IMPACT OF REPEATED INVASIVE PROCEDURES DURING NEONATAL INTENSIVE CARE ON BRAIN MICROSTRUCTURE, GROWTH, NEURODEVELOPMENT AND BEHAVIOR IN CHILDREN BORN VERY PRETERM

by

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ABSTRACT

Background
As part of their lifesaving care in the neonatal intensive care unit (NICU), infants born very preterm (between 24 and 32 weeks gestation), undergo frequent invasive procedures that induce pain and stress, during a period of rapid brain development. We examined whether repeated exposure to invasive procedures was associated with altered brain development, and thereby poorer neurodevelopmental and behavioral outcome in children born very preterm. We also explored whether parent interaction moderates long-term effects of invasive procedures on child behavior.

Methods
Data were collected from two prospective cohorts of infants born ≤32 weeks gestation between February 2001–July 2004 and March 2006–January 2009. Neonatal data were recorded from birth to term-equivalent age. Infants in the 2006–2009 cohort were scanned sequentially, once near birth and again at term-equivalent age. Infants in the 2001–2004 cohort were followed-up at 18 months corrected age (CA), and again at 7.5 years of age, when they underwent an MRI. At 18 months CA, parents of the 2001–2004 cohort completed questionnaires and participated in a recorded play session with their child, from which the parent-child interaction was later coded. All statistical analyses were adjusted for known neonatal and clinical confounders.

Results
In a series of 4 studies, greater exposure to invasive procedures in the NICU was associated with slower postnatal body and head growth, and slower growth was associated with delayed cerebral cortical maturation. Among the preterm children exposed to a higher number of invasive procedures, more positive parental interaction was associated with fewer anxious/depressive behaviors reported at 18 months CA. Furthermore, greater exposure to invasive procedures was related to poorer white matter maturation at 7.5 years, and together these factors predicted lower IQ.

Conclusion
Greater exposure to invasive procedures was associated with slower body and head growth, altered brain maturation and poorer outcomes, after adjustment for clinical confounders. It is necessary that pain management strategies be evaluated for the extent that they are brain protective, in order to minimize the long-term impact of ongoing pain/stress in the NICU. Furthermore, interventions should address the parent-child relationship in order to improve later outcomes.
This thesis entitled, “Impact of repeated invasive procedures during neonatal intensive care on brain microstructure, growth, neurodevelopment and behavior in children born very preterm,” summarizes the research I conducted during my 6 years in the Graduate Program in Neuroscience at the University of British Columbia, under the supervision of Professors Ruth E. Grunau and Steven P. Miller. The University of British Columbia/Children’s and Women’s Health Centre of British Columbia Research Ethics Board approved these studies (certificate numbers for each of the studies listed below: H08-00125, H05-70579, H01-70017, H06-03696, respectively), and parents provided written informed consent. Child assent was obtained when children were seen at 7.5 years of age. Neonatal brain imaging and neurodevelopmental outcome data was acquired from 2 prospective cohorts of infants admitted to the neonatal intensive care unit at the British Columbia’s Children’s and Women’s Hospitals between February 2001 – July 2004, and March 2006 – January 2009. Results from this data led to four first author publications.

A version of Chapter 2 has been published in Vinall J, Miller SP, Chau V, Brummelte S, Synnes A, Grunau RE (2012). Neonatal pain in relation to postnatal growth in infants born very preterm. Pain, 153(7):1374-81. I conducted the statistical analyses and drafted the manuscript. Professors Grunau and Miller conceptualized and designed the study. All authors contributed to the interpretation of the data and provided critical review of the manuscript for publication.

