SHORT-TERM EFFECTS OF REPEATED NEONATAL ORAL SUCROSE TREATMENT AND PAIN ON HIPPOCAMPAL AND SERUM INFLAMMATORY CYTOKINE LEVELS AND MICROGLIA DENSITY IN MOUSE PUPS

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

SHORT-TERM EFFECTS OF REPEATED NEONATAL ORAL SUCROSE TREATMENT AND PAIN ON HIPPOCAMPAL AND SERUM INFLAMMATORY CYTOKINE LEVELS AND MICROGLIA DENSITY IN MOUSE PUPS

submitted by Fermin Hoq in partial fulfilment of the requirements for the degree of Master of Science in Neuroscience

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**Abstract**

**Background:** In the neonatal intensive care unit, preterm infants experience 7-17 clinically required but painful procedures daily. Oral sucrose is the standard treatment for minor procedural pain, but the combined short-term cumulative effects of sucrose treatment for pain on brain development are unknown. Using a neonatal mouse paradigm, previous studies found that during the 1st week of life, repeated pain and/or sucrose exposure impaired short-term memory and reduced regional and white matter structure volumes in adulthood, including the corpus callosum, fimbria, and the hippocampus. The objective of this study was to determine whether repeated neonatal pain and/or sucrose exposure altered pro/anti-inflammatory markers, specifically IL-1β, IL-6, and TNF-α, in hippocampal tissue and serum of 8-day old mouse pups. Hippocampal microglial density of male mouse pups was also examined.

**Methodology:** Using a previously established neonatal mouse paradigm, neonatal mice were randomly assigned to receive water or 24% oral sucrose prior to being handled or needle-pricked, 10X/day from postnatal day (P) 1-6. Blood and hippocampal tissue were collected at P8 and assayed for various cytokines (e.g. IL-1β, IL-6, and TNF-α). In addition to cytokine levels, microglial density was assessed in the hippocampus of P8 male mice.

**Results:** Although no sex effects were evident, a significant group effect was found for several inflammatory cytokines. Hippocampal IL-10 levels were significantly lower in sucrose + handling ($p<0.01$), water + handling ($p<0.05$), and water + needle-prick exposed mice ($p<0.05$) compared to controls. In serum, IL-1β levels were significantly greater in
mice that were exposed to sucrose + handling compared to controls ($p<0.01$), sucrose + needle-prick ($p<0.01$), and water + needle-prick ($p<0.01$) groups, while mouse pups exposed to sucrose + needle-prick showed significantly lower serum IL-5 levels compared to controls ($p<0.05$). Preliminary microglia density descriptive analysis showed a trend suggesting that in 8-day old males, any treatment and/or intervention exposure alter microglial cell counts in the hippocampus.

**Conclusion:** My findings add to evidence of adverse effects of neonatal repeated exposure to pain and sucrose, with implications for the use of oral sucrose for pain in clinical practice based on the neuroinflammatory responses to these early-life stressors.
Lay Summary

In Canada, over 30,000 babies are born premature each year. Due to medical necessity, these infants are admitted into the neonatal intensive care unit where they experience 7-17 minor but painful procedures daily. Upon discharge, this can accumulate to 300-400 procedures in total. Based on the negative developmental outcomes associated with early-life pain, these painful experiences must be managed. Sweet-tasting solutions, such as sucrose, are provided as temporary pain relief for preterm infants during minor painful procedures, but little is known about its safety in combination with pain, especially in accumulation. In this study, I investigated the effects of early-life sucrose treatment for pain on inflammatory markers in the brain and blood of mouse pups. I found that several inflammatory markers were altered in mouse pups exposed to sucrose and/or pain. These findings support the idea that using sweet-tasting solutions (sucrose) for pain could lead to harmful outcomes.
Preface

All experiments and analyses presented in this thesis were performed under the guidance and supervision of Dr. Manon Ranger. This thesis was revised and approved by Dr. Manon Ranger, Dr. Kiran K. Soma, and Dr. Annie V. Ciernia.

All animal experiments and procedures presented in this thesis were conducted in Dr. Ranger’s lab at the BC Children’s Hospital Research Institute (BCCHR). The neonatal mouse treatment and intervention administration were performed in the Transgenic Facility of the Centre for Molecular Medicine and Therapeutics (CMMT) at BCCHR by me alongside Dr. Rujun Kang (senior lab technician in Dr. Ranger’s lab). Brain, serum, and additional tissue collection was performed in the CMMT Transgenic Facility by me with the help of Dr. Kang. All animal work was approved by the UBC Animal Care Committee (certificate #A19-0306 and #A19-0289).

Right hemisphere hippocampus and serum cytokine levels were all assessed by me. Hippocampal microglia density staining and imaging were done by myself, while cell counts were analyzed independently by myself and Dr. Kang. All other experiments and the preparation of this thesis were completed by me.

My initial research findings were presented in oral format at the 55th Annual Meeting of the International Society for Developmental Psychobiology (ISDP) in November 2022 in San Diego (California, USA) and poster format at the 2023 Annual Scientific Meeting of the Canadian Pain Society in May 2023 in Banff (Alberta, Canada). My abstract from the ISDP meeting is published: Hoq F, Kang R, Ciernia A, Soma K, Ranger M. (2022). Alterations in Pro/Anti-Inflammatory Markers in Hippocampus and Blood After Repeated Sucrose and
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<th>Description</th>
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<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>BCCHR</td>
<td>BC Children’s Hospital Research Institute</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>Cat.</td>
<td>Catalogue</td>
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<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
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<tr>
<td>CMMT</td>
<td>Centre for Molecular Medicine and Therapeutics</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<td>E#</td>
<td>Embryonic day #</td>
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<tr>
<td>EEG</td>
<td>Electroencephalography</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELS</td>
<td>Early-life stress</td>
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<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>GA</td>
<td>Gestational age</td>
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<tr>
<td>HFCS</td>
<td>High fructose corn syrup</td>
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<tr>
<td>HFS</td>
<td>High fat and sugar</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic–pituitary–adrenal</td>
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<tr>
<td>Iba1</td>
<td>Ionized calcium-binding adaptor molecule 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IL-1β</td>
<td>Interleukin-1 beta</td>
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<td>IL-12p70</td>
<td>Interleukin 12p70</td>
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<tr>
<td>KC/GRO</td>
<td>Keratinocyte chemoattractant/human growth-regulated oncogene</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>MDI</td>
<td>Mental Developmental Index</td>
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<tr>
<td>MEG</td>
<td>Magnetoencephalography</td>
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<tr>
<td>Met</td>
<td>Methionine</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MSD</td>
<td>Meso Scale Discovery</td>
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<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
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<tr>
<td>NBRS</td>
<td>Neurobiological Risk Score</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<tr>
<td>NICU</td>
<td>Neonatal intensive care unit</td>
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<tr>
<td>NNS</td>
<td>Non-nutritive suckling</td>
</tr>
<tr>
<td>O.C.T.</td>
<td>Optimum cutting temperature</td>
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</table>
P#  Postnatal day
PBS  Phosphate-buffered saline solution (pH 7.4)
PBST  Phosphate-buffered saline solution (pH 7.4) with 0.3% Triton X-100
PCA  Postconceptual age
PDI  Psychomotor Developmental Index
PFA  Paraformaldehyde
PFC  Prefrontal cortex
PIPP  Premature Infant Pain Profile
RCT  Randomized control trial
RK  Dr. Rujun Kang
RMS  Repeated maternal separation
Rpm  Revolutions per minute
SAH  S-adenosylhomocysteine
SAM  S-adenosylmethionine
SH  Sucrose and handling
SN  Sucrose and needle-prick
Th1  T helper type 1 cells
Th2  T helper type 2 cells
TNF-α  Tumor necrosis factor alpha
Val  Valine
VGAT  Vesicular GABA transporter
VGLUT1  Vesicular glutamate transporter 1
VGLUT2  Vesicular glutamate transporter 1
| WH  | Water and handling |
| Wks | Weeks             |
| WN  | Water and needle-prick |
| #-G | #-gauge           |
Acknowledgements

I would like to thank Dr. Manon Ranger for taking me under her wing and providing me with the opportunity to complete my Master of Science in Neuroscience under her guidance. Dr. Ranger has provided me with several opportunities to learn, practice, and enhance my skills as a scientist and has supported my insights and ideas for future projects. Because of her guidance, I was able to produce research using novel laboratory techniques to better understand the effects of repeated neonatal sucrose and pain exposure on the brain. I would also like to extend my gratitude towards my supervisory committee: Dr. Kiran K. Soma and Dr. Annie V. Ciernia. Both Dr. Soma and Dr. Ciernia have provided me with valuable feedback and enhanced my scientific literacy throughout my graduate studies for oral and poster presentations, and for the completion of this thesis.

I would like to provide a special thank you to Dr. Rujun Kang, the senior laboratory technician of the Ranger Lab at BCCHR. Throughout my graduate studies, Dr. Kang has played a pivotal role in my research endeavors. Her expertise and guidance have been invaluable, and her ongoing support has significantly contributed to my growth and development in my academic journey and as a researcher. Without her mentorship and assistance, I would not be where I am in today.

I would like to acknowledge the Healthy Starts Catalyst Grant and Innovation Canada Grant obtained by Dr. Manon Ranger, both of which have provided financial support to undergo the projects outlined in this thesis.

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Your endless love and support have been a guiding light in my journey, and I am truly fortunate to have you in my life. Thank you for pushing me to reach my full potential and for always being there for me. Your presence and faith in me have meant the world, and I am forever grateful for your love and support.
Dedication

To my beloved family Ma, Baba, and Nohor, I am filled with profound gratitude for the unwavering support, prayers, and boundless love you have showered upon me. Words cannot capture the depth of my appreciation for all that you have done for me. Your presence in my life has been a source of strength and inspiration. I hold each of you dear to my heart, and the love I feel for you is immeasurable. Thank you for being there for me, for believing in me, and for enriching my life with your unwavering love and support. I cherish each of you dearly.
Chapter 1: Introduction

1.1 Preterm Birth Prevalence

Preterm birth is defined as any fetus born at less than 37 full weeks gestational age (GA) (World Health Organization, 2022). Preterm birth can be further classified into 3 categories: extremely preterm (<28 weeks GA), very preterm (28 to 32 weeks GA), and moderate to late preterm (32 to 37 weeks GA) (World Health Organization, 2022). Globally, the preterm birth rate is 10.6% (Chawanpaiboon et al., 2019) with most industrialized countries displaying an increasing trend in preterm birth rates (Goldenberg et al., 2008). From 2005-2014, annual preterm birth rates in Canada (excluding Quebec) ranged from 8.0-8.3% (Public Health Agency of Canada, 2017). Due to their physiological immaturity, hemodynamic instability, and inability to self-regulate (e.g. thermoregulation, respiration), preterm infants are often admitted into the Neonatal Intensive Care Unit (NICU) where they are exposed to several painful, but clinically required procedures (Dekker et al., 2021; Johnston et al., 2011; Pereira et al., 2016).
1.2 Procedural Pain in the Neonatal Intensive Care Unit

Preterm infants are born during a period where they are less able to cope with their extra-uterine environment. Over the last decade, preterm neonatal survival rates have drastically increased (Ricci et al., 2022; Synnes & Hicks, 2018). In Canada from 2004-2017, survival of preterm infants born 23-25 weeks GA increased from 7.3% to 12.9%, while survival of those born 24-26 weeks GA increased from 32% to 60% (Lee et al., 2020), but this increase is accompanied by exposure to medically required but stressful and painful procedures in the NICU (Reviewed in Cruz et al., 2016; Roofthooft et al., 2014). In the NICU, preterm infants receive an average of 7-17 clinically required but painful procedures daily (Reviewed in Cruz et al., 2016), ranging from daily routine stressful events such as adhesive removals and diaper changes, to more substantial painful interventions, including wound treatments, chest tube insertions, and lumbar punctures (Witt et al., 2016). One of the most frequent skin-breaking painful procedures experienced by preterm neonates in the NICU include heel lance and venipuncture for blood collection (Shah & Ohlsson, 2011; World Health Organization, 2010). In the NICU, newborn infants are subject to blood draws to monitor, diagnose, and manage potential illnesses, such as sepsis (Reviewed in Persad et al., 2021; Reviewed in Shah & Ohlsson, 2011). These tests provide valuable information on the health of critically ill neonates and offer insight regarding necessary treatments if results deviate from within normal limits. Therefore, these painful procedures (i.e. blood draws) are clinically required to provide adequate care for premature infants.

On average, infants born preterm experience 8 blood draws per day within the first week of life alone in comparison to full-term neonates, who experience an average of 0.7 blood draws daily (Madsen et al., 2000; Puia-Dumitrescu et al., 2019). Unfortunately, the most common
blood-drawing procedures are encountered by preterm infants at rates of 100% for heel lancing and 97.3% for venipuncture daily (Reviewed in Kassab et al., 2019). Given the drastic difference in pain exposure, specifically blood collection in preterm versus full-term neonates, it brings into question the developmental consequences of this exposure during a period of critical neural development.

**Nociceptive Development in the Preterm Infant**

Noxious stimuli are believed to modify the nervous system’s structural and functional organization in preterm and full-term term infants (Ruda et al., 2000; Reviewed in Steinbauer et al., 2022). The effects of noxious stimuli in preterm neural, structural, and functional modifications are especially concerning since preterm neonates display heightened pain sensitivity in comparison to full-term infants (Fitzgerald, 2005). Preterm neonatal sensory circuits display greater excitability and larger receptive fields that become more fine-tuned postnatally. This occurs through the refinement of afferent excitatory input and maturation of inhibitory processes (Fitzgerald, 2005). Given the presence of immature sensory circuits, pain delivered to small areas may invoke a more wide-spread sensitivity and sensation (Andrews & Fitzgerald, 1994). Translational animal models of late gestational life show that low threshold mechanosensory A fibre terminals overlap with C fibres, which are mainly responsible for nociceptive input (Fitzgerald & Beggs, 2001). This may suggest that to the human neonate, non-painful stimuli can be perceived as painful (Fabrizi et al., 2011). Although these nociceptive thresholds rise with increasing postconceptional age (Andrews & Fitzgerald, 1999; Fitzgerald, 2005), descending inhibitory input remains minimal until postnatal day 22-24 due to underdevelopment (Bremner & Fitzgerald, 2008; Fitzgerald & Koltzenburg, 1986; Heinricher et
al., 2009; Koch et al., 2012), and plastic sensory afferents, including those involved in nociception (Ren & Dubner, 2007), can be altered throughout neural circuitry development.

Pain can be considered a primitive experience in neonates. In preterm infants, nociceptive activity is processed at lower processing levels of the central nervous system in regions of the brainstem and spinal cord (Reviewed in Brewer & Baccei, 2020; Reviewed in Fitzgerald, 2005). It has been established through electroencephalography (EEG) that preterm infants can perceive noxious stimuli beginning at 29-30 weeks GA (Reviewed in Lee et al., 2005), but the afferent neurons that detect noxious stimuli and innervate the spinal cord are present as early as embryonic day 14 in rodents, and between the 8-19th week of gestation in humans (Anand & Hickey, 1987; Jackman & Fitzgerald, 2000). Functional thalamocortical connections are required for fetal awareness of noxious stimuli (Reviewed in Brewer & Baccei, 2020; Reviewed in Fitzgerald, 2005). Although thalamocortical connections begin around 24 weeks gestation, these connections are functionally immature and remain in the subplate zone for a period of time. From 24-26 weeks GA, thalamocortical axons begin entering the cortical plate and creating synapses with layer IV cortical neurons, and by 36 weeks, a dramatic increase in associative connections commence with the cortex. Thus, as the nervous system develops, nociceptive signals reach higher-order processing and can be discriminated from other types of sensory inputs by postnatal day 14 (Reviewed in Brewer & Baccei, 2020; Chang et al., 2016; Reviewed in Ranger et al., 2021). However, prior to this critical developmental milestone, infants will have difficulties discerning between various types of sensory input due to their immature neural circuitry (Fabrizi et al., 2011). Given preterm infants demonstrate underdeveloped descending inhibitory inputs, exposure to noxious stimuli in early life can have adverse effects on the developing brain (e.g. decreased functional connectivity) (Tortora et al., 2019), and with a
greater rate of hospitalization in the NICU for this population, exposure to repeated early life noxious (i.e. painful) stimuli may pose negative short- and long-term neurodevelopmental impacts, requiring further investigation.

*Animal models of early-life stress*

Animal models of early-life stress (ELS) have been created to reproduce aspects of early-life stress experienced by humans. Several rodent models of ELS exist, including prenatal stress exposure (Lemaire et al., 2000), chronic ELS (Ivy et al., 2010), limited bedding and nesting (Reviewed in Walker et al., 2017), prenatal alcohol exposure (Reviewed in Bodnar & Weinberg, 2013; Raineiki et al., 2017), and chronic or periodic maternal separation (Huot et al., 2002; Nishi, 2020). Importantly, neonatal procedural pain in rodent pups is also considered an early-life stressor and is suggested to adversely influence neurodevelopment (Reviewed in Mooney-Leber & Brummelte, 2017).

When rats and mice are born, they are neurologically immature and completely dependent on their mothers (Biran et al., 2012). The first week of brain development in rodents (P0-P7) corresponds to preterm cerebellar, striatum, limbic, and glial development in the human neonate from 24-32 weeks GA (Biran et al., 2012; Semple et al., 2013). The neurodevelopmental trajectory and maternal dependency of rodents mimic development in preterm infants, making them a reliable preclinical model to study conditions in human preterm neonates (Tremblay et al., 2017). Based on the parallel neurodevelopmental trajectory in mice and preterm infants, using mice as an animal model to explore the consequences of repetitive procedural pain and non-pharmacological pain management on the developing brain can provide further insight on the clinical outcomes of sucrose use for procedural pain in the NICU.
The following section will provide an overview on the use of oral sucrose, a non-
pharmacological analgesic and the recommended standard of care in NICUs worldwide, for the
management of mild to moderate procedural pain (Reviewed in Stevens et al., 2016).
1.3 Neonatal Pain Management and Oral Sucrose Use in Preterm Infants

Given the plasticity of sensory afferents, larger receptive fields in preterm neonates, and hypersensitivity to sensory stimuli (Andrews & Fitzgerald, 1994; Fitzgerald, 2005; Ren & Dubner, 1996), repeated pain experienced in early life may have long-lasting developmental impacts, making early pain exposure critical to mitigate. The optimal method of pain management is to reduce the total number of painful procedures experienced by neonates, but this may not be feasible for preterm infants in the NICU who receive medically required stressful and possibly painful procedures (Witt et al., 2016).

To manage procedural pain, oral sucrose is considered the standard of care worldwide and is used as a non-pharmacological analgesic for minor procedural pain relief (Reviewed in Stevens et al., 2016, 2018). Research on the safety of sucrose in managing pain of preterm infants is often focused on isolated painful events (e.g. heel lance, venipuncture), and its safety and efficacy have been reported if administered only for isolated procedures (Reviewed in Stevens et al., 2016). Newborns that are provided sucrose for painful procedures display lower mean pain scores than those provided a placebo (Taddio et al., 2008), and preterm infants given oral sucrose prior to a heel lance or venipuncture display significant reductions in crying time and in pain behaviours, as assessed by their Premature Infant Pain Profile (PIPP) score compared to infants given no intervention or placebo (Reviewed in Stevens et al., 2016).

In rats, sucrose-induced analgesia emerges at postnatal day (P) 3, peaks between P7-P13, then declines until absent at P17 (Anseloni et al., 2002). However, it is difficult to discern whether sucrose provides a true analgesic effect involving developmentally mature antinociceptive brain structures, or whether its gustatory effects on the immature rat pup dampen behavioural pain responses. In P10 rat pups, intraoral infusion of sucrose activated ascending
gustatory pathways and regions of the brainstem involved in antinociception, including the rostroventrolateral medulla, periaqueductal grey, locus coeruleus, and the nucleus raphe magnus (Anseloni et al., 2005). However, a P10 rodent model will have bypassed the developmental trajectory of a preterm neonate (Biran et al., 2012; Semple et al., 2013), thus sucrose activation of these antinociceptive regions from a preterm-equivalent standpoint (i.e. P0-P7) remains unclear. It has also been suggested that sucrose mediates the effects of pain through endogenous opioid pathways, which are activated by sweet tastes (Blass & Watt, 1999). The belief of sucrose’s effects via opioid-mediation are based on taste-induced analgesia in humans and animal models. Behavioural analgesic effects are terminated immediately after suckling (an orotactile form of analgesia) has ceased in contrast to oral sucrose, whose behavioural analgesic effects are maintained even after ingestion is complete (Blass et al., 1989; Smith et al., 1990). In women who were on methadone during pregnancy, their newborn infants fail to exhibit taste-induced analgesia (i.e. a reduced behavioural response to pain) and were not calmed by oral sucrose (Blass et al., 1994). In rodent models, it has been established that young and adult rats provided with oral sucrose solution exhibit a reduced behavioural pain response after a tail-flick test. Of these rats, those provided with naloxone, a non-selective opioid receptor agonist, or with the μ1-opioid receptor antagonist naloxonazine, followed by oral sucrose treatment, displayed a reduced sucrose-induced behavioural pain response after the tail-flick test (de Freitas et al., 2012; Rebouças et al., 2005).

Although clinical studies suggest sucrose to dampen the behavioural pain response (Reviewed in Stevens et al, 2016), little is known about the safety of repeated exposure to sucrose on neurodevelopment in preterm neonates, especially in very preterm infants born <28 weeks GA (Johnston et al., 2002). Importantly, no studies have shown any neuroprotective
effects of sucrose in this population and there is a growing concern about the short- and long-term effects of sucrose exposure in preterm infants (Holsti & Grunau, 2010; Keels et al., 2016). This existing knowledge gap requires a need for mechanistic research to understand how sucrose interacts with pain to affect brain development.
1.4 Developmental Impacts of Neonatal Pain Exposure and/or Oral Sucrose

Early pain experiences have been linked to several adverse neurodevelopmental outcomes in preterm infants (Brummelte et al., 2012; Chau et al., 2019; Grunau et al., 2005; Holsti et al., 2006; Ranger et al., 2013). Although oral sucrose administration in humans is suggested to dampen an infant’s behavioural response to pain, the cerebral nociceptive response in the brain shows no such evidence (Slater et al., 2010). Oral sucrose reduces behavioural pain scores, but it is possible that the calming effects are based on sedative properties rather than analgesic (Reviewed in Stevens et al., 2016). Physiological stability and reduced cortisol levels fail to be achieved using oral sucrose to treat procedural pain in the first week of life (Boyer et al., 2004; Reviewed in Stevens et al., 2016). Cortical responses (i.e. electroencephalography data) in newborn infants also fail to display dampened nociceptive-specific regional responses when oral sucrose was administered for a heel lance procedure (Slater et al., 2010). Most significantly, oral sucrose administration for procedural pain in preterm infants was shown to increase plasma adenosine triphosphate oxidative stress markers, which may contribute to cellular injury (Asmerom et al., 2013).

