate (NMDA) to mediate the normal information processing. Upon an HI insult, the deprivation of glucose and oxygen within the brain leads to the deterioration of energy metabolism (Thornton and Hagberg, 2015; Zhao et al., 2007). The low levels of adenosine triphosphate (ATP) cause failure of the energy-dependent cell membrane ion channels that maintain cell integrity, which results in an acute intracellular influx of calcium (Ca^{2+}) and sodium and cell membrane depolarization, as well as accumulation of extracellular glutamate (Li et al., 2017). This significant increase in intracellular Ca^{2+} will then activate the calpains which will cleave the cytoskeleton proteins, the membrane receptors and transporters. Ultimately, this process will induce swelling of the organelles and the cytoplasm, as well as the rupture of the plasma membrane of the cell (Martin and Henry, 2013; Tovar-y-Romo et al., 2016). The necrotic cells will release their intracellular container: organelles and nucleus (DNA, RNA, nucleotides) as well as DAMPs such as IL-1α, the high-mobility group box 1 (HMGB-1), ATP, uric acid, and heat shock proteins. This release of DAMPs will induce the recruitment and activation of neutrophils, dendritic cells as well as macrophages, and promote an important inflammatory response. Indeed, the activation of inflammasomes and the transcription of the nuclear factor NFκB, via the binding of DAMPs to their Toll-like receptors (TLRs), Nod-like receptors (NLRs) induce the synthesis and release of pro-inflammatory cytokines, including

IL-1β) (Pisetsky, 2011; Sangiuliano et al., 2014) (see also paragraph 3.3). This inflammation can eventually lead to apoptosis or necrosis of nearby cells.

3.2.2. Programmed necrosis

Necroptosis is an early cell death pathway (occurring within the 6 h of injury) which is triggered by inflammatory mediators led by Tumor necrosis factor (TNF)-α and TNF family death receptor (TNFR): TNFR-1, FAS, and TLR, namely TLR-3 and TLR-4 (**Figure 5**). The recruitment of adapter proteins such as tumor necrosis factor receptor type 1-associated death domain protein (TRADD) et fas-associated protein with death domain (FADD) by the ligands mentioned above, as well as the receptor interacting protein-1 (RIP-1) will induce the activation of caspase-8 and t-Bid, and lead to death by apoptosis. On the other hand, if caspase-8 is inhibited, TRADD will facilitate the interaction and activation of RIP-1 and RIP-3, followed by the recruitment and phosphorylation of mixed lineage kinase domain-like protein (MLKL), thus forming the necrosome (Kaiser et al., 2013; Mandal et al., 2014; Tovar-y-Romo et al., 2016; Walsh, 2014). The necrosome will then induce necroptosis of the cell by increasing the level of reactive oxygen species (ROS) and/or by inducing mitochondrial fission via dynamin-related protein 1 (Drp1). Normally, Drp-1 regulates mitochondrial dynamics (homeostasis between mitochondrial fusion and fission) by facilitating fission of the mitochondria. During intense cellular stress, there will be an intensification of the translocation of Drp1 to the mitochondria, inducing its fragmentation and leading to cell death (**Figure 5**) (Pradeep et al., 2014). All the mechanisms by which MLKL induces cell death are not totally elucidated. The induction of necroptosis via non-mitochondrial mechanisms is still under study. Furthermore, the release of mitochondrial ROS within the cytoplasm of the neuron can induce the activation of the inflammasome.

Necroptosis has been discovered quite recently and is a hot topic since then. It has been described in several pathologies, including neurodegenerative diseases such as amyotrophic lateral sclerosis (Ito et al., 2016; Morrice et al., 2017; Re et al., 2014). Recent studies indicated that necroptosis is a neuronal death process involved in NE. It was shown in preclinical models that cerebral expression of TNF-α, as well as RIP-3 was triggered by LPS plus HI exposure (Savard et al., 2015, 2013). Besides, it was also shown that necrostatin-1—a RIP-1 inhibitor—administered after HI injury in P7 mice was neuroprotective: this inhibitor is able to prevent forebrain injury, as well as attenuates oxidative stress and mitochondrial dysfunction (Raul Chavez-Valdez et al., 2012; R Chavez-Valdez et al., 2012; Northington et al., 2011). To date, no study describes any effect of HT on necroptosis in NE.

3.2.3. Apoptosis/autophagy and anoikis cell death

The secondary phase of injury (from 24 h to 72 h post-insult) implicates mainly apoptosis, anoikis and autophagy cell deaths. Many preclinical studies characterized the involvement of apoptotic cell death in the genesis of NE – due to pure HI or combined with infection/inflammation – and provided evidence in favor of neuroprotective strategies targeting apoptotic pathways (Baburamani et al., 2017; Carlsson et al., 2011; Chauvier et al., 2011; Hallin et al., 2006; Jia et al., 2019; Nijboer et al., 2011; Zhu et al., 2010). It is described in the literature that apoptotic and autophagic cell death pathways crosstalk, and that autophagy can block apoptosis by sequestration of mitochondria. It is still debated if autophagy just after NE is a neuroprotective mechanism by limiting the release of cell content and the inflammation (Mariño et al., 2014; Xie et al., 2016). Anoikis is another form of apoptosis triggered by cell detachment from the extracellular matrix. Anoikis is induced by increased matrix metallopeptidases (MMPs), including MMP-9 and

activation of Fas receptor, which initiates the apoptosis cascade (Grossmann, 2002). This cell death was assessed following HI plus inflammation: Savard and colleagues describe an overexpression of MMP-9 after HI plus inflammation injury (Savard et al., 2015, 2013). They also showed that the use of an MMP-9 competitive inhibitor can limit the size of the brain injury (Savard et al., 2015). Recently, a clinical study reported elevated MMP-9 concentration in the serum of term newborns suffering from HIE (Salah et al., 2019). Higher MMP-9 level was observed in more severe cases of HIE, suggesting serum MMP-9 to be a predictor of important neurological sequelae and severity in NE (Salah et al., 2019).

3.3. IL-1β-driven neuroinflammation in LPS+HI-induced HIE and NAIS

IL-1β is a cytokine with many biological effects, including the activation of several pro-inflammatory mechanisms. It is expressed as an inactive pro-protein (pro-IL-1β) and must be cleaved into IL-1β by caspase-1 through the activation of the inflammasome, in order to exert its functions (Allan et al., 2005). For instance, the recognition of DAMPs by an intracellular receptor called Nod-like receptor (NLR) will activate the inflammasome, which controls the maturation and expression of IL-1β and IL-18. The main NLRs present in the central nervous system (CNS) are NLRP1 and NLRP3 (Schroder and Tschopp, 2010). IL-1β binds to its receptor, IL-1R1, which then binds with the adapter protein of the IL-1β receptor (IL-1RAcP) to carry out the intracellular signal. Signal transmission is via IL-1R1, NF-κB factors and Mitogen-activated protein kinase (MAPK) (Korherr et al., 1997) (**Figure 5**). When stimulated with IL-1β in vitro, all CNS cells respond conventionally by the NF-κB and MAPK pathways by expressing secondary inflammatory factors (prostaglandin E2 (PGE2), IL-6, nitric oxide). However, CNS cells have been shown to respond differently to IL-1β; neurons will use the p38MAPK pathway while

astrocytes will use the NF-κB pathway (Srinivasan et al., 2004). The ligand-receptor system has an interesting feature: there is an endogenous antagonist – called the IL-1Ra – expressed by the same cells which inhibits the signaling of the receptor (Allan et al., 2005).

Il-1β can also be produced in response to molecular motifs from pathogens, called pathogen-associated molecular patterns (PAMPs) that activate Toll-like receptors (TLR) or NLR, and regulate the expression of NFκB and MAPK (Brough et al., 2011). Besides, LPS is known to bind TLR-4 and activate IL-1β synthesis through the activation of myeloid differentiation primary response gene (MyD) 88 and NFκB (Bowie and O'Neill, 2000) (**Figure 5**). DAMPs released from dead cells can also activate the production of IL-1β (Brough et al., 2011). Exposure to LPS and/or HI results in release of PAMPs and DAMPs and, when combined, can lead to over-activation and overexpression of IL-1β (Brough et al., 2011) (**Figure 5**). Furthermore, it has been shown recently that necroptosis mediators (*i.e.* RIP3 and MLKL, see paragraph 3.2.2) can also activate NLRP3 and induce the expression of IL-1β, following LPS administration (Lawlor et al., 2015). Altogether, this will favor the establishment of a proinflammatory state, mediated by IL-1β and subsequent neurotoxic mechanisms such as IL-1β-induced other inflammatory mediators and/or exacerbation of the excitotoxicity (see paragraph 3.2.1)

3.3.1. Effects of IL-1β on neural cells

IL-1β has multiple effects on glial cells via the IL-1R1 (Allan et al., 2005). The most responsive cells to IL-1β are astrocytes (Allan et al., 2005; Chen and Swanson, 2003). IL-1β promotes astrogliosis (proliferation of astrocytes) and their activation (Chen and Swanson, 2003). Reactive astrocytes can help neurons by regulating the concentrations of ions and transmitters. They can also be a source of pro-inflammatory cytokines and become neurotoxic (Chen and Swanson, 2003).

A wide range of genes (around 1400) are express during activation of astrocytes by IL-1β, including MMPs, cytokines (IL-6), chemokines, growth factors (such as nerve growth factor (NGF)), adhesion molecules and receptors (Allan et al., 2005). Microglia also responds directly to IL-1β via IL-1R1 (Allan et al., 2005). This response results in the release of inflammatory neurotoxic mediators such as ROS and reactive nitrogen species (RNS), lipid mediators (PGE2), cytokines (TNFα) and chemokines (monocyte chemoattractant protein (MCP)-1) (Basu et al., 2004). At the neuronal level, IL-1β has been shown to be neurotoxic (Allan et al., 2005). IL-1β allows calcium to enter the NMDAR which is involved in neuronal death (see paragraph 3.2).

It has been shown by our group and others that HI ± inflammation induced an increase in the number and activity of astrocytes and microglia within the brain (Hagberg et al., 2015; Savard et al., 2013). However, Savard and colleagues did not identify these cells as major productor of IL-1β in their LPS+HI model (Savard et al., 2013). Instead, they described that neuronal cells were the only cell type with increased expression of IL-1β very early (namely 4 h) after the exposition to LPS+HI. NLRP1 was also expressed in neurons following LPS+HI insult (Savard et al., 2015). Microglia and astrocytes were more associated with an increase TNF-α expression 48 h after LPS+HI exposure (Savard et al., 2013).

3.3.2. Effect of IL-1β on endothelial cells and blood brain barrier disruption

In vitro studies have described that IL-1β can activate endothelial cells leading to the overexpression of intercellular adhesion molecule (ICAM)-1 and the vascular cell adhesion molecule (VCAM)-1 (Thornton et al., 2010). This effect has been confirmed in *in vivo* studies of focal ischemic stroke (Wong et al., 2019). After a stroke, IL-1β was able to activate brain endothelial cells through binding with IL-1R1, leading to the upregulation of ICAM-1, VCAM-1

and chemokines. Ultimately, this will result in neutrophils adhesion and infiltration within the brain parenchyma (Wong et al., 2019). It has been also described that the infiltration of neutrophils following ischemic injury will amplify the cerebral response and exacerbate the disruption of the blood brain barrier (BBB), leading to increase brain injuries (Jin et al., 2010). After deleting specifically IL-1R1 in the brain endothelium of mice, Wong and colleague observed a significant reduction of the disruption of the BBB, as well as the reduction of the infarct volume and neurological improvements (Wong et al., 2019). In line with these results, Savard and colleague described increased of cytokine-induced neutrophil chemoattractant (CINC-1) and MCP-1, as well as disruption of the BBB, in their model of inflammatory-sensitized HI (Savard et al., 2015). IL-1 blockade was able to restore the BBB and prevent neutrophils infiltration (Savard et al., 2015).

3.4. IL-1/IL-1Ra balance in the brain

IL-1β is expressed constitutively and in small amounts in the brain (Allan et al., 2005; Vitkovic et al., 2000). IL-1β has long been described as having physiological activity on the brain (Allan et al., 2005). Studies uncovered an important role for IL-1β in sleep, neuronal plasticity, neurogenesis, and modulation of learning and memory (Liu and Quan, 2018; Mantovani et al., 2019; Vitkovic et al., 2000). But IL-1 probably have many other direct or indirect impacts on physiological activities within the brain (Liu and Quan, 2018). For a particular example, studies demonstrated that knock out mice for IL-1R have decreased sleep periods, synaptic plasticity deficits and poor spatial memory (Avital et al., 2003; Liu and Quan, 2018). IL-1Ra overexposure can also have deleterious effects on brain morphology and can results in behavioural impairments or memory deficits (Goshen et al., 2007; Spulber et al., 2011, 2009). Hence, modulation of the IL-1 system needs to be tightly controlled in order to avoid harmful impact on the brain.

3.5. hrIL-1Ra effects in term brain injury and perinatal inflammation

IL-1Ra is an endogenous ligand that prevents the activation of the IL-1R1 by blocking both IL-1α and IL-1β. A human recombinant called Anakinra (or Kineret®) is actually used to treat rheumatoid arthritis and chronic inflammatory conditions, including those affecting pregnant mothers and newborns (Dinarello and van der Meer, 2013; Rosenzweig et al., 2014). In neonates, the recommended dose is between 1-8 mg/kg per day (see appendix #1). However, due to change of species, this compound will be twelve-times less effective when administrated in rats (Quiniou et al., 2008).

We already discussed that HI ± inflammation induce major postnatal brain injury through the release of inflammatory mediators, especially IL-1β. Therefore, the administration of IL-1Ra may be a potential therapeutic intervention of brain injury. Several preclinical studies have shown the neuroprotective effects of hrIL-1Ra in postnatal brain injury models. It has been shown in rodent models of HI that hrIL-1Ra reduced the extent of brain damage (Hagberg et al., 1996; Hu et al., 2005; Martin et al., 1994). Furthermore, Hu and colleagues found a reduction of caspase-3-induced cell death and NFκB activation following the administration of hrIL-1Ra. More recently, Savard and colleagues demonstrated the neuroprotective effect of hrIL-1Ra in their model of inflammatory-sensitized HI injury. In their studies, they found that hrIL-1Ra reduced the extent of brain injury by histologic and MRI observations (**Figure 6**) (Savard et al., 2015, 2013). For this study, hrIL-1Ra was used at the dose of 200 mg/kg. This dose has already been demonstrated to be the most effective in adult model of stoke (McCulloch et al., 2019; Relton et al., 1996; Savard et al., 2013).

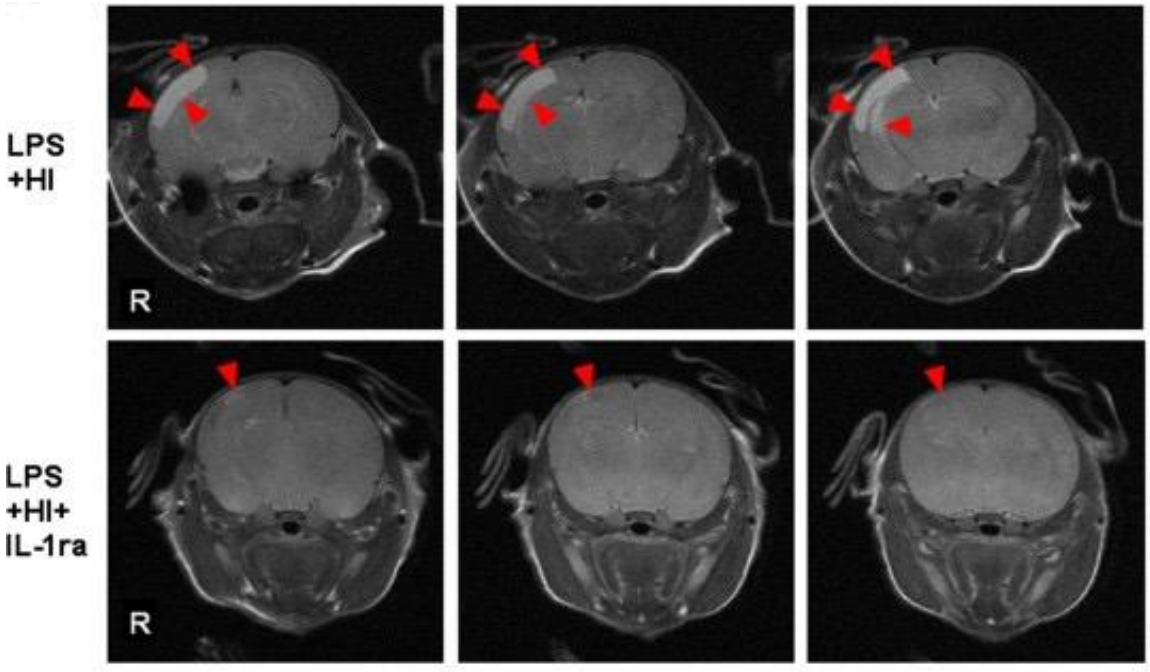

Figure 6. Neuroprotective effect of hrIL-1Ra observed on brain MRI in an inflammatory-sensitized HI rat model. The treatment with IL-1Ra decreased the extent of brain lesion (*i.e.* hyper-signal area) in the right cortex. Figure reproduced with permission from (Savard et al., 2013), copyright from Creative Commons Attribution License (CC BY).

Besides, hrIL-1Ra reduced LPS+HI-induced mortality, decreased the activation of caspase-3 and RIP-3-induced cell death, as well as improved motor behavioural impairments (Savard et al., 2015). Interestingly, a recent study uncovered that IL-1Ra – in addition to its effect on inflammatory pathway – is able to inhibit caspase 8 and 9; therefore, preventing apoptosis and necroptosis (Spinello et al., 2019).

In other preclinical studies, hrIL-1Ra was found to have beneficial effect in LPS-induced chorioamnionitis in fetal sheep (Berry et al., 2011), as well as in neonatal inflammation-induced

neuronal injury and long-term neurobehavioural deficits in adult rats (Lan et al., 2015; Pang et al., 2015).

In human, a randomised phase II trial of IL-1Ra in adult ischemic stroke showed promising results (Emsley et al., 2005). The administration of IL-1Ra was safe and well tolerated in acute stroke. Clinical outcomes after the stroke were more favourable in the IL-1Ra-treated group (Emsley et al., 2005). Despite promising results in preclinical studies, to date no clinical trial has been assessed to test its safety and efficacy in perinatal brain injury. Furthermore, no studies have yet been conducted to assess the efficacy of IL-1Ra in combination with HT.

4. Therapeutic HT

4.1. HT in HIE

Since 2010, therapeutic HT became the standard treatment for newborns suspected of having HIE (Edwards et al., 2010). Specific eligibility criteria are shown in the **Table 3**. This treatment consists in treating the affected newborn within 6 hours after birth. The whole body of the newborn is placed on HT to lower the body temperature to 33-34 °C. HT is maintained for 72 h, before a gradual rewarming (0.5 °C per hour) of the newborn. Neuroprotective mechanisms of HT are a decrease in: (*i*) brain energy metabolism, (*ii*) the accumulation of neurotoxic amino acids (glutamate), (*iii*) the release of ROS and RNS (Alva et al., 2013), and (*iv*) the activity of the apoptosis and necrosis pathways (Gancia and Pomero, 2010; Shankaran, 2012).

A. Cord pH ≤7.0 or base deficit ≥−16, OR
B. pH 7.01 to 7.15 or base deficit −10 to −15.9 on cord gas or blood gas within 1 h AND
1. History of acute perinatal event (such as but not limited to cord prolapse, placental abruption or uterine rupture) AND
2. Apgar score ≤5 at 10 minutes or at least 10 minutes of positive-pressure ventilation
C. Evidence of moderate-to-severe encephalopathy, demonstrated by the presence of seizures OR at least one sign in three or more of the six categories shown in Table 1.

Table 3. Eligibility criteria for HT treatment. The Canadian Pediatric Society indicates in their guidelines that infants who benefit from HT are term newborns (≥36 weeks of gestation) with HIE who are ≤6 hours old and who meet either treatment criteria A or B, and also meet criteria C (Lemyre and Chau, 2018).

Preclinical studies in rodents first demonstrated beneficial effects of HT on HI: these studies noted alleviation in energy metabolism with the improvement in ATP level (Williams et al., 1997), a decrease in acidosis, as well as a reduction in brain damage (Laptook et al., 1995; Thoresen et al., 1996; Williams et al., 1997; Yager et al., 1993). The potential effect of HT on neuroinflammatory cytokines expression has been poorly investigated up to now in preclinical models as well as in term newborns (see paragraph 4.3.5).

Following this work, six randomized controlled therapeutic trials were carried out in order to verify and better characterize neuroprotective effects of HT in human newborns (Azzopardi et al., 2009; Gluckman et al., 2005; Jacobs et al., 2013; Shankaran et al., 2005; Simbruner et al., 2010; Zhou et al., 2010). These clinical trials used two different cooling methods: selective head cooling (only the head is placed in HT) or whole-body HT. No significant difference in terms of speed and

stability of cooling was observed between these two methods (Gluckman et al., 2005; Shankaran, 2012; Wassink et al., 2019; Zhou et al., 2010). The different teams measured the survival rate of newborns as well as neurodevelopmental disabilities resulting from NE (the main outcome was based on a composite score integrating these two variables). The most convincing beneficial effect have been observed in infants with moderate encephalopathies (Gluckman et al., 2005; Gunn et al., 2008) (see **Table 1** for the criteria of encephalopathy according to severity). Evidence of safety and efficacy of HT for newborns with mild and severe NE is still insufficient. Further studies on this particular point is needed (Chawla et al., 2020; Edwards et al., 2010; Kariholu et al., 2020; Nair and Kumar, 2018; Perretta et al., 2019; Wassink et al., 2019) (see also paragraph 4.3.1).

The long-term beneficial effects of HT were further clarified by longer-term follow-up studies of cohorts of patients previously cited. These studies have shown that the risk of CP in HIE-exposed newborn is reduced by 15% with treatment with HT (Azzopardi et al., 2014; Jary et al., 2015) (**Table 4**). In addition, the authors noticed that children treated with HT were less at risk of developing severe motor disabilities. More children in the HT-treated *versus* untreated group survived without neurological problems (**Table 4**) (Azzopardi et al., 2014).