A version of Chapter 3 has been published in Vinall J, Grunau RE, Brant R, Chau V, Poskitt KJ, Synnes AR, Miller SP (2013). Slower postnatal growth is associated with delayed cerebral cortical maturation in preterm newborns. Science Translational Medicine, 5:168ra8. I identified and acquired diffusion parameters from 8 cortical regions of interest on the scans of 95 infants born very preterm that underwent MRI imaging at approximately 32 and 40 weeks postmenstrual age. In addition, I contributed to the statistical analyses, and drafted the manuscript. Professors Miller and Grunau conceptualized and designed the study. Drs. Poskitt and Chau contributed to the acquisition of data. Professor Rollin Brant conducted the linear mixed effect modeling. All authors contributed to the interpretation of the data and provided critical review of the manuscript for publication.

A version of Chapter 4 has been published in Vinall J, Miller SP, Synnes AR, Grunau RE (2013). Parent behaviors moderate the relationship between neonatal pain and internalizing behaviors at 18 months corrected age in children born very prematurely. Pain, 154(9):1831-39. I introduced the concept of Emotional Availability to the Grunau and Miller labs and applied the Emotional Availability Scale to 145, 5-minute recordings of parent-child interactions. I conducted the statistical analyses, and drafted the manuscript. Professor Grunau conceptualized and designed the overall study. I interpreted the results of the parent-child interaction data, and all of the authors provided critical review of the manuscript for publication.
A version of Chapter 5 has been published in Vinall J, Miller SP, Bjornson BH, Fitzpatrick KPV, Poskitt KJ, Brant R, Synnes AR, Cepeda IL, Grunau RE (2014). Invasive procedures in preterm children: brain and cognitive development at school age. Pediatrics, 133(3):412-21. I identified and acquired diffusion parameters from 7 white matter regions of interest on the scans 50 children very preterm that underwent MRI imaging at 7 years of age. With the help of Professor Rollin Brant, I conducted the statistical analyses. Moreover, I drafted the manuscript. Professor Ruth Grunau generally conceptualized the study. The white matter regions of interest were acquired based on previous publications by Professor Steven Miller, and Professor Miller advised the analysis and interpretation of the brain imaging data. Drs. Bjornson and Poskitt, as well as Mr. Fitzpatrick contributed to the acquisition of data. All authors contributed to the interpretation of the data and provided critical review of the manuscript for publication.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\lambda_1$</td>
<td>Eigenvalue 1 (Axial Diffusivity)</td>
</tr>
<tr>
<td>$\lambda_{2&amp;3}$</td>
<td>Eigenvalue 2&amp;3 (Radial Diffusivity)</td>
</tr>
<tr>
<td>5HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>AGA</td>
<td>Appropriate Weight for Gestational Age</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CA</td>
<td>Corrected Age</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CBCL</td>
<td>Child Behavior Checklist</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin-Releasing Factor</td>
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<tr>
<td>DTI</td>
<td>Diffusion Tensor Imaging</td>
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<tr>
<td>EA</td>
<td>Emotional Availability</td>
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<tr>
<td>EEG</td>
<td>Electroencephalography</td>
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<tr>
<td>ELGA</td>
<td>Extremely Low Gestational Age</td>
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<tr>
<td>FA</td>
<td>Fractional Anisotropy</td>
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<tr>
<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<td>FSIQ</td>
<td>Full Scale IQ</td>
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<tr>
<td>GA</td>
<td>Gestational Age</td>
</tr>
<tr>
<td>GENLIN</td>
<td>Generalized Linear Model</td>
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<tr>
<td>HC</td>
<td>Head Circumference</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary Adrenal</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor 1</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence Quotient</td>
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<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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<tr>
<td>IUGR</td>
<td>Intrauterine Growth Restriction</td>
</tr>
<tr>
<td>IVH</td>
<td>Intraventricular Hemorrhage</td>
</tr>
<tr>
<td>LMEM</td>
<td>Linear Mixed Effect Model</td>
</tr>
<tr>
<td>MDI</td>
<td>Mental Development Index</td>
</tr>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NGF1-A</td>
<td>Nerve Growth Factor 1-A</td>
</tr>
<tr>
<td>NMDA</td>
<td>Glutamate N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal Intensive Care Unit</td>
</tr>
<tr>
<td>PMA</td>
<td>Postmenstrual Age</td>
</tr>
<tr>
<td>PRI</td>
<td>Perceptual Reasoning Index</td>
</tr>
<tr>
<td>PRSI</td>
<td>Processing Speed Index</td>
</tr>
<tr>
<td>PSI</td>
<td>Parenting Stress Index III</td>
</tr>
<tr>
<td>PVHI</td>
<td>Periventricular Hemorrhagic Infarction</td>
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<tr>
<td>PVL</td>
<td>Periventricular Leukomalacia</td>
</tr>
<tr>
<td>SES</td>
<td>Socioeconomic Status</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for Gestational Age</td>
</tr>
<tr>
<td>SNAP-II</td>
<td>Score for Neonatal Acute Physiology II</td>
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<tr>
<td>VCI</td>
<td>Verbal Comprehension Index</td>
</tr>
<tr>
<td>VLGA</td>
<td>Very Low Gestational Age</td>
</tr>
<tr>
<td>WISC-IV</td>
<td>Wechsler Intelligence Scale for Children– 4th Edition</td>
</tr>
<tr>
<td>WMI</td>
<td>White Matter Injury</td>
</tr>
<tr>
<td>WRMI</td>
<td>Working Memory Index</td>
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## GLOSSARY