To the best of my knowledge, there is only one clinical study examining the short-term effects of sucrose administration during the first week of life in very preterm infants. Johnston and colleagues (2002) found that for preterm infants born <31 weeks postconceptional age, a greater number of sucrose doses predicted reduced alertness and orientation at 36 weeks, and poorer motor development and vigor at 36 and 40 weeks. More recently, greater exposure to glucose (another oral sugar solution) for procedural pain in very preterm neonates <28 weeks GA
was associated with poorer mental and psychomotor development at 18 months corrected age (Schneider et al., 2018).

Sucrose and/or pain exposure have also been linked to long-term adverse outcomes. In humans, a greater number neonatal invasive procedures were correlated with smaller amygdalar, thalamic, and hippocampal subregional volumes at 8 years of age (Chau et al., 2019). Rodent models further substantiate adverse outcomes of neonatal pain and/or sucrose exposure. Tremblay and colleagues (2017) found that the volume of white matter structures (corpus callosum, fimbria), the cerebellum, and the hippocampus were reduced with exposure to early-life repeated sucrose for procedural pain compared to controls. Behavioural testing in adult mice also revealed that neonatal repetitive sucrose exposure is correlated with poorer short-term memory compared to controls (Ranger et al., 2019). Therefore, the effects of repeated procedural pain in combination with sucrose exposure creates further concern over their cumulative effects on short- and long-term developmental, structural, and behavioural outcomes. Nevertheless, research exploring the underlying mechanisms linked to these adverse outcomes have not yet been investigated.

Tables 1.1 and 1.2 provide an overview of studies investigating pain and/or sucrose (or other sugar) exposure and their short- and long-term effects in clinical studies and animal models. These tables are not meant to provide an exhaustive review of literature in the field, rather they present seminal pre-clinical and clinical studies investigating the effects of pain and/or sucrose on neurodevelopment in these populations.
Table 1.1 Short and long-term effects of neonatal pain and/or sucrose (or glucose) exposure in humans.

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<thead>
<tr>
<th>Author, Year</th>
<th>Study Design</th>
<th>Participants</th>
<th>Methods</th>
<th>Results</th>
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<tr>
<td><strong>HUMAN SHORT-TERM EFFECTS</strong></td>
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<tr>
<td>Fitzgerald et al., 1989</td>
<td>Randomized control trial (RCT)</td>
<td>Preterm infants 27-32 wks GA n=17</td>
<td>3 treatment groups: (1) control group, (2) heel lance only, (3) heel lance and EMLA (anesthetic) cream</td>
<td>Greater frequency of heel lances resulted in hypersensitivity and sensitization in the region of the heel lance compared to controls</td>
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<tr>
<td></td>
<td></td>
<td>No sex specified</td>
<td>Heel lance provided every 4 hours for 1-3 days on one foot</td>
<td>Flexion reflex threshold is reduced to half of the control group when tested on the heel-lanced foot</td>
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<td></td>
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<td>Flexor reflex test on the plantar surface of the foot that received the heel lance</td>
<td>EMLA group reduced hypersensitivity and increased the flexion reflex threshold</td>
</tr>
<tr>
<td>Grunau et al., 2005</td>
<td>Crossover trial</td>
<td>Preterm infants &lt;32 wks GA n=87</td>
<td>Number of painful procedure (heel lance)</td>
<td>In infants ≤28 wks GA, greater frequency of neonatal procedural pain exposure correlated with reduced cortisol response during stress and reduced NFSC score at 32 wks post conceptual age</td>
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<tr>
<td></td>
<td></td>
<td>Males (54%)</td>
<td>Neonatal Facial Coding System (NFCS), plasma cortisol to stress, and cardiac reactivity to pain taken before, during, and after blood collection</td>
<td>Repeated neonatal exposure to procedural pain was associated with down-regulated hypothalamic-pituitary-adrenal (HPA) axis activity</td>
</tr>
<tr>
<td>Smith et al., 2011</td>
<td>Prospective study</td>
<td>Preterm infants &lt;30 wks GA n=44</td>
<td>Repeated stressful procedures and interventions (e.g. nursing activities, peripheral venous and arterial access, central vascular access, ventilation, medical procedures, surgery)</td>
<td>Greater frequency of neonatal stressors was associated with reduced width of frontal and parietal lobes, alterations in temporal lobe functional connectivity, and abnormal motor and neurobehavioural development</td>
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<td></td>
<td></td>
<td>Males (44%)</td>
<td>Neonatal Intensive Stressor Scale</td>
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<td>Magnetic resonance imaging (MRI)</td>
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<td>Author, Year</td>
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| Brummelte et al., 2012 | Prospective  | Preterm infants 24-32 wks GA n=86 Males (48%) | • Repeated skin-breaking procedures (e.g. heel lance, intravenous/central line insertion)  
  • White matter fractional anisotropy  
  • N-acetylaspartate to choline ratios (NAA/choline) | • Higher frequency of repeated neonatal procedural pain was associated with reduced white matter fractional anisotropy and subcortical gray matter NAA/choline |
| Johnston et al., 2002 | Prospective  | Preterm infants <31 wks postconceptional age (PCA) n=107 No sex specified | • Repeated invasive procedures (e.g. heel lance) or noninvasive but uncomfortable procedures (e.g. endotracheal tube suctioning)  
  • 24% oral sucrose or sterile water for each procedure  
  • Neurobehavioral assessments at 32-, 36-, and 40-wks PCA  
  • Neonatal Acute Physiology  
  • Neurobiological Risk Score (NBRS) at 2 weeks and discharge | • Greater sucrose exposure predicted reduced motor development and vigor at 36- and 40-wks PCA, lower alertness and orientation at 36-wks PCA, and greater NBRS scores at 2 wks PCA  
  • Greater frequency of invasive procedures predicted elevated NBRS scores at 2 wks PCA and at discharge in water group |
| Asmerom et al., 2013  | RCT          | Preterm neonates <31 wks GA n=131 Males (52%) | • 3 groups: (1) controls, (2) heel lance + placebo + non-nutritive sucking (NNS), and (3) heel lance + sucrose + NNS  
  • Plasma markers of ATP degradation (hypoxanthine, xanthine, and uric acid) and oxidative stress (allantoin) immediately prior to and after heel lance  
  • Pain – Premature Infant Pain Profile (PIPP) | • Hypoxanthine and uric acid were significantly elevated in neonates who received sucrose with heel lance  
  • Significant correlation between greater PIPP scores and plasma allantoin levels in neonates who received sucrose with heel lance |
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<th>Author, Year</th>
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<tr>
<td>Grunau et al., 2009</td>
<td>Prospective cohort study</td>
<td>Infants at 8 and 18-months corrected age born ≤32 wks GA or full term</td>
<td>• Number of skin-breaking procedures (e.g. heel lance)</td>
<td>Greater number of skin-breaking procedures were associated with poorer cognition and motor function at 8 and 18-months corrected age</td>
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<tr>
<td></td>
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<td><em>n</em>=116 (8 months), <em>n</em>=102 (18 months)</td>
<td>• Neurodevelopment (cognitive and psychomotor)</td>
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<td></td>
<td></td>
<td>Males (48%)</td>
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<tr>
<td>Ranger et al., 2013</td>
<td>Prospective cohort study</td>
<td>7-8 years born very preterm (24-32 wks GA)</td>
<td>• Number of skin breaking procedures (e.g. heel lance)</td>
<td>Greater neonatal pain-related stress was associated with reduced cortical thickness in various cerebral regions, most notably the frontal and parietal lobes</td>
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<td></td>
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<td><em>n</em>=42</td>
<td>• Cortical thickness (MRI)</td>
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<td>Males (38%)</td>
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<tr>
<td>Doesburg et al., 2013</td>
<td>Prospective cohort study</td>
<td>7 years born very preterm (24-32 wks GA)</td>
<td>• Number of skin-breaking procedures</td>
<td>Alterations in background cortical rhythmicity were associated with cumulative neonatal pain-related stress</td>
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<tr>
<td></td>
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<td><em>n</em>=54</td>
<td>• Functional brain activity (magnetoencephalography [MEG])</td>
<td>Alterations in spontaneous brain oscillations were negatively correlated with visual-perceptual abilities</td>
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<tr>
<td></td>
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<td>Males (61%)</td>
<td>• Visual-perceptual abilities</td>
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<td>Author, Year</td>
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<tr>
<td>Pain only</td>
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| Vinall et al., 2014 | Prospective cohort study | 7 years born very preterm (24-32 wks GA) n=50 Males (42%) | • Number of invasive procedures  
• Child cognition  
• White matter fractional anisotropy (MRI) | • Greater frequency of invasive procedures was associated with reduced white matter fractional anisotropy |
| Brummelte et al., 2015 | Prospective cohort study | 7 years born very preterm (24-32 wks GA) n=77 Males (45%) | • Total number of skin-breaking procedures from birth to term corrected age  
• Saliva samples to measure cortisol levels  
• Diurnal cortisol at home for two non-school days | • Greater neonatal procedural pain-related stress was associated with lower cortisol levels on the day of testing and reduced diurnal cortisol at home, primarily in boys |
| Gaspardo et al., 2018 | Retrospective study | Toddlers aged 18-36 months born <34 wks GA, <1,500g n=62 Males (49%) | • Number and type of painful procedures during hospitalization in the NICU: extremely stressful, very stressful, slightly stressful  
• Total neonatal pain-related stress  
• Temperament | • Greater neonatal pain-related stress was associated with reduced effortful control temperament in toddlers |
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| Chau et al., 2019    | Longitudinal study        | ~8 years old born very preterm (<32 wks GA)  
|                      |                            | $n=57$                                                                        | • Number of neonatal invasive procedures  
• Hippocampal, amygdala, and thalamic volumes (MRI)  
• Genotyping for the catechol-O-methyltransferase ($COMT$) $Val^{158}Met$ (rs4680) variant and brain derived neurotrophic factor (BDNF) $Val^{66}Met$ (rs6265) variant  
• Cognitive performance, behaviour, and visual-motor outcomes | • Greater frequency of neonatal painful procedures was associated with reduced amygdala and thalamic volumes  
• Interaction with the $COMT$ genotype predicted reduced hippocampal subregional volumes  
• Greater number of surgeries, days of ventilation, and lower GA correlated to reduced subcortical volumes  
• Reduced regional brain volumes were associated with poorer cognitive, visual-motor, and behavioural outcomes |
| Schneider et al., 2018 | Prospective study        | Infants at 18-months corrected age born very preterm (<30 wks GA)  
|                      |                            | $n=51$                                                                        | • Number of invasive procedures  
• 30% oral glucose 4x/day  
• Regional and total brain volume (MRI)  
• Functional connectivity (resting-state fMRI)  
• Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDi) | • Greater frequency of invasive procedures was associated with slower growth of the thalamus, basal ganglia, and total brain volumes in females  
• Greater frequency of invasive procedures was negatively associated with functional connectivity between the thalamus and the sensorimotor cortices  
• Greater frequency of procedural pain and glucose resulted in poorer MDI and PDI scores in females |
Table 1.2 Short and long-term effects of neonatal pain and/or sucrose exposure in rodents.

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<th>Author, Year</th>
<th>Study Design</th>
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<tr>
<td><strong>RODENT SHORT-TERM EFFECTS</strong></td>
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<td><em>Pain only</em></td>
<td>Mooney-Leber et al., 2018</td>
<td>RCT Sprague-Dawley rat pups (P4) Exact n not specified No sex specified</td>
<td>• Repetitive painful procedures (needle-prick) from P1-P4 • Maternal separation for 30 minutes • Frontal cortex and hippocampal neurotransmitter and brain metabolite analysis (magnetic resonance spectroscopy) • Serum corticosterone</td>
<td>• Neonatal pain and maternal separation increased serum corticosterone and reduced glutamate levels in the frontal cortex and hippocampus</td>
</tr>
<tr>
<td><em>Sucrose only</em></td>
<td>Beilharz et al., 2016</td>
<td>RCT Sprague-Dawley rats (P13-15) n=36 (12 rats/group) Males (100%)</td>
<td>• Access to 1) chow (controls), 2) chow with 10% sucrose solution, or 3) chow diet high in fat and sugar (HFS diet) • Object and place recognition (memory) • mRNA expression of pro-inflammatory cytokines (interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α) (RT-PCR)</td>
<td>• HFS diet or sucrose diet alone impaired hippocampal-dependent memory in the first week on the diet • Sucrose diet elevated IL-1β and TNF-α in white adipose tissue compared to the HFS diet • In the hippocampus, sucrose diet displayed higher IL-1β expression than controls and higher TNF-α expression than the HFS diet</td>
</tr>
<tr>
<td><em>Pain and Sucrose</em></td>
<td>Anseloni et al., 2002</td>
<td>RCT Sprague-Dawley rat pups (P0-P21) n=131 No sex specified</td>
<td>• 7.5% intraoral sucrose solution • Complete Freund’s adjuvant (CFA) to induce inflammation • Thermal stimuli (thermal withdrawal response). • Mechanical stimuli (cutaneous flexion withdrawal response)</td>
<td>• Intraoral sucrose elevated withdrawal latencies in inflamed animals exposed to mechanical stimuli, and thermal fore- and hindpaw stimulation • Intraoral sucrose significantly reduced hyperalgesia in inflamed rats that received thermal stimulation on fore- and hindpaw compared to naïve rats</td>
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<td>Author, Year</td>
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<td>Anand et al., 2007</td>
<td>RCT</td>
<td>Neonatal Long-Evans hooded rat pups  n=71  Males (35%)</td>
<td>• Paw-pad stimulation using needle-prick or cotton tip 1X, 2X, or 4X/day from P0-P7  • Cognition (radial arm maze)  • Neuroactivation (Fos protein) and cell death (FluoroJade-B)</td>
<td>Repetitive neonatal pain was associated with heightened neuronal excitation and greater cell death in several cortical and subcortical areas, including the amygdala and hippocampus at P1 and P7</td>
</tr>
<tr>
<td>Mooney-Leber &amp; Brummelte, 2020</td>
<td>RCT</td>
<td>Adult Sprague-Dawley rats (P79-P126)  n=79  Males (48%)</td>
<td>• Repetitive painful procedures (needle-prick) from P1-P4  • Behavioural testing (e.g. open field test, Morris water maze, restraint stress testing)  • Serum corticosterone (ELISA)</td>
<td>Early life pain enhanced spatial learning regardless of sex  In females, early life pain altered HPA recovery from an acute stressor</td>
</tr>
<tr>
<td>Timmerman et al., 2021</td>
<td>RCT</td>
<td>Adult Sprague-Dawley rats (P8 or P104-126)  n=160  Males (50%)</td>
<td>• Randomized into 5 groups: 1) touch control, 2) pain, 3) touch + isolation, 4) pain + isolation, 5) unhandled  • Painful procedure and maternal separation protocol described by Mooney-Leber et al. (2018)  • Reelin and cell proliferation marker (Ki67)</td>
<td>Exposure to either neonatal pain or maternal isolation individually increased reelin levels in the dentate gyrus of adult females</td>
</tr>
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<td>Author, Year</td>
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| Hsu et al., 2015     | RCT          | Adolescent (P30) and adult (P60) Sprague-Dawley rats  
\(n=38\) (adolescent),  
\(n=38\) (adult)  
Males (100%) | • 30-day access to (1) 11% sucrose solution, (2) 11% high fructose corn syrup (HFCS-55) solution, or (3) an extra bottle of water (control)  
• Spatial learning and memory, anxiety  
• Brain and liver IL-1β and interleukin-6 (IL-6) protein levels (Immunoblotting and ELISA) | • In adolescent rats, sucrose and HFCS-55 intake impaired hippocampal-dependent spatial learning and memory  
• IL-1β and IL-6 were increased in the dorsal hippocampus of adolescent rats given HFCS-55 relative to controls  
• Liver IL-1β levels were elevated in adolescent-exposed sucrose and HFCS-55 rats |
| Ramirez-Contreras et al., 2021 | RCT          | C57BL/6J mice (16 weeks)  
\(n=7-10\) mice/group/sex  
Males (50%) | • Neonatal mice received sterile water or 24% oral sucrose; 10x/day from P1-P6  
• Tissue collection at wk 16  
• Growth assessment (body weight)  
• Insulin-like growth factor-1 (IGF1) and fibroblast growth factor (FGF21)  
• Liver water-soluble choline metabolites (e.g. free choline, betaine)  
• Liver S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations | • Sucrose-treated mice gained less weight during the treatment period and were smaller at weaning than water-treated mice  
• Adult sucrose-treated females had smaller tibias and lower serum IGF-1 than adult water-treated females  
• Adult sucrose-treated mice had lower liver SAM, phosphocholine, and glycerophosphocholine  
• Sucrose-treated females had lower liver free choline and higher liver betaine compared with water-treated females |
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<th>Author, Year</th>
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<tr>
<td>Nuseir et al., 2015</td>
<td>RCT</td>
<td>• Chronic pain with needle-prick on paws from P0 to postnatal week 8</td>
<td>• Chronic pain impairs short-term memory, but oral sucrose prevents this impairment</td>
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<tr>
<td></td>
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<td>• Sucrose or paracetamol analgesia prior to needle-prick</td>
<td>• Sucrose increases serum β-endorphin and enkephalin</td>
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<tr>
<td></td>
<td></td>
<td>• Spatial learning and memory</td>
<td>• Chronic pain decreases hippocampal BDNF, but oral sucrose use prevents this reduction</td>
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<td>• Pain withdrawal reflex</td>
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<td></td>
<td>• Brain and blood neurotrophin and endorphin (ELISA)</td>
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<tr>
<td>Tremblay et al., 2017</td>
<td>RCT</td>
<td>• Mouse pups received sterile water or 24% oral sucrose 2 minutes prior to interventions: needle-prick, tactile pressure, or handling; 10x/day from P1-P6</td>
<td>• Mice that received sucrose prior to a needle-prick exhibited the smallest brain volumes in white matter structures (corpus callosum, fimbria), cerebellum, and hippocampus</td>
</tr>
<tr>
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<td>C57BL/6J mice P85-P95</td>
<td>• Regional brain volumes (MRI)</td>
<td>• Needle-prick only had no effect on brain volumes</td>
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<tr>
<td></td>
<td>n=109</td>
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<td></td>
<td>Males (46%)</td>
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<td></td>
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<tr>
<td>Ranger et al., 2019</td>
<td>RCT</td>
<td>• Same experimental model as Tremblay et al., 2017</td>
<td>• Sucrose/handling group had poorer short-term memory compared to water/handling group</td>
</tr>
<tr>
<td></td>
<td>Adult C57BL/6J mice (P60-P85)</td>
<td>• Behavioural testing in adulthood (P60-P85) (e.g. Morris Water Maze, sugar preference)</td>
<td>• Sucrose group consumed less sugar-water compared to controls or mice in water/pain group in adulthood</td>
</tr>
<tr>
<td></td>
<td>n=106</td>
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<td></td>
<td>Males (47%)</td>
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1.5 The Hippocampus and Early Development

As described in prior sections, prenatal and early postnatal life are critical developmental periods for neural circuit formation through excitatory and inhibitory input, synaptogenesis, synaptic pruning, myelination, and neurogenesis (Reviewed in Malave et al., 2022). In human neonates, the last trimester of gestation involves several cellular events that guide the brain’s cortical development and anatomical organization (Dimitrova et al., 2021). However, when an infant is born prematurely (i.e. 2-4 months early), their brain development is highly malleable, making it vulnerable to external stressors (Kostović & Judaš, 2010; Malave et al., 2022). Evidence exhibits delays in cortical development (Ball et al., 2013; Dimitrova et al., 2021), reduced grey and white matter volumes (Reviewed in Counsell & Boardman, 2005; Thompson et al., 2022), and heightened vulnerability to volumetric reductions within the hippocampus (Peterson et al., 2000) in children born preterm.

The hippocampus is a subcortical structure located deep within the temporal lobe that undergoes significant changes during childhood (Anand & Dhikav, 2012; Paquette et al., 2020) and is particularly vulnerable in preterm infants (Abernethy et al., 2002), especially those that experience early-life stressors (Lammertink et al., 2022). The hippocampus is a highly plastic brain structure involved in learning, memory, and neuroendocrine responses to stress (Jacobson & Sapolsky, 1991), yet exhibits high vulnerability with the potential to be damaged by a variety of stimuli (Anand & Dhikav, 2012). The developmental trajectory of the hippocampus begins as early as gestational week eight (Bajic et al., 2010) and spans from infancy to early adolescence (Gómez & Edgin, 2016; Uematsu et al., 2012). Hippocampal development during infancy is robust and considered a critical period for neural and functional development (Uematsu et al.,
Regional hippocampal volumes continue to increase non-linearly until 9-11 years old (Uematsu et al., 2012).

During fetal development, hippocampal CA1, CA2, and CA3 regions are already distinct at 11.5 weeks GA and by the 32nd to 34th week, CA2 and CA3 neurons will have undergone rapid enlargement while synaptic pruning continues postnatally (Arnold & Trojanowski, 1996). Myelination in the hippocampus is first observed at 39 weeks and continues to develop dramatically until 9 months, whereas neuroblast migration and cytoarchitectural maturation occur later in the hippocampus versus other regions of the neocortex (Arnold & Trojanowski, 1996). In preterm neonates, rapid hippocampal development and elevated vulnerability to noxious stimuli may lead to adverse developmental consequences when exposed to early-life stressors. Adverse effects may also persist in NICU practices after repeated neonatal procedural pain, whether or not sucrose administration is involved.