Variable	Hypothermia Group	Control Group	Relative Risk (95% CI)	P Value
	no./total no. (%)			
Grade of disability*				
No disability	65/96 (68)	37/83 (45)	1.52 (1.15–2.00)	0.002
Mild disability	10/96 (10)	15/83 (18)		
Moderate disability	8/96 (8)	11/83 (13)		
Severe disability	13/96 (14)	20/83 (24)		
Moderate or severe disability	21/96 (22)	31/83 (37)	0.59 (0.37–0.94)	0.03
Cerebral palsy†	21/98 (21)	31/86 (36)	0.59 (0.37–0.95)	0.03
Score on Gross Motor Function Classification System‡				
No abnormality	76/98 (78)	49/83 (59)	1.31 (1.07–1.62)	0.01
Level 1–2	6/98 (6)	13/83 (16)		
Level 3–5	16/98 (16)	21/83 (25)	0.65 (0.36–1.15)	0.14
Score on Manual Ability Classification System§				
No abnormality	75/98 (77)	51/83 (61)	1.25 (1.02–1.53)	0.04
Level 1–2	4/98 (4)	8/83 (10)		
Level 3–5	19/98 (19)	24/83 (29)	0.67 (0.40–1.13)	0.16
Visual impairment not corrected by eyeglasses	7/98 (7)	10/82 (12)	0.59 (0.23–1.47)	0.31
Blindness	1/98 (1)	1/82 (1)	0.84 (0.05–13.17)	1.00
Hearing impairment	4/98 (4)	8/83 (10)	0.42 (0.13–1.36)	0.15

* Two children in the hypothermia group could not be classified. P=0.002 for trend.
† Two children in the hypothermia group and two in the control group could not be classified and were subsequently found not to have cerebral palsy.
‡ Scoring on the Gross Motor Function Classification System is as follows: level 1, able to walk independently but may have some gait abnormalities; level 2, able to walk in most settings but with only minimal ability to perform gross motor skills such as running and jumping; level 3, walks with handheld assistive device and when seated may need seat belt for balance; level 4, requires physical assistance or powered mobility and needs adaptive seating; and level 5, severely limited in mobility, with limited ability to maintain antigravity head and trunk postures. P=0.01 for trend.
§ Scoring on the Manual Ability Classification System is as follows: level 1, handles objects easily and successfully; level 2, handles most objects but with somewhat reduced quality or speed of achievement; level 3, handles objects with difficulty; needs help to prepare or modify activities; level 4, handles a limited selection of easily managed objects in adapted situations; level 5, does not handle objects and has severely limited ability to perform even simple actions. P=0.04 for trend.

Table 4. Assessment of the efficacy of HT in children (6-7 years old) who survived an HIE at birth. Children in the hypothermia group had significantly reduced rates of cerebral palsy (21% *vs.* 36%) and moderate or severe disability (22% *vs.* 37%) and had significantly better scores for manual ability and gross motor function. Figure reproduced with permission from (Azzopardi et al., 2014), Copyright Massachusetts Medical Society.

In a small prospective clinical study, HT may decrease the severity and spectrum of CP in survivors of HIE at 24 months – with only one third of CP cases being severe (Jary et al., 2015). However, in this study, HT did not decrease the total number of CP cases in the cohort (Jary et al., 2015). Long-term follow-up and other studies are required to confirm the finding of a reduction of severity of CP in HT-treated newborns.

4.2. HT in NAIS

No treatment – aside from supportive therapy – is available for newborns with NAIS. Recent studies highlight the fact that HT could beneficiate to those newborns (Gancia and Pomero, 2012). It has been described in adult rodent models of ischemic stroke that HT was able to reduce the infarct size by 44% (van der Worp et al., 2007). However, preclinical studies assessing the efficacy of HT in NAIS are still missing. In clinic, it happened that neonates treated with HT and initially thought to have HIE were later diagnosed with NAIS (Adami et al., 2016; Michoulas et al., 2011; Ramaswamy et al., 2004). A single-center prospective cohort study shows for the first time a potential beneficial effect of HT in human newborns in which NE and NAIS coexist (Harbert et al., 2011). Indeed, Harbert and colleagues reported an absence of seizures in HT-treated neonates with NAIS as compared to untreated patients (Harbert et al., 2011). Further research is necessary to further assess the beneficial effect of HT in NAIS.

4.3. Limitations of HT

4.3.1. Limited clinical efficacy of HT

Several clinical studies indicate that HT confers only a partial neuroprotection. More than 50% of newborns treated with HT will experience mild to severe neurological sequelae such as intellectual disabilities, disorders of the learning, autism spectrum disorder or attention deficit disorder (Azzopardi et al., 2014; Davidson et al., 2015). These studies also showed that the efficacy of HT would be limited in severe cases of NE (Azzopardi et al., 2014; Davidson et al., 2015; Edwards et al., 2010). Besides, HT reduces the risk of mortality and morbidity by only 11% (from 58 to 47%) (Azzopardi et al., 2014; Davidson et al., 2015). Data from registries including the Canadian CP registry show that HT prevents only 4% of CP cases (Garfinkle et al., 2015). These findings have also been observed in preclinical studies performed in rat pups and piglets (Haaland et al., 1997; Sabir et al., 2012; Yager et al., 1993).

Almost all clinical data regarding the effect of HT on death and disabilities are combining together newborns having moderate and those having severe HIE (Azzopardi et al., 2014; Natarajan et al., 2016; Shankaran et al., 2005). However, there is not much evidence of the efficacy of HT for newborns suffering from severe HIE (Nair and Kumar, 2018). In the CoolCap study, death and severe disabilities (*i.e.* unfavourable outcome) were reported in 89% of HT-treated and 100% of untreated newborns suffering from severe HIE encephalopathy (Gunn et al., 2008). In contrast, HT-treated neonates with moderate encephalopathy had improved outcomes compared to untreated neonates. Unfavourable outcomes were reported in 31% in the HT-treated, compared with 64% in the untreated group (Gunn et al., 2008). Besides, HT reduced seizure burden in newborns with moderate, but not severe HIE (Srinivasakumar et al., 2013).

In addition, other clinical and preclinical studies have questioned the efficacy of HT when NE was the result of an infection/inflammation and HI combination (Johnson et al., 2016; Mir et al., 2015;

Osredkar et al., 2014; Wintermark et al., 2010). In a prospective study of placental histology relative to MRI outcomes, HT was less protective for infants with NE whose placentas exhibited chorioamnionitis (Wintermark et al., 2010). In adult clinical studies, HT has been found to be ineffective, and potentially harmful in the presence of severe meningitis or sepsis (Itenov et al., 2018; Mourvillier et al., 2013).

The limitations of neuroprotective efficacy of HT to combat pure HI, and the doubts about its effectiveness in mechanisms combining several insults such as inflammation and HI, require to study new therapies associated with HT in order to increase its therapeutic benefit, and further reduce the impact of NE on the survival of newborns and future neurological disabilities (Benninger et al., 2020) (see paragraph 4.4).

4.3.2. Optimization of HT protocol

The clinical limitation of HT can be partly due to the fact that the HT protocol still needs to be optimized. Indeed, the timing of HT induction after birth as well as the optimal temperature and duration of HT are still being questioned (Wassink et al., 2019).

The timing of induction of HT was set at 6 h maximum after birth, according to the results of preclinical studies indicating that HT was no longer effective after 6 h in moderate cases of HIE; and that HT is deleterious when induced after 12 h of delay in severe cases of HIE (Sabir et al., 2012). However, initiating HT within the 6 h time window can be difficult, especially in case of transportation of the neonate or delay in the diagnosis of HIE. A recent randomized clinical trial questioned the beneficial effect of late HT (from 6 to 24 h after birth) (Laptook et al., 2017). Data suggested that the induction of HT after 6 h still results in a reduction of death and disabilities (Laptook et al., 2017). However, data in this study have been criticized and raised controversy

(Bourque and Dietz, 2019, 2018; Laptook et al., 2019; Quintana et al., 2017; Walløe et al., 2019). Hence, further work is still required to clearly answer this question, before changing HT criteria in clinic.

Recent studies have sought to evaluate the effectiveness of HT when it was deeper (30 or 32°C instead of 33°C) and/or lasted for longer (24, 48 or even 120 h) compared to the classic protocol (Lee et al., 2010; Wood et al., 2016). Thomas Wood et al. have demonstrated that HT levels of 33.5°C, 32°C and 30°C are effective and safe in rat pups (Wood et al., 2016). However, Lee and colleagues did not find any differences between their HT protocol: neither prolonged the HT duration nor decreased the temperature (from 33 to 30°C) were found to further improve neurological outcomes (Lee et al., 2010). A clinical study evaluating the same question did not show any difference in terms of safety (cardiac arrest, persistent acidosis, thrombosis or bleeding of blood vessels and mortality) from prolonged HT (120 h) compared to conventional HT (72 h); similarly for HT at 32 °C versus 33.5 °C (Shankaran et al., 2014). The HT protocol still requires fine-tuning to be optimal.

4.3.3. Limitation in assessing the neuroprotective effect of HT in preclinical studies

HT was implemented as a standard of care based on previous preclinical work (Laptook et al., 1995; Thoresen et al., 1996; Williams et al., 1997; Yager et al., 1993). However, in these studies HT was modestly effective, and the criteria of neuroprotective effect mainly based on histological measures of the extent of brain damage. Very few studies used optimal tools such as MRI to visualize the integrality of brain injury, and magnetic resonance spectroscopy (MRS) or positron emission tomography (PET) to measure the brain metabolism (Ahn et al., 2018; Doman et al.,

2018; Lee et al., 2010; Traudt et al., 2013). Different imaging techniques are now available in animal model of postnatal brain injury (Lodygensky et al., 2008). Besides, imaging findings can be correlated to histopathology and behavioural evaluations, providing more information regarding HT efficacy.

4.3.4. Effect of HT on pharmacokinetic and pharmacodynamic parameters of drugs

Studies have questioned the effects of HT on the pharmacokinetics and pharmacodynamics of other drugs used in combination with HT, or in the therapeutic window of HT in the clinic. In particular, it has been reported that HT could interfere with plasma concentration, volume of distribution and the elimination of certain drugs, creating unexplained and potentially toxic effects in newborns (de Haan et al., 2012; Ezzati et al., 2017; Pokorna et al., 2015; van den Broek et al., 2010; Zhou and Poloyac, 2011). This includes morphine, agonists of α and β adrenergic receptors, and antiepileptic drugs which are often used in newborns suffering from HIE. As an example, a recent preclinical study demonstrated that dexmedetomidine (a selective agonist of the $\alpha 2$ adrenergic receptor used as a sedative) in combination with HT induces major cardiac and neurotoxic effects in a piglet model of HIE (Ezzati et al., 2017). Three recent clinical studies revealed alteration of pharmacokinetic and/or pharmacodynamic parameters of ampicillin, morphine, and melatonin following HT procedure on HIE newborns (Balduini et al., 2019; Cies et al., 2017; Favié et al., 2019). Information is still lacking for many medications regarding the impact of HT on pharmacokinetics and pharmacodynamics in newborns.

4.3.5. Effect of HT on HIE-induced cytokines release

Clinical studies only suggest an effect of HT on the peripheral immune responses in human newborns with HIE; some showed contradictory findings. HT was associated with increased median levels of IL-6, IL-8, IL-10 and MCP-1 as compared to untreated neonates (Jenkins et al., 2012). Some patterns of cytokines and chemokines expression were more associated with good or adverse outcomes (Jenkins et al., 2012). In contrast, Róka and colleagues found that HT was associated with a reduction of serum IL-6 and IL-4 (Róka et al., 2013). Besides, Chalak et al. found no difference in cord/serum cytokines levels during HT and rewarming (Chalak et al., 2014).
Another study made an association between a lower serum concentration of some pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-2 and favorable outcome at 24 h of HT treatment (Orrock et al., 2015). Given the conflicting results and small group tested, more data on cytokines profiles in larger cohorts of newborns with HIE will be needed to answer this question.

Preclinical studies of HIE ± inflammation provide conflicting results concerning the role of HT in modulating pro/anti-inflammatory cytokine production. In their model of 10-day old rat pups with HIE, Yuan and colleagues observed a significant decrease of IL-1β expression after a 24 h HT period (Yuan et al., 2014). They did not show any effect of HT on other inflammatory molecules they tested, namely: TNF-α, MCP-1 and interferon-γ (Yuan et al., 2014). In a similar model, Parks and colleagues did not found any significant difference in IL-1α, IL-1β, IL-6 and TNF-α levels in the cerebrospinal fluid (CSF) of treated with HT versus untreated animals (Park et al., 2015). These results were also confirmed by our study which found that the neuroprotective effect of HT was independent of the IL-1 system (including IL-1β, TNF-α, IL-1Ra and MMP-9) but can be partly explain through its effect on antioxidant enzymes (Chevin et al., 2016). Besides, two other preclinical studies observe a pro-inflammatory effect after HT treatment. In an in vitro study on

the effect of temperature on cytokines release from microglia, Matsui and Kakeda described a down-regulation of IL-10 (i.e. an anti-inflammatory cytokine) after HT treatment (Matsui and Kakeda, 2008). More recently, Rocha-Ferreira and colleagues observed a systemic pro-inflammatory status after the rewarming of HT in their piglet model of HIE (Rocha-Ferreira et al., 2017).

Additional work is needed to explore whether HT should be supplemented with anti-inflammatory treatment(s) for maximum benefit.

4.4. Other therapeutic approaches

Since HT is now the standard treatment for NE, other potential treatments should be evaluated in combination with HT (Davidson et al. 2015). None of these therapies - some are in phase I - III clinical trials in humans - have yet translated into routine care in neonates with NE.

4.4.1. Erythropoietin

Erythropoietin (EPO) is a hormone that acts as a growth factor for red blood cells (Steensma, 2007). EPO is now routinely used as a treatment for anemia in preterm infants. In the adult or newborn brain, EPO is able to bind to its receptor (EPO receptor) on neurons and glial cells, and thus promote the expression of anti-apoptotic genes but also attenuate ROS and the inflammatory response due to HI, and increase neurogenesis (Davidson et al., 2015; Oorschot et al., 2020; Robertson et al., 2012). Although recent studies indicate that human recombinant EPO (hrEPO) has neuroprotective effects after HIE, the efficacy of EPO in combination with HT is still debated (Fan et al., 2013; Fang et al., 2013; Traudt et al., 2013). Two phase I and II clinical trials investigating the combined effect of hrEPO and HT in children with HIE are in progress

(NCT01471015 / NCT01913340). These studies examine the safety, pharmacokinetics and effectiveness of treatment combining HT and hrEPO. The Neonatal Erythropoietin And Therapeutic Hypothermia Outcomes in Newborn Brain Injury (NEATO) study (NCT01913340) showed interesting preliminary results on the beneficial effect of EPO administration with HT on brain injury and improvement of motor function at 1 year old (Frymoyer et al., 2017; Mulkey et al., 2017; Razak and Hussain, 2019). Two phase III studies are ongoing in the US and Australia (NCT02811263 / ACTRN12614000669695) to investigate further the efficacy of EPO in addition to HT for newborns suffering from HIE (Juul et al., 2018; Oorschot et al., 2020).

4.4.2. Xenon

Xenon is a noble gas that acts as a non-competitive antagonist of the NMDA (Davidson et al., 2015). The neuroprotective effects of xenon involve the activation of anti-apoptotic factors and the inhibition of the permeability of the mitochondrial membrane (Lobo et al., 2013). Studies in rodents and piglets have shown that xenon in combination with HT has additive effects (Chakkarapani et al., 2010; Hobbs et al., 2008; Ma et al., 2005). The combination of HT and xenon was evaluated in two phase II clinical trials (TOBYXeNCT00934700 and CoolXenon2-NCT01545271). TOBYXe has shown that xenon has no additive effect on neuroprotection compared to HT after neonatal asphyxia (Azzopardi et al., 2016).

4.4.3. Allopurinol

Allopurinol is a xanthine oxidase inhibitor, reducing the production of ROS, such as superoxide. Superoxide radicals damage mitochondria resulting in secondary energy failure and apoptosis affecting neurons and glial cells after HIE (see paragraph 3.1). Human, compared to rodents, has

little xanthine oxidase circulating in the blood (Robertson et al., 2012). Few clinical studies on human HIE suggested a neuroprotective effect of allopurinol (Annink et al., 2017). The beneficial allopurinol in combination with HT is currently assessed in a larger clinical trial (phase III) (Maiwald et al., 2019).

RATIONALE

NE and subsequent CP resulting from HI or inflammatory plus HI remain highly prevalent and lead to significant mortality, morbidity, and social costs. Few neuroprotective treatments are available against neonatal asphyxia: they are limited to symptomatic care and HT, leaving about 50% of patients with neurological sequelae (Azzopardi et al., 2014). Recent evidence shows that the benefit of HT remains to be determined when NE results from inflammatory-sensitized HI, i.e. HI combined to perinatal infection/inflammation.

Our work, and that of others uncovered that interleukin-1 (IL-1) is at the apex of the inflammatory cascade generating brain injury in NE (Quiniou et al., 2008; Rosenzweig et al., 2014; Savard et al., 2015, 2013). We recently showed that HT fails to counteract the IL-1 pathway (Chevin et al., 2016). The lack of effect of HT in alleviating IL-1 signaling, which drives core lesions of HI-induced infarcts, supports a neuroprotective benefit of IL-1 blockade as a targeted add-on therapy to HT. Previous evidence demonstrated that HT can alter the pharmacokinetic and pharmacodynamic parameters of drugs and induced unexpected or adverse effects (de Haan et al., 2012; van den Broek et al., 2010; Zhou and Poloyac, 2011). IL-1 blockade by IL-1 receptor antagonist (IL-1Ra) is effective and safe for the treatment of chronic inflammatory conditions, including those affecting pregnant women or newborns. Given that IL-1Ra is not currently approved for the treatment of NE and has not yet been tested in combination with HT – a mandatory standard of care –, further pre-clinical evaluation of its safety and efficacy is necessary before considering human therapeutic trials.

We **hypothesized** that HT alone or in combination with IL-1Ra will provide a neuroprotective effect in our rat model of inflammatory-sensitized HI. We further hypothesized that IL-1Ra will potentiate HT in treating forms of NE that are presently refractory to the current therapies.

Considering the potential effect of HT on pharmacokinetic and pharmacodynamic of other drugs, we wanted to assess if this could be the case with IL-1Ra. **Aim 1** will examine the safety of the combination of HT plus IL-1Ra in order to find the appropriate dose of IL-1Ra we can use in this model. **Aim 2** will investigate the efficacy of HT with or without IL-1Ra in NE resulting from inflammatory-sensitized HI. This aim will specifically investigate the added value of IL-1Ra to HT on mortality, brain injuries, as well as short and long-term motor improvements in rats.

PREFACE TO CHAPTER II

Neonatal encephalopathy and subsequent cerebral palsy resulting from hypoxia-ischemia (HI) or inflammatory-sensitized HI remain very prevalent and lead to significant mortality and morbidity. Few neuroprotective treatments are available against neonatal asphyxia: they are limited to symptomatic care and hypothermia (HT), leaving about 50% of patients with neurological sequelae. We recently showed that HT fails to counteract the interleukin-1 (IL-1) system (Chevin et al., 2016), which play a key role in neonatal encephalopathy. This supports a potential neuroprotective benefit of IL-1 receptor antagonist (IL-1Ra) as targeted add-on therapy to HT. However, few evidence already demonstrated that HT can alter the pharmacodynamic and pharmacokinetic of other drugs, inducing unexpected or adverse effect (van den Broek et al., 2010; Zhou and Poloyac, 2011). In line with these results, we aimed in manuscript 1 to further investigate the safety and efficacy of IL-1Ra in combination with HT, in our model of inflammatory-sensitized HI.

CHAPTER II: MANUSCRIPT 1

Effect of hypothermia on interleukin-1 receptor antagonist pharmacodynamics in inflammatory-sensitized hypoxic-ischemic encephalopathy of term newborns.

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J Neuroinflammation. 2018 Jul 30; 15(1):214

Abstract

Background: Hypothermia is increasingly tested in several neurological conditions, such as neonatal encephalopathy, stroke, traumatic brain injury, subarachnoid hemorrhage, spinal cord injury, and neurological outcomes of cardiac arrest. Current studies aim to increase benefits of hypothermia with new add-on therapies including immunomodulatory agents. Hypothermia has been shown to affect the metabolism of commonly used drugs, including those acting on neuroimmune pathways.

Objective: This study focuses on the effect of hypothermia on Interleukin-1 receptor antagonist pharmacodynamics in a model of neonatal encephalopathy.

Methods: The effect of hypothermia on *(i)* the tissue concentration of the Interleukin-1 receptor antagonist, *(ii)* the Interleukin-1 inflammatory cascade, and *(iii)* the neuroprotective potential of Interleukin-1 receptor antagonist, has been assessed on our rat model of neonatal encephalopathy resulting from inflammation induced by bacterial compound plus hypoxia-ischemia.

Results: Hypothermia reduced the surface of core and penumbra lesions, as well as alleviated the brain weight loss induced by LPS+HI exposure. Hypothermia compared to normothermia significantly increased (range: 50-65%) the concentration of the Interleukin-1 receptor antagonist within the central nervous system. Despite this increase of intracerebral Interleukin-1 receptor antagonist concentration, the intracerebral Interleukin-1-induced tumor necrosis factor-alpha cascade was upregulated. In hypothermic condition, the known neuroprotective effect of Interleukin-1 receptor antagonist was neutralized (50 mg/kg/12 h for 72 h) or even reversed (200 mg/kg/12 h for 72 h) as compared to normothermic condition.

Conclusion: Hypothermia interferes with the pharmacodynamic parameters of the Interleukin-1 receptor antagonist, through a bioaccumulation of the drug within the central nervous system and

a paradoxical upregulation of the Interleukin-1 pathway. These effects seem to be at the origin of the loss of efficiency or even toxicity of the Interleukin-1 receptor antagonist when combined with hypothermia. Such bioaccumulation could happen similarly with the use of other drugs combined to hypothermia in a clinical context.

Introduction

Pure hypoxia-ischemia (HI) and inflammatory-sensitized HI are the most prevalent clinical scenarios underlying neonatal encephalopathy (NE) of term newborns, one of the leading causes of neonatal death or cerebral palsy [1]. Neuroprotective treatments available against NE of term newborns consist in symptomatic cares and hypothermia (HT) [2, 3]. Ongoing researches focuses on new add-on therapies in combination to HT to increase its neuroprotective effect [2, 4]. However, recent evidence demonstrated that HT can alter the pharmacokinetic and pharmacodynamic parameters of drugs and induces unexpected and sometimes adverse effects [5–8]. Our team and others recently showed that HT fails to counteract the IL-1 system [9, 10], which plays a key role in NE [11–14]. Interleukin-1 receptor antagonist (IL-1Ra) has already demonstrated a protective perinatal efficacy on several organs, especially the brain, exposed to inflammation induced by bacterial compounds and/or HI [11, 12, 15, 16]. These results support a potential neuroprotective benefit of IL-1Ra as a targeted add-on therapy to HT. An initial step in evaluating the effect of IL-1Ra in combination with HT is to test the effect of HT on its pharmacodynamics in this physiopathological context. Our hypothesis is that HT modifies the pharmacodynamic parameters of IL-1Ra under perinatal inflammatory and/or HI conditions. Our objectives will test the effect of HT on *(i)* the tissue concentration of IL-1Ra, including the central nervous system; *(ii)* the inflammatory cascade of the IL-1 system; and *(iii)* the neuroprotective potential of IL-1Ra.