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CA</td>
<td>The age of the child from the expected date of delivery</td>
</tr>
<tr>
<td>Chronological Age</td>
<td>Age calculated from birth</td>
</tr>
<tr>
<td>ELGA</td>
<td>Infants born from 24 to 28 weeks GA</td>
</tr>
<tr>
<td>Full-term</td>
<td>Infants born from 39 to 41 weeks GA</td>
</tr>
<tr>
<td>GA</td>
<td>Age calculated from the first day of the last normal menstrual period and the day of delivery</td>
</tr>
<tr>
<td>PMA</td>
<td>Age calculated from the first day of the last menstrual period and birth (GA) in addition to the age calculated from birth (chronological age)</td>
</tr>
<tr>
<td>Term-equivalent age</td>
<td>The expected date of delivery (i.e. 40 weeks PMA)</td>
</tr>
<tr>
<td>Very preterm</td>
<td>Infants born from 24 to 32 weeks GA</td>
</tr>
<tr>
<td>VLGA</td>
<td>Infants born from 29 to 32 weeks GA</td>
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DEDICATION

To Adam Miller
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Preterm Birth

Approximately 8% of all infants born in Canada are born less than 37 weeks gestational age (GA; Canadian Institute for Health Information 2012). Of these infants, approximately 15% are born very preterm (between 24 to 32 weeks GA; Canadian Institute for Health Information 2009). Advances in neonatal care have greatly improved infant survival, and reduced the number of severe disabilities (e.g. blindness, non-ambulatory cerebral palsy, developmental delay), particularly for infants born extremely low gestational age (between 24 to 28 weeks GA; Doyle et al. 2011; Moore et al. 2012). However, preterm children have more cognitive, motor and behavioral problems relative to children born full-term (~40 weeks GA) that appear early in life, and persist to adulthood (Anderson, Doyle, Victorian Infant Collaborative Study Group 2003; Doyle, Casalaz, Victorian Infant Collaborative Study Group 2001; Doyle and Anderson 2010; Grunau, Whitfield, Fay 2004; Johnson et al. 2009; Loe et al. 2011; Marlow et al. 2005; Marlow et al. 2007; Spittle et al. 2009). The birth of a preterm child causes substantial stress for families (Brummelte et al. 2011a; Garel, Dardennes, Blondel 2007; Glazebrook et al. 2007; Poehlmann and Fiese 2001; Singer et al. 2003; Thomas, Renaud, Depaul 2004), and puts considerable strain on health, educational and social systems (Johnston et al. 2014). In Canada, between 1996 and 2006, the cost to support children born preterm was estimated as $587.1 million (Johnston et al. 2014). To improve quality of life for the children and their families, it is imperative that we find ways to optimize the developmental outcomes of this vulnerable population of infants.
1.2 Invasive Procedures during Neonatal Care