Early-life stress and the hippocampus

The hippocampus is involved in cognition and emotional regulation during stress, and exposure to early-life stress (ELS) can alter hippocampal development and neural plasticity (Hanson et al., 2015; Lajud & Torner, 2015). Exposure to ELS during sensitive developmental windows can perpetuate and exacerbate its negative effects on the hippocampus by impeding proper structural and functional development in both human and rodent neonates in the short- (Anand et al., 2007; Zhang et al., 2002) and long-term (Hanson et al., 2015; Reviewed in Lajud & Torner, 2015; Teicher et al., 2012; Youssef et al., 2019). ELS, including repeated neonatal pain exposure and maternal separation, has been associated with increased apoptotic cells (Zhang et al., 2002), elevated cell death (Anand et al., 2007), and reduced volumes (Teicher et al., 2012;
Zhang et al., 2002) in the hippocampus. In addition to structural changes, stress generates a heightened inflammatory state that trigger microglia to induce alterations in the brain’s pro-inflammatory cytokine profile (Frank et al., 2007; Sugama et al., 2007). In rodents, these stress-related cytokine alterations influence neuroinflammatory responses in both male and female hippocampi (Diz-Chaves et al., 2012, 2013).

Early-life stressors experienced by preterm neonates, including repeated pain and/or sucrose exposure, also have implications on hippocampal development in humans and rodent models. In very preterm children (<32 weeks GA) with the COMT 158Met/Met minor allele, a greater number of neonatal invasive procedures was associated with smaller right hemisphere hippocampal volumes at school age after controlling for clinical neonatal risk factors (Chau et al., 2019). Thirty day exposure to either a high fructose or high sucrose diet in rodents was shown to impair hippocampal-dependent spatial learning and memory (Hsu et al., 2015). When investigating the inflammatory effects of sugar exposure, pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) were increased in the dorsal hippocampus for juvenile rats given fructose relative to controls (Hsu et al., 2015). Similar results were observed in adult rats fed a sucrose diet alone, with impaired hippocampal-dependent memory during the first week on the diet (Beilharz et al., 2016). Rats that received the sucrose-enriched diet also displayed elevated hippocampal markers of inflammation, with greater mRNA expression of interleukin-1 beta (IL-1β) compared to controls, and greater tumor necrosis factor alpha (TNF-α) expression compared to those on the high fat and sucrose combination diet (Beilharz et al., 2016).

Exposure to repeated skin-breaking procedures have already been associated with significant reductions in total and regional brain volumes with (Schneider et al., 2018; Tremblay
et al., 2017) and without (Anand et al., 2007; Brummelte et al., 2012; Chau et al., 2019; Ranger et al., 2013; Smith et al., 2011; Vinall et al., 2014) exposure to sucrose or other sugars. Moreover, the ingestion of sucrose and other sugars induce pro-inflammatory cytokine alterations in the rodent hippocampus (Beilharz et al., 2016; Hsu et al., 2015). This brings into question whether repeated sucrose exposure in combination with pain is associated with an altered inflammatory profile in the hippocampus of preterm infants and whether previously reported adverse neurodevelopmental outcomes can be linked to such alterations.
1.6 Inflammation and Pro- and Anti-Inflammatory Cytokines

Neuroinflammation is a broad term that encompass several definitions (Paolicelli et al., 2022). For the purpose of this thesis, neuroinflammation will be termed as a variety of cellular responses to tissue damage or infection that induce innate and adaptive responses from resident (e.g. Microglia) and infiltrating immune cells (e.g. Leukocytes) in the brain (Paolicelli et al., 2022). Neuroinflammatory responses occur in the central nervous system (CNS), resulting in tissue damage (DiSabato et al., 2016), recruitment of immune cells including infiltrating leukocytes and microglia (Carson et al., 2006), and cellular apoptosis (DiSabato et al., 2016). Downstream effects of neuroinflammation have been associated with decreases in synaptic function and consequently, cognitive function (Nolan et al., 2005), and such inflammatory actions are mediated by cytokines that encompass various interleukins and chemokines (Galgani et al., 2022).

Cytokines, such as IL-1β, IL-6, and TNF-α, are signalling proteins that immune cells secrete in response to inflammation that trigger both protective and damaging cellular responses (Reviewed in Arango Duque & Descoteaux, 2014; Dugue et al., 2017; Reviewed in Lumertz et al., 2022). Yet, these functions are critical in the regulation of immune responses in health and disease (Reviewed in Arango Duque & Descoteaux, 2014). In systemic circulation, cells can release cytokines directly into the blood (Zhang & An, 2007), while in the CNS, astrocytes and microglia are the primary producers (Freidin et al., 1992). There are two classes of cytokines: pro-inflammatory and anti-inflammatory (Reviewed in Arango Duque & Descoteaux, 2014). While there lacks a unanimous consensus on the divisive categorization of cytokine functions within the scientific community, this falls beyond the scope of this thesis. Nevertheless, it is important to acknowledge that some researchers caution against employing this dichotomous
classification in the context of the neuroinflammatory response to stressors. For the purpose of this thesis, I will continue to describe cytokines in accordance with their prevailing characterization, namely as pro- and anti-inflammatory.

Pro-inflammatory cytokines are involved in up-regulating inflammatory reactions, while anti-inflammatory cytokines control and modulate pro-inflammatory responses (Zhang & An, 2007). Alterations in the brain’s pro-inflammatory cytokine profile can stimulate neuronal apoptosis (Reviewed in Lumertz et al., 2022), while anti-inflammatory cytokine production is an essential mechanism required to counteract damage that transpires from the initiation of neuroinflammatory processes (Lobo-Silva et al., 2016). Although elevated pro-inflammatory states have been implicated in several pathologies, acute stressors such as acute physiological stress (Reviewed in Kim & Maes, 2003), can also result in elevated peripheral cytokine expression in healthy adult humans (Steptoe et al., 2007), which may suggest early-life stress to pose similar effects.

Early-life stress and cytokines

Early-life stress impacts the neurodevelopment of infants (Provençal & Binder, 2015) and is believed to be a risk factor for systemic inflammation in adulthood (Danese et al., 2007). Individuals who have experienced early-life stressors show altered inflammatory states and immune function in adulthood (Reviewed in Agorastos et al., 2019; Coolen & Grattan, 2019). Studies have suggested that abnormalities in neuroendocrine-immune functioning that arise from early-life stress contribute to a pro-inflammatory phenotype in adults (Elenkov, 2008; Powell et al., 2009), and may prime a more robust inflammatory response in adulthood (Romero-Sandoval et al., 2008). CNS inflammation contributes to neuronal damage, astrogliosis, and
oligodendrocyte loss, and processes involved in these functions contribute to amplified neuronal cell death (Reviewed in Magalhães et al., 2018). The adverse outcomes associated with neuroinflammation may result in life-long functional impairments in preterm neonates (Reviewed in Magalhães et al., 2018) and it is essential to determine the inflammatory molecules contributing to these consequences.

Cytokine signalling is involved in inflammatory pain, as well as other forms of early-life stress (Zhang & An, 2007). Certain pro-inflammatory molecules, including IL-1, IL-6, and TNF-α, are considered biomarkers for adverse neonatal outcomes (e.g. sepsis, necrotizing enterocolitis, length of stay in NICU) and are associated with fetal inflammatory response syndrome, a systemic inflammatory response resulting from the activation of the innate immune response (Cordeiro et al., 2016; Jung et al., 2020). A literature review found that animals who experienced early-life stress (e.g. maternal separation, limited bedding) displayed increased pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the brain but no changes with anti-inflammatory IL-10 (Reviewed in Lumertz et al., 2022). In addition, alterations in cytokine levels were observed to be most prominent in the hippocampus of adult animals that experienced extended ELS. Studies investigating the inflammatory signatures of ELS mainly focus on pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. However, various cytokines involved in systemic and neural functioning are also involved in inflammatory processes and are important for processing pathological pain (Zhang & An, 2007). Table 1.3 provides an overview of the short- and long-term effects of ELS on cytokine expression. This table does not provide an exhaustive overview of ELS on inflammatory cytokine expression in humans and rodents, rather relevant studies investigating ELS on inflammatory signaling in the brain, and the behavioural implications associated with such changes.
Table 1.3 Short and long-term effects of early-life stress on cytokine expression in humans and rodents.

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Study Design</th>
<th>Participants</th>
<th>Methods</th>
<th>Cytokines assessed</th>
<th>Results</th>
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<tbody>
<tr>
<td><strong>HUMAN CYTOKINE EFFECTS</strong></td>
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<tr>
<td>Redlich et al., 2015</td>
<td>Prospective study</td>
<td>Healthy adults $n=409$ Males (52%)</td>
<td>• Genomic DNA extraction to detect IFN-γ SNPs (rs1861494 and rs2069718) • Emotional face processing (fMRI) • Childhood trauma</td>
<td>IFN-γ</td>
<td>• IFN-γ genetic variant (rs1861494) moderated the effects of ELS on emotional processing in the amygdala, resulting in a greater neural response to emotional faces</td>
</tr>
<tr>
<td>Carpenter et al., 2010</td>
<td>Prospective study</td>
<td>Healthy adults $n=69$ Males (39%)</td>
<td>• Childhood trauma • Plasma IL-6 (high-sensitivity ELISA)</td>
<td>IL-6</td>
<td>• Moderate to severe childhood maltreatment was positively correlated with greater acute release of IL-6 and greater overall IL-6 concentrations in plasma compared to controls</td>
</tr>
<tr>
<td>Pace et al., 2006</td>
<td>Prospective study</td>
<td>Healthy adults $n=28$ Males (100%)</td>
<td>• Childhood maltreatment • Psychosocial stress • Plasma IL-6 (enzyme-linked immunosorbent assay)</td>
<td>IL-6</td>
<td>• Males with major depressive disorder who also experienced childhood maltreatment displayed exaggerated IL-6 release in response to an acute psychosocial stressor</td>
</tr>
<tr>
<td>Hartwell et al., 2013</td>
<td>Prospective study</td>
<td>Healthy adults $n=38$ Males (47%)</td>
<td>• Number of early traumatic experiences • Plasma pro-inflammatory cytokine levels (multiplex bead array assay)</td>
<td>IL-1β, IL-6, and TNF-α</td>
<td>• Greater number of early traumatic experiences were correlated with greater IL-6, IL1-β, and TNF-α levels</td>
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<tr>
<td><strong>RODENT CYTOKINE EFFECTS</strong></td>
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<tr>
<td>Pinheiro et al., 2015</td>
<td>RCT</td>
<td>Male Wistar rats $n=15$ Males (100%)</td>
<td>• Pups were exposed from P1-P14 to: i) no maternal deprivation (controls), or ii) daily 180-minute period of deprivation • Cytokine assays (cytometric bead array)</td>
<td>IL-10, TNF-α</td>
<td>• Maternal deprivation increased IL-10 and TNF-α levels in the hippocampus in adulthood</td>
</tr>
<tr>
<td>Author, Year</td>
<td>Study Design</td>
<td>Participants</td>
<td>Methods</td>
<td>Cytokines assessed</td>
<td>Results</td>
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<tr>
<td>Bodnar et al., 2016</td>
<td>RCT</td>
<td>Female Sprague-Dawley rat pups (P1, P8, and P22) n=7-11 per group Males (0%)</td>
<td>Dams randomly assigned to i) alcohol-containing liquid diet, ii) maltose dextrin (sham) liquid diet, or iii) pellet diet (control) from gestational day 0-21. Tissue collection (prefrontal cortex [PFC], hippocampus, hypothalamus, spleen, serum) at P1, P8 or P22. Multiplex cytokine measurements</td>
<td>IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, KC/GRO, and TNF-α</td>
<td>Serum TNF-α levels were higher in pups exposed to prenatal alcohol at P8 compared to sham and control diets. Hippocampal IFN-γ and IL-1β were elevated in P8 pups exposed to prenatal alcohol compared to sham and control diets. IL-4 was elevated in prenatal alcohol pups at P8 compared to controls.</td>
</tr>
<tr>
<td>Roque et al., 2016</td>
<td>RCT</td>
<td>Male Sprague-Dawley rat pups (P1-P15) n not specified Males (100%)</td>
<td>Repeated maternal separation (RMS) 3 hours/day from P1-P15. On P15, pups i) were sacrificed, or ii) experienced an acute stressor then sacrificed. Pro-inflammatory cytokine mRNA expression (ELISA)</td>
<td>IL-1β, IL-6, TNF-α</td>
<td>Animals sacrificed immediately after RMS had a 2-fold increase in hippocampal IL-1β mRNA compared to controls. After RMS and an acute stressor, IL-6 and TNF-α mRNA expression was increased in the hypothalamus.</td>
</tr>
<tr>
<td>Giridharan et al., 2019</td>
<td>RCT</td>
<td>Male Wistar rat pups (P1-P10) n not specified Males (100%)</td>
<td>RMS 3 hours/day from P1-P10. Inflammatory cytokine levels (multiplex fluorescent immunoassay)</td>
<td>IFN-γ, IL-5, IL-6, IL-10, TNF-α</td>
<td>In the PFC, IFN-γ, IL-5, IL-6, IL-10, and TNF-α were elevated in RMS compared to controls. In the hippocampus, IFN-γ and TNF-α were elevated in RMS compared to controls.</td>
</tr>
<tr>
<td>Wang et al., 2020</td>
<td>RCT</td>
<td>Male Wistar rats (P27, P76) n=80 Males (100%)</td>
<td>RMS 4 hours/day from P2-P20. Tissue collection (PFC, hippocampus) at P27 or P76. Pro-inflammatory cytokine levels (ELISA)</td>
<td>IL-1β, IL-6, TNF-α</td>
<td>At P27, RMS rats expressed elevated IL-1β and IL-6 levels in the hippocampus, and elevated IL-1β, IL-6, and TNF-α levels in the PFC compared to controls. At P76, RMS rats expressed elevated IL-1β levels in the hippocampus compared to controls.</td>
</tr>
</tbody>
</table>
Cytokines represent a large and diverse group of pro- and anti-inflammatory molecules grouped into families based on their or their receptors’ structural homology (Reviewed in Ramesh et al., 2013). The following sections (Sections 1.6.1 to 1.6.10) provide a short overview of 10 inflammatory cytokines involved in inflammatory signaling in rodents and humans as similarly investigated by Bodnar et al. (2016). These 10 cytokines were examined in this thesis project and further detailed in subsequent chapters in the hippocampus of P8 mice (Table 1.4). Although the main cytokines of interest are IL-1β, IL-6, and TNF-α, properties of all 10 cytokines, including the contexts in which they are released, their presence in the hippocampus, sex-based differences in expression, and expression in ELS will be assessed.

Table 1.4. Summary of Pro- and Anti-Inflammatory Cytokines

<table>
<thead>
<tr>
<th>Pro-Inflammatory</th>
<th>Anti-Inflammatory</th>
<th>Pro- and Anti-Inflammatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ, IL-1β, IL-2, IL-6, KC/GRO, IL-12p70, TNF-α</td>
<td>IL-4, IL-10</td>
<td>IL-5</td>
</tr>
</tbody>
</table>

Note: Cytokine categorization described above is derived from most prevalent classifications reported in the literature (Reviewed in Gordon et al., 2023; Wu et al., 2021)
1.6.1 Interleukin-1 beta (IL-1β)

IL-1β is a pro-inflammatory cytokine that plays a critical role in the body’s inflammatory response by mediating pathogen resistance (Maes et al., 1998) and exacerbating damage induced in acute tissue injury, which affects virtually all cells and organs (Garlanda & Jaillon, 2016; Lopez-Castejon & Brough, 2011). Systemically, IL-1β is produced by various cells of the immune system, including monocytes, macrophages, and B lymphocytes (Garlanda & Jaillon, 2016; Lopez-Castejon & Brough, 2011). In the brain, IL-1β is directly produced by cells in the CNS including neurons and microglia (Giulian et al., 1986; Hetier et al., 1988; Yao et al., 1992). In purified microglia cultures from second-trimester human fetal brains, inducing inflammation using lipopolysaccharides (LPS) resulted in increased IL-1β mRNA expression (Lee et al., 1993), suggesting IL-1β expression being generated by inflammatory processes.

IL-1β is widely distributed throughout the brain, particularly in the hippocampus, and plays a role in neuronal proliferation, differentiation, cell death, and long-term potentiation associated with learning and memory in the hippocampus (Dong et al., 2015; Lechan et al., 1990; Tsai, 2017). By exposing rodents to a LPS challenge to activate a peripheral immune response, hippocampal IL-1 mRNA and protein levels become elevated in the hippocampus (Layé et al., 1994). Although IL-1β has not been investigated in early life repeated exposure to pain or a combination of pain and sucrose, it has been associated with elevated expression in the hippocampi of rodents fed sugar-enriched diets (Beilharz et al., 2016; Hsu et al., 2015) and other forms of ELS (i.e. maternal separation and prenatal alcohol exposure) (Bodnar et al., 2016; Roque et al., 2016; Wang et al., 2020). In the hippocampus, P13-P15 rats exposed to an 8-day sucrose diet displayed greater hippocampal IL-1β mRNA expression compared to controls (Beilharz et al., 2016), and adolescent rats provided 30-day access high-fructose corn syrup
displayed elevated IL-1β protein levels in the dorsal hippocampus (Hsu et al., 2015). With exposure to prenatal alcohol, hippocampal IL-1β protein levels were elevated in female P8 rat pups in comparison to shams and controls (Bodnar et al., 2016). In RMS, animals sacrificed immediately after exposure had a 2-fold increase in hippocampal IL-1β mRNA expression compared to controls at P10 (Roque et al., 2016), and similar expression patterns were also observed at P27 and P76 (Wang et al., 2020). Provided the evidence, it is apparent that there is a relationship between pro-inflammatory IL-1β with sugars and ELS independently.

As with several cytokines, it has also been suggested that IL-1β expression in the hippocampus is sex-dependent, and adult female mice displayed elevated IL-1β mRNA expression after experiencing chronic adolescent stress post-LPS administration, but not males (Bekhbat et al., 2019).
1.6.2 Interleukin 6 (IL-6)

IL-6 is a well-studied pro-inflammatory cytokine (Maes et al., 1998; Ng, 2003) and systemically, is involved in inducing the maturation of B-cells into antibody-generating cells involved in the body’s immune response (Erta et al., 2012). IL-6 is also associated with the production of red blood cells, bone metabolism, and embryonic development (Hirano, 2021). In the CNS, neurons, astrocytes, and microglia are involved in IL-6 production and in response to inflammation and injury, these cells will release IL-6 in abundance (Erta et al., 2012).

Alongside IL-6 production, microglia also respond directly to IL-6 stimulation, resulting in heightened microglial reactivity (West et al., 2019). It has been shown that inducing inflammation using LPS resulted in increased IL-6 mRNA expression in purified microglia cultures from second-trimester human fetal brains (Lee et al., 1993).

IL-6 expression is also implicated in hippocampal growth and developmental delays. IL-6 mRNA has been found in rat hippocampal neurons and Purkinje cells, and is suggested to contribute to hippocampal function and neuronal differentiation (Gadient & Otten, 1994). High serum IL-6 levels in adults have also been associated with reduced hippocampal volumes (Kakeda et al., 2018) and elevated plasma IL-6 protein levels in preterm neonates correlated with later developmental delays (Leviton et al., 2013; O’Shea et al., 2012, 2013; Silveira & Procianoy, 2011). IL-6 production is also heightened in several chronic inflammatory diseases, including Parkinson’s disease, amyotrophic lateral sclerosis (ALS), and traumatic brain injuries (Kumar et al., 2016; Müller et al., 1998; Sekizawa et al., 1998).

Sugar-enriched diets have been associated with alterations in IL-6 protein levels. Adolescent rats provided with 30-day access high-fructose corn syrup displayed elevated IL-6 protein levels in the dorsal hippocampus compared to controls (Hsu et al., 2015). With ELS
exposure, elevations in hippocampal IL-6 levels are most notable during repeated maternal separation (RMS) (Giridharan et al., 2019; Roque et al., 2016; Wang et al., 2020). At P10 and P27, RMS exposure was correlated with elevated IL-6 mRNA expression in the PFC of rats (Giridharan et al., 2019; Wang et al., 2020). RMS also elevated IL-6 mRNA expression in the rat hippocampus at P27 (Wang et al., 2020). Although a direct relationship has not been investigated in early life pain and/or sucrose exposure, IL-6 sensitivity to ELS may suggest alterations in expression under these conditions.

Sex-based differences in IL-6 expression have been noted in human and animal studies, where plasma IL-6 levels at baseline are elevated in adult women compared to men (O'Connor et al., 2007), and rodent models displayed greater IL-6 expression in the female ventral hippocampus compared to males (Porcher et al., 2021).
1.6.3 Tumor necrosis factor alpha (TNF-α)

TNF-α is one of the most well-studied pro-inflammatory cytokines implicated in several inflammatory pathways (Maes et al., 1998; Ng, 2003). TNF-α is involved in cell survival, proliferation, differentiation, and apoptosis (Wang & Lin, 2008). Systemically, it is produced by macrophages and monocytes in response to acute inflammation, resulting in cellular apoptosis (Idriss & Naismith, 2000). However, B cells and natural killer cells of the immune system can also produce TNF-α in limited quantities (Sellati & Sahay, 2014). In the CNS, TNF-α is involved in regulating the permeability of the blood-brain-barrier, synaptic plasticity, and injury-mediated microglial activation (McCoy & Tansey, 2008; Merrill, 1991). It is also implicated in Alzheimer’s disease, depression, and anxiety (Clark et al., 2010).