Material and methods

Rat model

Our preclinical model was designed as previously described [9, 14, 15]. Briefly, pups at postnatal day (P) 5-7 were obtained from Charles River Laboratories (Saint-Constant, QC). At P12, they received a single intraperitoneal (ip) injection of lipopolysaccharide (LPS: 50 µg/kg diluted in 50 µL of pyrogen-free saline) from *E. coli* (Sigma-Aldrich, ON). HI was induced 4 h after LPS administration by permanent ligation of the right common carotid artery followed by 8% O_2 exposure at 36°C for 1.5 h [9, 15, 17]. HT was induced 30 min after hypoxia, as previously described [9]. Briefly, pups were kept on a hot plate at 32°C in order to lower their core body temperature until 32.5°C±0.5°C (Fig. 1). HT was maintained in a reproducible manner for 4 h. LPS+HI and LPS+HI+IL-1Ra pups stayed with the dam during the time their peers underwent HT [9].

Human recombinant (hr) IL-1Ra was used at a concentration of 50 or 200 mg/kg (diluted in 50µl of pyrogen-free saline). Both doses are commonly used in the perinatal preclinical context to protect the organs against inflammation and/or HI [15, 16]. The first injection was given ip, 30 min before LPS injection. Five other injections were given every 12 h thereafter (Fig. 1). The end of hypoxia referred to as 0 h. Pups were euthanized at 4 h (which correspond to the end of HT), 24 h (P13) or 8 days (P20) post-HI. A total of 181 pups were included in the study. Pups were randomized in 5 experimental groups, namely: 35 pups in LPS+HI condition, 32 pups in LPS+HI+HT condition, 24 pups in LPS+HI+IL-1Ra (50 mg/kg) condition, 52 pups in LPS+HI+HT+IL-1Ra (50 mg/kg), 7 pups in LPS+HI+HT+IL-1Ra (200 mg/kg). Among all pups subjected to LPS+HI±HT±IL-1Ra (n=181), the mortality rate was 17% (death occurred for all

pups during hypoxia, except for 3 pups who died within 10 h following hypoxia). No significant difference was observed in the mortality rate between all experimental groups.

The experimental protocol was approved by the Institutional Animal Care Committee of the McGill University (protocol #2015-7691) in accordance with the Canadian Council on Animal Care guidelines: http://www.ccac.ca/en_/standards/guidelines.

Cerebrospinal fluid (CSF) collection

CSF was collected by cisternal puncture of anesthetized rat pups at 4 or 24 h post-HI, as described [18, 19]. The mean volume of CSF collected was 28 µl (range: 10-45 µl) with 96% of successful collection. CSF samples were kept frozen at -80°C. Immediately after CSF collection, rat pups were euthanized by decapitation, and their forebrain rapidly removed and frozen by immersion in methylbutane on dry ice.

Histology

The brains were removed and fixed (paraformaldehyde 4%, glutaraldehyde 0.1%) at room temperature, paraffin-embedded, and cut in 5 µm slices using a microtome, as described [9, 15]. Hematoxylin-eosin (H&E) staining was performed to visualize brain injuries. Coronal sections were scanned and the surface of the hemispheres were located at the epicenter of the infarct (Bregma from -2.30 mm to -2.50 mm), as previously described [9, 14, 15]. Core *versus* penumbra areas of brain infarcts were defined as previously described [9, 15]. Briefly, core injuries were associated with infarcted areas bearing cavitary lesions, whereas penumbra injuries were identified as regions surrounding the core where pycnotic neurons and/or loss of normal neuronal architecture were observed [9, 15].

ELISA

Protein extracts were prepared from right hemisphere forebrains as previously described [9, 14, 15]. ELISAs were performed on these protein extracts using ELISA Kits (R&D System, MN, USA), as previously described [9, 14, 15].

Behavioral test

The open field test was used to determine spontaneous locomotor activity and exploratory behavior of juvenile rats (P20), as described previously [20]. The following parameters were assessed in the open field apparatus using Any-Maze Video Tracking System™ (IL, USA) software: total distance traveled during the test period, mobile time, time in the center, and number of square visited.

Data analysis

Statistical analyses were performed using IBM Statistics 24 (SPSS) and GraphPad software version 6.02. The data are presented as the mean ± standard error of the mean (SEM). Normality were assessed across experimental conditions. Data were analyzed by independent samples t-test or one-way analysis of variances (ANOVA) with a Tukey's HSD test. Mann-Whitney U test was used when data were not normally distributed. Male and female data were combined, because no significant interaction was observed between sex and treatment. The statistical significance level was set at $p \leq 0.05$.

Results

Effect of HT on hrIL-1Ra titers within the tissues of interest

At 4 h post-HI, HT did not modify the titer of hrIL-1Ra, at the dose of 50 mg/kg, within the organ tested, namely: plasma, liver, CSF and right forebrain hemisphere exposed to LPS+HI (Fig. 2). At 24 h post-HI, HT induced a significant increase (50 to 65%) of the hrIL-1Ra titers within the plasma, CSF and right forebrain hemisphere exposed to LPS+HI (Fig. 2A-C).

Effect of HT+hrIL-1Ra (50 mg/kg) on the inflammatory cascade-induced by LPS+HI exposure

IL-1Ra administration interferes with the autocrine loop of IL-1β synthesis and shuts down the downstream inflammatory cascades including TNF-α production [11, 15, 21, 22]. In HT conditions at 4 and 24 h post-HI, hrIL-1Ra (50 mg/kg) failed to counteract these pathways (Fig.3), or conversely induced paradoxical upregulations of the IL-1β production at 4 h post-HI (Fig. 3B), and of the TNF-α production at 24 h post-HI in the LPS+HI-exposed right hemisphere (Fig. 4A).

Dose-dependent neurotoxic effect of hrIL-1Ra added to HT

HT alone exerted a neuroprotective effect on the extent of LPS+HI-induced core (Fig. 5A) and penumbral injuries (Fig. 5B-D). HT also protected against the loss of brain weight observed in such condition (Fig. 5E). hrIL-1Ra at the dose of 50 mg/kg did not provide any neuroprotective added value when combined to HT (Fig. 5A-D). hrIL-1Ra at the dose of 200 mg/kg increased LPS+HI-induced penumbral - but not core – injuries (Fig. 5B). Open field experiments in juvenile rats (P20) did not show any difference between LPS+HI+HT *versus* LPS+HI+HT+hrIL-1Ra (50 mg/kg) conditions (Fig. 6A-D).

Discussion

Our results showed that HT altered the pharmacodynamic parameters of hrIL-1Ra in our model of NE-induced by inflammation plus HI. HT increased the concentration of hrIL-1Ra (at 24 h post-HI) within the LPS+HI-exposed plasma, CSF, and forebrain. Paradoxically, this effect was not associated with an IL-1Ra-induced anti-inflammatory effect on the IL-1 system. We also observed a lack of effectiveness of the combination of hrIL-1Ra with HT, as compared to sole hrIL-1Ra in the same model of LPS+HI-induced NE [14, 15].

According to the pharmacokinetic study performed in a rat model of arthritis [23], and also taking into account the short half-life (4-6 h) of IL-1Ra, it is unlikely that an accumulation of IL-1Ra would be due in our experimental design to the repeated administration of IL-1Ra every 12 h. We hypothesize that the blood brain barrier (BBB) dysfunction induced by LPS+HI exposures might increase over time, with a more important BBB leak at 24 h (allowing the IL-1Ra to diffuse within the brain) as compared to 4 h post-HI. Few studies dealt with the impact of HT on the pharmacokinetic and pharmacodynamic of drugs used in the human neonatal context. However, it was shown that several drugs - *e.g.* isoflurane, morphine, ligands of β1 and β2 adrenoreceptors - had reduced metabolism and clearance on HT as compared to non-HT condition [5, 6]. Affinity between ligands and their cognate receptors as well as alterations of downstream signaling are also reported on HT [5, 6, 8]. Our results suggest that the bioaccumulation of hrIL-1Ra within the brain and CSF in LPS+HI+HT condition might result from a decreased clearance of hrIL-1Ra and/or from a decreased affinity of hrIL-1Ra for the IL-1R, and also possibly from the blockade of the IL-1R signaling pathway. hrIL-1Ra is rapidly eliminated (half-life of 4-6 h) mainly by the kidney through glomerular filtration (GFR) [24]. It is known in human studies that the GFR is decreased

under hypothermic condition [5, 6]. Besides, acute kidney injury can be associated to HI encephalopathy in the term neonate [25, 26]. Hence, HI could potentially affect the renal filtration, especially in the HT condition, and decrease the clearance of IL-1Ra.

The increased hrIL-1Ra bioaccumulation in HT condition might explain the switch from protective [14, 15] to toxic effects of our highest dose of hrIL-1Ra (200 mg/kg/12 h for 72 h). hrIL-1Ra (200 mg/kg/12 h for 72 h) might reach in HT condition a toxic concentration within the brain inducing non-specific ligand-receptors interactions deleterious for neural cells.

This study has some limitations. The concentration of hrIL-1Ra was assessed only at 4 and 24 h post-HI. In future experiments blood samples could be taken at additional time-points to study in more detail the pharmacology of this drug. However, to our knowledge this is the first study focusing on the pharmacology of IL-1Ra in neonatal rats.

Conclusion

Our study addresses for the first time the impact of HT on hrIL-1Ra pharmacodynamics. HT might decrease the clearance of hIL-1Ra, inducing its bioaccumulation and loss of efficiency within the brain [11, 14, 15, 22, 27]. According to this hypothesis, current and future studies aiming to develop HT therapies - as already performed in neurological conditions, such as neonatal encephalopathy, stroke, traumatic brain injury, subarachnoid hemorrhage, spinal cord injury, and neurological outcomes of cardiac arrest [28–30] - should take into account the pharmacokinetic and pharmacodynamic impact of HT and the inherent modification of the safety profile of drugs.

List of abbreviation

CSF: cerebrospinal fluid

GFR: glomerular filtration

HI: hypoxia-ischemia

HT: hypothermia

hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist

IL: interleukin

ip: intraperitoneally

LPS: lipopolysaccharide from *E.coli*

NE: neonatal encephalopathy

P: postnatal day

SEM: standard error of the mean

TNF-α: tumor necrosis factor α

Ethic approval

Our research protocol was approved by the Ethic Committee from the Research Institute of the McGill University Health Center (#2015-7691).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the Canadian Institute of Health Research, Foundation of Stars, Montreal Children Hospital Foundation (Hoppenheim Fund), and Heart and Stroke Foundation of Canada. This project has been made possible by Brain Canada Foundation through the Canada Brain Research Fund, with the financial support of Health Canada and the funding partners. The views expressed herein do not necessarily represent the views of the Minister of Health or the Government of Canada.

Author's contribution

MC and CG carried out the experiments. MC performed the statistical analyses and drafted the manuscript. CG edited the manuscript. GS conceived the study, coordinated the project, and further edited the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Marie-Julie Allard for statistical advice and Dr Pia Wintermark for helpful discussion. We would like to than Noha Gerges for editing the manuscript.

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Figures

Figure 1

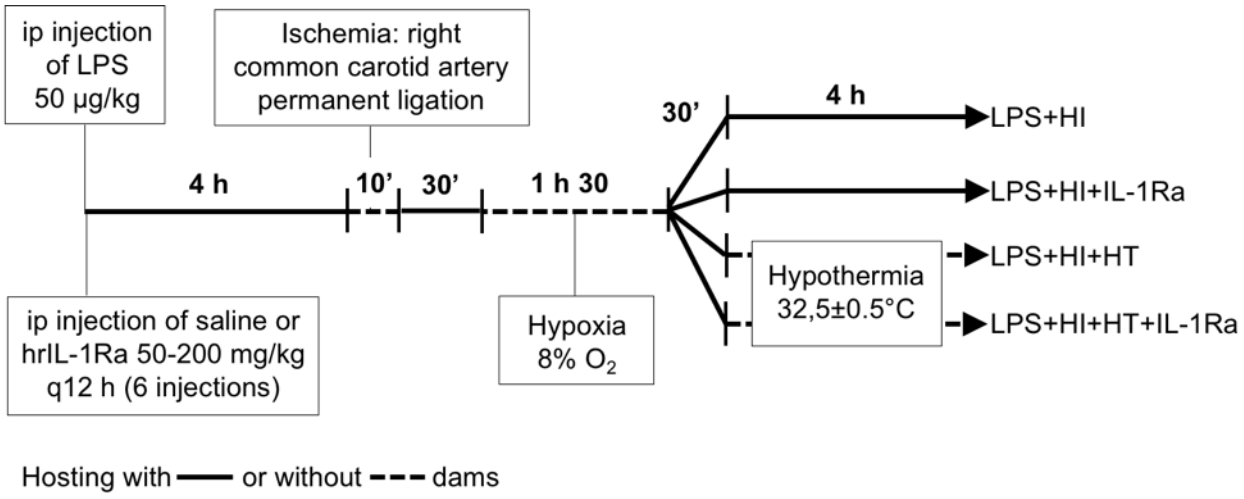

Figure 1. Experimental design.
The first hrIL-1Ra (50-200 mg/kg) or saline injection was administrated 30 min before the ip injection of LPS from *E.coli* (50µg/kg) in pups at P12. Four hours later, the right common carotid artery was ligated, and hypoxia was induced (8% O_2 for 1.5 h). Rat pups were subjected or not to hrIL-1Ra (50-200 mg/kg q12 h from P12 to P14) and treated or not by HT (32.5°C±0.5°C for 4 h). *Abbreviations:* HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist; ip: intraperitoneally; LPS: lipopolysaccharide from *E.coli*; P: postnatal day.

Figure 2

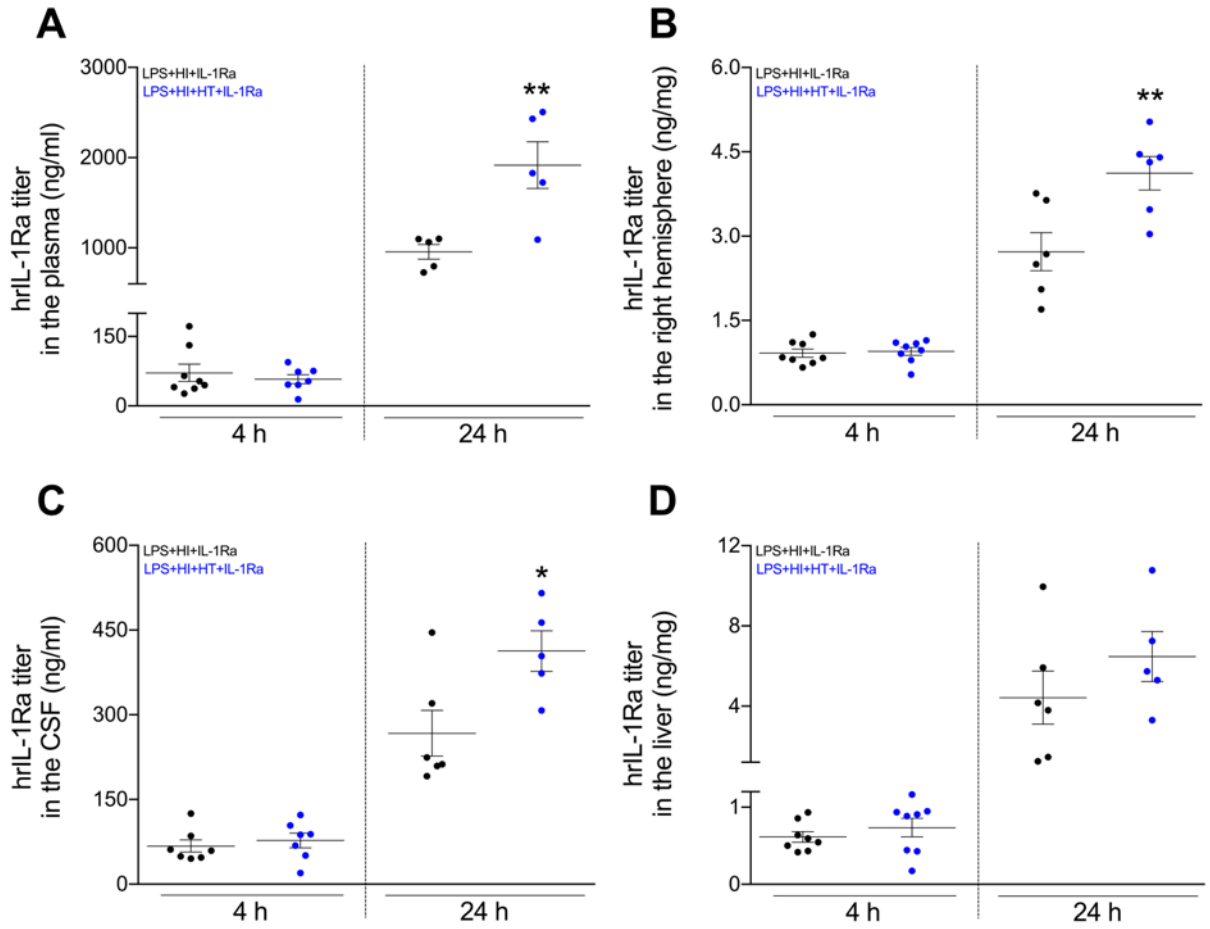

Figure 2. hrIL-1Ra titers within tissues of interest from pups exposed to LPS+HI+IL-1Ra±HT. hrIL-1Ra titers measured by ELISA at 24 h post-HI were increased within the plasma (**A**), right cerebral hemisphere (**B**), and CSF (**C**) in LPS+HI+HT+IL-1Ra (50 mg/kg) as compared to LPS+HI+IL-1Ra (50 mg/kg) condition. The concentrations of hrIL-1Ra were similar in both conditions at 4 h post-HI, as well as at 24 h post-HI within the liver (**D**). The number (n) of rats used was: LPS+HI+IL-1Ra (n=5-8 from 4 litters), LPS+HI+HT+IL-1Ra (n=5-8 from 4 litters). The bars indicate the mean ± SEM. *p≤0.05, **p≤0.01; Independent T-test. *Abbreviations:* CSF: cerebrospinal fluid; HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist; LPS: lipopolysaccharide from *E.coli*.

Figure 3

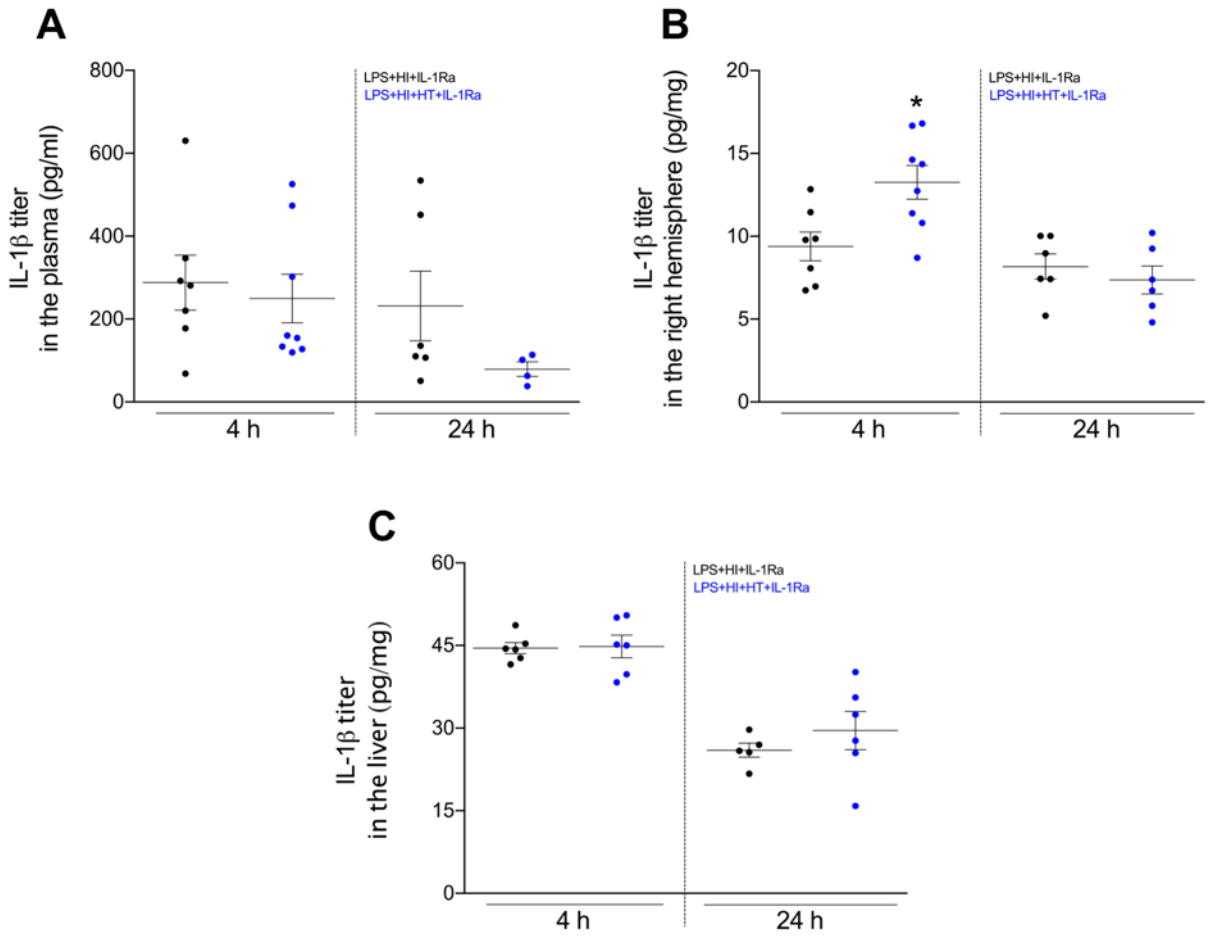

Figure 3. IL-1β expression within tissues of interest from pups exposed to LPS+HI+IL-1Ra±HT. IL-1β concentration measured by ELISA at 4 h and 24 h post-HI within the plasma (**A**), right cerebral hemisphere (**B**), and liver (**C**) in LPS+HI+IL-1Ra (50 mg/kg) and LPS+HI+HT+IL-1Ra (50 mg/kg) conditions. HT increased the expression of IL-1β within the right hemisphere at 4 h post-HI (**B**). The number (n) of rats used was: LPS+HI+IL-1Ra (n=5-7 from 4 litters), LPS+HI+HT+IL-1Ra (n=4-8 from 4 litters). The bars indicate the mean ± SEM. *p≤0.05; Independent T-test. *Abbreviations:* HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human

recombinant of interleukin-1 receptor antagonist; IL-1β: interleukin-1β; LPS: lipopolysaccharide from *E.coli*.