The majority of infants born very preterm now survive as a result of advances in medical care, however, they must develop outside of the protective intrauterine environment during the third trimester of “fetal” life, which is a critical period of physiological immaturity and vulnerability. They are susceptible to a number of pathophysiological conditions for which invasive medical interventions are required. A recent North American survey of 14 Canadian neonatal intensive care units (NICU) revealed that in a single week, 580 neonates underwent over 17,500 painful/stressful procedures (Johnston et al. 2011). On average, infants undergoing neonatal intensive care required 4 to 14 procedures per day (Brummelte et al. 2012; Carbajal et al. 2008; Doesburg et al. 2013; Grunau et al. 2005; Johnston et al. 2011; Simons et al. 2003a). For infants born very preterm, repeated exposure to invasive procedures occurs during a period of rapid brain and stress system development (Brummelte et al. 2012; Brummelte et al. 2015; Doesburg et al. 2013; Grunau, Weinberg, Whitfield 2004; Grunau et al. 2005; Grunau et al. 2007; Ranger et al. 2013; Smith et al. 2011; Zwicker et al. 2013). There is an increasing amount of evidence which suggests that exposure to greater number of invasive procedures in the NICU leads to altered brain and neurodevelopmental outcomes in children born very preterm.

1.3 History of Pain Management in the NICU

Concerns regarding the long-term effects of pain in very preterm infants are relatively recent. Prior to the 1980’s, infant surgery was routinely conducted with little to no anesthesia (Schechter, Allen, Hanson 1986). This was partly due to concerns about the safety of anesthesia (e.g. respiratory suppression), but also because it was believed that newborns could not feel pain, and that surgery could be safely accomplished using oxygen and a paralytic (Rodkey and Pillai
Riddell 2013). Therefore, preterm infants such as the famous Jeffery Lawson, born February 1985, at 26 weeks GA, were not anesthetized for surgery. Jeffery died 5 weeks after his surgery. The effect of this case was that Jill Lawson, and other parents began to advocate for the management of infant pain in the NICU (Rodkey and Pillai Riddell 2013). In 1987, Anand and colleagues published a seminal paper, which demonstrated for the first time that preterm infants treated with fentanyl for anesthesia, in addition to nitrous oxide and muscle relaxants, had decreased hormonal responses after surgery compared to the standard care (non-fentanyl treated) group (Anand, Sippell, Aynsley-Green 1987). This study by Anand and colleagues changed surgical practice in newborns. Concurrently, Grunau and Craig published the first quantified measure of infant behavior (Neonatal Facial Coding System), which greatly facilitated pain measurement for research in infants (Grunau and Craig 1987). Moreover, specific facial actions then became incorporated into multidimensional scales for clinical assessment (e.g. Stevens et al. 1996). Furthermore, Anand and Hickey published a critical literature review proposing that the human fetus has cortical and subcortical brain regions necessary for pain detection, as well as neurochemical systems associated with pain transmission (Anand and Hickey 1987). These events combined led to a rapid expansion in the field of pediatric pain research.

1.4 Pain Transmission and Modulation

In order to understand the impact of repeated exposure to invasive procedures during neonatal intensive care on brain microstructure, growth, neurodevelopment and behavior in children born very preterm, it is important to address how the pain system operates in adults, and how it differs in infants born very preterm.
1.4.1 Transduction, Transmission and Central Nociceptive Processing

Peripheral tissue injury activates high-threshold sensory receptors of the somatosensory nervous system that are capable of transducing and encoding noxious stimuli along specialized nerve cells called nociceptive neurons (Fitzgerald 2005; Renn and Dorsey 2005; International Association for the Study of Pain 1979; Melzack and Wall 1965; Willis and Westlund 1997). Nociceptive neurons consist of small diameter A-delta (myelinated, fast transmission), and C-fibres (unmyelinated, slow transmission; Konietzny et al. 1981; Ochoa and Torebjork 1989). The likelihood of nociceptive neurons firing an action potential is influenced not only by adequate input (e.g. strong mechanical, thermal or chemical stimuli; Willis and Westlund 1997), but also by the numerous chemical agents produced or released at an injury site. Prostaglandins, leukotrienes, bradykinin, substance P, cytokines, and serotonin can both activate nociceptors, and sensitize nociceptors to subsequent stimuli (Woolf and Costigan 1999), which can result in allodynia (pain due to a stimulus that does not normally provoke pain; International Association for the Study of Pain 1979).