In the brain, TNF-α is predominantly produced by neurons and glial cells, especially microglia (Muhammad, 2020). In purified microglia cultures from second-trimester human fetal brains, inducing inflammation using LPS resulted in increased levels of TNF-α mRNA expression (Lee et al., 1993). It is also involved in the morphological development and functioning of the hippocampus (Golan et al., 2004). Young mice deficient of TNF-α exhibited accelerated maturation in the dentate gyrus, smaller dendritic trees in the CA1 and CA3 regions of the hippocampus, and significant improvements in spatial memory-based behavioural task performance (Golan et al., 2004). After experiencing peripheral nerve injury, TNF-α is also up-regulated in the hippocampus of adult male mice while brain derived neurotrophic factor (BDNF), a protein involved in the regulation of synaptic plasticity, is reduced (Liu et al., 2017). Peripheral nerve injury has been associated with memory deficits in rodents due to the upregulation of TNF-α in the hippocampus (Ren et al., 2011). Because upregulation of TNF-α is associated with reductions in BDNF expression (Xu et al., 2015), it is possible that the observed
memory deficits linked to the reduction in BDNF levels are influenced by TNF-α expression. Adult mice that received sucrose (with or without pain exposure) in their first week of life also displayed poorer memory compared to controls (Ranger et al., 2019). Although it is yet to be investigated, memory deficits associated with sucrose exposure may also be associated with changes in TNF-α expression in the hippocampus induced by repeated oral sucrose exposure.

Elevated TNF-α mRNA and protein levels have been associated with sucrose exposure and ELS. In rats provided a sucrose-enriched diet, hippocampal TNF-α is significantly increased compared to controls (Beilharz et al., 2016). RMS exposure for the first two weeks of life in neonatal rats also correlated with elevated TNF-α levels in the PFC at P10, and heightened mRNA expression in the hypothalamus at P15 and hippocampus in adulthood (Giridharan et al., 2019; Pinheiro et al., 2015; Roque et al., 2016).

TNF-α also exhibits sex-based differences in expression, with greater astrocytic TNF-α expression in adult females compared to males in adulthood after LPS stimulation in human astrocytes (Goldstein et al., 2021).
1.6.4 Interleukin 5 (IL-5)

Systemically, IL-5 has been implicated in several inflammatory processes including eosinophil maturation, proliferation, activation, and migration (Reviewed in Pelaia et al., 2019). Systemic changes in IL-5 expression have also been associated with major depressive disorder, as well as chronic pain (Elomaa et al., 2012; Merriwether et al., 2021).

In the brain, IL-5 has been implicated in inducing cellular proliferation and upregulating microglial metabolism (Liva & de Vellis, 2001). The role of IL-5 in brain injury is still unknown, but elevated expression was observed during apoptosis initiating inflammation in rodent models (Siva Sai Sujith Sajja et al., 2014). In cultured mouse microglia, stimulation using pro-inflammatory IFN-γ triggered the production of IL-5 mRNA, yet there was an absence of IL-5 receptors in microglia, suggesting that IL-5 production in the brain modulates the interaction between brain cells and immune cells in the CNS (Sawada et al., 1993). In animal models of chronic pain, it has been suggested that IL-5 acts as an anti-inflammatory cytokine and its release induces analgesic effects (Merriwether et al., 2021).

Alterations in the expression of IL-5 mRNA and protein are also associated with ELS exposure, where RMS-exposed rat pups expressed elevated IL-5 mRNA expression at P10 compared to controls (Giridharan et al., 2019). Furthermore, female rats exposed to prenatal alcohol in the PFC exhibited elevated IL-5 levels at P8 (Bodnar et al., 2016). However, sex-effects on IL-5 expression were not investigated in these studies.

IL-5 expression may be sex-dependent, but triggers of sex-based differences in IL-5 release during immature development remains unclear. Cognitive bias is described as repetitive shifts towards negative or threatening information, and aspects of cognitive bias are modulated by neuroinflammatory processes and hippocampal neurogenesis (Clelland et al., 2009; Reviewed
in Miskowiak & Carvalho, 2014). Male rats with negative cognitive bias expressed greater levels of IL-5 in the hippocampus at adolescence (P40) compared to females (Hodges et al., 2022), but whether these results are consistent in systemic circulation remain unclear.
1.6.5 Interleukin 10 (IL-10)

IL-10 is a potent anti-inflammatory cytokine (Maes et al., 1998; Ng, 2003; Zhang & An, 2007) that plays an essential role in the resolution of inflammation (Sabat et al., 2010). Systemically, the major producers of IL-10 include helper T cells, monocytes, macrophages, and dendritic cells (Iyer & Cheng, 2012; Ouyang et al., 2011), while microglia are one of the major sources of IL-10 production in the CNS (Ledeboer et al., 2002). IL-10 is essential in promoting the survival of neurons and glia (Strle et al., 2001), and is elevated in several CNS diseases, including multiple sclerosis (Brate et al., 2021), Alzheimer’s disease (Zhang et al., 2011), and neuropathic pain (Deng et al., 2021).

Functions of IL-10 involve repressing the expression of pro-inflammatory cytokines including the IL-1 family, IL-6, and TNF-α (Zhang & An, 2007). It can also function to up-regulate other anti-inflammatory cytokines and down-regulate pro-inflammatory cytokine receptors (Zhang & An, 2007). Cultured mouse microglia produce IL-10 mRNA and receptors, which suppresses LPS-induced pro-inflammatory cytokine production, suggesting that CNS-derived IL-10 is an inhibitory regulator of pro-inflammatory cytokine activity (Mizuno et al., 1994). IL-10 is also implicated in hippocampal functioning as chronically stressed mice exhibited significant reductions in hippocampal IL-10 levels (Labaka et al., 2017), which may suggest IL-10 production as sensitive to external stressors. IL-10 alterations have only been implicated in RMS models of ELS, with neonatal RMS exposure inducing elevated IL-10 mRNA expression in the PFC of P10 rats (Giridharan et al., 2019), and increased IL-10 protein levels in the adult rat hippocampus (Pinheiro et al., 2015).

IL-10 is also noted to display sex-based differences in expression, although inconsistent. In the developing brain of male and female rats, anti-inflammatory IL-10 expression was
significantly elevated in the female hippocampus compared to males at all assessed ages (P0, P4, P60) (Schwarz et al., 2012). However, opposing results were observed after cognitive bias testing and LPS stimulation. Male rats expressed greater levels of IL-10 in the hippocampus at adolescence (P40) when displaying negative cognitive bias compared to female rats (Hodges et al., 2022), and LPS stimulation reduced IL-10 production in astrocytes of female rats compared to males (Goldstein et al., 2021). It is unclear whether there are biological differences in IL-10 expression throughout development, or whether alterations in sex-based expression of IL-10 in the brain are dependent on the presence of external stressors.
1.6.6 Interferon gamma (IFN-γ)

IFN-γ is a pro-inflammatory cytokine (Maes et al., 1998; Ng, 2003) that plays a critical role in the recognition and elimination of pathogens, as well as regulation of cellular proliferation and apoptosis (Kak et al., 2018). IFN-γ is noted to display antiproliferative, antiviral, and immunomodulatory processes through the stimulation of lymphocytes and phagocytes (Gordon et al., 2023; Pestka et al., 1987) and is the main pro-inflammatory cytokine released by T helper type 1 (Th1) cells systemically. Helper T cells are regarded as the most prolific cytokine producers systemically and work to destroy intracellular parasites and maintain autoimmune responses (Berger, 2000). IFN-γ also produces an inhibitory effect on the proliferation of B cells, suggesting that IFN-γ reduces the production of antibodies (Rabin et al., 1986). Most notably, IFN-γ has been implicated in cancer immunotherapy. Increases in IFN-γ have been associated with the elimination of cancer cells, and resistance to cancer immunotherapy was partially regulated through IFN-γ signaling (Chen et al., 2009; Peng et al., 2012).

Under normal circumstances, IFN-γ does not bypass the blood-brain barrier and therefore is undetectable in the CNS (Fabry et al., 1994). However, in response to CNS infections (e.g. bacterial meningitis, fungal infections), the number of T-cells that cross the blood-brain barrier significantly increase, triggering CNS release of IFN-γ (Hickey, 1991), although the CNS cell-type responsible for its release is unclear. IFN-γ is not often investigated in the CNS due to its undetectability, but elevations in its expression are present in several autoimmune diseases, including multiple sclerosis and experimental autoimmune encephalomyelitis, which is mediated by T-cell infiltration into the CNS (Fabry et al., 1994; Robinson et al., 2014). It has also been shown that IFN-γ triggers microglia surveillance, and with the presence of specific neurological
infections (i.e. *Toxoplasma gondii*), IFN-γ can be released by microglia (Reviewed in Monteiro et al., 2017).

Prenatal alcohol exposure and RMS models of ELS have exhibited alterations in rat hippocampal IFN-γ, where prenatal alcohol exposure increased protein levels at P8 (Bodnar et al., 2016), and RMS increased mRNA expression at P10 (Giridharan et al., 2019). Although studies have not assessed the impacts of procedural pain in neonates on IFN-γ expression, systemic IFN-γ was found to be elevated in preterm infants who experienced systemic infection or necrotizing enterocolitis (Ng, 2003). Sex-based differences in IFN-γ have also been made apparent. In a study investigating sex differences in inflammation in association to negative cognitive bias, juvenile male rats (P40) expressed greater IFN-γ expression in the hippocampus compared to females (Hodges et al., 2022).
1.6.7 Interleukin 2 (IL-2)

IL-2 is a pro-inflammatory cytokine (Ng, 2003) that plays a role in the growth and development of peripheral immune cells, cellular apoptosis (Dembic, 2015), and T cell growth (Dembic, 2015). Systemically, IL-2 is released from activated T lymphocytes (Dembic, 2015) and can penetrate through the blood-brain-barrier (Hanisch & Quirion, 1995). In the nervous system, IL-2 modulates neural and endocrine functions and has been implicated in neuronal cell growth and survival, transmitter and hormone release, and CNS activity (Hanisch & Quirion, 1995). IL-2 is believed to regulate sleep and arousal, memory, movement, as well as modulate neuroendocrine functions, and is involved in homeostasis in both the brain and the immune system (Hanisch & Quirion, 1995; Petitto et al., 2012). The pathological effects of IL-2 on the brain have been recognized in Alzheimer’s disease, where hippocampi of individuals suffering from Alzheimer’s disease exhibited elevated IL-2 protein levels compared to controls (Araujo & Lapchak, 1994).

Neurons and glial cells can synthesize IL-2 (Hanisch & Quirion, 1995). IL-2 and its receptors can be found in several brain regions, including the hippocampus. However, levels generally remain low in the hippocampus (Araujo et al., 1989; Hanisch & Quirion, 1995; Petitto & Huang, 2001). The localization of IL-2 receptors in the hippocampus may influence learning, memory, and typical hippocampal functioning (Petitto et al., 2012). Repeated administration of IL-2 in aging mice resulted in memory deficits and neuronal damage specifically to the hippocampus (Nemni et al., 1992). Furthermore, in IL-2 knockout mice, hippocampal BDNF was significantly reduced compared to controls, while nerve growth factor (NGF) was increased (Petitto et al., 1998; Petitto & Huang, 1994). BDNF plays a critical role in the synaptic plasticity of learning and memory (Miranda et al., 2019) while NGF is a key molecule involved in the
maintenance of sensory neurons, including those involved in pain transmission (Petruska & Mendell, 2009). Therefore, it is possible that IL-2 works in combination with BDNF and NGF to influence memory deficits observed in aging mice (Nemni et al., 1992). To my knowledge, ELS-based alterations in IL-2 mRNA and protein levels, as well as sex-differences in IL-2 expression, have not been reported in the literature.
1.6.8 Interleukin 12p70 (IL-12p70)

IL-12p70 is a pro-inflammatory cytokine (Ethuin et al., 2003) that is composed of 2 subunits: p35 and p40 (IL-12p40 is the biologically inactive form). When p35 and p40 subunits are combined, IL-12p70, the biologically active subtype of IL-12, is produced (Gee et al., 2009). The IL-12 family, including IL-12p70, plays an important role in the early inflammatory response and promotes the production of Th1 immune cells (Hsieh et al., 1993). Systemically, IL-12p70 is mainly produced by macrophages and dendritic cells, and is one of the first cytokines released to initiate the immune response when an antigen is detected (Trinchieri, 2003). IL-12p70 also promotes the production of IFN-γ, another pro-inflammatory cytokine (Eley, 2009). In the CNS, microglia express IL-12 receptors on their surface and produce IL-12p70 in response to microglial receptor stimulation by IL-12 (Taoufik et al., 2001). One component of the IL-12p70, the p40 homodimer, has been implicated in pain intensity and antinociception in rat models of neuropathic pain (Chen et al., 2013). Various administered doses of the IL-12p40 subunit reduced mechanical hyperalgesia for up to 4 hours in neuropathic pain models (Chen et al., 2013), which may suggest a role of the IL-12 family in nociceptive sensation.

IL-12 has been implicated in the pathogenesis of various autoimmune diseases, including multiple sclerosis, autoimmune encephalomyelitis, and neuropathic pain (Chen et al., 2013; Leonard et al., 1995; Smith et al., 1997; Windhagen et al., 1995). There remains a paucity of research investigating IL-12p70 and its receptor localization in the brain, expression patterns with ELS exposure, and sex-based differences in expression.
1.6.9 Keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO)

Keratinocyte chemoattractant (KC), also referred to as CXCL1/2, is the mouse homologue of human growth-related oncogenes (GRO) (Semple et al., 2010; Son & Roby, 2006). It is a pro-inflammatory chemokine (Wu et al., 2021) that attracts immune cells to sites of inflammation (Turner et al., 2014). As previously stated, cytokines represent a large and diverse group of pro- and anti-inflammatory molecules that can be categorized into families according to their structural similarities or receptor characteristics. Chemokines are a subfamily of cytokines and represent a group of secreted proteins involved in facilitating cell migration (Reviewed in Ramesh et al., 2013). Inflammatory chemokines such as KC/GRO are produced in response to inflammation-inducing stimuli and target cells involved in the innate and adaptive immune system to induce an immune response (Reviewed in Ramesh et al., 2013) Systemically, neutrophils, macrophages, and epithelial cells release KC/GRO (Wu et al., 2021). It has been suggested that KC/GRO acts as a neutrophil chemoattractant, which is important for inducing the directional migration of neutrophils (Laffin et al., 2019; Moser, 2015). Systemically, induction of KC/GRO production by hepatocytes is stimulated through LPS as well as pro-inflammatory IL-1 and TNF cytokines (Son & Roby, 2006). Although research does not primarily focus on the effects of KC/GRO in the brain, one study in neonatal rats found KC levels to be significantly up-regulated in microglia following LPS induced pro-inflammatory microglia triggering (Serdar et al., 2020). Research is also lacking on the effects of ELS on central and systemic expression of KC/GRO, as well as sex differences in its expression.
Interleukin 4 (IL-4)

IL-4 is an anti-inflammatory cytokine (Maes et al., 1998; Ng, 2003; Zhang & An, 2007) produced systemically by mast cells, T helper type 2 (Th2) cells, and white blood cells, including eosinophils and basophils (Gadani et al., 2012). Systemically, IL-4 functions in the regulation of antibody production, red blood cell generation, and the development of effector T cells (Brown & Hural, 1997). It plays a significant role in modulating the immune response and inhibits the secretion of several pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α (Anovazzi et al., 2017).

In the CNS, microglia express IL-4 receptors and when exposed to IL-4, exhibit anti-inflammatory properties (Lambert et al., 2008; Nolan et al., 2005; Park et al., 2005). IL-4 receptors are also localized in the hippocampus (Maher et al., 2005). In the brain, changes in IL-4 are most notably involved in age-related processes. In naïve male rodents, IL-4 levels have been shown to decrease with age (Gadani et al., 2012; Maher et al., 2005; Nolan et al., 2005). In the aging hippocampus of rodents, IL-4 is suggested to promote neuroplasticity and long-term potentiation (LTP) by counteracting changes induced by pro-inflammatory cytokines (Boccardi et al., 2019). Increases in hippocampal pro-inflammatory cytokines (e.g. IL-1β, IL-6) in aging rats have been associated with decreases in anti-inflammatory IL-4 levels, which is believed to contribute to deficits in LTP (Maher et al., 2005). This was noted specifically in rats challenged with LPS to induce inflammation. Normally, hippocampal IL-4 expression decreases with age, but by increasing IL-4 concentrations in the hippocampus of rats administered LPS, age-related LTP impairment was reversed (Nolan et al., 2005). However, contradicting evidence showed age-related IL-4 expression in male and female rats being increased in the adult brain compared
to the developing brain (Schwarz, et al., 2012), making the neurodevelopmental trajectory of IL-4 expression unclear.

IL-4 is also suggested to display sex-based differences in expression, but results are inconsistent. In rats that displayed negative cognitive bias, males expressed greater levels of IL-4 in the hippocampus at adolescence (P40) compared to females (Hodges et al., 2022). However, regardless of early life stress experiences, another study found that female mice expressed higher circulating IL-4 expression compared to males at P25 (Grassi-Oliveira et al., 2016), presenting contradicting results. Although both results suggest IL-4 induces sex-specific expression patterns in the hippocampus and systemic circulation, the context in which these differences emerge are unclear and the pathways involved in their sex-based expression in the brain and serum remain elusive.

Overall, there are several cytokines involved in neuroinflammatory processes, and a subset of those described (e.g. IL-1β, IL-6, TNF-α) can be linked to the effects of ELS on neurodevelopment. However, the relationship between early life repeated pain exposure and/or sucrose and the neuroinflammatory effects of these cytokines, as well as their mechanisms of release have not been investigated in the immature and vulnerable developing brain. The following section further describes the relationship between pro- and anti-inflammatory cytokine levels and microglia responsivity.
1.7 Microglia and Inflammatory Cytokine Release

Microglia are a subtype of glial cell that are associated with neural imbalances in homeostasis, neural circuitry formation and plasticity, and disease states in the CNS (Reviewed in Salter & Beggs, 2014). Extensive research has been focused on microglia revealing their diverse and complex functions and properties. The roles and functions of microglia can be investigated in several neurological pathologies, all of which cannot be thoroughly covered. For this thesis, the role of microglia will be investigated in the literature describing ELS and neurodevelopment, as well as its relationship to pro- and anti-inflammatory markers already described and subsequently investigated in this thesis.

Neuroinflammation is mediated by cytokines and chemokines produced by CNS glia, including microglia (DiSabato et al., 2016). Microglia are the resident immune cells of the CNS that monitor their surrounding microenvironments and respond to disturbances in homeostasis (Hanisch, 2002; Perry & Teeling, 2013). Depending on the region, microglia comprise between 0.5-16.6% of the total cell population in the brain (Lawson et al., 1992) and are involved in establishing functional brain connectivity during postnatal brain development (Reviewed in Paolicelli et al., 2022). During development, microglia are critical for synaptic pruning and refining neuronal connectivity (Salter & Beggs, 2014). Phenotypically, microglia undergo major transformations during postnatal brain development, and they have been suggested to develop distinctly in male and female brains (Bordt et al., 2020; Schwarz, Sholar, et al., 2012; Schwarz, Bilbo, et al., 2012). Male rats at postnatal day 4 (P4) displayed greater microglia cell density in hippocampal regions CA1, CA3, and the dentate gyrus, while females displayed elevated surveillant microglial morphology between P30-P60 in the same brain regions (Schwarz et al., 2012).
In steady state conditions, microglia are less responsive but continuously survey their microenvironments (Paolicelli et al., 2022; Perry & Teeling, 2013). When homeostasis is disturbed, microglia respond by assisting in specific immune reactions and can initiate a potent inflammatory response. Previous literature label microglia in the absence of threats as “resting” and responsive microglia as “active,” but these terms are now considered outdated (Paolicelli et al., 2022) As per updated nomenclature, microglia are termed as homeostatic in the absence of a threat and surveillant when reactive or responding to threats (Paolicelli et al., 2022). The recommended nomenclature will be utilized throughout this thesis, even when describing previous studies that use the outdated terminology.

Microglia play a significant role in innate immunity as well as developing and maintaining the neuroinflammatory response in the CNS by displaying elevated proliferation and greater microglial density in response to threats (Gomez-Nicola et al., 2013). Microglia are triggered (i.e. more responsive to threats) very early in response to injury based on changes in the brain’s structural integrity, or even subtle changes in their microenvironment (Kreutzberg, 1996). It has been suggested that exposure to neonatal injury can “prime” microglial responses resulting in an increased intensity, spatial distribution, and duration of microglial response to injuries experienced later in adulthood (Romero-Sandoval et al., 2008). This primed response predisposes individuals to enhanced sensitivity to painful events, but this change is not solely mediated by microglial alterations (Beggs et al., 2012; Hains et al., 2010). Furthermore, stress can induce alterations in pro-inflammatory cytokine expression by increasing microglial surveillance (Bollinger et al., 2016), and exposure to ELS is also believed to prime microglia in stimulating the release of proinflammatory cytokines (Weber et al., 2015). When microglia are
triggered, they produce and release several pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α (Reviewed in Lumertz et al., 2022).

It is well established that ELS can disrupt the normal, sexually dimorphic maturation of microglia and alter the responsiveness to future immune or environmental stressors (Reviewed in Burke et al., 2016; Hanamsagar & Bilbo, 2017; Reviewed in Perry & Holmes, 2014). Microglia are densely populated in the hippocampus of the adult mouse brain (Lawson et al., 1990), and neuroinflammation and ELS are observed to alter microglial density and reactivity in the hippocampus. In adult mice, administering LPS to induce acute inflammation significantly increased microglial density in various cortical regions, including the hippocampus (Furube et al., 2018; Lawson et al., 1990). Furthermore, in an ELS model using maternal separation, both repeated and single-episode maternal separation resulted in elevated microglial density and greater microglia responsiveness in the hippocampal hilus and CA3 regions in rat pups (Roque et al., 2016; Saavedra et al., 2017). Microglial responsiveness was measured morphologically, with large somas and amoeboid bodies, and thicker and short processes (microglia types IV-V) being considered more responsive (Diz-Chaves et al., 2012; Roque et al., 2016). This effect was also shown at various timepoints during adolescence and adulthood, in addition to altered microglial morphology in the hippocampus (Delpech et al., 2016). Maternal separation also perturbed the maturation of microglia in the developing hippocampus (Delpech et al., 2016; Réus et al., 2019). Interestingly, microglial-neuronal signaling in response to pain is suggested to be sexually dimorphic, where microglia in female rodents with peripheral nerve injuries do not contribute to mediating pain hypersensitivity but are involved in males (Reviewed in Miller et al., 2005; Sorge et al., 2015).
An increase in hippocampal microglial density after ELS exposure (Roque et al., 2016; Saavedra et al., 2017) may be correlated with a modified neuroinflammatory profile (Reviewed in Lumertz et al., 2022). Although ELS effects on microglial density in the hippocampus have been investigated in RMS, there remains a paucity of research investigating the impact of repetitive early-life pain and/or sucrose exposure on microglia morphology and function.