Figure 4

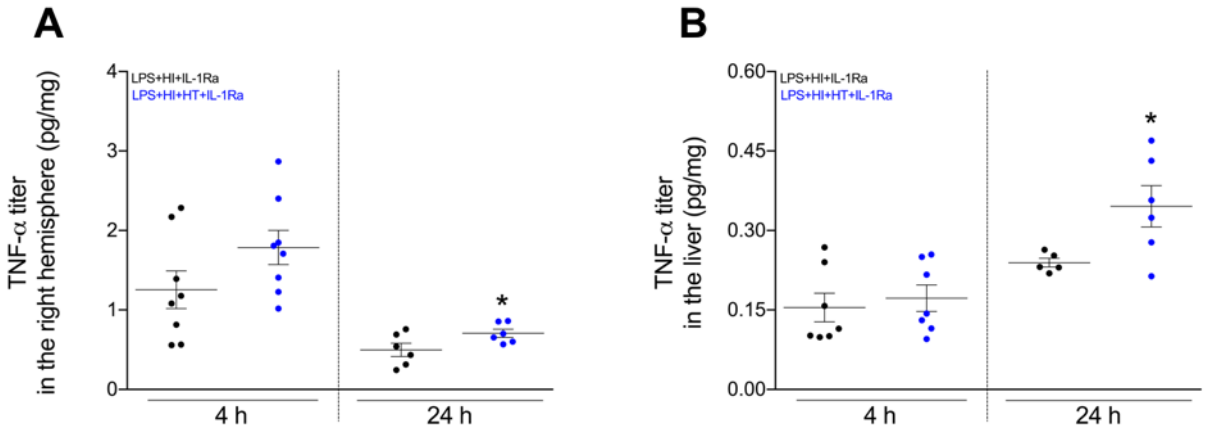

Figure 4. TNF-α titers within tissues of interest from pups exposed to LPS+HI+IL-1Ra±HT. TNF-α concentrations measured by ELISA were increased at 24 h post-HI within the right cerebral hemisphere (**A**) and the liver (**B**) in LPS+HI+HT+IL-1Ra (50 mg/kg) as compared to LPS+HI+IL-1Ra (50 mg/kg) conditions. The TNF-α titers were similar in both conditions at 4 h post-HI. The number (n) of rats used was: LPS+HI+IL-1Ra (n=5-8 from 4 litters), LPS+HI+HT+IL-1Ra (n=6-8 from 4 litters). The bars indicate the mean ± SEM. *p≤0.05; Independent T-test. *Abbreviations:* HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist; LPS: lipopolysaccharide from *E.coli*; TNF-α: tumor necrosis-α.

Figure 5

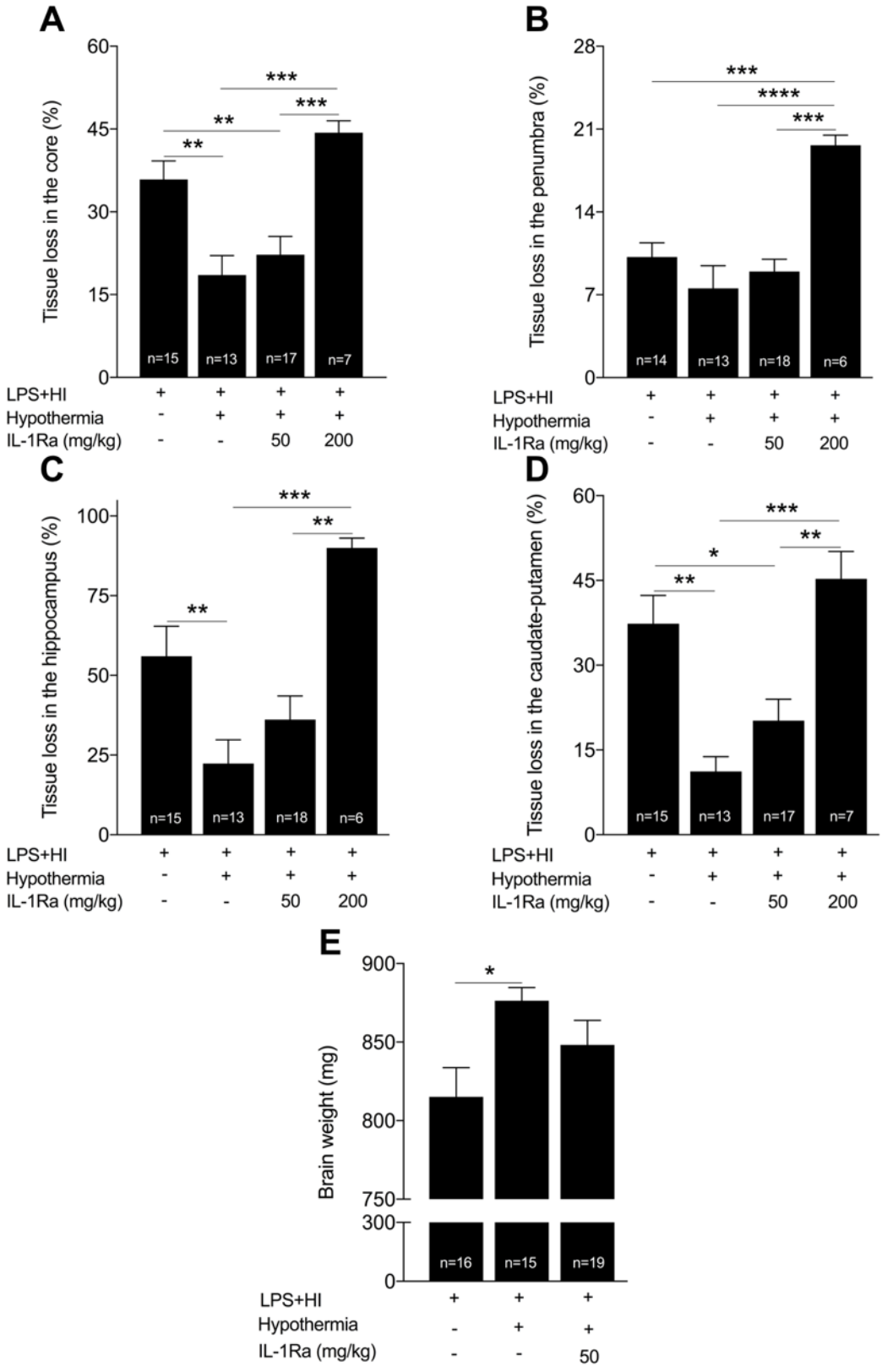
A
Tissue loss in the core (%)
60
45
30
15
0
**
**
***
***
n=15
n=13
n=17
n=7
LPS+HI + + + +
Hypothermia - + + +
IL-1Ra (mg/kg) - - 50 200
B
Tissue loss in the penumbra (%)
28
21
14
7
0
***
****
***
n=14
n=13
n=18
n=6
LPS+HI + + + +
Hypothermia - + + +
IL-1Ra (mg/kg) - - 50 200
C
Tissue loss in the hippocampus (%)
100
75
50
25
0
**
***
**
n=15
n=13
n=18
n=6
LPS+HI + + + +
Hypothermia - + + +
IL-1Ra (mg/kg) - - 50 200
D
Tissue loss in the caudate-putamen (%)
60
45
30
15
0
**
*
***
**
n=15
n=13
n=17
n=7
LPS+HI + + + +
Hypothermia - + + +
IL-1Ra (mg/kg) - - 50 200
E
Brain weight (mg)
900
850
800
750
300
0
*
n=16
n=15
n=19
LPS+HI + + +
Hypothermia - + +
IL-1Ra (mg/kg) - - 50

Figure 5. Comparison of the extent of brain injuries between LPS+HI±HT±IL-1Ra conditions. Comparisons of the extent of core and penumbra injuries (within the neocortex, hippocampus, and caudate-putamen) between pups exposed to LPS+HI±HT±IL-1Ra (50-200 mg/kg) by H&E staining of the right forebrains at P20. HT reduced the surface of core and penumbra lesions (**A-D**), as well as alleviated the brain weight loss observed after LPS+HI exposure (**E**). The surface of core and penumbral lesions were similar in LPS+HI+HT+IL-1Ra (50 mg/kg) as compared to LPS+HI+HT condition (**A-D**). HT+hrIL-1Ra (200 mg/kg) increased the extent of penumbra injury as compared to the LPS+HI condition (**B**), as well as core and penumbral injuries as compared to LPS+HI+HT and LPS+HI+HT+IL-1Ra (50 mg/kg) (**A-D**). The number (n) of rats used was: LPS+HI (n=14-16 from 9 litters), LPS+HI+HT (n=13-15 from 9 litters), LPS+HI+HT+IL-1Ra 50 mg/kg (n=17-19 from 9 litters), LPS+HI+HT+IL-1Ra 200 mg/kg (n=6-7 from 3 litters). The bars indicate the mean ± SEM. *p≤0.05, **p≤0,01, ***p≤0,001, ****p≤0,0001; One-way ANOVA. *Abbreviations:* HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist; LPS: lipopolysaccharide from *E.coli*.

Figure 6

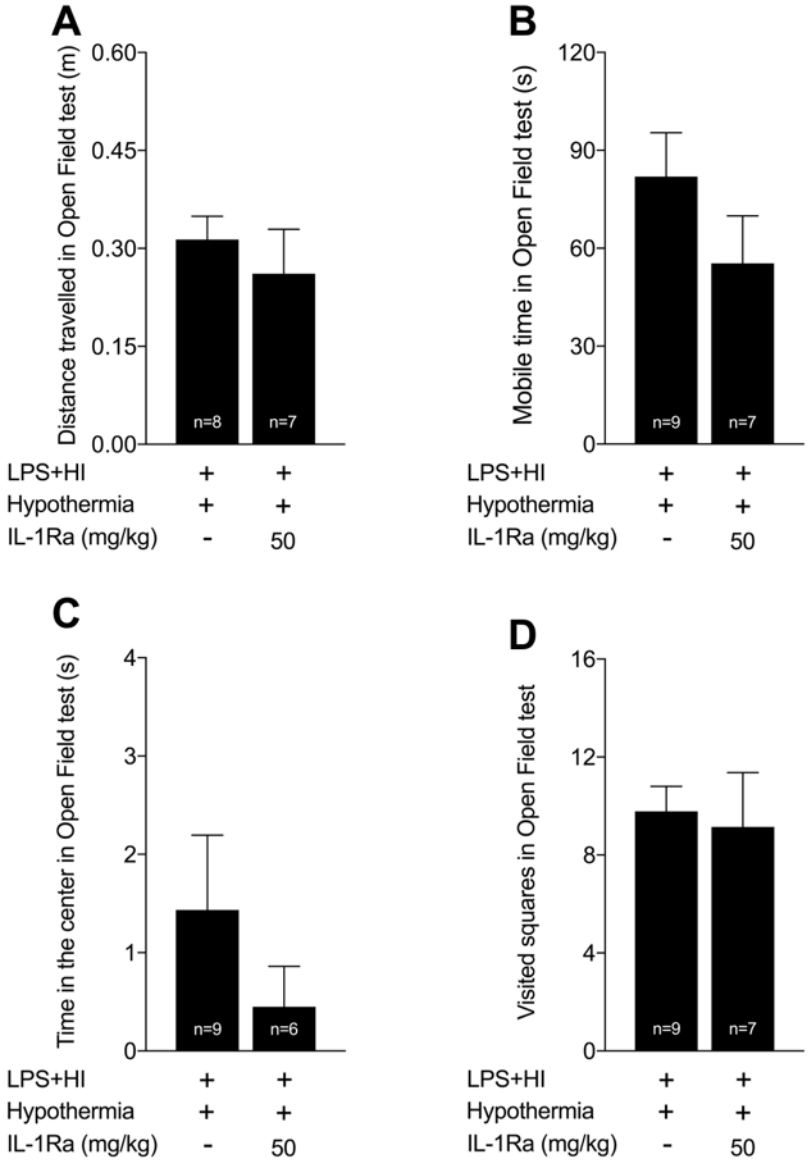

Figure 6. Open Field experiment at P20 in pups exposed to LPS+HI+HT±IL-1Ra (50 mg/kg). No difference was observed between the two conditions for the different Open Field parameters tested: the distance travelled (**A**), the mobile time (**B**), the time in the center (**C**), and the visited squares in the apparatus (**D**). The number (n) of rats used was: LPS+HI+HT (n=8-9 from 6 litters),

LPS+HI+HT+IL-1Ra 50 mg/kg (n=6-7 from 5 litters). Independent T-test. *Abbreviations:* HI: hypoxia-ischemia; HT: hypothermia; hrIL-1Ra: human recombinant of interleukin-1 receptor antagonist; LPS: lipopolysaccharide from *E.coli*.

PREFACE TO CHAPTER III

Neuroprotective treatment available against neonatal encephalopathy of term newborns consist in hypothermia (HT), leaving 50% of treated newborns with long-term sequelae (Azzopardi et al., 2014; Davidson et al., 2015). Ongoing researches focuses on new add-on therapies in combination to HT to increase its neuroprotective effect (Davidson et al., 2015; Hassell et al., 2015). Among them, interleukin-1 receptor antagonist (IL-1Ra) has already demonstrated a protective perinatal efficacy on several organs, especially the brain, exposed to inflammation and/or hypoxia-ischemia (Berry et al., 2011; Leitner et al., 2014; Rosenzweig et al., 2014; Savard et al., 2015, 2013). In their model of inflammatory-sensitized hypoxic ischemic encephalopathy, Savard and colleagues already demonstrated a neuroprotective effect of sole IL-1Ra (Savard et al., 2015, 2013). IL-Ra has been shown, in particular, to reduce the mortality and improve the motor behaviour of rats (Savard et al., 2015). The following research letter aimed to add a piece of information concerning the efficacy of IL-1Ra combined with HT on these specific parameters.

CHAPTER III: MANUSCRIPT 2

Added value of interleukin-1 blockade to hypothermia in the treatment of neonatal encephalopathy.

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Am j Obstet Gynecol. 2020 Sep;223(3):458-460.

Objective

Hypoxic-ischemic encephalopathy (HIE) occurs in 2-9 per 1000 term newborns. To date, the only evidence-based treatment is therapeutic hypothermia (HT), which leaves however 50% of patients with long-term disabilities (1). Beside hypoxia-ischemia (HI), perinatal inflammation is an important determinant of HIE injuries (2,3). However, there is few evidence in favor of an anti-inflammatory role of HT in HIE (4). This suggests that anti-inflammatory drugs, such as interleukin-1 receptor antagonist (IL-1Ra) – whose neuroprotective effect is well established preclinically in neonatal encephalopathy due to combined inflammatory and HI aggressions (3,5) – might have an added value on sole HT (4). Using this inflammatory plus HI model of neonatal encephalopathy, we studied the efficacy of IL-1Ra plus HT *vs* sole HT on mortality and motor outcome.

Study Design

Our established rat model of HI plus inflammation was used, as described (3,4). Briefly, postnatal day 12 (P12) pups – corresponding to term human newborns – received a single intraperitoneal (ip) dose of 50 µg/kg of lipopolysaccharide (LPS) of *E.coli*. HI was induced 4 h after LPS administration by permanent ligation of the right common carotid artery followed by 8% O_2 exposure at 36 °C for 1.5 h.

HT (32.5 °C ± 0.5 °C) was induced 30 min after hypoxia during 4 h. Human recombinant IL-1Ra (50 mg/kg/12 h for 72 h) was started 30 min before LPS injection. Rat pups were randomly allocated to LPS+HI+HT (n=63) *vs* IL-1Ra+LPS+HI+HT (n=87) groups. The mortality rate was calculated in each group. To avoid the litter effect, motor outcome was tested in only 1 to 2 survivors per litter (n=10) of both sexes at P14. Travelled distance in 1 min was measured in the

Open Field apparatus, and duration of effective upswing (Figure, C) was assessed by Elevated Body Swing Test (3). χ^2 and unpaired t-test (IBM Statistics 25 (SPSS)) were used to analyze data. The statistical significance level was set at $p<0.05$.

The experimental protocol was approved by the Institutional Animal Care Committee of the McGill University (protocol #2015-7691) in accordance with the Canadian Council on Animal Care guidelines.

Results

During hypoxia, 12 out of 63 pups died in the LPS+HI+HT *vs* 7 out of 87 in the IL-1Ra+LPS+HI+HT groups (19% *vs* 8%, p=0.045; Figure, A). No mortality occurred thereafter. In the Open Field apparatus, there was an increased distance travelled in the IL-1Ra+LPS+HI+HT *vs* LPS+HI+HT conditions (1.4 m *vs* 0.8 m, p=0.043; Figure, B). The amount of time spent in the effective upswing position in the elevated body swing test was increased in the IL-1Ra+HT-treated compared to the HT-treated groups (17.1 s *vs* 13.1 s, p=0.016; Figure, C).

Conclusions

This is the first study showing a benefit of IL-1Ra as add-on therapy to HT in neonatal encephalopathy. This benefit was demonstrated on both mortality and motor outcome. Evaluation of other cytokines such as IL-6 and TNF-α that might still participate to injuries would be important to study. This finding paves the way for novel human therapeutic trials taking advantage of this preclinical cumulative effect of IL-1Ra plus HT in neonatal encephalopathy to further alleviate its heavy burden of mortality and morbidities.

Declaration

The authors report no conflict of interest.

Research reported in this publication was supported by grants from the Canadian Institute of Health Research, Foundation of Stars, Montreal Children Hospital Foundation (Hoppenheim Fund), Heart and Stroke Foundation of Canada, Université Jean Monnet de Saint-Étienne and Région Auvergne-Rhône-Alpes, France. This project has been made possible by Brain Canada Foundation through the Canada Brain Research Fund, with the financial support of Health Canada and the funding partners (M Chevin). The views expressed herein do not necessarily represent the views of the Minister of Health or the Government of Canada.

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Figure

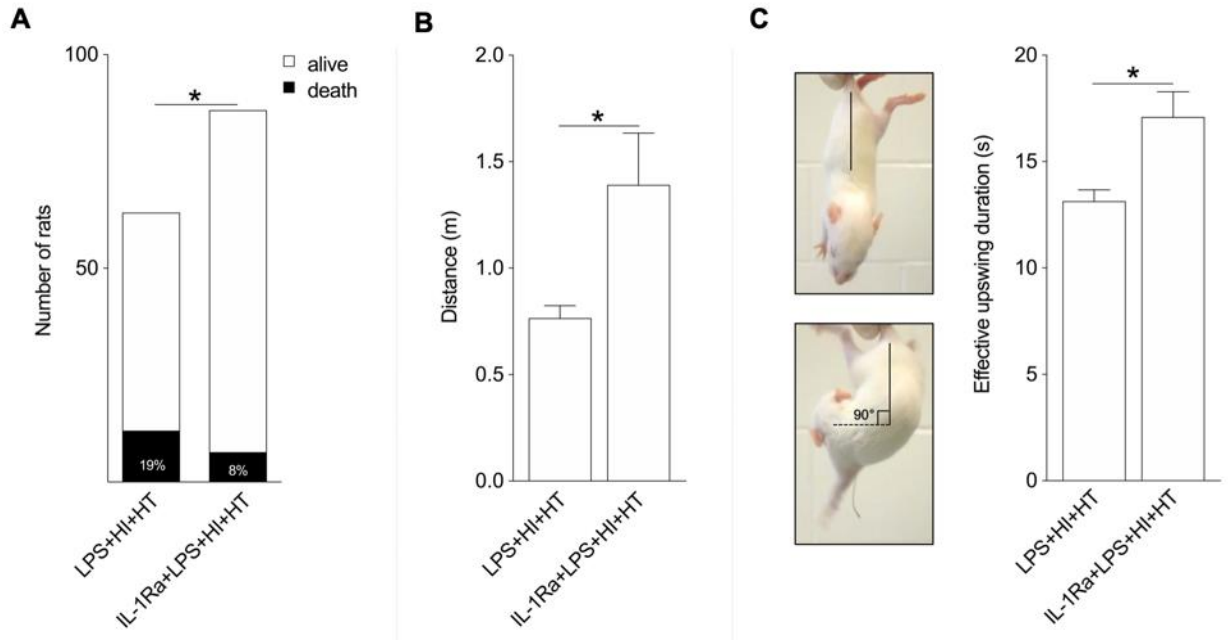

Figure. IL-1Ra+HT compared to sole HT improved mortality and motor outcome.

(**A**) Mortality rates in LPS+HI+HT- *vs* IL-1Ra+LPS+HI+HT- exposed pups at P12. Number (N) of rats: n=63 in the LPS+HI+HT group, n=87 in the IL-1Ra+HI+HT group. *p<0.05, using a χ^2 test. (**B**) Distance travelled (m) during 1 min in the Open Field at P14. N of rats: n=9 in the LPS+HI+HT group, n=7 in the IL-1Ra+LPS+HI+HT group. Data are expressed as mean±SEM. *p<0.05, using a two-tailed unpaired t-test. (**C**) Duration (s) of effective upswing by survivors during 30 s in the EBST. The threshold was set at an angle of 90° for an efficient upswing. N of rats: n=7 in each group. Data are expressed as mean±SEM. *p<0.05, using a two-tailed unpaired t-test. *Abbreviations: HI: hypoxia-ischemia; HT: therapeutic hypothermia; IL-1Ra: interleukin-1 receptor antagonist; LPS: lipopolysaccharide of E.coli.*

PREFACE TO CHAPTER IV

Over the past decades, the Rice-Vannucci model has been extensively used to recreate neonatal hypoxic-ischemic injury, which has been characterized through histological analysis and behavioral test. However, few studies used magnetic resonance imaging and positron emission topography to further characterize the extent of brain infarct and metabolism activity within the injured hemisphere. Furthermore, the neuroprotective effect of HT when neonatal encephalopathy (NE) results from infection/inflammation plus HI remains unclear (Osredkar et al., 2014; Wintermark et al., 2010).

On the other hand, our adapted Rice-Vannucci model combining inflammatory component exposure plus ischemia and global hypoxia seems also relevant to model neonatal arterial ischemic stroke (NAIS) (Gennaro et al., 2019). Perinatal infection/inflammation, peripartum asphyxia, and arterial occlusion of the carotid tree are the main determinants we used in our model to recreate brain injury (Giraud et al., 2017).

The following manuscript aimed to further assessed the neuroprotective effect of HT in inflammatory-sensitized brain injury, such as NE or NAIS.

CHAPTER IV: MANUSCRIPT 3

Benefits of hypothermia in neonatal arterial ischemic strokes: a preclinical study.

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Int J Dev Neurosci. 2020 Jun;80(4) :257-266.