Sensory neuron cell bodies are located in the dorsal root ganglia (Bourne, Machado, Nagel 2014). These neurons project from the dorsal root ganglia to the spinal cord, entering laterally through Lissauer’s tract, and extending vertically in this tract for several spinal segments before synapsing onto second-order neurons (Earle 1952). The gray matter of the spinal cord consists of 10 laminae, 6 of which are in the dorsal horn. The laminae of the dorsal horn can be grouped into the superficial layers (laminae I and II) and the deep layers (laminae III-VI). A-delta fibers terminate lamina I, II and V, whereas C-fibers terminate in lamina II (Traub and Mendell 1988).
The dorsal horn is not simply a relay station, but rather is a network of neurons through which inputs from the periphery are transduced and modulated by local, and descending, excitatory and inhibitory mechanisms (Woolf and Salter 2000). Excitatory synaptic transmission is mediated by glutamate acting on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate ligand-gated ion channels. Dampening of this signal may occur through segmental and descending activation of inhibitory neurons, which co-release glycine and γ-aminobutyric acid (Chery and De Koninck 1999). Modulation of the nociceptive transmission involves activation of intracellular signaling cascades, which facilitate excitatory synaptic responses and depress inhibition (Woolf and Salter 2000). These sequences of cellular events can lead to the occurrence of central sensitization, which involves increased responsiveness of nociceptive neurons within the central nervous system to normal or subthreshold afferent input (International Association for the Study of Pain 1979; Woolf 2011). Unlike peripheral sensitization, central sensitization can increase sensitivity in non-inflamed tissue by changing the response elicited by normal input long after the initial stimulus has disappeared (Woolf 2011). The nociceptive transmission leaves the dorsal horn via second-order neurons, which cross the midline, and ascend through the ventrolateral quadrant of the contralateral half of the spinal cord to connect with nuclei within the thalamus. Lamina I spinothalamic tract neurons project to the posterolateral, ventral medial, ventral posterior, ventral posterior inferior and medial dorsal nuclei of the thalamus. (Craig 2003). Lamina V spinothalamic tract neurons axons terminate in ventral posterior, ventral posterior inferior, ventrolateral and the intralaminar nuclei of the thalamus (Craig 2003).

Projections from the ventral posteromedial nucleus and ventroposterolateral nucleus synapse directly with the primary somatosensory cortex (Bourne, Machado and Nagel 2014; Gingold et al., 1991; Kenshalo et al., 1980). The primary somatosensory cortex is somatotopically
organized (Jones, Kulkarni, and Derbyshire 2003), and demonstrates a graded response according to intensity of noxious stimulus (Chudler et al., 1990; Kenshalo et al., 1988; Kenshalo and Isensee, 1983; Lamour et al., 1983). Therefore, the primary somatosensory cortex is thought to be involved in the location and discrimination of pain. The secondary somatosensory cortex receives input from the posteromedial, ventroposterolateral nuclei as well as the primary somatosensory cortex, and it also responds according to the magnitude of noxious stimuli (Bourne, Machado and Nagel 2014; Stevens et al. 1993). The insula receives input from the primary and secondary somatosensory cortex, the ventral posterolateral, dorsomedial and intralaminar nuclear groups (Bourne, Machado and Nagel 2014; Robinson 1997). In addition, it projects to limbic structures such as amygdala and perirhinal cortex (Bourne, Machado and Nagel 2014; Shi and Cassell, 1998). The insula has been shown to be involved in both the discriminatory and motivational-affective responses to pain (Bourne, Machado and Nagel 2014; Schnitzler and Ploner 2000). Finally, the anterior and middle cingulate cortices receive projections from the venterolateral, medial and intralaminar thalamic nuclei and are involved in the affective or motivational responses to pain (Baleydier and Mauguiere, 1980; Bourne, Machado and Nagel 2014). With the advancement of noninvasive neuroimaging techniques in humans, researchers are acquiring a better understanding of how sensory neurons, the spinal cord, and higher centers of the brain act in concert to contribute to the processing of nociceptive stimuli.