Determining the immune signature, alterations in microglial density, and sex differences of mice exposed to repetitive neonatal pain and/or sucrose will enhance our understanding of the immediate consequences linked to repeated neonatal pain and/or sucrose exposure. By investigating such effects, results may offer valuable insights into potential clinical implications that may emerge in the NICU.
Chapter 2: Rationale and Hypothesis

Overview

Most infants born prematurely will be admitted to the NICU due to significant changes in their post-uterine environment during a period of physiological immaturity and hemodynamic instability (Johnston et al., 2011). In the NICU, preterm infants can receive an average of 7-17 clinically required but painful procedures daily (Reviewed in Cruz et al., 2016). Repeated exposure to neonatal pain is an early-life stressor and has been implicated in several adverse neurodevelopmental outcomes in both humans and animal models, as summarized in Tables 1.1 and 1.2.

Given the susceptibility of this immature and highly vulnerable population to the detrimental consequences of recurring pain exposure, especially with their heightened sensitivity to pain (Fitzgerald, 2005), it is important to mitigate the experience of pain during these painful procedures. To manage procedural pain in preterm infants, oral sucrose is used as a non-pharmacological analgesic and is considered the standard of care for neonatal procedural pain relief (Stevens et al., 2001, 2016). However, repeated exposure to procedural pain in the NICU places preterm infants at risk of accumulated sucrose exposure. If a preterm infant experiences 10 painful procedures daily and is provided the recommended sucrose dose (0.5ml/dose), it is nearly equivalent to providing a 10kg 1-year old child half a can of Coke Classic (22g or 4 tablespoons of sucrose) each day (Holsti & Grunau, 2010). Recently Stevens and colleagues (2018) showed that a very small dose of sucrose (0.1 ml) appeared to be as equally effective at reducing pain in neonates during a single painful procedure compared to larger doses (i.e. 0.5 to 1ml/dose), while also acknowledging the need for further studies examining the long-term
effects of cumulative sucrose use (Stevens et al., 2018). For preterm infants, the cumulative
effects of sucrose and/or pain in the brain have been associated with several macro- and micro-
structural alterations throughout development (see Tables 1.1 and 1.2), but the mechanistic
pathways explaining why and how these effects come about have yet to be studied.

Other forms of ELS, such as repeated maternal separation (RMS) and prenatal alcohol
exposure, have adverse impacts on brain development, specifically in the hippocampus (e.g.
Bodnar et al., 2016; Giridharan et al., 2019; Pinheiro et al., 2015; Roque et al., 2016; Wang et al.,
2020), making the hippocampus a region of interest for the effects of ELS. RMS has been
implicated in alterations in inflammatory cytokine levels (Giridharan et al., 2019; Pinheiro et al.,
2015; Roque et al., 2016; Wang et al., 2020), where IL-1β, IL-6, and TNF-α levels were elevated
in the hippocampus of infant and adult rats. Further implications of RMS include increased
microglial density and perturbed microglial maturation in the hippocampus (Delpech et al., 2016;
Reviewed in Dutcher et al., 2020; Roque et al., 2016). Similar inflammatory protein findings to
RMS have been observed with prenatal alcohol exposure, where infant female rats exposed to
prenatal alcohol displayed greater hippocampal IL-1β levels compared to controls at P8 (Bodnar
et al., 2016). Furthermore, rodents on sucrose or fructose-enriched diets exhibited elevated IL-
1β, IL-6, and TNF-α expression in the hippocampus (Beilharz et al., 2016; Hsu et al., 2015),
suggesting possible neuroinflammatory outcomes associated with sugar consumption. The
negative effects on the hippocampus’ microstructure observed in various models of ELS and
high sugar consumption brings into question whether other ELS paradigms (i.e. repeated early
life pain and/or sucrose exposure) will show parallel outcomes.
Figure 2.1 provides a summary of various subtypes of ELS and their relationship with inflammatory cytokines IL-1β, IL-6, and TNF-α. This figure shows previously investigated effects of ELS on hippocampal development and their alterations in the hippocampus’ inflammatory signature, while highlighting the lack of research exploring the hippocampal inflammatory profile after repeated neonatal pain and/or sucrose exposure.

Figure 2.1. Rodent models of early-life stress and its effects on inflammatory cytokines. Evidence of prenatal alcohol exposure, maternal separation, and limited bedding effects on macrostructure and specific inflammatory cytokines in the hippocampus. Structural effects on hippocampal volumes have been associated with repeated neonatal pain and/or sucrose exposure, but effects on hippocampal inflammatory cytokine profiles require further investigations or has not been examined in pre-clinical rodent models.
Research purpose

The purpose of my thesis project was to determine the short-term inflammatory and microstructural impacts in the hippocampus of repeated sucrose and/or pain exposure in neonatal mice. To address this, I used a neonatal mouse paradigm (Ranger et al., 2019; Tremblay et al., 2017) of ELS during the first week of life.

Research questions

The following three research questions were addressed in my thesis:

1. What is the effect of repeated neonatal pain and/or sucrose exposure on several inflammatory cytokine levels in the right hemisphere hippocampus and serum in 8-day old mice, with specific interest in cytokines IL-1β, IL-6, and TNF-α?

2. What sex effects are apparent after repeated neonatal pain and/or sucrose exposure on several inflammatory cytokine levels in the hippocampus and serum in 8-day old mice, with specific interest in cytokines IL-1β, IL-6, and TNF-α?

3. What is the effect of repeated neonatal pain and/or sucrose exposure on hippocampal microglia density in 8-day old mice?
Research Aims

The following three aims were addressed in my thesis:

1. Determine the inflammatory cytokine profile of 10 inflammatory cytokines in the right hemisphere hippocampus and serum of P8 male and female mice provided repeated neonatal 24% oral sucrose and/or pain exposure during the first week of life.

2. Assess sex differences of 10 inflammatory cytokine levels in the right hemisphere hippocampus and serum of P8 male and female mice provided repeated neonatal 24% oral sucrose and/or pain exposure during the first week of life.

3. Determine microglial cell density in the hippocampal CA3 region of male mice at P8 exposed to repeated neonatal 24% oral sucrose and/or pain during the first week of life.

Hypotheses

I formulated three hypotheses to address my research questions:

1. I hypothesized that there will be an altered inflammatory response observed in the hippocampus and serum of 8-day old mice after repeated neonatal sucrose and/or pain exposure, with a more robust alteration in the sucrose + needle-prick (SN) group. Specifically, there will be greater levels of IL-1β, IL-6, and TNF-α in the SN group compared to all the other groups.

2. I hypothesized that there will be sex-specific alterations in hippocampal and serum inflammatory cytokines in 8-day old mice exposed to repeated neonatal sucrose and/or pain.
3. I hypothesized that there will be alterations in microglia density in the hippocampus of 8-day old mice with exposure to repeated neonatal early-life sucrose and/or pain, with a more robust alteration in the SN group compared to all the other groups.
Chapter 3: Materials and Methods

3.1 Animals

The use of animals in this study was approved by the University of British Columbia Animal Care Committee (certificate #A19-0306 and #A19-0289) in accordance with the Canadian Council of Animal Care policies.

Mice (*Mus musculus*) of strain C57BL/6J were used for this study. The first week of brain development (P0-P7) in rodents corresponds to preterm cerebellar, striatum, limbic, and glial development in the human neonate from 24-32 weeks gestational age (Biran et al., 2012; Semple et al., 2013). The use of *Mus musculus* as an animal model for preterm infant brain development has also been utilized in other studies investigating repeated sucrose exposure for procedural pain (Ranger et al., 2019; Tremblay et al., 2017). In addition, postnatal day 8 (P8) was selected to evaluate early-life inflammatory profiles in C57BL/6J mice based on microglia activity and findings from prenatal alcohol exposure in offspring of female rats (Bodnar et al., 2016). P8 is a developmental period when microglia are more active and display a higher level of cytokine production in early development (Schwarz, Bilbo, et al., 2012), and only P8 rat pups exposed to prenatal alcohol displayed an elevated cytokine profile in the hippocampus (Bodnar et al., 2016), indicating P8 as a developmental period of interest.
3.2 Breeding Protocol

All breeding protocols were run by Dr. Rujun Kang (RK; senior laboratory technician and manager). At 6-7 weeks of age, one male and two female mice were paired in their own cage for breeding. Each breeding pair was provided with environmental enrichment hangers and nesting materials to promote good nest building and proper litter care. Animals were housed in a 14/10-hour light/dark cycle, as per the Tremblay et al. (2017) experimental protocol. Mice were checked daily by the Centre for Molecular Medicine and Therapeutics (CMMT) Transgenic Facility’s animal care technicians and animal cages were changed weekly in a Baker or Biobubble HEPA-filtered hood.

For six consecutive mornings after set-up of the breeding cages, each female was weighed and checked for a positive pregnancy using the presence of a copulatory plug. Females with a copulatory plug were group housed until E17. At E17, each female was caged with an ICR/CD1 nulliparous non-nursing mouse for animal experiments (#A19-0289). CD1 mice were matched with C57BL/6J mothers as a “nanny” to help reduce cannibalism and promote litter survival (based on previous study protocol [Ranger et al., 2019; Tremblay et al., 2017]). Mouse colonies were monitored daily for new births by RK, myself, or a CMMT Animal Care Facility technician during the last week of gestation.

Females that did not display a copulatory plug were returned to the main colony but continued to be monitored for weight changes for three weeks. If these females conceived and gave birth, the dams and their litter were housed in a separate cage from their day of birth (P0). Female breeders can be used for further breeding after a minimum of six weeks post-parturition. Stud males remained in their home cage for at least two days prior to being used for breeding again, if required.
3.3 Experimental Design

At birth (P0), sex of mouse pups were determined visually based on a spot of dark pigmentation on the scrotum for males, and lack of visible pigmentation in the anogenital region in females (Wolterink-Donselaar et al., 2009). Animals of both sexes were randomly assigned to one of 6 experimental groups or to the undisturbed control group (see Table 3.1 for a list of experimental and control groups, and their respective procedures). Animals were tattooed using the Ketchum green animal tattoo paste (#329A Ketchum Manufacturing Inc) using the CMMT Animal Care Facility mouse tattooing legend for experimental group identification (see Appendix A: Detailed Methodology).

Table 3.1 Mouse pup control and experimental groups.

<table>
<thead>
<tr>
<th>Treatment and Intervention</th>
<th>Experimental Group Abbreviation</th>
<th>Experimental Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C</td>
<td>Pups were not tattooed and left undisturbed from P0 until tissue collection at P8.</td>
</tr>
<tr>
<td>Sucrose + Needle-prick</td>
<td>SN</td>
<td>Pups received 24% oral sucrose 2 minutes prior to a needle-prick with a 30-gauge (G) needle on the ventral paw pad, 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
<tr>
<td>Water + Needle-prick</td>
<td>WN</td>
<td>Pups received sterile water 2 minutes prior to a needle-prick with a 30-G needle on the ventral paw pad, 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
<tr>
<td>Sucrose + Handling</td>
<td>SH</td>
<td>Pups received 24% oral sucrose 2 minutes prior to being held on the palm of the researcher 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
<tr>
<td>Water + Handling</td>
<td>WH</td>
<td>Pups received sterile water 2 minutes prior to being held on the palm of the researcher 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
<tr>
<td>Sucrose + Tactile pressure</td>
<td>Not used for cytokine and microglia analysis</td>
<td>Pups received 24% oral sucrose 2 minutes prior to being gently pressed on the ventral paw pad with a cotton swab, 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
<tr>
<td>Water + Tactile pressure</td>
<td>Not used for cytokine and microglia analysis</td>
<td>Pups received sterile water 2 minutes prior to being gently pressed on the ventral paw pad with a cotton swab, 10x/day from P1-P6. Tissue collection at P8.</td>
</tr>
</tbody>
</table>
Animal experiments commenced from P1-P6. Administration of treatment (24% oral sucrose, sterile water) and intervention (needle-prick, pressure, handling) on mouse pups followed experimental protocols as outlined by Tremblay et al. (2017) and Ranger et al. (2019). Figure 3.1 illustrates treatment and intervention administration used for these experiments.

**Figure 3.1 Experimental treatment and intervention administration in neonatal mouse pups from P1-P6.** Obtained from Tremblay et al. *Pain;158*(8):1586 (2017). A) Experimental treatment (24% oral sucrose, water) administration using a 10μl pipette. B) Experimental intervention tactile pressure applied with a sterile cotton swab on the ventral surface of the paw pad. C) Experimental intervention needle-prick applied with a sterile 30-G needle on the ventral surface of the paw pad. Handling intervention is not shown in this diagram.
On the day of experiments, home cages of dams and nanny mice had access to food and HydroGel (ClearH₂O Product Code 70-01-1082) for water ad libitum. Transfer cages, where dams and nanny mice were transferred during experimental procedures on mouse pups, also contained HydroGel, but no food. For the first experimental procedure of the day from P1-P6, mouse pups were weighed to determine the dosage of 24% oral sucrose or sterile water administration required. Administered sucrose dosages were based on standard dosage recommendations for very preterm human neonates, which was 0.5ml per dose (Stevens et al., 2016). For this experiment, 0.1-0.2g of sucrose per kg body mass was provided to mouse pups, and doses were adjusted daily based on body mass (Ranger et al., 2019; Tremblay et al., 2017). See Table 3.2 for dosage of 24% oral sucrose or sterile water provided based on daily weights of mouse pups.

Table 3.2 Administered intervention dosage (sucrose or sterile water) for mouse pups during the experimental protocol

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Sucrose or sterile water dosage (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0-1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5-1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>2.0-2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>2.5-2.9</td>
<td>2.5</td>
</tr>
<tr>
<td>3.0-3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>3.5-3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>4.0-4.4</td>
<td>4.0</td>
</tr>
<tr>
<td>4.5-4.9</td>
<td>4.5</td>
</tr>
</tbody>
</table>
The 24% sucrose solution used in this experiment was created by weighing 24g of pure sucrose (Sigma #S7903) and adding sterile water to a final volume of 100ml. The solution was then heated evenly using a hot plate and filtered with a 125ml filter bottle. The final 24% sucrose solution was aliquoted and stored in 4°C for long-term storage.

From P1-P6, experimental procedures were conducted 10x per day from 8am to 6pm with a minimum of 30-45 minutes between experiments. Mothers and CD1 nanny mice were taken from their home cage, relocated to a secondary (transfer) cage, and placed outside of the experimental procedure room to ensure they were unable to smell or hear the distressed litter (ultrasonic vocalizations). In the experimental procedure room, pups were removed from their home cage and placed onto a heating pad to maintain body temperature. A two-minute timer was set, and all animals received their assigned treatment (24% oral sucrose, sterile water). After 2-minutes, pups were administered their intervention (handling, needle-prick, tactile pressure). Tactile pressure and needle-prick interventions were rotated between all four paws for each experimental round. At the end of each experimental round, pups were placed back into their home cages and covered with nesting material where they originally laid. The C57BL/6J dam and CD1 nanny mouse were transferred back to their home cage, reunited with the litter and placed outside of the procedure room. Dams and nanny mice were not separated from their home cage and litter for longer than 5 consecutive minutes. This process was repeated 10x/day from P1-P6, with a minimum of 30-45 minutes in between each round of experimental procedures over a 10-hour period. On P7, animals were left undisturbed. At P8, pups were weighed and tissue collection for cytokine and microglial cell density analysis commenced. Figure 3.2 illustrates a simplified experimental protocol from P1 until tissue collection.
Figure 3.2 C57BL/6J mouse pup experimental groups and protocol from P1-P8. From P1-P6, mouse pups received their randomly assigned treatment and intervention 10x/day over a 10-hour period. Experimental groups in this thesis project included SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), and WN (water + needle-prick). On P7, pups were left undisturbed. At P8, pups were sacrificed for brain tissue and serum collection. Birth (P0), tattooing protocol at P0, and pups in the touch (tactile pressure) and control groups are not shown on this timeline.

Although multiple treatment and intervention groups were generated in the larger experimental protocol, for this thesis work, only mouse pups assigned to needle-prick and handling intervention groups were used for cytokine levels (see Section 3.4.3) and microglial density (see Section 3.7.2) assessments.
3.4 Tissue Collection for Cytokine Assessment

At P8, mouse pups were deeply anesthetized using isoflurane, then euthanized via decapitation in under 17 minutes. With the mouse pup inside, the anesthesia induction chamber was filled with oxygen at a rate of 1L/minute with 3% isoflurane for 10-15 minutes. Adequate anesthesia was assessed within the induction chamber through loss of the righting reflex and depressed respiratory rate, and outside of the induction chamber via loss of withdrawal response to a toe pinch. Once adequate anesthesia was reached, a 30-G insulin needle was inserted through the diaphragm and into the heart for blood collection. Blood was drawn and placed in a 1.5ml Eppendorf tube and later centrifuged to isolate serum. Using a pipette, serum was transferred to a new 1.5ml Eppendorf tube, placed on ice for the remainder of tissue collection, then stored in the -80°C freezer while the remaining red blood cells were disposed. After blood collection, mouse pups were decapitated and whole brains were dissected and wrapped in aluminum foil, labelled, and flash frozen in dry ice for the remainder of tissue collection prior to storage in the -80°C freezer. Brain collection was completed in two minutes post-decapitation. Brain and serum samples remained in the -80°C freezer until tissue analysis (e.g. total protein and inflammatory cytokine assays).

3.4.1 Sample Preparation

Right hemisphere hippocampal tissue was dissected by senior laboratory technician RK using a variety of sizes of fine forceps (Fine Science Tools; North Vancouver, BC) on the Nikon Zoom Stereomicroscope (Nikon product #SMZ800N). The hippocampus was identified by first separating the whole brain into left and right hemispheres to produce two sagittal sections. The cerebellum was removed, and each sagittal section was positioned to situate the prefrontal cortex.
dorsally and the dentate gyrus ventrally. The midbrain and brainstem regions were carefully dissected, providing visualization of the hippocampus. The hippocampus was then removed using the Dumont #5 Forceps (Fine Science Tools, Item #11251-10), placed in a labelled 1.5ml Eppendorf tube, and frozen in -80°C for later protein analysis.

Prior to conducting brain cytokine assays, several 2ml bead ruptor tubes (ThermoFisher, tubes Cat. #02-681-344, caps Cat. #02-681-363) were prepared for hippocampal tissue homogenization and protein extraction. Bead ruptor tubes were filled with approximately 150 0.5mm diameter glass beads (BioSpec.com, Cat. #11079105) to homogenize P8 right hemisphere hippocampal tissue. 50µl of ice-cold lysis buffer composed of mini protease inhibitor cocktail tablets (1 tablet/5µl lysis buffer; Thermo Scientific, Cat. #A32955), 500mM Tris HCl, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS, and 1mM PMSF (Sigma, Cat. #329-98-6) were pre-filled into each bead ruptor tube and placed on ice. Right hemisphere hippocampal tissue was weighed while frozen and placed into the pre-filled bead ruptor tube. Due to the variability in hippocampal tissue weights and the subsequent lysates obtained after homogenization, it was necessary to measure analyte concentrations per milligram of tissue during protein analysis of solid tissue. Practice protocols were conducted as described in this section to test various tissue to lysis buffer ratios that yield cytokine levels within observable detection limits of the multiplex assay. It was determined that a 75mg tissue/500µl lysis buffer ratio (1mg tissue/6.67µl lysis buffer) provided cytokine levels within observable levels of detection. Additional lysis buffer was added to each bead ruptor tube based on tissue weight until a 75mg tissue/500µl lysis buffer ratio was achieved. Tissue weights that required less than 50µl lysis buffer were not included in the samples processed for cytokine levels. Bead ruptor tubes containing hippocampal tissue and sufficient volumes of lysis buffer were homogenized using
the Bead Ruptor 24 (Omni International Inc, Kennesaw, GA) in 4-5 cycles (speed 2.10 – 3.10 for 5 seconds each cycle) with 1 minute on ice in between cycles. Samples were then collected and placed to rest on ice for 10 minutes. Following the rest period, samples were centrifuged at 1400g for 10 minutes at 4°C. Supernatants were collected and transferred into newly labelled 1.5ml microcentrifuge tubes and total protein analysis commenced immediately after transfer.

Serum samples were thawed on ice and centrifuged at 2000g for 3 minutes as recommended per the Meso Scale Discovery (MSD) V-PLEX Proinflammatory Panel 1 Mouse Kit protocol. Samples were then placed back on ice after centrifuging prior to inflammatory protein analysis.

### 3.4.2 Measurement of Total Protein

Measurement of total protein was analyzed only for hippocampal tissue samples. Serum total protein levels were not conducted using Bicinchoninic Acid (BCA) assays due to the presence of interfering substances such as lipids that could bind to the BCA reagent and interfere with colour development during incubation, resulting in inaccurate protein concentration measurements (Shen, 2019). Additionally, the MSD V-PLEX Proinflammatory Mouse Panel 1 used for cytokine detection is calibrated specifically for serum samples, but not for tissue supernatants (i.e. hippocampal tissue). Consequently, assessment of total protein levels was not required for serum samples but were necessary to evaluate total protein levels in hippocampal tissue samples.

Total protein concentration for hippocampal tissue was measured using the Pierce BCA Protein Assay Kit (Thermofisher USA, Cat. #23225) as per the manufacturer’s instructions. The Pierce BCA Protein Assay Kit includes two 500ml bottles of BCA Reagent A, 25ml of BCA Reagent B, and ten vials of 1ml 2mg/ml Albumin Standard Ampules (Bovine Serum Albumin,
BSA). The concentration of total protein for hippocampal lysates were calculated against a serially diluted BCA standard curve. To establish the standard curve, 2mg/ml stock solution of BSA was serially diluted to provide a working range of 75-2000μg/ml and added to a 96-well plate in duplicates. Original homogenized lysate obtained from the right hippocampal tissue preparation (75mg tissue/500μl lysis buffer) was subsequently diluted with additional lysis buffer to result in a final ratio of 20mg tissue/500μl lysis buffer. The need for a lower ratio of hippocampal tissue to lysis buffer for the BCA assay is due to protein concentrations of the original sample preparation exceeding the detection range of the BCA assay standard curve. However, a greater ratio is required for the MSD multiplex assay; 10μl of the newly diluted hippocampal lysates (now 20mg tissue/500μl lysis buffer) were placed in a 96-well plate in duplicates alongside the BSA standard curve. 200μl of BCA working reagent (50 parts BCA Reagent A:1 part BCA Reagent B) were added to each well and placed in a 37°C incubator for 30 minutes. After incubation, absorbance of the hippocampal samples were measured using the iMark Microplate Absorbance Reader (Bio-Rad Laboratories, product #1681135) at 562nm. The BCA Assay standard curve and total protein levels were automatically plotted using the Bio-Rad Laboratories Microplate Manager v. 6.3.