Abstract

Background: There is currently no targeted treatment available for neonatal arterial ischemic strokes (NAIS). Epidemiological studies demonstrated that perinatal infection/inflammation, *peripartum* hypoxia, and occlusion of the internal carotid tree are the main determinants of NAIS. The well-established benefit of therapeutic hypothermia (HT) in neonatal encephalopathy due to diffuse hypoxia-ischemia provides a rationale for the potential use of HT as a neuroprotective strategy in NAIS.

Methods: We used a rat model to reproduce the most prevalent human physiopathological scenario of NAIS. The neuroprotective effect of HT was measured by morphometric magnetic resonance imaging, [^{18}F] fluorodeoxyglucose (FDG) metabolic activity by positron emission tomography/computed tomography, and behavioral tests.

Results: HT (*i*) prevented the occurrence of 44% of NAIS cases, (*ii*) reduced the volume of strokes by 37%, (*iii*) enhanced [^{18}F] FDG metabolic activity within the territory of the occluded carotid artery, and (*iv*) improved motor behavior. Both morphometric and metabolic techniques showed consistently that HT provided a neuroprotective effect located in the motor cortex, hippocampus and caudate-putamen.

Conclusion: Through combining anatomical, metabolic imaging and behavioral studies, our study provides evidence of neuroprotective effects of HT in NAIS. These results are potentially translational to human NAIS.

Introduction

NAIS affects up to 1-2/6,000 newborns and results in lifelong sequelae, such as cerebral palsy and epilepsy, as well as cognitive and behavioral impairments (Dunbar and Kirton, 2018; Fluss et al., 2019; Giraud et al., 2017). Clinical studies show that perinatal infection/inflammation – often due to chorioamnionitis – is the main independent risk factor of NAIS (Giraud et al., 2017; Martinez-Biarge et al., 2013; Sorg et al., 2019). To date, no controlled clinical trials have addressed the safety and efficacy of HT in NAIS (Austin et al., 2013; Harbert et al., 2011). The few preclinical studies assessing the effect of HT in inflammatory-sensitized hypoxic-ischemic (HI) brain injuries provided inconsistent results (Chevin et al., 2018, 2016, Osredkar et al., 2015, 2014). Hence, the potential benefit of HT in NAIS remains to be determined.

Using a preclinical model, our aim was to test the efficacy of HT in treating NAIS and reducing the likelihood of its unfavorable outcomes (Chevin et al., 2018, 2016; Savard et al., 2015). We hypothesized that HT would lessen the effects of NAIS on: (*i*) the volume of stroke measured by magnetic resonance imaging (MRI), (*ii*) the [^{18}F] FDG metabolic activity measured by positron emission tomography/computed tomography (PET/CT), and (*iii*) the motor behavior at a juvenile age.

Materials and Methods

Rat model

The experimental protocol was approved by the Institutional Animal Care Committee of McGill University (protocol #2015-7691) in accordance with the Canadian Council on Animal Care Guidelines: http://www.ccac.ca/en_/standards/guidelines. As attempts to model neonatal selective occlusion of the middle cerebral artery were difficult (Gennaro et al., 2019), we adapted the Rice-

Vanucci rat model (Chevin et al., 2018, 2016, Savard et al., 2015, 2013) (perinatal inflammation, unilateral common carotid occlusion, and global hypoxia) to reproduce the most prevalent human physiopathological scenario of NAIS. A total of 35 Lewis pups at postnatal day (P) 6 – from five different litters – were obtained from Charles River Laboratories (Kingston, NY). (Chevin et al., 2018) At P12 (corresponding to the level of rat brain development equivalent to that of a term human newborn (Patel et al., 2014; Towfighi et al., 1997)), pups received a single intraperitoneal (ip) injection of lipopolysaccharide (LPS: 50 μg/kg diluted in 50 μL of pyrogen-free saline) from *E. coli* (Sigma-Aldrich, ON). HI was induced 4 hours (h) after LPS administration by permanent ligation of the right common carotid artery (RCCA) followed by 8 % O_2 exposure at 36 °C for 1.5 h (Fig. 1A) (Brochu et al., 2011; Chevin et al., 2016; Savard et al., 2015). A control (CTL) group underwent ip saline injection followed by a sham surgery consisting of common carotid artery exposure without ligation, and without hypoxia and HT (Fig. 1A). HT was induced 30 min after hypoxia, as previously described (Chevin et al., 2018, 2016). Pups were kept on a hot plate at 32 °C for four hours in order to lower their core body temperature to 32.5±0.5 °C (Fig. 1B) (Buckley et al., 2015; Chevin et al., 2018, 2016). Among all pups subjected to LPS+HI (n=25), the mortality rate was 20 %. Death occurred for all pups during hypoxia, except for one pup who died within 24 h following hypoxia. The remaining pups (n=20) were randomly assigned in either the LPS+HI or LPS+HI+HT group. No mortality occurred in CTL animals (n=10). Three rats died during imaging acquisitions. Therefore, the following rats were excluded from the study: one LPS+HI during the MRI, one LPS+HI+HT during the MRI, and one LPS+HI+HT during the PET-CT. All experimenters were blind to experimental conditions during outcome assessments.

MRI and PET/CT of the rat brain

MRI and PET/CT scans were performed at the Research Institute of the McGill University Health Centre Small Animal Imaging Labs (http://rimuhc.ca/small-animal-imaging-labs). MRI and PET-CT parameters were adapted for rat pups using previously described methods from our group (Assadian et al., 2008; Grand'maison et al., 2013; Thompson et al., 2014). MRI scans were performed at P26-28 using the 7T Bruker BioSpec 70/30USR system (Bruker Biospin, Ettlingen, Germany) and a rodent brain surface coil. All the pups were placed and positioned in the same way with the same landmarks in order to obtain a reproducible brain scan between each animal. MRI studies were performed under 1.8-2 % isoflurane with the respiration rate controlled at 55-60 breaths/min. Rat's body temperature was maintained at 37±0.3 °C using the Small Animal Instruments Inc. system (Stony Brook, NY). Anatomical images were acquired using a 3D balanced Steady-State free precession (bSSFP) sequence with repetition time=5.2 mm, echo time=2.6 mm, flip angle=30*, matrix size=192x192x192 mm, NEX=4, field of view=44.41x44.41x22.20 mm, spatial resolution=231x231x116 µm, and acquisition time 28 min. PET/CT experiments were performed one day after the MRIs on a nanoScan PET/CT for small animals (Mediso Medical Imaging Systems, Budapest, Hungary). The radiotracer FDG was performed by a bolus injection in the tail vein (9.16±0.94 MBq in 200 µl), and all animals were kept awake for 45 min before the scan. Afterwards, the animal was placed in the scanner under anesthesia (isoflurane 2 %), with the brain at the center of the field-of-view. Each PET/CT session consisted of a 30 min PET emission scan, followed by a 10 min CT transmission scan. Temperature and heart rate were monitored throughout the procedure using the Mediso system. Images were reconstructed using expectation maximization, ordered subset expectation maximization, then normalized and corrected for scatter, dead time, and decay.

Analysis of MRI and PET/CT imaging data

Image analysis was performed using the SPM8 software package (https://www.fil.ion.ucl.ac.uk/spm), as well as an image analysis toolbox developed in house based on Mathworks' MATLAB (https://www.mathworks.com). Medical imaging software AMIDE (Molecular Imaging Vol. 2, No. 3, July 2003, pp. 131–137) and MRIcron (http://www.mccauslandcenter.sc.edu/mricro/) were also used for the purpose of generating illustrations. In order to conduct image analysis, all DICOM images from both MRI and PET/CT scanners were converted into NIFTY format.

Measure of the volume of the stroke

The volume of the stroke was calculated using MRI anatomic images at P26, i.e. 14 days after exposure to LPS+HI. The boundaries of the lesion area were readily identified and could be easily segmented on the MRI volumes using our image processing toolbox.

Group lesion maps

Using SPM8, each 3D MRI data were realigned (sampling distance: 0.1 mm, interpolation 7th degree B-spline) on a reference MRI scan (from a CTL MRI scan, which was chosen as the reference due to being considered as the most representative scan). Subsequently, the boundaries of the lesion were manually delineated on a slice-by-slice basis on the realigned individual anatomic images to create a binary lesion mask using the MRIcron software (Dinomais et al., 2015). Group lesion maps were then created by summing all individual realigned lesion maps (Dinomais et al., 2015) to identify damaged regions common to multiple rats. This process was done separately for the LPS+HI and LPS+HI+HT (Fig. 2B, Supporting Fig. I and II) groups. Group

lesion maps in LPS+HI and LPS+HI+HT rats were overlaid on our reference 3D MRI brain scan. All the 3D MRI data and lesion maps obtained from these steps were verified and checked by two of the authors (MC, MD).

Region-of-Interest (ROI) analysis of PET images

ROIs were adapted from a rat brain atlas on SPM8 that was modified to provide standard template images in MRI (T1 and T2), PET, as well as CT image format (Valdés-Hernández et al., 2011). In order to conduct ROI analysis, we performed a nonlinear transformation of the CT images to the atlas using SPM8. The transformations from the CT scans were applied to the correspondingly co-registered PET scans to map the PET images to the atlas. In order to normalize for difference of [^{18}F] FDG uptake between animals, standardized uptake value response (SUV_r) was measured based on the average activity obtained in the ROIs and normalized on the reference region of the cerebellum (Assadian et al., 2008).

Behavioral tests

An open field test was used to measure spontaneous locomotor activity and exploratory behavior of the juvenile rats (P20-35), as previously described (Chevin et al., 2018; Girard et al., 2009). The following parameters were assessed in the open field apparatus using Any-Maze Video Tracking System™ (IL, USA) software: total distance traveled during the test period, mobile time, time in the center, and the number of squares visited. The turning-in-alley test – classically used in rodent models of stroke (Chu et al., 2004; Zou et al., 2006) – was performed at P40 to measure a shift of lateralization due to motor impairment (Savard et al., 2015, 2013). Rats were placed facing the end of a closed alley. The amount of time required for the animal to turn around and face the open end

of the alley, as well as the specific turning direction were recorded repeatedly (10 times per rat) (Chu et al., 2004; Savard et al., 2015, 2013).

Data analysis

Statistical analyses were performed using IBM Statistics 25 (SPSS) and GraphPad software version 8.02. Normality of data were assessed across experimental conditions by Shapiro-Wilk test. Male and female data were combined, as no significant interaction was detected between sex and treatment. There was no difference in sex distribution between the experimental groups. Data were analyzed by unpaired t-test (MRI volumes) or one-way analysis of variances (ANOVA). When significant, pairwise comparisons were performed using a Tukey's HSD test. For the PET-scan analysis, group average differences were calculated using SPSS, and a False Discovery Rate of q=0.05 was applied in order to correct for multiple comparisons. A Spearman correlation was used to assess the linear correlation between the latency to turn in the turning in alley test and the volume of brain infarct in MRI, as normality assumptions were not met. Chi-square tests for independence were used to assess the difference of proportions between samples. In the case of multiple t-tests, we used the Bonferroni t correction to avoid type I error. Data were presented as the mean±standard deviation (SD) or mean±95% confidence intervals for Chi-square tests, with the statistical significance level being set at $p<0.05$.

Results

Effect of HT on LPS+HI-induced stroke.

A 2.7-fold decrease of the volume of the stroke was observed in the LPS+HI+HT compared to LPS+HI groups: respectively 86.3±17.5 mm^3 *versus* 32.2±12.5 mm^3, p=0.02 (Fig. 2A). Nine out

of nine brains presented a stroke in the MRI in the LPS+HI group *versus* five out of nine brains in the LPS+HI+HT group (χ^2: p=0.08). There was no sex difference in terms of volume of stroke within the experimental groups (Fig. 2A). Group lesion maps showed that in the LPS+HI group, lesions appeared more extensive than in the LPS+HI+HT group (Fig. 2B). Group lesion maps demonstrated that the epicenter of strokes was located in the LPS+HI condition to the caudate-putamen, the hippocampal CA2 and CA3 areas, as well as the somatosensory and part of the adjacent motor (M1) cortex (Fig. 2B, see also supporting figure I). In LPS+HI+HT condition, group lesion maps showed that the epicenter of strokes was restricted to part of the primary somatosensory cortex (S1), but spared the hippocampus, the motor cortex, and the caudate-putamen as compared to LPS+HI condition (Fig. 2B, see also supporting figure II).

Effect of HT on neurometabolic activity.

As compared to CTL, LPS+HI induced a decrease in the mean [^{18}F] FDG activity within the RCCA territory; namely, the primary somatosensory cortex, the motor cortex, the internal capsule, the hippocampus and the caudate-putamen ($p<0.01$; Fig. 3 A, B). Following LPS+HI exposure, HT-treated rats had improved metabolic activities in the motor cortex, the hippocampus, and the caudate-putamen – but not in the primary somatosensory cortex and internal capsule (data not shown) – as compared to untreated rats ($p<0.05$; Fig. 3C).

Effect of HT on motor behavior.

Whatever the experimental conditions, a linear correlation (p=0.003) was observed between the volume of stroke and the latency to turn in the turning in alley test (Fig. 4A). The required time for turning was significantly prolonged in the LPS+HI as compared to the CTL group (13.5 s

versus 6.7 s, $p<0.001$; Fig. 4B). After LPS+HI exposure, this required time was decreased in HT-treated compared to untreated rats ($p=0.06$; Fig. 4B). Furthermore, a change in lateralization was observed in the turning in alley test: the proportion of rats turning on the right *versus* left side was 59 % in the CTL group compared to 38% in the LPS+HI group ($p<0.05$; Fig. 4C). Following LPS+HI, the proportion of rats turning on the right *versus* left side was 64 % in the HT-treated group as compared to 38 % in the untreated animals ($p<0.01$; Fig. 4C). Hence, HT fully prevents the right/left shift of LPS+HI-exposed animals in the turning in alley test as the LPS+HI+HT rats performed identically to the CTL group (Fig. 4C). Open field tests performed between P20 and P35 did not show any significant difference between the experimental groups (data not shown).

Discussion

Our results show a neuroprotective effect of HT in NAIS, preventing the occurrence of 44 % of the stroke cases. HT reduced the volume of the stroke and improved the brain's metabolic activity, as well as the animal's motor behavior. The beneficial effect of HT against NAIS was observed on both MRI and PET/CT imaging in the RCCA territory. In addition, MRI as well as [^{18}F] FDG metabolism show that HT provides a consistent neuroprotective effect between both techniques in the spatial distribution, namely in the motor cortex, the hippocampus, and the caudate-putamen of the lesioned hemisphere. In line with these results, the turning in alley test shows an improvement of the motor behavior in HT-treated as compared to untreated rats. These results are highly relevant and potentially translational to human NAIS in which 30 to 40 % of neonates will further develop a unilateral spastic form of cerebral palsy (Wu et al., 2006).

Four preclinical studies demonstrated inconsistent results regarding the benefit of HT in inflammatory-sensitized HI brain lesions (Chevin et al., 2018, 2016, Osredkar et al., 2015, 2014). Among them, two studies showing no benefit of HT in LPS+HI-induced stroke (Osredkar et al., 2015, 2014) present some limitations, namely: (*i*) the use of rodents with a level of brain maturation equivalent to preterm human newborns, which does not exactly match with the gestational age of NAIS, which affects term newborns (Patel et al., 2014); (*ii*) the assessment of the neuroprotective effect of HT subjected to potential sample biases due to measures of surface – but not volume – of only a few selected forebrain sections; (*iii*) the absence of morphometric and metabolic imaging outcome measures, and (*iv*) the absence of neurobehavioral outcome measures (Osredkar et al., 2015, 2014). Combining cutting-edge imaging technologies and quantified behavioral studies, our study provides compelling evidence of the neuroprotective effect of HT in NAIS, with promising potential for human newborns.

In contrast with the two previous studies dealing with the neuroprotective effect of HT in HI-exposed pups, we did not observe any sex difference in any of the parameters we assessed (Fan et al., 2013; Smith et al., 2016). However, these studies focused on pups at P7, which were exposed to pure HI *i.e.* a different experimental design as compared to ours.

As HT is already used as a standard of care in neonates suffering diffuse neonatal HI encephalopathy, the therapeutic translation of HT to NAIS would, therefore, be facilitated by a repurpose of indication. Consistent with the idea of a randomized clinical trial assessing the safety and efficacy of HT in NAIS, a single-center prospective cohort study recently shows for the first

time a potential beneficial effect of HT in human newborns in which neonatal encephalopathy and NAIS coexist (Harbert et al., 2011).

While presenting innovative and promising findings, our study has some limitations. Motor behavior was based on turning in alley and open field tests. Further evaluation of motor and cognitive functions might refine the assessment of the behavioral effect of HT. Our model also does not strictly reproduce the ecological physiopathological event of *in utero* chorioamnionitis leading to NAIS. Such modeling is not feasible in rodents since the level of maturation of immediately postnatal pups is equivalent to very preterm brain development; hence, not matching with the timing of NAIS affecting term human newborns. Due to the efficient anastomosis of the circle of Willis in pups, our experimental design requires adding hypoxic stress to the ischemia-induced by RCCA ligation to trigger a stroke. However, the triple-hit (namely, bacterial (LPS) component exposure plus focal ischemia plus global hypoxia) used in our experimental design is relevant with the most prevalent causal pathway of human NAIS featured by the sequence of chorioamnionitis (Giraud et al., 2017; Martinez-Biarge et al., 2013; Sorg et al., 2019), *peripartum* asphyxia identified in up to 26 % of NAIS (Fluss et al., 2019; Martinez-Biarge et al., 2013; Michoulas et al., 2011; Ramaswamy et al., 2004; Sorg et al., 2019), and arterial occlusion of the carotid tree (Fluss et al., 2016; Husson et al., 2016; Kirton et al., 2011; Suppiej et al., 2019).

Conclusion

This is the first preclinical study showing a benefit of HT in NAIS. Through analysing cases of treated and untreated juvenile rats, the benefit of HT was demonstrated with structural and functional brain assessments, as well as motor behavior. Our findings provide a potential avenue

– among others (Chang et al., 2005; Felling et al., 2006; Ferriero et al., 2019) – for novel human therapeutic trials in NAIS to further alleviate its heavy burden morbidities and reduce its negative effects (Dunbar and Kirton, 2018; Fluss et al., 2019).

Acknowledgment

We are very grateful to all members of the Small Animal Imaging Labs of the Research Institute of the McGill University Health Centre for their help with the acquisition of imaging data. We would like to thank Randy Pinsky and Marie-Julie Allard for editing the manuscript.

Funding

Research reported in this publication was supported by grants from the Canadian Institute of Health Research (CIHR), The Foundation of Stars, The Montreal Children's Hospital Foundation (Hoppenheim Fund), and The Heart and Stroke Foundation of Canada; *La Région Auvergne-Rhône-Alpes* and Université Jean Monnet at Saint-Étienne, France. This project has been made possible by Brain Canada Foundation through the Canada Brain Research Fund and Fonds de Recherche du Qébec-Santé, with the financial support of Health Canada and its funding partners (MC). The views expressed herein do not necessarily represent those of the Minister of Health or the Government of Canada.

Conflict of interest

The authors declare that they have no competing interests.

Ethic approval statement

The experimental protocol was approved by the Institutional Animal Care Committee of McGill University (protocol #2015-7691) in accordance with the Canadian Council on Animal Care Guidelines: http://www.ccac.ca/en_/standards/guidelines.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supporting information

Supporting figure I (TIFF): LPS+HI group lesion maps.

Group lesion maps of our LPS+HI rats (n=9), overlaid on our reference 3D MRI brain scans on the axial, sagittal, and coronal axes. Distance between images=1 mm.

Supporting figure II (TIFF): LPS+HI+HT group lesion maps.

Group lesion maps of our LPS+HI+HT rats (n=5), overlaid on our reference 3D MRI brain scans on the axial, sagittal, and coronal axes. Distance between images=1 mm.

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Figures

Figure 1

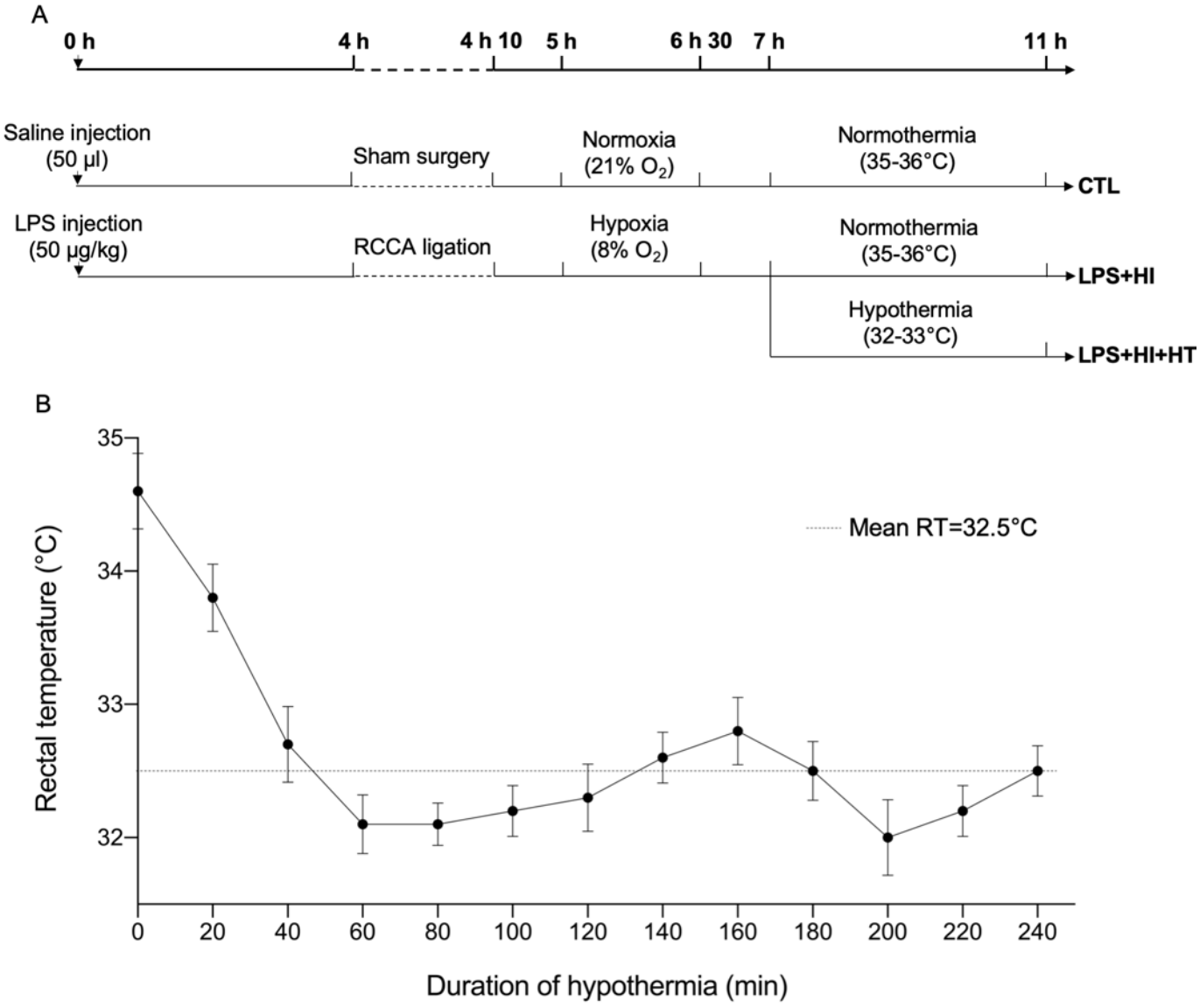


Figure 1. Experimental design.