1.5 Cortical Processing of Centrally Transmitted Nociceptive Activity

Functional magnetic resonance imaging (fMRI) and positron emission tomography have been used to study regional responses to noxious stimuli in the brain.
1.5.1 Imaging Responses to Noxious Stimuli in Adults

Many researchers have attempted to identify ascending nociceptive systems and their central nervous system targets (e.g. primary and secondary somatosensory cortices, insula, anterior cingulate cortex, and thalamus; Apkarian et al. 2005; Bushnell, Ceko and Low 2013; Duerden and Albanese 2013; Iadarola and Coghill 1999; Willis and Westlund 1997), in addition to showing that these cortical and subcortical brain areas correlate with the modality and intensity of noxious input (Atlas et al. 2014; Apkarian et al. 2005; Coghill et al. 1999; Wager et al. 2013). For example, in a recent fMRI study involving 114 healthy adult participants, Wager and colleagues (2013) found a highly sensitive and specific neurologic signature of physical pain. It included both medial (e.g., “affective”; anterior cingulate cortex) and lateral (e.g., “sensory”; somatosensory cortices) “pain systems” that were consistent across individuals (Wager et al. 2013). Although pain has relatively well-defined primary targets, pain is a multidimensional experience, with sensory, cognitive, and evaluative aspects. Therefore, other networks may be simultaneously activated during invasive procedures, and may influence how the brain responds to noxious stimuli. The salience network comprising of the anterior insula, midcingulate cortex, temporoparietal junction, and dorsolateral prefrontal cortex is more strongly activated when an individual is paying attention to the noxious stimulus (Kucyi et al. 2012; Seeley et al. 2007). In contrast, the default mode network (posterior cingulate cortex/precuneus, medial prefrontal cortex, lateral parietal lobe, and areas within the medial temporal lobe) becomes activated when a person is at rest, and unaware of the noxious stimulus (Andrews-Hanna et al. 2014; Kucyi et al. 2013). Moreover, the fronto-striatal pathway, connecting ventromedial prefrontal cortex and nucleus accumbens, and the antinociceptive pathway, connecting the medial prefrontal cortex
and periaqueductal gray, are involved in the modulatory aspects of pain (Beccera 2008; Borsook et al. 2010; Petrovic et al. 2000; Tracey et al. 2002; Fields 2007; Valet et al. 2004). Therefore, in adults the processing of nociceptive activity involves a complex integration of brain networks involved in the cognitive, emotional, and sensorimotor aspects of pain.

1.5.2 Imaging Responses to Noxious Stimuli in Infants and Children

In comparison to the extensive study of pain processing in adults, very few studies to date have examined the cortical processing of noxious stimuli in children and infants. Hohmeister and colleagues (2010) demonstrated that children born full-term and children born very preterm seen between the ages 11 to 16 years showed activation in several of the same brain regions known to be activated in response to noxious stimuli in adults. However, children born very preterm demonstrated greater activation within these regions compared to children born full-term (Hohmeister et al. 2010). Exposure to mild/moderately painful heat in children born very preterm also led to signaling in several regions that were not activated in full-term controls, including the thalamus, anterior cingulate cortex, cerebellum, basal ganglia, regions known to be involved in the sensory, affective, and cognitive aspects of pain. Repeated exposure to pain/stress in the first weeks of life may have led to increased afferent drive to CNS sites that respond to noxious input (LaPrairie and Murphy 2007; Ranger et al. 2013). Increased connectivity along pathways associated with pain/stress signaling (LaPrairie and Murphy 2007; Ranger et al. 2013) may in part explain why children and adolescents born very preterm showed greater brain activation compared to their term-born peers in response to noxious stimulation.