3.4.3 Multiplex Immunoassay (MSD Proinflammatory Mouse Panel 1)

The V-PLEX Proinflammatory Panel 1 Mouse Kit (Meso Scale Discovery, Cat. #K15048D-2) was used to simultaneously assess 10 inflammatory cytokines in hippocampal tissue and serum. The V-PLEX Proinflammatory Panel 1 Mouse kit included five specialized Proinflammatory Panel 1 (mouse) 96-well plates, 50ml Diluent 41 (Cat. #R50AH-2), 25ml Diluent 45 (Cat. #R50AI-2), 50ml Read Buffer T 4X (Cat. #R92TC-3), one vial (375μl) of each
of the ten SULFO-TAG Mouse Antibodies (IL-1β, IL-6, TNF-α, IL-5, IL-10, IFN-γ, IL-2, IL-12p70, KC/GRO, and IL-4), and five vials of the multi-analyte lyophilized calibrator blend (Cat. #C0048-2).

Initially, the calibrator blend was reconstituted and serially diluted 3-fold using Diluent 41 as recommended per the MSD V-PLEX Proinflammatory Panel 1 Mouse Kit protocol. 50µl of 3-fold serially diluted calibrator were then added to the V-PLEX plate in duplicates. Stock concentrations of reconstituted calibrator for each assessed inflammatory marker can be found in Table 3.3.

### Table 3.3 List of V-PLEX Proinflammatory Panel 1 Mouse Kit concentration assignments for multi-analyte lyophilized calibrator.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stock concentration of reconstituted calibrator (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>890</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1590</td>
</tr>
<tr>
<td>IL-2</td>
<td>2260</td>
</tr>
<tr>
<td>IL-4</td>
<td>1660</td>
</tr>
<tr>
<td>IL-5</td>
<td>947</td>
</tr>
<tr>
<td>IL-6</td>
<td>4680</td>
</tr>
<tr>
<td>IL-10</td>
<td>3030</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>27500</td>
</tr>
<tr>
<td>KC/GRO</td>
<td>1780</td>
</tr>
<tr>
<td>TNF-α</td>
<td>594</td>
</tr>
</tbody>
</table>

Obtained from [https://www.mesoscale.com](https://www.mesoscale.com)

To ensure cytokine levels fell within the optimal range of detection, all total protein concentrations recorded from the BCA assay (20mg tissue/500µl lysis buffer) were adjusted to reflect protein concentrations of the original 75mg tissue/500µl lysis buffer samples (3.75X dilution). Total protein concentrations obtained from the BCA assay were multiplied by 3.75 to obtain the original 75mg tissue/500 µl lysis buffer protein concentration.
To conduct V-PLEX cytokine analysis, 45µl of sample per well was required as per standard MSD V-PLEX protocol, but first, the initial 75mg tissue/500µl lysis buffer hippocampal tissue supernatants had to be diluted with Diluent 41 at a minimum 2-fold dilution. As determined through a previous trial run, 75mg/45µl total protein concentration must be maintained per well for V-PLEX cytokine testing after dilution. To calculate the volume required to obtain 75mg total protein for each well in µl, 75mg was divided by each sample’s total protein concentration per µl. The remaining volume was brought to a final volume of 100µl (with the additional 10µl to account for pipetting error) with Diluent 41. 45µl of the newly diluted 75mg tissue/500µl lysis buffer plus Diluent 41 samples were pipetted onto a plate in duplicates. For serum, a 3-fold dilution was created using Diluent 41, as recommended by the MSD V-PLEX protocol, to obtain a final volume of 100µl. 45µl was added into each well in duplicates. Each plate was then sealed with an adhesive plate seal and placed on the shaker overnight at 100rpm in 4°C.

The following day, seals were removed and each plate was washed 3X with 150µl PBST. 25µl detection antibody solution was then added to each well. A list of each detection antibody provided with the MSD V-PLEX kit alongside their detection limits is shown in Table 3.4. Each plate was sealed and incubated at room temperature with light shaking (100-120rpm) for 2 hours. Following the incubation period, each plate was washed 3X with 150µl PBST, and 150µl of 3X Read Buffer T was added to each well. Each plate was then immediately read for cytokine levels using the MESO QuickPlex SQ 120MM (MSD, Rockville, MD; Cat. #A11AA-0) plate reader.
Table 3.4 List of V-PLEX Proinflammatory Panel 1 Mouse Kit analytes and typical detection limits

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Lowest level of detection</th>
<th>Lowest level of quantitation</th>
<th>Upper level of quantitation</th>
<th>Dynamic range</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.04</td>
<td>0.39</td>
<td>570</td>
<td>0.04 - 570</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.11</td>
<td>0.72</td>
<td>1,030</td>
<td>0.11 - 1,030</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.22</td>
<td>1.03</td>
<td>1,570</td>
<td>0.22 - 1,570</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.11</td>
<td>0.818</td>
<td>1,060</td>
<td>0.11 - 1,060</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.06</td>
<td>0.302</td>
<td>590</td>
<td>0.06 - 590</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.61</td>
<td>7.61</td>
<td>3,140</td>
<td>0.61 - 3,140</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.94</td>
<td>7.26</td>
<td>2,030</td>
<td>0.94 - 2,030</td>
<td>pg/ml</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.95</td>
<td>179</td>
<td>20,600</td>
<td>9.95 - 20,600</td>
<td>pg/ml</td>
</tr>
<tr>
<td>KC/GRO</td>
<td>0.24</td>
<td>3.29</td>
<td>1,230</td>
<td>0.24 - 1,230</td>
<td>pg/ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.13</td>
<td>0.98</td>
<td>403</td>
<td>0.13 - 403</td>
<td>pg/ml</td>
</tr>
</tbody>
</table>

Obtained from [https://www.mesoscale.com](https://www.mesoscale.com)

Concentrations were automatically assessed by the MSD Discovery Workbench software v. 4.0 and mean concentrations for each duplicate were provided. These values were inputted into GraphPad Prism 9 for graphing and statistical analysis. Cytokine levels in hippocampal tissue were expressed as pg cytokine/mg tissue and in serum as pg/ml.

### 3.4.4 Statistical Analysis

Weights of mouse pups (experimental groups SH, SN, WH, WN) were measured daily from P1 to P8, excluding the rest day at P7. Control animals were only weighed on the day of tissue collection (P8) to maintain them as true, undisturbed controls. Weight data from P1-P8 were assessed using a two-way repeated measures analysis of variance (ANOVA) between experimental groups SH, SN, WH, and WN, with experimental group as the between-subject factor and postnatal age as the within-subject factor. A Tukey post hoc test was used to adjust for multiple comparisons. To determine weight differences at P8 between controls and experimental groups, a one-way ANOVA was conducted.
The objective of this study was to determine if sex, treatment (water, sucrose), and/or intervention (needle-prick, handling) affect cytokine levels in the hippocampus and serum of mouse pups. Cytokine levels were graphically plotted using a QQ plot to visually examine for data normality and outliers, then statistically assessed using a Shapiro-Wilk test. Assuming normality, cytokine data were initially analyzed using two sets of two-way ANOVAs to determine effects of sex, treatment, and/or intervention. The first two-way ANOVA analyzed whether sex and experimental group (treatment and intervention combined; 5 groups including control) had an independent or a combined interactive effect on cytokines levels. The second two-way ANOVA analyzed whether treatment and intervention had an independent or a combined interactive effect on cytokines levels (controls not included). If sex and/or an interaction term between sex and experimental group were deemed not significant, males and females were combined within treatment and intervention groups to further assess group effects, and a separate one-way ANOVA was run to examine cytokine levels between experimental groups and controls. Multiple comparisons were corrected with a Tukey post hoc analysis. A $p$-value <0.05 was considered statistically significant in all statistical analyses, unless otherwise described. Based on visual inspection, outliers in hippocampal IL-1β and IL-6, and serum IL-6, KC/GRO, and IL-2 were found. A Grubbs’ test (significance level $\alpha=0.05$) was used to confirm these outliers and were removed if greater than 3 standard deviations from the mean. All statistical analyses were conducted using GraphPad Prism 9.
3.5 Tissue Collection for Microglial Density

At P8, mouse pups were placed under deep anesthesia using isoflurane then euthanized via perfusion for brain tissue collection as described in Section 3.4. Once adequate anesthesia was reached, the mouse pup was laid on its back on a wax-lined tray and secured by pinning the limbs away from the body using 20-G needles. The chest cavity was opened by cutting through the abdominal wall and up through the ribs starting from 0.5 cm below the end of the sternum. A small hemostat was used to clamp the sternum upwards to expose the chest cavity. With the heart still beating, the right atrium was clipped using surgical forceps and the left ventricle was pierced at its base using the Surflo 27-G needle part of the Winged Infusion Set (Fisher Scientific, Product #22-289912). First, 10ml of 4% paraformaldehyde (PFA) was perfused into the left ventricle at a flow rate of 12ml/minute and traveled throughout the body to rinse out circulating blood through the right atrium, followed by 10ml of ice-cold phosphate-buffered saline solution (PBS, pH 7.4) administered at same rate in the same region of the heart. Once perfusion was complete, the body of the mouse pup became stiff. The mouse pup was subsequently decapitated, and whole brain tissue was collected.

To store tissue for immunohistochemistry, brain tissue had to be transferred into a gradient sucrose solution overnight starting at 20% in 4ºC. The gradient of sucrose solution was incrementally increased daily until reaching a final storage concentration of 30% sucrose. The tissue was then stored long-term in the fridge at 4ºC.
3.6 Immunohistochemistry (Iba1 and DAPI)

3.6.1 Sample Preparation

Whole brains stored in 30% sucrose solution were removed from solution, placed on an imaging slide, and the olfactory bulb was removed and discarded using a single-edge razor blade. The remaining brain was embedded using Tissue-Tek optimum cutting temperature (O.C.T.) Compound (Cat. #25608-930) and coronally cryosectioned into 30µm slices. Slices were then placed into a 24-well plate with PBS at 4°C for immunostaining. The Allen Mouse Brain Atlas (https://mouse.brain-map.org/static/atlas) was utilized to determine the optimal hippocampal imaging location including the CA3 brain region using the P7 mouse brain atlas. Hippocampal slices used for imaging were acquired at a depth between 1550-2410µm from the anterior surface of the brain. A maximum of five cryosections of 30µm brain slices were placed into each well. The immunostaining protocol (described below) was completed over 2 consecutive days and all steps were done at room temperature unless otherwise stated.

Day 1 Immunostaining Protocol

Free-floating immunostaining was conducted for microglia visualization and cell counts in the CA3 region of the hippocampus in both hemispheres. In a 12-well plate, 2.5ml of PBS was added to each well and three to four brain slices from male P8 mouse pups of a given sample were placed within a single well. Brain slices were washed in PBS on a shaker at 100rpm 3X for 5 minutes each. After washing, slices were transferred into 0.01M PBS + 0.5% Triton X-100 solution to permeabilize for 5 minutes without shaking. The next step involved washing brain slices in 0.01M PBS + 0.3% Triton (PBST) wash buffer 3X for 5 minutes each on a shaker at 100rpm. Following the wash, brain slices were transferred into 300µl blocking solution (0.01M
PBS, 0.3% Triton X-100, 5% Normal Goat Serum [NGS], 0.02% NaN₃) for 1 hour on a shaker at a low speed of 100rpm. Once blocking was complete, brain slices were placed into a 24-well plate in 500µl primary antibody solution composed primarily of ionized calcium-binding molecule 1 (Iba1) antibody (Iba1 antibody 1:500; Wako Chemicals USA, Product # 019-19741), 0.01M PBS, 0.3% Triton X-100, 2% Normal Donkey Serum, and 0.02% NaN₃, then sealed with parafilm and placed in the 4°C cold room on a shaker at a gentle speed (between 100-120rpm) overnight.

Day 2 Immunostaining Protocol

A 12-well wash plate was filled with 2.5ml PBST wash buffer. Brain slices that remained in primary antibody overnight were obtained from the shaker in the 4°C cold room and washed in the PBST wash buffer 3X for 5 minutes. Brain samples were transferred to a 24-well plate with 500µl secondary antibody solution composed of Alexa 488 1:2000 (Thermoscientific, Cat. #A21206), DAPI 1:2000 (Sigma, Cat. #D9452), 0.01M PBS, 0.3% Triton X-100, 2% Normal Donkey Serum, and 0.02% NaN₃, and sealed with parafilm. The plate was placed on a shaker for 2 hours at 90rpm. Due to photosensitivity of the secondary antibody solution, the plate was covered with aluminum foil for all steps moving forward. After incubation, samples were washed in PBST wash buffer 3X for 5 minutes, then a final wash was done in PBS. Each brain section was then mounted onto a coverslip and left to dry for 30-45 minutes under a dark cover to prevent photodecomposition. Slides were labelled with the applicable sample numbers. Once dry, a drop of mounting media (Fluoromount G, Southern Biotech Cat. #0100-01, or Fluoroshield with DAPI, Thermoscientific, Cat. #501128966) was placed onto slides and coverslips were carefully positioned on top of the slide, with a maximum of six coverslips per
The slides were placed in a slide box overnight at room temperature to continue drying. After 24 hours, the dry slides were stored in a slide box at 4°C for long-term storage (until imaging).

### 3.6.2 Imaging and Measurement of Microglial Cell Density

Two researchers (myself, RK) were blinded to treatment and intervention of each sample and performed microglial cell counts and analysis independently.

**Image acquisition**

All images were taken in the CMHT/BC Children’s Hospital Research Institute (BCCHR) Imaging Core Facility.

Z-stack images were acquired using a Leica SP5 Confocal Microscope at 40X magnification using objective EC “Plan-Neofluar” 40x/1.30 Oil DIC M27 with a 1.30 numerical aperture (Carl Zeiss Microscopy, Item #420462-9900-000). For all images, a 40X/0.75 Leica objective lens was used. The base and height of the dissector were set at 387.5 X 387.5 µm² and 10-20µm respectively. Image parameters had a guard space depth of 0.34µm. The image matrix was acquired at 512 x 512 pixels, a pixel scaling of 0.386 x 0.386µm, and a depth of 8 bit. Confocal images were collected in Z-stacks with a slice-distance of 0.4µm. Figure 3.3 provides an example of hippocampal CA3 localization in the right hemisphere of a male P8 mouse pup. Note that although Figure 3.3 provides an example of microglia imaging in the right hemisphere, images used to assess microglia density were obtained from both hemispheres.
Figure 3.3 Example of image localization of the hippocampal CA3 region. Blue fluorescence represents DAPI staining of neurons, while green fluorescence represents Iba1 staining of microglia. The image on the left depicts neurons in the right hemisphere hippocampus and a section of the dentate gyrus at 20X magnification. The image on the right focuses on the right hippocampal CA3 region, magnified at 40X, to display microglia in a singular plane within CA3. Z-stack images were obtained from the same region (right hemisphere hippocampal CA3) of each male mouse for microglial cell count.

Semi-manual 3D object counter using FIJI/ImageJ imaging software

Bilateral hippocampal CA3 Z-stack images acquired from the Leica SP5 Confocal Microscope were imported into the FIJI/ImageJ software for microglia cell counts and filter size based on microglial cell type was set to 30-50 pixels (Leyh et al., 2021; Young & Morrison, 2018). Using the green (Iba1-positive cell) channel, the FIJI/ImageJ software 3D object counter plugin v2.0 was mapped to display objects (i.e. microglia) and centroids. The software plugin was also employed to establish individual sample fluorescent thresholds for microglial cell counts, as microglia fluorescence varied across hippocampal Z-stacks. By determining the appropriate threshold for each sample, microglia cell counts can be established. To establish
microglia thresholds, Z-stacks were independently examined between myself and RK, with Iba1-positive (Iba1+) cells identified through manual observation. Following completion, results of independently assessed microglia thresholds for each Z-stack were cross-referenced between both observers, and threshold discrepancies greater than 10% were re-evaluated based on judgement. Total microglia cell counts calculated based on threshold assignment was then divided by the volume of each image stack (387.5 X 387.5 X 10.0-20.1µm) to obtain Iba1-positive cells/mm³.

3.6.3 Statistical Analysis

Inferential statistical analyses were not conducted for microglial cell density due to low sample size (n=2-4 male mice per group). Results from this experiment are in the preliminary stages and descriptive statistics were used to assess for trends in microglial density between experimental groups and controls.
Chapter 4: Hippocampal and Serum Inflammatory Cytokine Levels and Microglia Density in P8 Mice Treated with Neonatal Sucrose ± Procedural Pain

The objective of this study was to investigate the effects of sex, treatment (24% oral sucrose, sterile water), and/or intervention (needle-prick, handling) on cytokine levels in the right hemisphere hippocampus and serum of P8 mouse pups. Additionally, this study assessed bilateral hippocampal microglia density exclusively in male pups at P8. The subsequent sections of this chapter provide an overview of the experimental samples, describe weight distribution and differences between experimental groups, and present the results of statistical analyses on cytokine protein levels based on sex, treatment, and/or intervention.

4.1 Sample Description

C57BL/6J mice were randomly assigned to the control group or one of 4 experimental groups: SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), and WN (water + needle-prick). Table 4.1A-C provide a breakdown of the sample size and sex distribution of each experimental group for hippocampal cytokine analysis, serum cytokine analysis, and hippocampal microglial density respectively.
Table 4.1 Sample description of P8 C57BL/6J mouse pups within control and experimental groups for studies investigated in this thesis

A) Hippocampal cytokine analysis

<table>
<thead>
<tr>
<th>Sample size (n)</th>
<th>Control</th>
<th>SH</th>
<th>SN</th>
<th>WH</th>
<th>WN</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>24</td>
<td>23</td>
<td>20</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>58%</td>
<td>50%</td>
<td>52%</td>
<td>60%</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

B) Serum cytokine analysis

<table>
<thead>
<tr>
<th>Sample size (n)</th>
<th>Control</th>
<th>SH</th>
<th>SN</th>
<th>WH</th>
<th>WN</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>15</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>38%</td>
<td>64%</td>
<td>47%</td>
<td>45%</td>
<td>56%</td>
<td></td>
</tr>
</tbody>
</table>

C) Hippocampal microglia density

<table>
<thead>
<tr>
<th>Sample size (n)</th>
<th>Control</th>
<th>SH</th>
<th>SN</th>
<th>WH</th>
<th>WN</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Cytokine levels and microglia cell counts were assessed for experimental groups SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick), and C (controls). A) Hippocampal cytokine analysis, B) Serum cytokine analysis, C) Hippocampal microglia density.
Body weight

Body weight was measured daily in mice of all experimental groups from P1-P8 prior to euthanasia, apart from the rest day at P7. Animals in the control group were not weighed until the day of euthanasia (P8) to maintain them as true, undisturbed controls. There were no significant differences in weight between mouse pups in experimental groups SN, SH, WN, and WH at any postnatal age (Figure 4.1) as revealed by a two-way repeated measures ANOVA. A Tukey post hoc analysis further revealed no significant differences in weight from P1-P8 between experimental groups. At P8, no significant differences in weight were uncovered between control, SN, SH, WN, and WH groups (Figure 4.1), as revealed by a one-way ANOVA. Note that sample size range for weight data is greater than cytokine and microglial density combined. This is due to weight data for additional control mice and female mice collected for subsequent microglia cell counts being included in weight change analysis.

![Change in Mice Weight from Postnatal Day 1 to 8](image)

**Figure 4.1 Change in mice weight from postnatal day 1 to 8.** Weight changes were monitored for experimental groups SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick) from postnatal day 1 to 8. Control group (C) weights were measured only at postnatal day 8. Values presented as mean ± SEM. n=26-37 mice per group.
4.2 Right Hemisphere Hippocampal Inflammatory Cytokine Levels in P8 Mice

Data were deemed normally distributed visually using QQ plots and through statistical analysis with a Shapiro-Wilk test. Grubbs’ test identified a single outlier in hippocampal IL-1β (WH group) and IL-6 (SN group) respectively, which deviated significantly from the rest of the data. To maintain the robustness of my analysis, these two outliers were removed from the datasets. IFN-γ protein levels were undetectable in all hippocampal samples, thus excluded in further analyses. A set of two-way ANOVAs revealed no significant sex-based differences in right hemisphere hippocampal cytokine levels across all experimental groups and controls. Therefore, sex was not considered in subsequent statistical analyses, and both male and female cytokine data were combined for experimental and control groups. This first set of two-way ANOVAs revealed experimental group effects for cytokines IL-5 and IL-10, which were further assessed with a second two-way ANOVA as described in Chapter 3. The second set of two-way ANOVAs demonstrated that repeated exposure to experimental treatment (24% sucrose or water) and intervention (needle-prick or handling) had no independent or interactive effect on hippocampal cytokine levels, but this set of analyses did not include control groups. Controls were not included here to assess whether administered treatments or interventions had independent effects on inflammatory cytokine levels.