(**A**) CTL animals who received saline ip injection followed by a sham surgery, did not undergo hypoxia nor hypothermia. LPS from *E.coli* (50 µg/kg) was administered by ip injection in rat pups at P12. Four hours later, the RCCA was ligated, and hypoxia was induced (8.1±0.1 % O_2) for one hour and a half. Pups were divided into groups, one of which was treated by HT (32-33 °C) for four hours. (**B**) Rat pups that underwent HT reached the targeted temperature (32.5±0.4 °C) 30±10 min after being isolated from the mother. Rectal temperature was measured every 20 min using a calibrated probe. Number (n) of LPS+HI+HT rats: n=10. *Abbreviations:* CTL, control; HI, hypoxia-ischemia; HT, hypothermia; ip, intraperitoneally; LPS, lipopolysaccharide of *Escherichia coli*; P, postnatal day; RCCA, right common carotid artery; RT, rectal temperature.

Figure 2

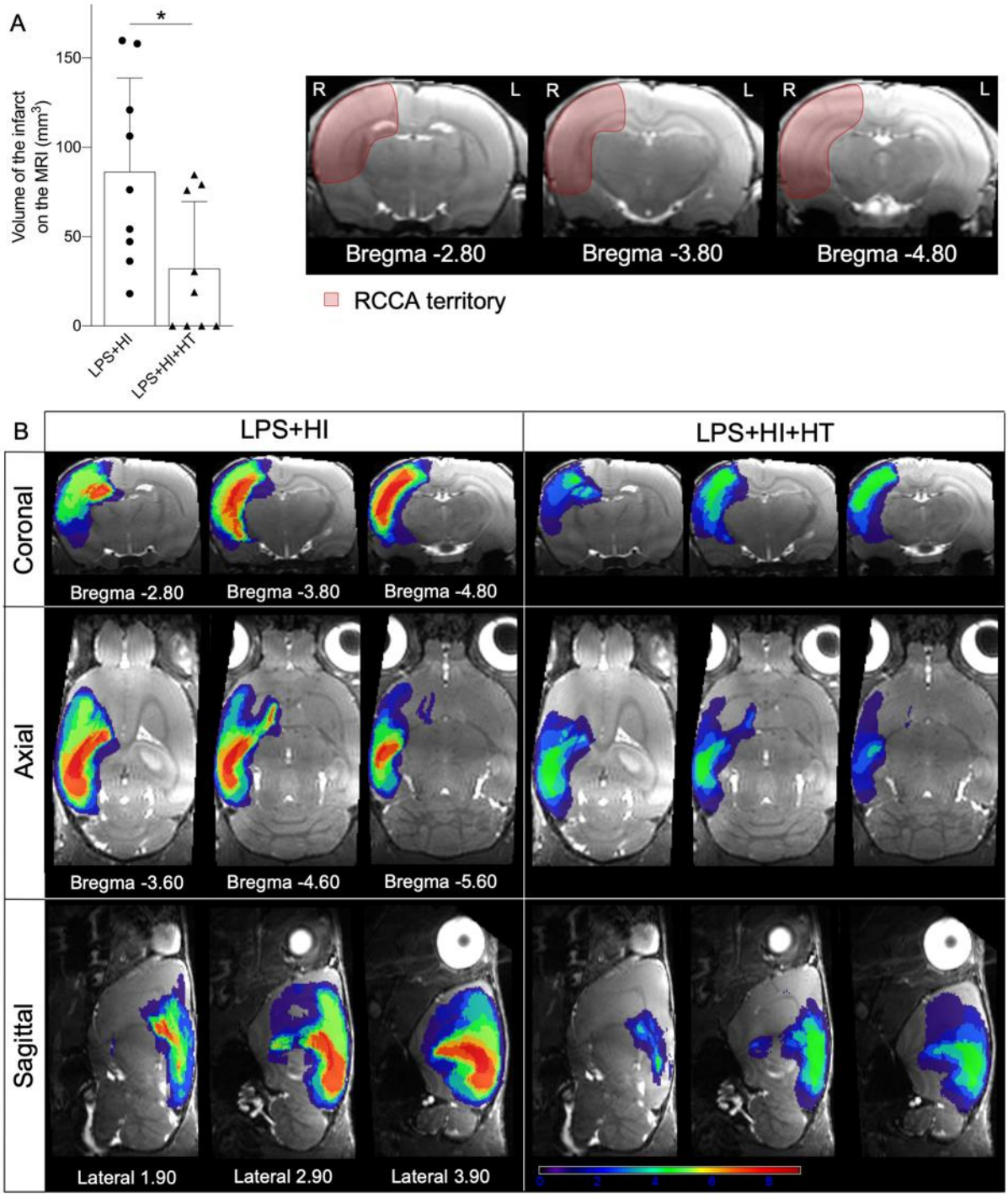

A
*
Volume of the infarct on the MRI (mm3)
150
100
50
0
LPS+HI
LPS+HI+HT
R
L
Bregma -2.80
Bregma -3.80
Bregma -4.80
RCCA territory
B
LPS+HI
LPS+HI+HT
Coronal
Axial
Sagittal
Bregma -2.80
Bregma -3.80
Bregma -4.80
Bregma -3.60
Bregma -4.60
Bregma -5.60
Lateral 1.90
Lateral 2.90
Lateral 3.90

Figure 2. Comparison of the volume of the stroke between LPS+HI±HT-exposed rats.

(**A**) After LPS+HI exposure, HT significantly reduced the volume of the stroke measured by MRI from 86.3±17.5 mm^3 to 32.2±12.5 mm^3. Following LPS+HI exposure, 44 % of HT-treated rats did not present stroke on the MRI, compared to 0 % in the untreated group. The bars indicate the mean±SD. * $p<0.05$; independent T-test. (**B**) Examples of lesion overlap plots for LPS+HI (n=9) and LPS+HI+HT (n=5; 4 animals had no stroke) group. The color range indicates the number of overlapping lesions by coding increasing frequencies from violet (n=1 rat) to red (n=9 rats). Distance between images: 1 mm. *Abbreviations:* HI, hypoxia-ischemia; HT, hypothermia; LPS, lipopolysaccharide of *Escherichia coli*; MRI, magnetic resonance imaging; RCCA, right common carotid artery.

Figure 3

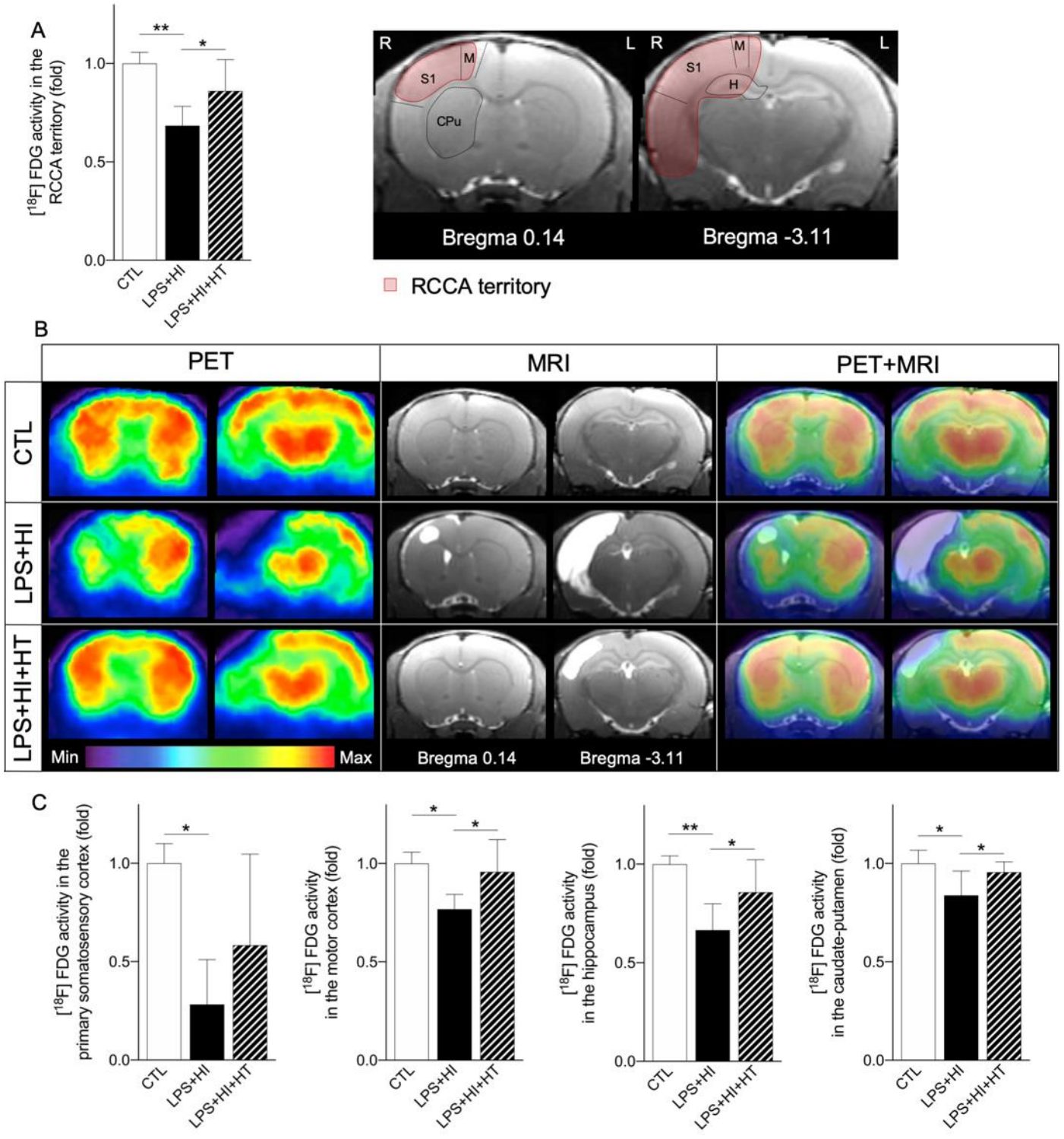

A
[18F] FDG activity in the RCCA territory (fold)
1.0
0.5
0.0
**
*
CTL
LPS+HI
LPS+HI+HT
R
L
M
S1
CPu
H
Bregma 0.14
Bregma -3.11
RCCA territory
B
PET
MRI
PET+MRI
CTL
LPS+HI
LPS+HI+HT
Min
Max
C
[18F] FDG activity in the primary somatosensory cortex (fold)
[18F] FDG activity in the motor cortex (fold)
[18F] FDG activity in the hippocampus (fold)
[18F] FDG activity in the caudate-putamen (fold)

Figure 3. Comparison of the [^{18}F] FDG metabolic activity within the rat brain between CTL and LPS+HI±HT-exposed rats.

(**A**) Mean [^{18}F] FDG activity in the RCCA territory in CTL (n=3), LPS+HI (n=9), and LPS+HI+HT (n=8) group: [^{18}F] FDG metabolism was reduced in the LPS+HI compared to CTL group. After LPS+HI exposure, HT improved [^{18}F] FDG activity in the RCCA territory. (**B**) Representative examples of PET, MRI, and co-registered PET-MRI images in the three experimental conditions. (**C**) Mean [^{18}F] FDG activity in the primary somatosensory cortex, the motor cortex, the hippocampus, and the caudate-putamen in CTL (n=3), LPS+HI (n=9), and LPS+HI+HT (n=8) group. Following LPS+HI exposure, HT-treated rats had improved [^{18}F] FDG metabolism in the motor cortex, the hippocampus, and the caudate-putamen. Data are expressed in fold increased with CTL being set at 1. The bars indicate the mean±SD. * $p<0.05$, ** $p<0.01$; one-way ANOVA, FDR=0.05. *Abbreviations:* CTL, control; CPu, caudate-putamen; H, hippocampus; HI, hypoxia-ischemia; HT, hypothermia; LPS, lipopolysaccharide of *Escherichia coli*; M, motor cortex; MRI, magnetic resonance imaging; PET, positron emission tomography; RCCA, right common carotid artery; S1, primary somatosensory cortex.

Figure 4

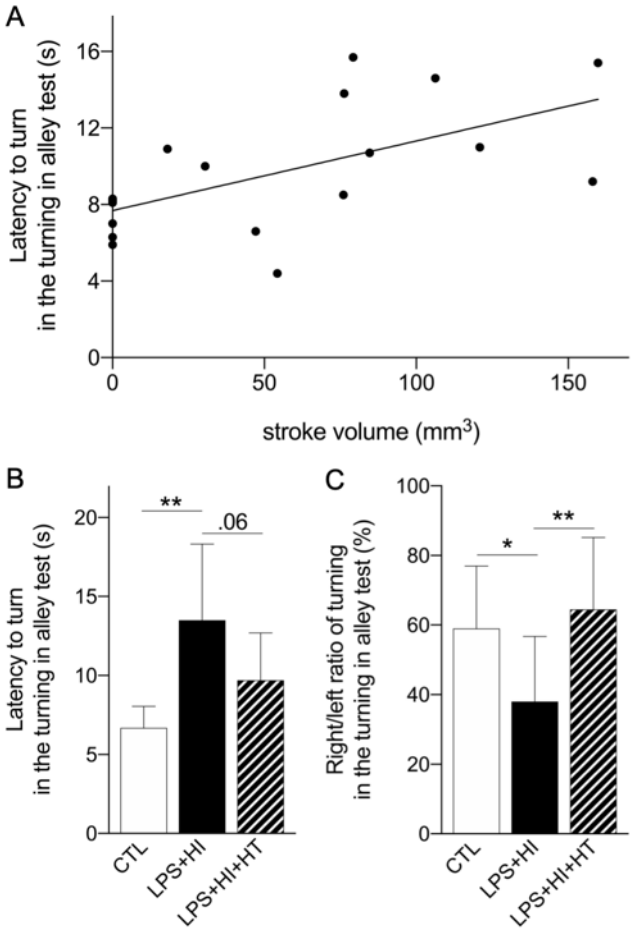
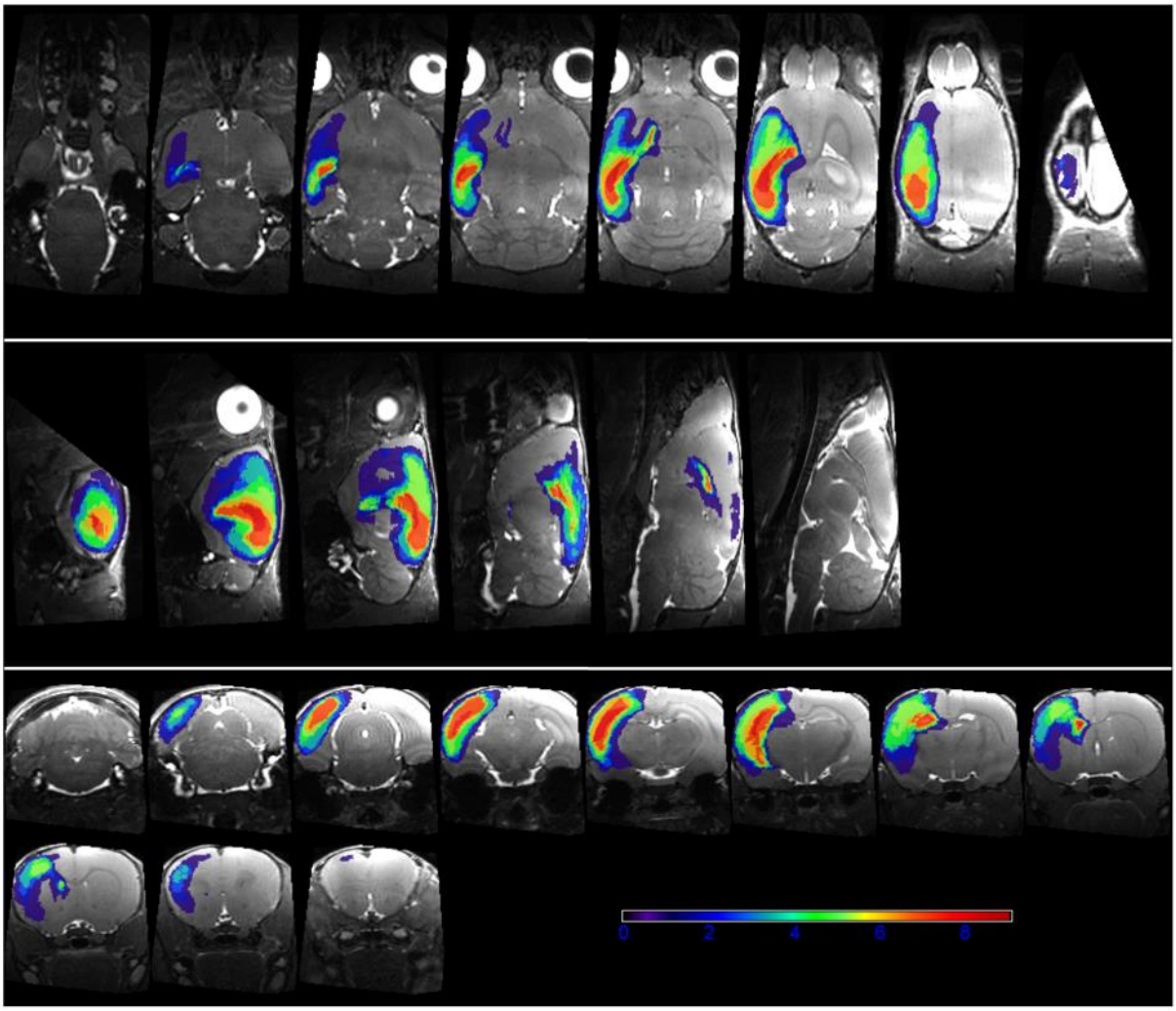


Figure 4. Comparison of the motor behavior between CTL and LPS+HI±HT-exposed rats. (**A**) A linear correlation was found between the volume of the stroke (mm^3) and the latency to turn in (s) using a Spearman correlation, $p<0.001$. Number (n) of rats: n=20, including CTL (n=3), LPS+HI (n=9), and LPS+HI+HT (n=8). (**B**) The required time for turning in alley was significantly prolonged in the LPS+HI compared to CTL group and decreased in HT-treated compared to untreated rats. (**C**) Following LPS+HI exposure, a shift in lateralization toward the left side was observed compared to CTL. This shift was fully prevented in HT-treated as compared to untreated animals. There was a mean of 10 trials per animal. The bars indicate the mean±SD (**B**) and the mean±95% confidence intervals (**C**). * $p<0.05$, ** $p<0.01$; one-way ANOVA.

Abbreviations: CTL, control; HI, hypoxia-ischemia; HT, hypothermia; LPS, lipopolysaccharide of *Escherichia coli*.

Additional files

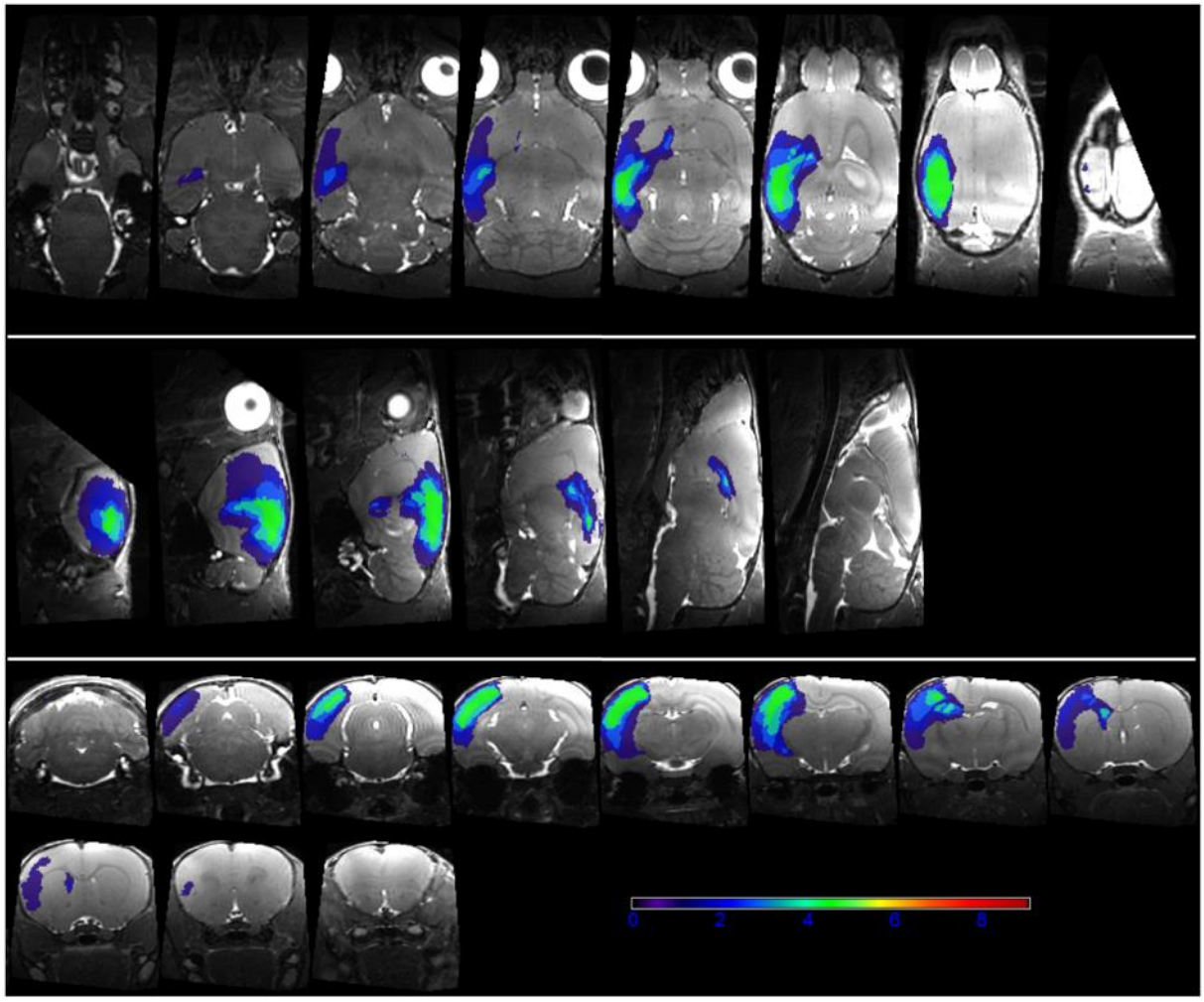
Additional figure I



Additional figure I. LPS+HI group lesion maps.