Recently, studies have used fMRI to study the responses of infants born full-term to noxious
stimuli (Goksan et al. 2015; Williams et al. 2014). First the feasibility of using fMRI to study nociceptive evoked brain activity in a healthy unsedated full-term newborn was established (Williams et al. 2014). Slater and colleagues then conducted a study to compare fMRI responses in healthy full-term newborns with those of adults. They found that full-term infants showed increases in activity in all but 2 (amygdala and the orbitalfrontal cortex) of the 20 regions that were active in adults in response to acute noxious stimulation (Goksan et al. 2015). The amygdala and orbitalfrontal cortex are involved in the emotional and cognitive processing of pain signals (Simons et al. 2014; Winston et al. 2014). Other brain structures known to be involved in emotional processing of pain, such as the anterior cingulate, were activated in both the full-term infants and the adults (Goksan et al. 2015). Therefore, it may be that no prior experience of pain is necessary to produce sensory and some emotional responses to pain/stress.

While thalamocortical connectivity is present earlier, the period between 35 and 37 weeks PMA is dominated by the growth of interhemispheric connections, which continue to develop after term-equivalent age (Kostovic and Judas 2010). Therefore, the complex integrated perceptual, emotional and cognitive pain experience is not likely possible until later in infancy. Moreover, the cognitive component that requires experience to contextualize pain may develop later in childhood.

The prenatal development of thalamocortical and cortico-cortical connections is a prolonged process that involves afferents projecting towards the cortex and forming transient, functional circuits with subplate neurons before proliferating into the cortical layers. At 24 and 25 weeks postmenstrual age (PMA), thalamocortical afferents are ‘waiting,’ in the subplate zone, just beneath the cortical plate (Kanold 2009; Kostovic and Judas 2010). Thus, at 24 weeks PMA the subplate neurons can receive extrinsic input from sensory and associative thalamic nuclei.
Between 26 and 28 weeks PMA, thalamocortical axons begin to penetrate the cortical plate (Kostovic and Judas 2010; Vasung et al. 2010), and evoked potentials can be recorded from the somatosensory cortex (Kostovic and Rakic 1990). However, fMRI has not been used to study activation in the brain in response to nociceptive stimulation in infants born very preterm. Given the immaturity of the brain and its connections, cognitive and affective processes associated with pain are unlikely to be developed at this early age. However, repeated exposure to invasive procedures during this critical period of brain development may lead to alterations in both structure and function of these developing regions.

1.6 Supraspinal Descending Control Pathways

Pain inhibitory pathways have also been well characterized. Inhibition of pain is initiated by the activation of the periaqueductal gray, which forms connections with the prefrontal cortex, anterior cingulate cortex, hypothalamus, and central nucleus of the amygdala (Fields 2004; Schweinhardt and Bushnell 2010). The periaqueductal gray connects with the rostral ventroventral medulla and dorosolateral pontine tegmentum, and these two regions project through the spinal cord (dorsolateral funiculus), to selectively target the nociceptive relay neurons housed in lamina I, II and V of the spinal cord (Fields 2004; Koch and Fitzgerald 2014).

While in neonatal rats the anatomical connections for nociceptive modulation are present at birth, descending inhibitory controls are not functionally active until postnatal day 21, beyond term-equivalent age in humans (Hathway et al. 2009a; van Praag and Frenk 1991). Up to postnatal day 21 in the rat, the rostroventral medulla of the brainstem exclusively facilitates spinal pain transmission (Hathway et al. 2009a). After this age there is a shift to inhibition of the pain signal (Hathway et al. 2009a). The delayed maturation of descending inhibition may therefore contribute to increased vulnerability of the immature somatosensory system to repeated
exposure to invasive procedures in infants born very preterm.