A set of one-way ANOVAs revealed that IL-5 protein levels were significantly greater in P8 mice exposed to water + handling compared to controls ($F_{(4,99)} = 2.786; p<0.05$) and water + needle-prick exposed mice ($F_{(4,99)} = 2.786; p<0.05$). No other experimental group displayed significant differences in IL-5 levels (Figure 4.2D).
IL-10 protein levels were significantly greater in control mice compared to the sucrose + handling ($F_{(4,99)} = 4.430; p<0.01$), water + handling ($F_{(4,99)} = 4.430; p<0.05$), and water + needle-prick exposed mice ($F_{(4,99)} = 4.430; p<0.05$). No significant differences were observed in IL-10 protein levels between mice in the control group and those in the sucrose + needle-prick group (Figure 4.2E). Significant differences remained evident after correcting for multiple comparisons using Tukey’s post hoc test. There were no statistically significant differences between experimental and control groups for protein levels of IL-1β, IL-6, TNF-α (Figures 4.2A-C), IL-2, IL-12p70, KC/GRO, and IL-4 (Figures 4.2F-I). Table 4.2 provides a summary of findings where statistically significant differences in cytokine protein levels in right hemisphere hippocampal samples were found. A complete summary of all one-way ANOVA statistics for the 9 assessed cytokines in the right hemisphere hippocampus can be found in Appendix B: Additional Statistical Analysis.
Figure 4.2 Inflammatory cytokine protein levels in the right hemisphere hippocampus after repeated sucrose and/or pain exposure in postnatal day 8 mice.
Cytokine levels were assessed for experimental groups SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick), and C (controls). Values presented as mean ± SEM. A) IL-1β, B) IL-6, C) TNF-α, D) IL-5, E) IL-10, F) IL-2, G) KC/GRO, H) IL-12p70, I) IL-4. *p<0.05, **p<0.01; n=18-24 mice per group.

Table 4.2 Summary of statistically significant differences in cytokine protein levels in right hemisphere hippocampal samples

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>F (4, 99)</th>
<th>Experimental Group</th>
<th>Adjusted p-value</th>
<th>Mean difference</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>2.786</td>
<td>C vs. WH</td>
<td>0.0334</td>
<td>-0.0747</td>
<td>-0.1456 to -0.0039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WH vs. WN</td>
<td>0.0491</td>
<td>0.0721</td>
<td>0.0002 to 0.1439</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.430</td>
<td>C vs. SH</td>
<td>0.0017</td>
<td>0.4163</td>
<td>0.1180 to 0.7146</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C vs. WH</td>
<td>0.0244</td>
<td>0.3408</td>
<td>0.0296 to 0.6520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C vs. WN</td>
<td>0.0138</td>
<td>0.3725</td>
<td>0.0531 to 0.6920</td>
</tr>
</tbody>
</table>

F-statistic was calculated using a one-way ANOVA. Adjusted p-values, mean differences, and 95% confidence intervals were calculated using the Tukey multiple comparisons test. SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick), and C (controls).
4.3 Serum Inflammatory Cytokine Levels in P8 Mice

Data were deemed normally distributed visually using QQ plots and through statistical analysis with a Shapiro-Wilk test. Grubbs’ test identified a single outlier in serum IL-6 (WH group), IL-2 (SH group), and KC/GRO (WH group) respectively, which deviated significantly from the rest of the data. To maintain the robustness of my analysis, these three outliers were removed from the datasets. IFN-γ protein levels were undetectable in all serum samples, thus excluded in further analyses. A first set of two-way ANOVAs revealed no significant sex-based differences in serum cytokine protein levels across all experimental groups and controls. Therefore, sex was not considered in subsequent statistical analyses, and both male and female cytokine data were combined for experimental and control groups. This first set of two-way ANOVAs revealed experimental group effects in cytokines IL-1β and IL-5, which were further assessed with a second set of two-way ANOVAs. The second set of two-way ANOVAs demonstrated that repeated exposure to experimental treatments and interventions had independent effects on IL-1β levels, but no independent or interactive effect on IL-5. However, this set of analyses did not include control groups, as described in Section 4.2.

A set of one-way ANOVAs revealed that IL-1β protein levels were significantly greater in P8 mice that were exposed to sucrose + handling compared to controls (F(4,52) = 6.131; p<0.01), sucrose + needle-prick (F(4,52) = 6.131; p<0.01), and water + needle-prick (F(4,52) = 6.131; p<0.01) groups. No significant differences were observed in serum IL-1β protein levels in the water + handling group compared to controls or any experimental group (Figure 4.3A). Additionally, IL-5 serum protein levels were significantly greater in the control group compared to levels in the sucrose + needle-prick (F(4,52) = 2.272; p<0.05) group. No other treatment and intervention groups showed significant differences in serum IL-5 protein levels.
(Figure 4.3D). Significant differences remained evident after correcting for multiple comparisons using Tukey’s post hoc test.

There were no statistically significant differences between experimental and control groups for protein levels of all the other inflammatory cytokines, i.e. IL-6 (Figure 4.3B), TNF-α (Figure 4.3C), or IL-10, IL-2, IL-12p70, KC/GRO, and IL-4 (Figures 4.3E-I). Table 4.3 provides a summary of the findings where statistically significant differences in cytokine protein levels in serum samples were shown. A complete summary of all one-way ANOVA statistics for the 9 assessed cytokines in serum samples can be found in Appendix B: Additional Statistical Analysis.
Figure 4.3 Inflammatory cytokine protein levels in serum after repeated sucrose and/or pain exposure in postnatal day 8 mice.
Cytokine levels were assessed for experimental groups SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick), and C (controls). Values presented as mean ± SEM. A) IL-1β, B) IL-6, C) TNF-α, D) IL-5, E) IL-10, F) IL-2, G) KC/GRO, H) IL-12p70, I) IL-4. *p<0.05, **p<0.01; n=8-15 mice per group.

Table 4.3 Summary of statistically significant differences in cytokine protein levels in serum samples.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>F</th>
<th>Experimental Group</th>
<th>Adjusted p-value</th>
<th>Mean difference</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>F (4,52) = 6.1310</td>
<td>C vs. SH</td>
<td>0.0066</td>
<td>-2.7960</td>
<td>-5.006 to -0.5847</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH vs. SN</td>
<td>0.0048</td>
<td>2.4800</td>
<td>0.6265 to 4.3340</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH vs. WN</td>
<td>0.0010</td>
<td>3.1490</td>
<td>1.0170 to 5.2800</td>
</tr>
<tr>
<td>IL-5</td>
<td>F (4,52) = 2.2720</td>
<td>C vs. SN</td>
<td>0.0483</td>
<td>39.4600</td>
<td>0.1914 to 78.7300</td>
</tr>
</tbody>
</table>

F-statistic was calculated using a one-way ANOVA. Adjusted p-values, mean differences, and 95% confidence intervals were calculated using the Tukey multiple comparisons test. SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), WN (water + needle-prick), and C (controls).
4.4 Hippocampal Microglia Density in Male P8 Mice

In this preliminary analysis, bilateral hippocampal microglia density was only assessed in males. Additionally, statistical comparisons of hippocampal microglia density between groups were not conducted due to a limited sample size ($n=2-4$ mice/group) and a wide range in density cell counts within each experimental group (especially in water treated mice). Results from this experiment are in the preliminary stages and used to explore trends in microglial density between groups. A trend suggested that in males, hippocampal microglial density is altered in all experimental groups compared to controls (see Figure 4.4). SN, WH, and WN males display elevated microglia compared to controls, whereas SH exhibit a slightly reduced microglia density compared to controls. For example, control males displayed a mean of 7203 cells/mm$^3$ compared to 9652 cells/mm$^3$ in WH males, with a mean difference of 2449 cells/mm$^3$.

**Treatment and Intervention Effect on Male Hippocampal Iba1+ Cells**

![Graph showing treatment and intervention effect on male hippocampal Iba1+ cells](image)

Figure 4.4 Male microglia density in the hippocampal CA3 region after repeated sucrose and/or pain exposure in postnatal day 8 mice. Experimental groups SH (sucrose + handling), SN (sucrose + needle-prick), WH (water + handling), and WN (water + needle-prick), and C (controls) were assessed. Values presented as mean ± SEM. $n=2-4$ mice per group.
4.5 Summary

Overall, there were no sex effects on cytokine protein levels in the right hemisphere hippocampus and serum of P8 mice in all groups. In the right hemisphere hippocampus, IL-5 protein levels were significantly greater in mouse pups that were exposed to water + handling compared to both controls and water + needle-prick exposed mice. IL-10 protein levels were significantly greater in control mice compared to the sucrose + handling, water + handling, and water + needle-prick exposed mice.

In serum, IL-1β protein levels were significantly greater in mouse pups that were exposed to sucrose + handling compared to controls, sucrose + needle-prick, and water + needle-prick. IL-5 protein levels were also significantly greater in the control group compared to pups in the sucrose + needle-prick group.

Finally, microglia density in the hippocampal CA3 region of male pups at P8 suggested that microglial cell counts are altered in all experimental groups compared to controls.
Chapter 5: Discussion

For my thesis research, I investigated the short-term effects of repeated neonatal pain and/or sucrose exposure on inflammatory cytokine levels in the hippocampus and serum of mice at postnatal day eight (P8). In addition, I also explored the effects of repeated neonatal pain and/or sucrose exposure on microglial cell density in P8 males. To my knowledge, no published studies have investigated the effects of repeated neonatal pain and/or sucrose exposure on the inflammatory cytokine profile in the brain or serum in mice. Using a translational neonatal mouse paradigm that mimics the use of oral sucrose solution to treat neonatal procedural pain in the NICU (Ranger et al., 2019; Tremblay et al., 2017), I was able to assess protein levels of nine inflammatory cytokines in hippocampal tissue and serum, as well microglial cell counts in the hippocampus of mice at P8. IFN-γ was undetectable in both tissue types and therefore not further analyzed. Experimental treatments (24% oral sucrose, sterile water) and interventions (needle-prick, handling) were administered from P1 to P6, and mouse pups were euthanized and whole brains and serum were collected at P8. Inflammatory cytokine levels were then assessed in the right hemisphere hippocampus and serum. Microglia density was assessed in the bilateral hippocampi of P8 males only.
Results overview

Data from my thesis studies did not support my hypothesis of sex differences in cytokine levels in the hippocampus or serum of P8 mice exposed to repeated neonatal pain and/or sucrose.

In the hippocampus, treatment and intervention exposure had significant effects on IL-5 and IL-10 cytokine levels, but alterations were not more robust in the sucrose + needle-prick (SN) group compared to other groups as originally hypothesized. In the hippocampus, results show that mouse pups exposed to water + handling (WH) had elevated hippocampal IL-5 levels compared to controls and water + needle-prick (WN) exposed mice at P8, while hippocampal IL-10 levels were significantly greater in control mice compared to the sucrose + handling (SH), WH, and WN animals. Further contradicting my hypothesis, treatment and intervention exposure had no significant effects on IL-1β, IL-6, and TNF-α levels in the P8 hippocampus.

In serum, treatment and intervention exposure had significant effects on IL-5 cytokine levels. In support of my hypothesis, systemic IL-5 protein levels were significantly lower in the SN group compared to controls. IL-1β protein levels were also significantly altered in serum of P8 mice, but these results were not more robust in the SN group as originally hypothesized. Contrary to my hypothesis, elevations in IL-1β levels were only found in mice exposed to SH compared to those in the control, SN, and WN groups. Further refuting my original hypothesis, treatment and intervention exposure did not have significant effects on IL-6 and TNF-α serum protein levels at P8.

Although statistical significance could not be reached for male hippocampal microglial cell density, possibly due to minimal sample size, a trend did support my hypothesis that microglia density is affected by repeated neonatal sucrose and/or pain exposure. Interestingly,
the observed trend suggests that any combination of treatment and intervention has an impact on microglia cell counts in the hippocampus of male mice at P8 in comparison to control pups.

*Sex-based differences in cytokine protein levels*

Contrary to my initial hypothesis, there were no sex-based effects on cytokine levels for any of the 9 analyzed inflammatory cytokines. More specifically, sex effects on IL-1β, IL-6, and TNF-α levels were not present in either tissue type at P8. This was an unexpected finding due to previous literature supporting sex-based differences in cytokine levels found in the hippocampus and serum of rodents of varying age.

In the hippocampus, IL-1β, IL-6, and TNF-α display significant sex differences in rodent models. IL-1β mRNA expression was elevated in adult female mice after experiencing chronic adolescent stress post-LPS administration, but the same effect was not observed in males (Bekhbat et al., 2019). Greater astrocytic TNF-α expression was shown in human adult female astrocytes compared to males after LPS stimulation (Goldstein et al., 2021), and age-related elevations in IL-6 levels were observed in the ventral hippocampus of adult female C57BL/6J mice (aged 18-24 months) compared to adult males and younger females (Porcher et al., 2021). In this same study, “young” aged mice assessed by Porcher et al. (2021) ranged from 2-5 months of age, and this age range failed to display sex differences in cytokine levels at the adolescent stage. In addition, microglial-neuronal signaling in response to pain has been suggested to be sexually dimorphic, where microglia in female rodents with peripheral nerve injuries do not contribute to mediating pain hypersensitivity unlike males (Reviewed in Mapplebeck et al., 2016; Miller et al., 2005; Sorge et al., 2015). Published evidence described above may suggest that in mice, the brain may not display sex-differences in inflammatory cytokine levels until
adulthood, or mechanisms underlying pain hypersensitivity may be acting along different sex-dependent pathways.

In plasma, sex-dependent differences in inflammatory cytokine levels are also observed in the literature. Plasma IL-1β concentrations were elevated in adult male mice exposed to chronic stress during adolescence, but only in those who received LPS administration prior to assessment (Bekhbat et al., 2019). Greater plasma IL-6 is also observed at “baseline” in adult females compared to male rats (O’Connor et al., 2007). To the best of my knowledge, no study has investigated sex-based differences in TNF-α levels in serum or plasma in rodent ELS models. In addition, there are no previous reports investigating sex-based differences in serum cytokine levels in P8 mice or very preterm infants after sucrose and/or pain exposure. It is possible that sex-based differences in cytokine levels, especially for my cytokines of interest, after repeated neonatal sucrose and/or pain emerge in later developmental periods, hence explaining why differences were not observed in peripheral circulation of the early-life mouse.

Hippocampal cytokine protein levels

Unexpectedly, higher levels of hippocampal IL-5 were observed in the WH group compared to controls and WN exposed mice at P8. Because IL-5 has been noted to display both pro- (Pelaia et al., 2019) and anti-inflammatory actions (Hodges et al., 2022; Merriwether et al., 2021; Pirola & Ferraz, 2017), higher levels in the “least adverse” experimental group (WH) as presented in my thesis may suggest that IL-5 acts through anti-inflammatory pathways with repeated neonatal sucrose and/or pain exposure in the brain. However, if this is how IL-5 activity is mediated in the brain, it would also be expected that the undisturbed control group would display higher IL-5 levels in comparison to the WH group. A human model of chronic pain has
suggested that IL-5 acts as an anti-inflammatory cytokine and its release induces analgesic effects (Merriwether et al., 2021), but this contradicts my findings because the needle-prick intervention groups displayed no significant differences in IL-5 levels compared to controls.

Furthermore, hippocampal IL-10 protein levels were significantly greater in control mice compared to the SH, WH, and WN animals. This was also an unexpected finding. To the best of my knowledge, there are no published studies investigating neonatal repeated sucrose and pain exposure on the brain’s inflammatory profile, and similar results have not been observed in other animal models of ELS. IL-10 has been described as a potent anti-inflammatory cytokine (Maes et al., 1998; Ng, 2003; Zhang & An, 2007) responsible for the prevention of inflammation (Sabat et al., 2010). Interestingly, IL-10 is also implicated in repressing inflammatory cytokines in the IL-1 family, IL-6, and TNF-α (Zhang & An, 2007). As my results show, exposure to any of the experimental treatment and intervention combinations of SH, SN, WH, and WN had no significant effects on IL-1β, IL-6, and TNF-α levels in the hippocampus. Because hippocampal IL-10 levels were shown to be significantly in lower in mouse pups in the SH, WH, and WN groups, it would be expected that these same groups exhibit elevated IL-1β, IL-6, and TNF-α levels, but this was not the case. It is possible that in the context of sucrose and/or pain exposure, IL-10 may not be directly influencing cytokine levels of my three inflammatory markers of interest. Based on my results, IL-10 levels in the SN group did not exhibit significant differences relative to controls, which may insinuate alternative inflammatory pathways involved in response to neonatal pain and sucrose exposure. In terms of ELS, IL-10 signalling may be exclusive to repeated neonatal sucrose or pain exposure in the hippocampus but not their combination.

Contrary to my initial hypotheses, exposure to any of the treatment and intervention combinations did not have a significant effect on hippocampal IL-1β, IL-6, and TNF-α levels
Based on studies investigating the effect of ELS exposure on IL-1β protein and mRNA levels (Bodnar et al., 2016; Roque et al., 2016; Wang et al., 2020), it was expected that pain and/or sucrose exposure would show parallel effects in the hippocampus. Rats that experienced repeated maternal separation (RMS) exhibited elevated IL-1β mRNA expression in the hippocampus at P15 (Roque et al., 2016), and in P8 rat pups that experienced prenatal alcohol exposure, IL-1β protein levels were elevated in the hippocampus (Bodnar et al., 2016). Furthermore, high sugar (fructose) consumption in juvenile rats produced elevated IL-1β and IL-6 levels in the dorsal hippocampus (Hsu et al., 2015). Although repeated neonatal pain exposure had not been directly assessed in any of the mentioned studies in relation to inflammatory markers, evidence strongly supports that sugar (i.e. fructose) and other models of ELS contribute to altered IL-1β and IL-6 inflammatory states in the hippocampus of rats.

Hippocampal TNF-α levels in my thesis also failed to show significant differences between groups. Previous studies have established that RMS until P10, P15, and P20 elevate TNF-α levels in the hippocampus, hypothalamus, and prefrontal cortex (Giridharan et al., 2019; Roque et al., 2016; Wang et al., 2020). Interestingly, when assessed at P8, prenatal alcohol exposure was not found to have the same effects as observed in RMS. Prenatal alcohol-exposed mice showed no significant differences compared to controls in hippocampal TNF-α protein levels (Bodnar et al., 2016). It is possible that altered hippocampal IL-1β, IL-6, and TNF-α cytokine levels reported in maternal separation ELS models and/or high sugar consumption are exclusively observed in rat models. Inflammatory processes in rat models may differ from mouse models of ELS as inflammatory effects in one rodent model may not be representative of the other. Importantly, it is plausible that changes are observed in later developmental periods, as Roque et al. (2016) implemented their ELS model up to when they measured cytokine levels at
P15, while Hsu et al. (2015) investigated 30-day sucrose exposure on cytokines at P30. Results observed in my thesis seem to suggest that IL-1β, IL-6, and TNF-α are not involved in inflammatory signalling associated with repeated neonatal sucrose and/or pain exposure, and if they were, these effects may either be specific to rat models of ELS or evident in later developmental stages (e.g. post-weaning, young adulthood).

*Serum cytokine protein levels*

In support of my hypothesis, IL-5 levels in the SN group were significantly lower compared to controls. In addition, IL-1β protein levels were significantly different in serum of P8 mice, but contrary to my hypothesis, it was mouse pups in the SH group that displayed heightened IL-1β levels compared to those in the controls, SN, and WN groups. Further refuting my initial hypothesis, none of the treatment and intervention exposures had significant effects on IL-6 and TNF-α serum levels at P8.

An interesting finding from my research was the significantly lower levels of IL-5 found in the SN group compared to controls. Although not a primary cytokine of interest, the results align with my hypothesis that SN exposure would produce the most robust cytokine response compared to mice in other experimental groups and/or controls. This is similar to results observed in RMS, where rats displayed elevated IL-5 protein levels at P10 compared to controls, but this was specific to the prefrontal cortex (Giridharan et al., 2019). Given IL-5 levels were significantly lower in the most adverse experimental condition (SN) in the study, my results may suggest that in the context of repeated neonatal exposure to sucrose and pain, IL-5 functions through anti-inflammatory pathways, thus dampening pro-inflammatory cytokine levels in the SN group.
IL-1β in the SH group showed the highest protein levels in comparison to controls, SN, and WN groups, and based on my initial hypothesis, sucrose treatment was expected to heighten IL-1β signalling in the serum. However, my initial hypothesis suggested this pattern of activation was expected to be equivalent to, if not more robust, in the SN group, which was believed to be my most adverse experimental condition. Based on Figure 4.3A, IL-1β levels in the SN group were very similar to those in the control group. This may suggest that additional inflammatory mechanisms, such as serum IL-5 levels which were lower in the SN group mouse pups in comparison to controls (see Figure 4.3E), may be contributing to the lower IL-1β serum levels found in the SN group (i.e. dampening effect). However, the means behind this potential interaction are unclear and would need to be further investigated.

Going against my initial hypothesis, treatment and intervention exposure had no significant effects on IL-6 and TNF-a levels in serum of P8 mice. Bodnar et al. (2016) did not observe significant differences in serum IL-6 protein levels in the P8 offspring of female rats exposed to prenatal alcohol either, but elevated TNF-α levels were observed in serum compared to controls. Interestingly when assessing IL-6 levels in healthy adult human males, plasma levels of IL-6 were higher in those who experienced early-life stress with and without an additional acute stressor (i.e. a second-hit) (Carpenter et al., 2010; Hartwell et al., 2013; Pace et al., 2006). This supports evidence published by Bilbo et al. (2005, 2009) where a subsequent stressor is required after ELS exposure in order to induce an inflammatory response. It is possible that depending on the age of assessment, inflammatory cytokine levels will vary given the effects of repeated sucrose and/or pain and P8 may not be a sufficient age to observe these effects in mice. Furthermore, differences in cytokine levels between ELS models in humans and rodents may
suggest that repeated neonatal sucrose and/or pain exposure in comparison to RMS, prenatal alcohol exposure, and childhood maltreatment affect different neuroinflammatory pathways.

*Comparison between hippocampus and serum inflammatory cytokine levels*

My thesis findings did not show any statistically significant effects of repeated neonatal treatment and/or intervention exposure on inflammatory cytokine levels for IL-2, IL-4, KC/GRO, and IL-12p70 in the hippocampus and serum. There is insufficient evidence from peer-reviewed literature supporting a relationship between these cytokines and ELS. Therefore, it is believed that IL-2, IL-4, KC/GRO, and IL-12p70 levels are expressed without being influenced by repeated exposure to sucrose and/or pain, or other form of ELS. Oddly, my results revealed undetectable IFN-γ levels in all P8 mouse samples in both the hippocampus and serum. IFN-γ levels have been shown to be elevated in early-life RMS at P10 (Giridharan et al., 2019) and prenatal alcohol exposure at P8 (Bodnar et al., 2016) in the prefrontal cortex and hippocampus respectively, but these effects are only observed in rats. This may indicate that even if IFN-γ is involved in the inflammatory signalling pathway in early-life sucrose and/or pain exposure, effects may only be observable in rat pups. Furthermore, IFN-γ protein detection in specific regions of the brain may be ELS-model dependent as well.