Group lesion maps of our LPS+HI rats (n=9), overlaid on our reference 3D MRI brain scans on the axial, sagittal, and coronal axes. Distance between images=1 mm.

Additional figure II



Additional figure II. LPS+HI+HT group lesion maps.

Group lesion maps of our LPS+HI+HT rats (n=5), overlaid on our reference 3D MRI brain scans on the axial, sagittal, and coronal axes. Distance between images=1 mm.

CHAPTER V: DISCUSSION AND CONCLUSION

SUMMARY OF FINDINGS

The general objective of this research was to investigate the neuroprotective effect of HT in combination or not with IL-1Ra in the treatment of NE resulting from inflammatory-sensitized HI. We specifically aimed to investigate **(1)** the effect of HT on pharmacodynamic parameters of IL-1Ra, and **(2)** the efficacy of HT with or without IL-1Ra in terms of mortality, and brain injury assessed by histology or imaging technics, as well as motor behaviour improvement at short and long-term.

Using our rat model of inflammatory sensitized-HI brain injury, I demonstrated that:

(1) HT modified the pharmacodynamics of IL-1Ra inducing an opposite effect than expected resulting in the paradoxical up-regulation of the innate immune response. This alteration led to an upregulation of pro-inflammatory cytokines within the brain.

(2) IL-1Ra (50 mg/kg) plus HT *vs* sole HT significantly decreased the mortality rate occurring during hypoxia as well as improved the short-term motor outcome. However, no added value of IL-1Ra to HT was observed in term of reduction of brain injury assessed by histology and imaging measures.

(3) Sole HT prevented the occurrence of 44% of brain injury, reduced the volume of the infarct by 37% on MRI, enhanced the brain metabolic activity within the carotid artery territory, and improved the long-term motor behavior.

The two first manuscript dealt with the safety and efficacy of the combination of HT with IL-1Ra in the treatment of LPS+HI -induced NE. The first manuscript revealed the effect of HT on some pharmacodynamic parameters of IL-1Ra leading to its bioaccumulation within the brain. These results could explain why we observed added value of IL-1Ra to HT on only certain outcome parameters but not on the surface of brain lesions at the tested doses. Manuscript two described that IL-1Ra (50 mg/kg) plus HT *vs* sole HT significantly decreased the mortality rate occurring during hypoxia and improved the short-term motor outcome in rats. The rationale of the third manuscript was to deepen investigation of the effect of HT in the context of NE resulting from inflammation plus HI. The use of cutting-edge imaging tools helped us to further characterize the neuroprotective effect of sole HT in our model.

COMPREHENSIVE DISCUSSION

The following sections will present a more detailed discussion of the findings, the limitations, and future direction of this thesis work.

1. HT effects on pharmacodynamic parameters of hrIL-1Ra

Manuscript 1 revealed an effect of HT on IL-1Ra concentration in our model. We observed a bioaccumulation of IL-1Ra under HT condition within the plasma, CSF and forebrain of LPS+HI exposed pups. Only few studies dealt with the impact of HT on the pharmacokinetic and pharmacodynamic of drugs used in the human neonatal context (de Haan et al., 2012; Pokorna et al., 2015; van den Broek et al., 2010; Zhou and Poloyac, 2011). In the same line, current evidence demonstrated that HT decreases the clearance of a variety of drugs used in neonates with NE, such as antibiotics (Choi et al., 2018; Cies et al., 2017), antiepileptics (van den Broek et al., 2012), and others (Balduini et al., 2019; Favié et al., 2020, 2019). Besides, to our knowledge, no other study focused on the impact of HT on the pharmacodynamics of hrIL-1Ra. We hypothesized that the bioaccumulation of hrIL-1Ra might result from a decreased of renal clearance under HT condition. In a preclinical pharmacology study on Lewis rats with arthritis, it has been shown that hrIL-1Ra is eliminated rapidly (elimination half-life of 4-6 h) mainly through the kidney by glomerular filtration (Kim et al., 1995; Liu et al., 2011). It has been shown in human studies that HT can reduce glomerular filtration (van den Broek et al., 2010; Zhou and Poloyac, 2011). hrIL-1Ra clearance is influenced by kidney function in human (Yang et al., 2003), and neonates suffering from HIE can have acute kidney injury (Durkan and Alexander, 2011; Sweetman et al., 2013). Hence, HIE and HT could both affect renal filtration and decrease the clearance of hrIL-1Ra.

Further work is necessary to assess the elimination of hrIL-1Ra and kidney function in our LPS+HI+HT condition.

1.1. hrIL-1Ra bioaccumulation and toxicity

Under HT condition, we demonstrated a bioaccumulation of hrIL-1Ra within the ipsilateral hemisphere and CSF of LPS+HI-exposed animals (Chevin et al., 2018). This was assessed with the mild dose (*i.e.* 50 mg/kg/12 h) of IL-1Ra. Regarding the short half-life (4-6 h) and the twelve-time effectiveness of hrIL-1Ra in rats as compared to human, we did not anticipated toxicity due to an overdose of the drug at this dose. This result could explain the toxicity observed in brain of rats exposed to the highest dose (*i.e.* 200 mg/kg/12 h) of IL-1Ra combined with HT.

Indeed, in this particular experimental group, we observed an increase in the penumbra injury (*i.e.* the region surrounding the loss of tissue with pycnotic neurons and/or loss of architecture), as well as a loss in the weight of pups during hrIL-1Ra administration and lasting up to the end of the study (**Figure 1**).

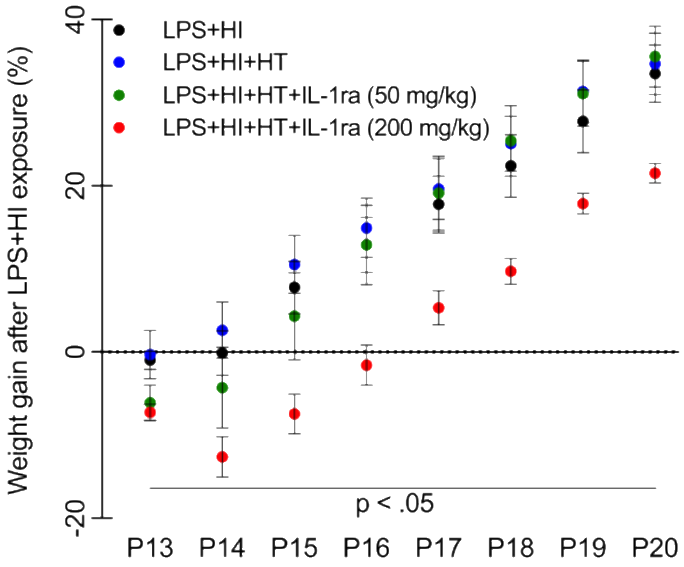


Figure 1. Weight gain of rat pups after LPS+HI exposure. Rats in the LPS+HI+HT+IL-1Ra (200

mg/kg) group lost more weight after LPS+HI exposure than in the other experimental conditions. They did not regain a normal weight before the end of the study (*i.e.* 8 days after exposition to LPS+HI). We did not observe the same pattern with rats treated with IL-1Ra at 50 mg/kg/12 h.
If we think about a potential translation of hrIL-1Ra plus HT from the rat to humans and regarding some change in the half-life and effectiveness between species: a dose of 50 mg/kg/12 h will be similar to a dose of 8 mg/kg/d in humans (which is the maximal dose recommended for neonates). A dose of 200 mg/kg/ 12 h will represent an approximate dose of 33 mg/kg/d in humans, which is above the maximal recommended dose of 8 mg/kg/d (see appendix #1). However, we decided to use this dose as it previously showed important neuroprotective effects in the same LPS+HI model (Savard et al., 2015, 2013). Besides, if we consider the impact of HT on pharmacodynamics of hrIL-1Ra, we suggest in the future to deeply study this effect on a longer timeframe (*i.e.* from 4 h to 72 h, for instance) and with a wider range of doses between 12 to 50 mg/kg/12 h (which represents dose varying between 1 to 10 mg/kg/d in neonates, as per recommendation in the appendix #1).

We hypothesized that this hrIL-1Ra bioaccumulation in HT condition might perturbate the IL-1/IL-1Ra balance within the brain (Spulber et al., 2009); therefore, inducing deleterious effect for neural cells. In keeping with these findings, experimental studies using transgenic mice chronically overexpressing human IL-1Ra within the CNS have observed alteration of the brain morphology (smaller volume of the cerebral cortex and hippocampus) and behavioural impairments (Spulber et al., 2011). Besides, this lifetime IL-1 blockade on mice was not able to prevent brain infarct induced by permanent focal ischemia (Oprica et al., 2004), in contrast with transient administration of IL-1Ra in the same model (McCann et al., 2016). To our knowledge, the mechanistic

explanation behind this deleterious effect of IL-1Ra overexpression is still unknown but could be due to non-specific ligand-receptors interactions or compensatory mechanisms (Oprica et al., 2004). Using the same transgenic mice in a model of traumatic brain injury, Tehranian and colleagues observed an increased in TNF-α levels in cerebral cortex as compared to wild type mice (Tehranian et al., 2002). They conclude that this could be due to a compensatory mechanism in response to the inhibition of IL-1R activation by an excess of IL-1Ra in the brain and subsequent reduction of IL-1 signalling (Tehranian et al., 2002). This early overexpression of TNF-α may contribute to neuronal death, in particular by necroptosis (see paragraph 3.2.2), and the subsequent inflammatory burst. This finding is in line with our results showing overexpression of IL-1β and TNF-α at 4 h and 24 h respectively within the ipsilateral hemisphere in the HT+hrIL-1Ra-treated rats (Chevin et al., 2018).

Another possible mechanism that could explain this toxicity can be through the blockade of NFκB. NFκB is known for its dual role in neuroprotection and neurotoxicity in the brain (Shih et al., 2015). In particular, data demonstrated a physiological role of NFκB in maintaining survival and proliferation of neurons (Bhakar et al., 2002; Shih et al., 2015). Using a "super-repressor" (*i.e.* non-degradable IκB), the permanent inhibition of NFκB in neurons lead to neuronal death and learning impairments in adult mice (Bhakar et al., 2002; Meffert and Baltimore, 2005), supporting that NFκB plays key roles in physiological neuronal function and neuronal networks. Overall, these findings support the fact that the dosage and administration window of IL-1Ra needs to be carefully targeted in order to avoid a harmful impact on the brain.

On the other hand, a larger dose effect study of hrIL-1Ra has been done in our model. We tested doses of 12.5 to 200 mg/kg/12 h for 72 h. Even with the small dose of 12.5 mg/kg combined with

HT – a dose we chose to test in the context of the potential toxicity due to bioaccumulation of hrIL-1ra on HT condition – we did not observe any benefit on histological measures of brain infarct. In contrast to the dose of 50 mg/kg, we did not find any reduction of the total loss of tissue for the dose of 12.5 mg/kg as compared to untreated rats (**Figure 2**).

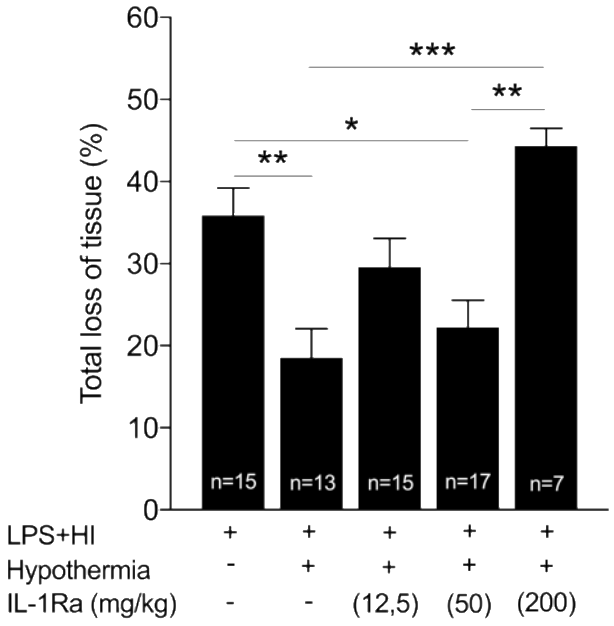


Figure 2. Histology measurements of the total loss of tissue induced by LPS+HI exposure. The total loss of tissue was significantly reduced by HT and HT+IL-1Ra (50 mg/kg) treatments, as compared with untreated rats. hrIL-1Ra did not provide any added value to HT at the doses we tested.

Note that the dose of hrIL-1Ra at 12.5 mg/kg added to HT reverse the neuroprotective effect of HT alone. This result is difficult to explain as we previously reported that HT did not counteract the IL-1 signalling (Chevin et al., 2016) but how exactly interacts hrIL-1Ra under low temperature is still unknown.

Histological measures as we did in this study can be challenged. These data are based on surface – and not volume – measures of two consecutive brain sections; thus, did not represent the entirety

of brain injury after LPS+HI exposure. HT plus hrIL-1Ra (50 mg/kg) results on brain infarct (*i.e.* no added value as compared to HT alone) should be taken with caution since they contradict the observed benefits of the combined treatment in term of mortality and short-term motor behaviour (**manuscript 2**). The combination of hrIL-1Ra to HT may still have an interest in neuroprotection if we refine the dose of hrIL-1Ra to administrate (around 50 mg/kg) and use imaging technics to more precisely analyze brain injuries.

1.2. IL-1Ra administration window

In our studies, the timing of injection of IL-1Ra (*i.e.* 30 min before LPS administration) was based on previous preclinical works on adult ischemic stroke (Loddick and Rothwell, 1996; McColl et al., 2007; Relton and Rothwell, 1992; Touzani et al., 2002) and previous studies from our laboratory (Savard et al., 2015, 2013). Such timing could be of interest for clinical purpose, as hrIL-1Ra is already approved in pregnant women having arthritis (see appendix 1). Therefore, using hrIL-1Ra prenatally when there is signs of infection-inflammation during pregnancy could be of interest to reduce the mortality occurring in those cases of infection-inflammation-induced neurodevelopmental disabilities (Chen et al., 2020; Chevin et al., 2020b). However, pre-administration of IL-1Ra could induce unexpected effects. As IL-1 signaling has also important roles in normal brain functions, its early blockade could interfere with neurophysiological processes (Liu and Quan, 2018; Mantovani et al., 2019; Spulber et al., 2009). It would be of interest to test if acute or delayed administration of IL-1Ra in combination with HT could have better effects in our model. It is known in the literature that a delayed administration of 3 h post insult of IL-1Ra still have neuroprotective effects after ischemic stroke in adult rats (Pradillo et al., 2017, 2012). In contrast, 24 h delayed IL-1Ra treatment did not provide any effect in the same model

(Girard et al., 2014).

Further clinical studies are needed to answer the potential effect of hrIL-1Ra during the pregnancy to alleviate mortality. Moreover, further preclinical works could be of interest to study the delayed administration of hrIL-1Ra after LPS+HI exposure.

1.3. Sex difference in HI±LPS±HT injury

There is more and more evidence in the literature of sex-difference in rodent models of HI injury (Charriaut-Marlangue et al., 2017; Netto et al., 2017). Most sex-differences were found on specific parameters such as behavioural responses, cell death pathway and oxidative stress (Netto et al., 2017). However, these results might be different depending on the age of animals due for instance to the immaturity, and rapidly evolving functions of hormonal systems between juvenile and adult animals, as well as between preterm and term newborn animals (Csernus, 1986). Throughout our studies, we did not find sex-dimorphic effect in any parameters we assessed. Although for some parameters, it might be due to a lack of power (*i.e.* the experimental numbers might not allow for statistic demonstration of any dimorphic effect); for others, such as brain histology (Chevin et al., 2018) we did not observe any difference between males and females. Interestingly, only one study combining endotoxin plus HI injury investigated sex-differences in their model (Fleiss et al., 2012). They observed a similar volume of tissue loss, number of glial cells, and cytokine expressions between females and males following LPS+HI exposure (Fleiss et al., 2012). Similarly, our previous works using inflammatory-sensitized HI injury did not bring evidence of sex-differences on the same parameters tested (Girard et al., 2012; Savard et al., 2015, 2013). Sex-differences vary according to the age, type of aggression (LPS or others) as well as treatment(s) administered after the HI insult. Only few preclinical studies investigated sex-dimorphic effects in

HI+HT models (Fan et al., 2013; Smith et al., 2016, 2015). These studies reported that females appear to have slightly more benefits from HT than male for specific behavioural tasks (*i.e.* motor learning) (Fan et al., 2013; Smith et al., 2015). Protective effects of HT were similar for males and females in terms of brain injury (Fan et al., 2013; Smith et al., 2016).

2. Effect of HT on inflammatory-sensitized HI

In preclinical studies investigating the neuroprotective effect of HT, NE is modelled using pure HI brain injury. However, in the clinical setting, the etiology of NE is often multifactorial. Evidence emphasized an important role of perinatal infection/inflammation in addition to HI events (Nelson, 2009; Nelson and Penn, 2015; Nelson and Willoughby, 2000). The benefit of HT in this context of infection/inflammation sensitized HI is still controversial.

Using the same inflammatory-sensitized HI model during a previous research project, I demonstrated a neuroprotective effect of HT in penumbra brain injury possibly through an increased in antioxidant enzymes (Chevin et al., 2016). We further described a protective effect of HT in the total loss of brain tissue, as well as in the reduction of the atrophy of the caudate-putamen and the hippocampus (Chevin et al., 2018), which are areas of the brain among the most exquisitely sensitive to HI. In contrast with these findings, Osredkar and colleagues found that HT was not neuroprotective in a similar model of inflammation-sensitized HI brain injury (Osredkar et al., 2015, 2014). However, their studies comport some limitations as they used P7 rat pups (*i.e.* rodents with a level of brain maturation equivalent to preterm human newborns) in contrast with HIE affecting full term newborns (Patel et al., 2014) and focused their work mainly on hippocampus (Osredkar et al., 2015, 2014). That is why, our results using P12 pups (corresponding to a level of maturation equivalent to full-term newborns) may be more suitable in term of brain injury of the

term neonate. Besides, their studies were restricted to surface measurements on few selected forebrain sections and did not include volumetric (*e.g.* by imaging) measures of brain lesions or neuro-behavioural outcome measures (Osredkar et al., 2015, 2014).

One of the aims of **manuscript 3** was to confirm the neuroprotective effect of HT observed previously in our model using powerful imaging technics allowing us to obtain morphometric and metabolic imaging outcome measures and tested the effect of HT on motor behavioural tasks. Interestingly, HT was found to limit the extent of the infarct and promote full recovery in 44% of treated rats, as well as improve the motor behaviour at juvenile age (Chevin et al., 2020a). Imaging data suggest that the effects are located to the motor cortex, hippocampus and caudate-putamen. In our study, the motor behaviour was assessed by turning in alley and open field tests. Beyond the improvement of the motor behaviour in HT-treated rats, the negative results in open field tests suggest that the general motor functions of the rats may not be affected after LPS+HI-induced brain lesion. The positive correlation found between the volume of brain infarct and the latency to turn suggest that cognitive functions may be altered. Indeed, rats with large brain infarct were still able to turn at the end of the alley; however, they will need more time to perform the test. This finding underlines the importance of assessing, in future work, specific motor task that required both motor and cognitive functions, such as Morris Water Maze, Foot Fault, and novel object recognition tests.

2.1. LPS+HI as a model of NAIS

Our adjusted Rice-Vannucci model can be used as a model of NAIS (Gennaro et al., 2019). As we confirmed in MRI, LPS+HI induced a consistent focal injury in the territory of the carotid artery (Chevin et al., 2020a). This model could be a good alternative to the one using transient or

permanent occlusion of the middle cerebral artery (MCAO), producing a high rate of mortality (Ashwal et al., 2007; Gennaro et al., 2019). One preclinical study comparing HI and transient MCAO models observed a similar volume of injury on MRI, with a greater cortical involvement in the HI model (Ashwal et al., 2007). In our model, we showed that lesions were located mainly in motor areas and can be correlated with the motor behaviour observed in the turning in alley test (Chevin et al., 2020a). Besides, the weakness on the left side we observed in the LPS+HI-exposed rats are relevant to hemiplegia often seen in human patients after NAIS (Chevin et al., 2020a, 2020b; Husson et al., 2010). It could be of interest to further study hemiplegia and other symptoms of CP in our model (Nelson, 2002; Wu et al., 2006).

2.2. Early diagnostic for an early intervention of NAIS

Neuroimaging using MRI is the gold standard to diagnose NAIS, which may, however, takes time to obtain in the clinical setting or not always feasible in instable neonates (Nevalainen et al., 2019; Wagenaar et al., 2019). Besides, it is crucial to shorten the diagnosis in order to initiate new therapeutic intervention, such as HT, to limit the extend of the infarct and/or promote recovery (Basu, 2014; Chevin et al., 2020a). As investigated in HIE clinical and preclinical studies, HT may not be effective after 10 h post insult (Laptook et al., 2017; Li et al., 2009; Sabir et al., 2012). HT was found to have deleterious effect after 12 h in a severe model of HIE (Sabir et al., 2012). An interesting option to shorten the diagnosis of NAIS in neonates could be the use of electrophysiological tests. Nevalainen and colleagues have shown the very good diagnostic sensitivity of electroencephalography (EEG) coupled with somatosensory evoked potentials (SEPs) at bedside. This SEP measure also have a strong early predictive value for progression to hemiplegia (Nevalainen et al., 2019). Another study demonstrated that the monitoring cerebral

activity and oxygenation, using EEG and near-infrared spectroscopy (NIRS), may provide useful information for early prognosis in NAIS (Wagenaar et al., 2019). Although these diagnostic technics could be relatively complicated to put in place at bedside in clinical daily practice (availability of the material and training is necessary). The two articles showed, however, that electrophysiology has its place alongside MRI as a diagnostic and prognostic tool for NAIS.

3. Future directions

By combining two individually effective treatments – IL-1Ra and HT – we hoped that two treatments would be better than one. We found a partial benefit of IL-1Ra as an add-on therapy to HT on mortality and short-term motor outcome. In future studies, it would be preferable to use imaging technics to evaluate neuroprotective effects more precisely and on the entire brain infarct.

3.1. Further evaluation of the effect of HT on IL-1Ra pharmacology

Results we obtained indicate that further study concerning IL-1Ra pharmacokinetics and pharmacodynamics in combination with HT is required. For instance, the use of radiolabelled IL-1Ra and PET imaging can be of interest to further study the biodistribution and metabolism of IL-1Ra in combination or not with HT (Cawthorne et al., 2011). Such study could inform us on the brain uptake of [^{18}F] IL-1Ra after LPS+HI-exposure, as well as its metabolism and excretion via the kidneys. Radiolabelled metabolites quantification can be easily done and compared throughout time in different organs, under HT condition or not. This could give us another way to quantify the amount of IL-1Ra entering the brain. This additional study should allow us to find an explanation to the alteration of some pharmacodynamics parameters by HT as well as find the lowest effective dose of IL-1Ra in our model.

3.2. Further evaluation of motor behaviour and CP-like symptoms

In our studies, the refined analysis of motor behaviour has not been carried out; therefore, we cannot conclude on the presence or not of hemiplegia and spastic or dystonic symptoms associated with CP. It could be of interest to further study this question as motor outcome seems to vary depending on the model of HI used. Rat model of HI injury did not present aberrant reflexes or severe spastic, or dystonic impairments observed in human patients; however, most of them did not report long-term motor functions or used premature (P4) or late preterm (P7) – instead of term – rat pups (Cavarsan et al., 2019; Fan et al., 2005; Rumajogee et al., 2016). The use of rodent models can be challenged due to important differences in terms of level of cortical development and its different implications in motor control, and the maturation of corticospinal tract with human (Clowry et al., 2014; Gennaro et al., 2019, 2017). In contrast, LPS or HI model of rabbit reported spasticity (Derrick et al., 2004; Saadani-Makki et al., 2008). It is of importance to evaluate more precisely motor anomaly in these models in order to better validate their relevance in CP (Cavarsan et al., 2019).

3.3. Evaluation of the effect of HT on necroptosis

Neuroprotective effects of HT were assessed through the size of the brain lesion (by histology or MRI measurements), brain metabolic activity, and behavioural studies. Further work needs to be done concerning the potential effect of HT on cell death pathways. Our laboratory is also interested to investigate the effect of HT on necroptosis (*i.e.* a caspase-independent form of cell death that can induced a massive neuronal loss) (Kaiser et al., 2013; Mandal et al., 2014; Tovar-y-Romo et al., 2016; Walsh, 2014). Only one preclinical study investigated the role of necroptosis after HI injury in mice at P7 (Northington et al., 2011). Our preliminary study revealed that key molecules

of necroptosis, namely MLKL and RIP-3, were overexpressed after LPS+HI-exposure (unpublished data). Phosphorylated forms of these markers were detected in the motor cortex, hippocampus and caudate-putamen of the ipsilateral hemisphere. A study is underway to investigate the potential effect of HT, in combination or not with a specific necroptosis blocker, on these necroptotic molecules.

3.4. Evaluation of the neuroprotective effect of HT after gram-positive bacteria + HI-exposure

Like our study, other preclinical models of inflammatory-sensitized HI-brain injury used LPS (a constituent of gram-negative bacterial membrane) to trigger inflammation. However, gram-positive bacteria, such as Group B Streptococcus (GBS) is the leading cause of neonatal sepsis (Fjalstad et al., 2016). It could be of interest to test whether gram-positive bacteria also sensitized the neonatal brain and if HT is neuroprotective in this context. One recent preclinical study investigated the sensitizing effect of TLR-2 activation combined with mild HI on an immature rat brain. HT was found to reduce the hippocampal and hemispheric area loss in this context (Falck et al., 2018, 2017). Our laboratory developed and characterized two models of end-gestational exposure to inactivate or activated GBS (Allard et al., 2019, 2017; Bergeron et al., 2016). To our knowledge, no preclinical study tested the effect of HT on infection – and not only inflammation – sensitized HI brain injury.

3.5. Benefits of HT in NAIS: a clinical study?

There is currently no targeted treatment for NAIS. Epidemiological studies of NAIS showed that

perinatal infection/inflammation, *peripartum* hypoxia, and occlusion of the internal carotid tree are the main determinants of NAIS (Fluss et al., 2019, 2016; Giraud et al., 2017; Kirton et al., 2011). The well-established benefit of HT in NE due to diffuse hypoxia-ischemia provides a rationale for the potential use of HT as a neuroprotective strategy in NAIS. Besides, 5 to 20% of term newborns suffering from NE treated by HT are found to have NAIS and conversely 10-15% of babies with NAIS share the biological criteria for HIE (Adami et al., 2016; Martinez-Biarge et al., 2016; Michoulas et al., 2011; Ramaswamy et al., 2004; Sorg et al., 2019). It could be of interest to compare NE *versus* NAIS patients – treated or not with HT – in terms of clinical outcomes, such as percent of CP, language delay, and other long-term outcomes.

4. Limitations

Rodents are considered a model of choice to study perinatal brain injuries, although no animal model can fully replicate human diseases or conditions. It is important to note the difference between rodents and humans in the overall complexity of brain organization, and the discrepancies in the rate of maturation (Rumajogee et al., 2016; Sarkar et al., 2019). For instance, our model does not strictly reproduce *antepartum* aggression, such as *in utero* infection/inflammation (*e.g.* chorioamnionitis), leading to brain injury. However, using fetuses or immediately postnatal pups is equivalent to very preterm brain development, which is in contradiction to the level of maturation of term human newborns suffering from NE or NAIS.

In our studies, we used human recombinant IL-1Ra instead of IL-1Ra from the rat. Although its efficacity is around 10 to 12 times less than in humans (Quiniou et al., 2008), hrIL-1Ra remains very specific and very used for IL-1 blockade in rats (Lan et al., 2015; Leitner et al., 2014; McCann et al., 2016; Quiniou et al., 2008; Savard et al., 2015, 2013). Furthermore, it allowed us to evaluate

the concentration and distribution of hrIL-1Ra within organs, including the brain, using ELISA and immunohistochemistry with monoclonal antibodies specific to human IL-1Ra (Chevin et al., 2018; Savard et al., 2015, 2013). Other concentration and timing of administration of hrIL-1Ra – in combination or not with HT – could be evaluated in future preclinical studies.

The pharmacology study of hrIL-1Ra was only based on the drug and pro-inflammatory cytokines concentrations at 4 and 24 h post-LPS+HI-exposure. These measurements were not assessed after the completion of hrIL-1Ra administration. Blood samples could be taken at additional time-points to study in more detail the pharmacology of this drug. Pharmacokinetic parameters were not investigated in our study. In future experiments, clearance of the drug and renal function of rats should be assessed under HT condition. However, to date, this is the first study dealing with the pharmacology of hrIL-1Ra in neonatal rats.

In our studies, motor behaviour was based on elevated body swing, open field, and turning in alley tests from P14 (2 days after the insult) to P40 (juvenile rats). Further evaluation of motor and cognitive functions might refine the assessment of the behavioural effect of HT with or without IL-1Ra. Other behavioural tests at adult age (P90-120) could be of interest to complement these studies.

CONCLUSION

In this thesis, I present three major findings:

(1) HT influences IL-1Ra pharmacodynamic parameters. HT induced an augmentation of the concentration of IL-1Ra, leading to a paradoxical upregulation of inflammatory cytokines within the brain (Chevin et al., 2018). This effect, which has been little studied, can have a significant impact on the safety of drugs used in neonates suffering from NE and treated by HT. In the same way, it could have an impact on current and future studies aiming to test the beneficial effect of HT in other neurological diseases (*i.e.* spinal cord injury, traumatic brain injury, adult and neonatal stroke, and others) (Han et al., 2015; Karnatovskaia et al., 2014).

(2) The combination of hrIL-1Ra to HT provides encouraging results in terms of mortality and short-term behaviour (Chevin et al., 2020b). These observations are clinically relevant, as further preventing mortality and morbidities arising from NE is still a research priority. These results also confirm the causal role of IL-1 in neuro-morbidities that currently remain uncontrolled in HT-treated neonates. At this point, further preclinical evaluation of hrIL-1Ra added to HT is still required before launching human clinical therapeutic trials.

(3) In contrast with previous preclinical observations, HT has major neuroprotective effects in our model of inflammatory-sensitized HI brain injury (Chevin et al., 2020a). Using cutting-edge imaging tools, we demonstrated that HT prevented the occurrence of brain infarct in 44% of cases; and reduced the extent of brain injury in other cases by 37%. HT alleviated brain metabolic activity in the affected territory and improved the long-term motor behaviour (Chevin et al., 2020a). These results open new research avenues on the potential benefit of HT in other postnatal brain injury sharing common determinants with NE, such as NAIS.

CHAPTER VI: BIBLIOGRAPHY

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CHAPTER VII: APPENDICES

Appendix 1: Monographie du médicament Anakinra, Kineret®

Anakinra
MONOGRAPHIE

anakinra (Kineret)

- Agent immunomodulateur
- Protéine obtenue par technologie recombinante

Posologie usuelle : injecter une dose SC DIE chaque jour au même moment

Tableau comparatif :

Monographie : 897

Codification :

Type de code	Code	Nom
Vigilance Santé	82040401	Anakinra (INHIBITEURS DE L'INTERLEUKINE)
AHFS	92:36	Antirhumatismaux modificateurs de la maladie
ATC	L04AC03	Anakinra

Effets secondaires

	Fréquence	Placebo	Note
céphalées	12.3%	8.8%	
diarrhée	7.1%	5.2%	
douleurs abdominales	5.3%	4.5%	
douleurs articulaires	5.6%	6.6%	
infection des voies respiratoires	13.8%	15.2%	
infections graves	1.8%	0.7%	
nausées	8.4%	6.4%	
neutropénie			
réaction au site d'injection	71.2%	28.5%	
sinusite	7.1%	5.6%	
symptômes pseudogrippaux	6%	5.2%	

Cette liste n'est pas exhaustive. Elle présente les effets secondaires les plus fréquents et les plus pertinents.

- Taux d'abandon pour effets secondaires : 6% (réactions au site d'injection)
- Les infections graves observées ont été essentiellement des infections bactériennes, comme la cellulite, la pneumonie et les infections ostéo- articulaires.
- Les asthmatiques seraient plus susceptibles de développer une infection sévère.
- On ignore si l'utilisation chronique du produit peut augmenter la fréquence du cancer.
- Les réactions au site d'injection ont typiquement été signalées au cours des 4 premières semaines de traitement et se manifestaient pour 2 à 4 semaines. L'apparition de réactions au site d'injection après le premier mois de traitement est exceptionnelle. Les réactions sévères sont rares. Les réactions au site d'injection les plus fréquemment rapportés incluent l'érythème, le prurit, les éruptions cutanées et la douleur.
- Les réactions au site d'injection ont été traitées à l'aide de corticostéroïdes topiques ou d'antihistaminiques, plus rarement à l'aide de corticostéroïdes oraux.
- Certains effets indésirables peu fréquents, mais potentiellement graves ont également été signalés. Les principaux sont : thrombocytopénie.

Indications et doses

arthrite goutteuse	100 mg SC DIE X 3 jours
* arthrite rhumatoïde (Adultes)	100 mg SC DIE
fièvre méditerranéenne familiale (Adultes)	100 mg SC DIE

* maladie inflammatoire multisystémique à début néonatal	3-8 mg/kg/jour SC DIE
péricardite (Adultes)	récurrente : 100 mg SC DIE données limitées
polyarthrite juvénile (Enfants)	

* indication officielle au Canada

Dose adulte

Arthrite rhumatoïde

Indiqué pour réduire les signes et symptômes de la polyarthrite rhumatoïde évolutive modérée à sévère.
Peut être utilisé seul ou en association avec d'autres agents antirhumatismaux modificateurs de la maladie, tel le méthotrexate.
Ne pas utiliser en association avec des agents bloquant l'action du facteur de nécrose tumorale, tel l'étanercept - risque largement accru d'infections sérieuses.

Dose usuelle : 100 mg SC DIE, administrer la dose à environ la même heure chaque jour.

Maladie inflammatoire multisystémique à début néonatal

Indiqué pour le traitement de la maladie inflammatoire multisystémique à début néonatal qui est un syndrome périodique associé à la cryopyrine.

Dose initiale : 1-2 mg/kg SC DIE, administrer la dose à environ la même heure chaque jour.
Ajustement de dose : 0.5-1.0 mg/kg par augmentation
Dosage d'entretien usuelle : 3-4 mg/kg/jour
Dose maximale: 8 mg/kg/jour

Dose pédiatrique

Maladie inflammatoire multisystémique à début néonatal

Indiqué pour le traitement de la maladie inflammatoire multisystémique à début néonatal chez les enfants > 8 mois et > 10 kg.

Dose initiale : 1-2 mg/kg SC DIE, administrer la dose à environ la même heure chaque jour.
Ajustement de dose : 0.5-1.0 mg/kg par augmentation
Dosage d'entretien usuelle : 3-4 mg/kg/jour
Dose maximale: 8 mg/kg/jour

Arthrite juvénile idiopathique

Données limitées
1-4 mg/kg/jour SC DIE (max: 200 mg/jour)

Appendix 2: Published review article as first co-author

Role of perinatal inflammation in neonatal arterial ischemic stroke.

Antoine Giraud [a,b,†], Clémence Guiraut [b,†], Mathilde Chevin [b,†], Stéphane Chabrier [c], Guillaume Sébire [b]

[a] EA 4607 SNA EPIS, Université Jean Monnet, Saint-Etienne, France.

[b] Child Neurology division, Department of Pediatrics, McGill University, Montreal, QC, Canada.

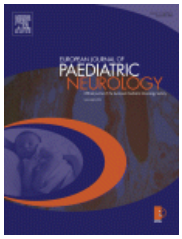
[c] French Center for Pediatric Stroke and Pediatric Rehabilitation Unit, Department of Pediatrics, Saint-Etienne University Hospital, Saint-Etienne, France.

† These authors have contributed equally as first authors

This article was submitted to Stroke, a section of the journal Frontiers in Neurology, as well as a part of an E-book entitled "Preventing developmental brain injury – from animal models to clinical trials".

Front Neurol. 2017; 8: 612.

Appendix 3: Copyright agreement for Figure 2 (CHAPTER I)



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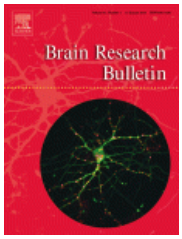

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Appendix 5: Ethical protocol 2015-7691 approval and protocol contact listing

De : eSiriusWebServer <darwin@mcgill.ca>
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À : Guillaume Sébire, Dr
Cc : Melanie Tremblay, Dr.
Objet : Darwin notification -- APPROVED GLEN FACC Annual Review for protocol 2015-7691

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***

The Annual Review for the following protocol was APPROVED:

Protocol Number: 2015-7691
Title: Interleukin-1 (IL-1) blockade along with hypothermia to prevent cerebral palsy arising from refractory neonatal encephalopathy.

eSirius

Protocol Search	Chevin, Mathilde/Sébire, Guillaume	PI

Protocol Search Results

List of Protocol Search

PI	Protocol #	Protocol Year	Protocol Title	Status	Approved	Protocol Expiry Date	3rd Yr Full Renewal Due Date
Sébire, Guillaume	2015-7675	5	Investigating the materno-fetal immune activation induced by group B Streptococcus leading to sex-specific neuro-behavioural impairments.	Approved (w/o Stipulation)	02/01/2019	02/01/2021	02/01/2022
Sébire, Guillaume	2015-7691	5	Interleukin-1 (IL-1) blockade along with hypothermia to prevent cerebral palsy arising from refractory neonatal encephalopathy.	Approved (w/o Stipulation)	02/01/2019	02/01/2021	02/01/2022
Sébire, Guillaume	2015-7699	1	Role of gestational inflammation in the physiopathology of the neonatal arterial ischemic stroke.	Un-Finished	/ /	/ /	/ /

Return to Protocol Search Filters

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Darwin Report

Protocol Contact Listing

Investigator Name	PI Email ID	PI Phone	Protocol No	Expiration Date	Protocol Status	Contact	Contact ID	Business Role	Contact Method	Phone	Cell Phone	Home Phone	Email	FACC	Department
Sébire, Guillaume	guillaume.sebire@mcgill.ca		2015-7691	02/01/2022	Approved (w/o Stipulation)	Chabrier, Stephane	260885076		Collaborator	5149341934 76124	4387285433		stephane.chabrier@mail.mcgill.ca		
Sébire, Guillaume	guillaume.sebire@mcgill.ca		2015-7691	02/01/2022	Approved (w/o Stipulation)	Chelabi, Khadidja	260789731		Other		4384997921		khadidja.chelabi@mail.mcgill.ca		
Sébire, Guillaume	guillaume.sebire@mcgill.ca		2015-7691	02/01/2022	Approved (w/o Stipulation)	Chevin, Mathilde	260698090	Researcher Staff Members	Graduate Student	5149341934 76124	819 640-3648		mathilde.chevin2@mail.mcgill.ca		
Sébire, Guillaume	guillaume.sebire@mcgill.ca		2015-7691	02/01/2022	Approved (w/o Stipulation)	Guiraut, Clémence	260688257		Research Assistant	5149341934 76124	8199435062		clemence.guiraut2@mail.mcgill.ca		
Sébire, Guillaume	guillaume.sebire@mcgill.ca		2015-7691	02/01/2022	Approved (w/o Stipulation)	Rimuhc, Sail		Animal Care	Other	76266			sail.rimuhc@mcgill.ca		

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Appendix 6: Published abstract in Perfectionnement en Pédiatrie

https://www.sciencedirect.com/science/article/pii/S2588932X2030005X

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Le syndrome d'alcoolisation fœtale (SAF) constitue la forme la plus sévère des troubles causés par l'alcoolisation fœtale (TCAF). Les enfants TCAF sont dépourvus de dysmorphies faciales permettant un diagnostic périnatal mais présentent des troubles du neurodéveloppement qui apparaitront progressivement avec l'âge. Ainsi, la majorité des TCAF échappe au diagnostic précoce. Des travaux du laboratoire ont mis en évidence l'existence d'un axe fonctionnel « placenta-cerveau » impliqué dans le contrôle de l'angiogenèse cérébrale. En particulier, l'alcool altère l'expression placentaire du PlGF et induit une désorganisation des microvaisseaux cérébraux, support de migration des interneurones GABA et des oligodendrocytes. Ces données suggèrent que l'altération de l'angiogenèse corticale induite par l'alcool pourrait contribuer au mauvais positionnement des interneurones GABA et des oligodendrocytes. De plus, en corrigeant les effets de l'alcool sur l'angiogenèse cérébrale, le PlGF pourrait être en mesure de prévenir les anomalies de migration de ces cellules nerveuses. Les données obtenues chez la Souris, révèlent que la répression placentaire du PlGF par électroporation in utero mime les effets de l'alcoolisation fœtale sur la désorganisation des microvaisseaux corticaux. À l'inverse, la surexpression placentaire de PlGF corrige les anomalies de l'angiogenèse cérébrale induites par l'alcool. Les données obtenues par Western blot indiquent que l'alcoolisation in utero affecte fortement l'expression de marqueurs du lignage oligodendrocytaire (Olig2, CNPase, MBP) à différents stades développementaux (E20, P2 et P15). De plus, l'étude morphométrique révèle une étroite interaction entre les oligodendrocytes en migration et les microvaisseaux corticaux. L'alcoolisation in utero ne modifie pas cette interaction mais altère la densité des oligodendrocytes. Ainsi, ces résultats suggèrent que les atteintes cérébrales induites par l'alcoolisation fœtale pourraient être liées à une angiogenèse anormale et potentiellement corrigées par la modulation placentaire de l'expression du PlGF.
Ethique Comité d'éthique en expérimentation animale n°54, autorisation n°01680.02.
Financements Financement doctoral ministériel, Fondation pour la Recherche en Alcoologie, Fondation Motrice, Fondation de France, FEDER, ANR et Normandie Valorisation.
Déclaration de liens d'intérêts Les auteurs n'ont pas précisé leurs éventuels liens d'intérêts.

https://doi.org/10.1016/j.perped.2020.01.004

Abstract 2

Effet neuroprotecteur de l'hypothermie dans l'infarctus cérébral artériel néonatal : une étude préclinique

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Introduction Aucun traitement n'est actuellement disponible pour l'infarctus cérébral artériel néonatal (NAIS). Les études épidémiologiques ont montré que l'infection/inflammation périnatale, l'hypoxie perpartum associées à l'occlusion d'une artère cervico-encéphalique sont les principaux déterminants du NAIS. L'effet neuroprotecteur de l'hypothermie thérapeutique (HT) dans l'encéphalopathie néonatale justifie son utilisation potentielle dans le NAIS.
Matériel et méthodes Nous avons utilisé notre modèle triple hit murin: inflammation périnatale, hypoxie et occlusion carotidienne afin de reproduire le scénario physiopathologique humain. Vingt ratons de souche Lewis ont reçu à J12 de vie—correspondant au développement cérébral du nouveau-né humain à terme—une injection intrapéritonéale (ip) de 50 µg/kg de lipopolysaccharide d'*E. coli* afin d'induire une inflammation. Quatre heures plus tard, l'artère carotide commune droite a été ligaturée puis les ratons ont été installés dans une enceinte hypoxique (FiO2 = 8%) durant 1,5 h. Dix d'entre eux ont été traités par HT (32,5 ± 0,4 °C) durant 4 h, tandis que les autres sont restés en normothermie. Dix contrôles ont reçu une injection ip de sérum physiologique suivie d'une procédure anesthésique avec incision cervicale sans ligature carotidienne et n'ont été soumis ni à l'hypoxie ni à l'HT. Les conséquences anatomiques (IRM) et fonctionnelles (TEP au 18F-FDG) ont été mesurées à J26–28. L'activité locomotrice et exploratoire des rats a été évaluée entre J20–40 (correspondant à l'adolescence chez l'humain).
Résultats L'HT (i) empêche la survenue du NAIS dans 44% des cas, (ii) réduit de 37% le volume de l'infarctus, (iii) augmente l'activité métabolique dans le territoire carotidien et (iv) améliore le comportement moteur. Les techniques morphométriques et métaboliques montrent l'effet neuroprotecteur de l'HT au sein du cortex moteur, de l'hippocampe et du striatum.
Conclusions Notre étude combinant imagerie anatomique et fonctionnelle et évaluation comportementale montre l'efficacité de l'HT dans le NAIS. Ces résultats ouvrent des perspectives quant à l'utilisation de l'HT dans le NAIS humain. L'HT étant déjà utilisée en pratique clinique, les essais seraient facilités par transfert d'indication.
Ethique The experimental protocol was approved by the Institutional Animal Care Committee of McGill University (protocol #2015–7691) in accordance with the Canadian Council on Animal Care Guidelines: http://www.ccac.ca/en_/standards/guidelines.
Déclaration de liens d'intérêts Les auteurs n'ont pas précisé leurs éventuels liens d'intérêts.

https://doi.org/10.1016/j.perped.2020.01.005

Abstract 3

Impact de l'âge des culots globulaires transfusés sur le stress oxydant de l'extrême prématuré

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