Exposure to repeated neonatal sucrose and/or pain did not result in significant differences in IL-1β, IL-6, and TNF-α levels in the hippocampus, as well as IL-6 and TNF-α in serum, as previously stated. However, it is possible that a subsequent immune challenge, or “second hit,” is required in adulthood to observe a heightened immune response (Bilbo et al., 2005; Bilbo & Schwarz, 2009). Exposure to infectious agents in early postnatal life can alter immune outcomes in adulthood in animal models (Bilbo et al., 2005). For example, it has been shown that adult rats
neonatally infected with *Escherichia coli* displayed impaired memory and altered IL-1β levels in the hypothalamus, but only in rats that received LPS in adulthood (Bilbo et al., 2005). Giridharan et al. (2019) also showed similar effects requiring a subsequent immune challenge after ELS, as rats at P15 exposed to RMS who also experienced an acute physiological stressor prior to sacrifice (i.e. a “second hit”) displayed elevated IL-6 levels in the hypothalamus compared to those who did not. This evidence suggests that a subsequent stressor after ELS exposure is required to induce a modified immune response. This may extend to repeated sucrose and/or pain exposure to produce significant inflammatory responses in various cytokines (i.e. elevated IL-1β, IL-6, and TNF-α levels). However, additional studies are required to support this premise.

Because inflammatory cytokines IL-1β, IL-6, and TNF-α did not exhibit a robust response in the SN group as originally hypothesized, it is also plausible to consider alternative mechanistic pathways that may be attributed to negative developmental outcomes associated with ELS exposure. For example, the administration of oral sucrose to preterm infants for procedural pain was found to elevate plasma adenosine triphosphate oxidative stress markers (Asmerom et al., 2013). This increase has the potential to cause cellular damage, and it may serve as a mechanistic pathway leading to a reduction in regional brain volumes previously reported (Tremblay et al., 2017). Furthermore, rats subjected to repetitive neonatal pain also exhibited heightened neuronal excitation and neuronal cell death in various cortical and subcortical regions, including the hippocampus (Anand et al., 2007). In addition to oxidative stress markers and neuronal cell death, neonatal pain and reduced maternal care in rat pups produced elevated corticosterone but decreased glutamate levels in the hippocampus and frontal cortex at P4 (Mooney-Leber et al., 2018). This combination also led to modified hypothalamic-pituitary-adrenal (HPA) axis recovery following an acute stressor in adulthood, but effects were observed only in adult female
rats (Mooney-Leber & Brummelte, 2020). Alternative mechanistic pathways involved in ELS outcomes can also include glutamatergic and GABAergic transport, as early-life RMS downregulated vesicular glutamate transporters 1 and 2 (VGlut1 and VGlut2) and GABA transporter (VGAT) in the hippocampus of adult rats (Martisova et al., 2012). These mechanistic pathways may contribute to the macrostructural and behavioural alterations previously reported in adulthood following repeated neonatal pain and/or sucrose exposure (Ranger et al., 2019; Tremblay et al., 2017), but further investigation is required.

*Microglia density*

As microglia density assessment in the hippocampus was preliminarily assessed, only trends in microglia density could be inferred. Based on my results, all experimental groups displayed alterations in microglia density compared to controls. Whereas 8-day old mouse pups in the SN, WH, and WN groups showed higher microglia cell counts compared to those in the control group, those in the SH group showed slightly lower microglia counts. In steady state conditions (i.e. controls), microglia are less responsive to challenges but continuously survey their micro-environments (Paolicelli et al., 2022; Perry & Teeling, 2013). With the repeated neonatal exposure to SN, WH, or WN, the disruption in homeostasis may have influenced microglia proliferative markers, therefore altering microglia density in the hippocampus. This could explain why microglia counts in SN, WH, and WN groups were greater than in control mice. However, reasons behind lower microglia density in the SH group compared to controls are unclear. If independently administering sucrose halts or downregulates microglia proliferation (or upregulates cell death markers) in the hippocampus, this would corroborate that oral sucrose provides no evidence of neuroprotective effects in the brain, further substantiating
the growing concerns behind its use in clinical practice (Holsti & Grunau, 2010; Keels et al., 2016). However, the SH group contained only two subjects at the time of assessment, so the small sample size does not provide sufficient data to accurately determine microglia density in this group and sucrose’s relationship with microglia proliferation.

In response to threats, microglia display elevated proliferation and greater density (Gomez-Nicola et al., 2013), and are often triggered very early in response to subtle changes in their microenvironment (Kreutzberg, 1996). Microglia responsivity is closely linked to IL-1β release in the brain (Madry et al., 2018; Reviewed in Paolicelli et al., 2022), and since significant alterations in hippocampal IL-1β levels after repeated sucrose and pain exposure were not observed in my results, this may suggest that microglia are not in their fully responsive or developmentally mature states at P8. In addition, microglia responsivity states can be defined by several intrinsic and extrinsic determinants (e.g. sex, genetic background), spatiotemporal context (e.g. age, environment), and layers of complexity (e.g. epigenomics, proteomics, phenomics). For example, mouse models of early postnatal development express elevated proliferative-associated microglia markers, which may contribute to elevated microglia density trends observed in my thesis (Reviewed in Paolicelli et al., 2022). On the other hand, adult mouse models exhibit homeostatic microglia signatures. If true, alterations in microglia density may suggest that neonatal sucrose and/or pain exposure influence microglia proliferation and/or cell death markers at P8 and could provide an avenue to investigate such changes.

My preliminary results suggest that exposure to seemingly insignificant stressors (i.e. water, handling) can induce a microglial response beyond its surveillant state and may promote microglial proliferation in the hippocampus. Despite being originally hypothesized that water and handling exposure would not produce as robust of a response in microglia density compared
to sucrose and pain, it is apparent that even mild stressors can lead to disturbances in microglial homeostasis. These deviations from microglial homeostasis generate a microglial response within the hippocampus, which may influence microglia cell proliferation.
5.1 Limitations

Although investigation on the effects of neonatal pain and/or sucrose on inflammatory responses in the hippocampus, serum, and hippocampal microglial density revealed interesting findings, there are several limitations in my thesis that must be acknowledged.

Inflammatory marker analysis

Studies that assessed hippocampal cytokine levels (e.g. IL-1β, IL-6, and TNF-α) after various forms of ELS (see Table 1.3) investigated bilateral hippocampal cytokine levels. When investigating cytokine protein levels of the hippocampus in this thesis, I only examined the right hemisphere due to limited tissue availability. Therefore, my findings are not generalizable to the whole hippocampus’ cytokine profile after repeated neonatal sucrose and/or pain exposure as results from other models of ELS are. Furthermore, it is possible that cytokine levels not being measured in both hemispheres explain the lack of statistical significance associated with neonatal pain and sucrose exposure seen in this thesis.

In addition to very specific tissue localization, the absence of a distinct relationship between hippocampal and serum cytokine levels makes it difficult to infer cytokine alterations in one tissue type using the other. Even if a specific immune signature exists in the P8 mouse hippocampus and serum after repeated neonatal sucrose and/or pain exposure, relative alterations in hippocampal tissue cannot infer alterations in serum cytokine levels, and vice versa. This becomes a drawback for future studies exploring potential brain biomarkers using serum cytokine levels of human preterm infants exposed to repeated neonatal pain and/or sucrose. If the inflammatory profile in serum is correlated to the brain, this relationship may provide insight on whether inflammatory pathways are involved in adverse neurodevelopmental processes observed
in human and animal subjects exposed to repeated neonatal sucrose and/or pain (e.g. Asmerom et al., 2013; Brummelte et al., 2012; Chau et al., 2019; Ranger et al., 2013; Smith et al., 2011; Vinall et al., 2014). However, the absence of a distinct relationship between hippocampal and serum cytokine levels observed in my thesis makes this difficult to determine.

Although duplicate intra-sample reliability was high for my data, inter-mice variability did also exist (e.g. outliers in hippocampal and serum IL-6 levels, hippocampal IL-1β). Maternal care (e.g. licking, grooming, time spent with offspring) was not measured in my thesis but could act as a buffer on the effects of my administered treatments and/or interventions on cytokine levels. Maternal care has been previously shown to produce direct effects on the development of neural systems involved in cognitive, emotional, and neuroendocrine responses to stress (Meaney, 2001). Low levels of licking and grooming from mothers have been associated with reduced hippocampal long-term potentiation and lower glucocorticoid and mineralocorticoid receptors in adult offspring (Champagne et al., 2008). It has also been associated with elevated HPA responses to stress (Liu et al., 1997; Weaver et al., 2004). For my thesis, the inability to measure the frequency and/or amount of maternal care could present a confounding variable that influenced the immune profile observed at P8. To the best of my knowledge, maternal care has not been previously investigated in relation to inflammatory cytokine levels, but it is possible that differences in maternal care that were not controlled in my experimental model contributed to the inter-animal variability that was observed in my data.
Microglia density

A major limitation pertaining to microglia density assessments were the sex and number of subjects analyzed. The analysis of microglia density had a limited sample size, with only 2 to 4 subjects per group, all of whom were males. The limited sample size prevents statistical analyses from being conducted due to a lack of statistical power, allowing only descriptive analyses and visual assessment of trends in microglia cell counts between groups. Furthermore, assessing only male subjects restricts the generalizability of my findings, and trends that are observed in male subjects may not be applicable to females. Microglial-neuronal signaling in response to pain has been suggested to be sexually dimorphic, where microglia in female rodents with peripheral nerve injuries do not contribute to mediating pain hypersensitivity unlike males (Reviewed in Mapplebeck et al., 2016; Miller et al., 2005; Sorge et al., 2015). If sex-based differences in microglia density are prevalent in the hippocampus, an exclusively male sample does not allow for further investigation of these differences.

For the assessment of microglia density in any brain region, 3D (Z-stack) images must be captured, and microglia cell counts assessed within the 3D image. Developmentally, the P8 mouse brain is much smaller in comparison to an adult mouse, so when assessing microglia in a developmentally immature structure (i.e. the hippocampus), the reduced size of the hippocampus means that Z-stacks obtained were also much smaller than normally assessed in adult rodent models. In addition to the reduced 3D image size, my assessment of microglia density was specific only to the CA3 region of the bilateral hippocampus. Although my findings are in the preliminary stages, localizing microglia cell counts in a very specific hippocampal region reduces the generalizability of microglia density trends throughout the hippocampus, so trends observed in CA3 may not be replicated in other hippocampal regions. Moreover, microglia cell
counts in my thesis were confined to a depth between 1550-2410µm from the anterior surface of the brain, again contributing to the lack of generalizability of my findings.

Lastly, to reduce background noise of 3D images, pixel sizes needed to be restricted to 30-50 pixels in the FIJI/ImageJ software, as this is the typical pixel size used for microglia cell counts (Leyh et al., 2021; Young & Morrison, 2018). However, the stringent pixel range could have resulted in the underestimation of microglia cell counts, as smaller microglia would not be accounted for by the 3D object counter software. To mitigate this, smaller microglia may need to be manually counted within Z-stacks, but employing such methods could introduce additional human error and will be time-consuming.
5.2 Future Directions

For future studies, several avenues can be explored in both inflammatory marker analysis and microglial density, which will help to further investigate the neurodevelopmental effects of sucrose and pain on brain development.

*Inflammatory marker analysis and microglia density*

To further explore the effects of repeated neonatal sucrose and pain exposure on inflammatory cytokine levels and microglia density, it would be valuable to investigate their long-term effects at different stages of development, including adolescence and adulthood. Exploring long-term effects will provide insight on whether repeated neonatal sucrose and/or pain exposure has short-term, temporary effects on inflammatory processes, or whether effects are persistent throughout the lifespan. In addition to these studies, if cytokine and microglia effects are present, also investigating implications on behaviour and cognitive function, such as memory, at the assessed developmental periods may provide information on whether altered inflammatory markers correlate with previously reported behavioural outcomes associated with early-life sucrose and/or pain exposure (Anand et al., 1999; Nuseir et al., 2015; Ranger et al., 2019).

It would also be beneficial to assess inflammatory cytokine levels and microglia density in different brain regions, such as the prefrontal cortex and hypothalamus, which have been implicated in altered cytokine profiles in other forms of ELS (repeated maternal separation, prenatal alcohol exposure) (Bodnar et al., 2016; Reviewed in Lumertz et al., 2022). By expanding the analysis to include additional brain regions, a more comprehensive understanding
of the impact of repeated neonatal sucrose and pain exposure on inflammatory processes and microglia density can be obtained.

As previously discussed, repeated neonatal sucrose and/or pain did not induce significant alterations in IL-1β, IL-6, and TNF-α levels in the hippocampus, and IL-6, and TNF-α in serum. It is possible that in order to observe an immune response for animals who have been exposed to ELS, a subsequent immune challenge, or “second hit,” may be required (Bilbo et al., 2005; Bilbo & Schwarz, 2009). Indeed, a study found that P15 rats exposed to RMS who also experienced a single stressful event prior to sacrifice (i.e. a “second hit”) displayed elevated IL-6 levels in the hypothalamus compared to those who did not receive the second-hit (Giridharan et al., 2019). The same pattern of results were observed in mice exposed to chronic adolescent stress. Hippocampal IL-1β mRNA expression was elevated in adult female mice that experienced chronic adolescent stress, but only after LPS administration, and plasma IL-1β concentrations were elevated in adult male mice under the same conditions (Bekhbat et al., 2019). This may suggest that the subsequent stressful event following ELS is needed to induce a heightened response in an already altered immune system. However, this may not be apparent at P8. When investigating later developmental ages (e.g. adolescence, adulthood), future directions can also investigate the introduction of a subsequent immune challenge to determine whether certain cytokine markers (e.g. IL-1β, IL-6, and TNF-α) are affected in the hippocampus and serum.

According to my thesis proposal, it was hypothesized that alterations in cytokine levels, particularly IL-1β, IL-6, and TNF-α, could explain the negative alterations that were previously shown in both brain structure and behaviour of adult mice exposed to neonatal pain and sucrose (Ranger et al., 2019; Tremblay et al., 2017). However, direct administration of these cytokines on long-term outcomes have not investigated. Although not ELS-specific, previous research has
shown that exogenous IL-1β administration in female mice aged 7-9 weeks was associated with
spatial learning impairment (Gibertini et al., 1995). Furthermore, systemic administration of
TNF-α increased cellular proliferation in the hippocampus, while repeated IL-6 infusion was
associated with enhanced microglial density in the hippocampus (Seguin et al., 2009). These
studies demonstrated a correlation between hippocampal cell density, behaviour, and peripheral
cytokine administration, making it a valuable model to explore the effects of repeated exposure
to exogenous cytokines on long-term brain development and behaviour. Such investigations
would provide researchers with stronger evidence to support a causal relationship between
inflammatory marker levels and long-term developmental outcomes. Furthermore, such studies
may determine whether adverse effects produced by repeated sucrose and/or pain exposure are
linked to these changes.

Other studies have also investigated C-reactive protein (CRP) in the brain and blood in
ELS models of prolonged maternal separation and prenatal restraint stress, both of which have
been associated with elevated blood CRP protein levels and hippocampal CRP mRNA in rodents
(Figueiredo et al., 2016; Li et al., 2021). CRP is a clinical marker of inflammation (Sproston &
Ashworth, 2018) so exploring the direct effects of sucrose and/or pain exposure on its expression
will provide insight on additional inflammatory effects that may be associated with their repeated
neonatal exposure. If exposure to sucrose and/or pain is producing an adverse inflammatory
response, CRP levels in the hippocampus (or other affected brain regions) and serum should be
altered alongside the already assessed inflammatory cytokines, which can substantiate the results
presented in this thesis.

Future studies can also investigate mechanistic pathways involving glutamatergic,
GABAergic, and HPA axis responsivity as assessed in repeated neonatal pain and other rodent
ELS models (Martisova et al., 2012; Mooney-Leber et al., 2018; Mooney-Leber & Brummelte, 2020). However, these mechanisms extend beyond the scope of my thesis and will not be further discussed.

Microglia density assessment

Assessing microglia responsivity is also a future direction that would provide valuable insights into functional microglial alterations and its potential short- and long-term implications on neurodevelopment. These avenues of investigation would contribute to a deeper understanding of the impact of early-life stressful experiences on microglia and their role in neurodevelopmental processes. These studies can also be investigated into adolescence and adulthood, with and without a subsequent immune challenge, to determine the long-term impacts of neonatal pain and/or sucrose exposure on the brain’s developmental trajectory.

The investigation of neonatal sucrose and/or pain exposure on alterations in hippocampal microglia density would greatly benefit from the identification of markers associated with these changes. As detailed in this thesis, microglia can be labelled using the Iba1 marker (see Section 3.6), and hippocampal colocalization of Iba1-positive cells with proliferative markers (e.g. Bromodeoxyuridine [BrdU] injections, Ki67 staining) (Hancock et al., 2009; Nkomozepe et al., 2019), or cell death markers (e.g. Caspase-3 staining) (Mengying et al., 2017) can be assessed. If alterations in the expression of these markers were to be evident in hippocampal microglia, this could significantly substantiate the findings presented in this thesis and offer valuable insights into possible mechanisms associated with alterations in microglia density in relation to neonatal sucrose and/or pain exposure.
5.3 Conclusion

Based on my findings using a previously established neonatal mouse model, I have shown that early-life repeated neonatal sucrose and/or pain exposure induces inflammatory alterations in IL-5 and IL-10 levels in the hippocampus, and IL-1β and IL-5 in serum. Given my findings, it is important to note that animal research cannot be directly applied to humans, even with the use of a translational mouse paradigm. Considering the widespread use of oral sucrose for procedural pain management in preterm infants and increasing evidence supporting the adverse effects of repetitive pain and/or sucrose exposure during a period of developmental vulnerability, it is important to approach the use of this standard care strategy with caution, especially in accumulative use. Continued investigation into the use of oral sucrose for procedural pain and its potential impact on long-term inflammatory processes is of utmost importance, especially given the possibility of adverse effects persisting into adulthood.
Bibliography


https://doi.org/10.1093/brain/awr288

https://doi.org/10.1016/j.bbr.2016.03.018

https://doi.org/10.1016/j.bbi.2018.12.005


https://doi.org/10.1155/2012/858929
https://doi.org/10.2307/1166096


https://doi.org/10.3389/fnagi.2018.00439

https://doi.org/10.1016/j.bbi.2016.05.022


Gee, K., Guzzo, C., Che Mat, N. F., Ma, W., & Kumar, A. (2009). The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. Inflammation & Allergy Drug Targets, 8(1), 40–52. https://doi.org/10.2174/187152809787582507


cortisol and behavioral reactivity in preterm infants in the NICU. *Pain, 113*(3), 293–300.

https://doi.org/10.1016/j.pain.2009.02.014


https://doi.org/10.1016/j.conb.2017.10.007

https://doi.org/10.1016/j.bbr.2008.11.050

https://doi.org/10.1002/glia.10161


https://doi.org/10.1023/a:1010983119125


https://doi.org/10.1016/j.cytogfr.2011.10.001

https://doi.org/10.1016/j.neubiorev.2022.104746


by neuroblastoma cells. *Brain Research, 650*(1), 140–145. https://doi.org/10.1016/0006-8993(94)90216-X


https://doi.org/10.1016/j.jpeds.2022.04.048


https://doi.org/10.1159/000485383


parameter and comparison with inflammatory and non-inflammatory central nervous system diseases. *Journal of the Neurological Sciences, 154*(2), 194–199.

https://doi.org/10.1016/S0022-510X(97)00228-1


https://doi.org/10.1016/j.pneurobio.2013.04.001


https://doi.org/10.1038/jcbfm.2009.240


https://doi.org/10.1002/14651858.CD001452.pub4


Appendix A: Detailed Methodology for Mouse Pup Tattooing

Figure A1 CMMT Mouse Core Transgenic Facility tattooing legend. Tattooing legend used to identify C57BL/6J mouse pups and their assigned experimental groups throughout the experimental protocol.
Appendix B: Additional Statistical Analysis

Table B1 Statistical values of 9 assessed cytokines in hippocampal samples using a one-way ANOVA.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>F statistic</th>
<th>Degrees of freedom</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.8310</td>
<td>4</td>
<td>0.1289</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.9225</td>
<td>4</td>
<td>0.4541</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.7981</td>
<td>4</td>
<td>0.5292</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.4300</td>
<td>4</td>
<td>0.0025</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.7860</td>
<td>4</td>
<td>0.0306</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.6630</td>
<td>4</td>
<td>0.1646</td>
</tr>
<tr>
<td>KC/GRO</td>
<td>0.1411</td>
<td>4</td>
<td>0.9665</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.7186</td>
<td>4</td>
<td>0.5812</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.4142</td>
<td>4</td>
<td>0.7980</td>
</tr>
</tbody>
</table>

A single outlier was removed for hippocampal IL-1β (WH group) and IL-6 (SN group) statistical analyses respectively. IFN-γ protein levels were undetectable in all samples and therefore not included in statistical analysis.

Table B2 Statistical values of 9 assessed cytokines in serum samples using a one-way ANOVA.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>F statistic</th>
<th>Degrees of freedom</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>6.1310</td>
<td>4</td>
<td>0.0004</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.1460</td>
<td>4</td>
<td>0.0885</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.5054</td>
<td>4</td>
<td>0.7319</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.7962</td>
<td>4</td>
<td>0.5331</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.2720</td>
<td>4</td>
<td>0.0739</td>
</tr>
<tr>
<td>IL-2</td>
<td>2.0370</td>
<td>4</td>
<td>0.1031</td>
</tr>
<tr>
<td>KC/GRO</td>
<td>1.5880</td>
<td>4</td>
<td>0.1916</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>2.3280</td>
<td>4</td>
<td>0.0683</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.0750</td>
<td>4</td>
<td>0.0974</td>
</tr>
</tbody>
</table>

A single outlier was removed for serum IL-6 (WH group), IL-2 (SH group), and KC/GRO (WH group) statistical analyses respectively. IFN-γ protein levels were undetectable in all samples and therefore not included in statistical analysis.