1.7 Pain Processing in Infants Born Very Preterm

Preterm infants have the nociceptive circuitry required to process pain signals, however, this system is functionally immature (Fitzgerald 2005; Fitzgerald and Walker 2009). Cutaneous receptive fields are large in the neonate, and peripheral sensory fibers are sensitive to tissue injury and have reduced peak firing frequencies (Andrews and Fitzgerald 1994; Beggs et al. 2002; Fitzgerald and Walker 2009; Granmo, Petersson, Schouenborg 2008; Jennings and Fitzgerald 1998; Li et al. 2009). Receptors innervated by Aδ fibers (myelinated, fast transmitting) are distinguishable in the preterm neonate (Andrews and Fitzgerald 1994; Jennings and Fitzgerald 1998; Li et al. 2009), however, C-fibre (unmyelinated, slow transmitting) evoked activities are not observable until approximately 28 weeks PMA (Jennings and Fitzgerald 1998). Axon terminals temporarily overlap in lamina II of the spinal cord with low-threshold tactile inputs, making it more difficult for neonates to discriminate between noxious and non-noxious stimuli (Beggs et al. 2002; Granmo, Petersson, Schouenborg 2008). Peripheral injury triggers a long-lasting increase in the excitability of spinal cord neurons, which manifests as a reduction in threshold (allodynia), and increase in responsiveness (hyperalgesia; Woolf 2011). The amplification of neural signaling in the spinal cord that generates pain hypersensitivity is referred to as central sensitization (Woolf 2011). Prior to 35 weeks PMA, infants demonstrate central sensitization and therefore, react more strongly to subsequent noxious and non-noxious stimuli (Andrews and Fitzgerald 1994; Fitzgerald, Millard, McIntosh 1989; Grunau et al. 2001; Holsti et al. 2005; Holsti et al. 2006; Walker, Tochiki, Fitzgerald 2009). Moreover, descending inhibition of nociceptive activity develops later, beyond term equivalent age (Hathway et al.
Given that infants born very preterm have reduced localization and specification to noxious stimuli, become sensitized to repeated noxious stimuli and lack descending inhibitory control, identifying, relieving, and preventing pain are very important aspects of NICU care.

1.8 Behavioural and Physiological Responses to Noxious Stimuli in the NICU

A wide variety of behavioral and physiological responses (e.g., facial actions, body movements, cry, heart rate, respiratory rate, blood pressure, and oxygen saturation) are used to identify pain in nonverbal patients (Holsti et al. 2008; Stevens, Johnston, Horton 1993). However, these indicators are not specific to pain, and may also represent agitation or distress. While all pain is stressful, not all stress is painful. This ambiguity in identifying pain in the NICU presents a challenge to both clinicians and researchers. Therefore, throughout this dissertation pain in infants born very preterm will be broadly defined as pain/stress. Responses to invasive procedures vary based on a number of clinical factors, for example, GA, illness severity, and prior exposures to pain/stress. The following will highlight why variation in pain/stress responses make it difficult for clinicians to discriminate and appropriately manage pain/stress in infants born preterm.

1.8.1 Behavioral Responses to Invasive Procedures

Infants born full-term demonstrate clear facial responses to pain/stress including eye squeeze, brow contraction, naso-labial furrow, taut tongue, and open mouth (Grunau and Craig 1987). Subsequently, these behavioral responses to pain/stress were confirmed in very preterm infants (Craig et al. 1993). However, infants born <28 weeks GA demonstrate fewer facial responses to invasive procedures (Craig et al. 1993; Gibbins et al. 2008; Holsti et al. 2006; Xia et al. 2002).
Greater PMA has been associated with an increase in the amplitude and likelihood of responding to an invasive procedure (Johnston and Stevens 1996; Johnston et al. 1999; Slater et al. 2009; Williams et al. 2009). In contrast, greater exposure to invasive procedures in the NICU has been associated with reduced behavioral responses in infants born preterm, after adjusting for GA (Grunau et al. 2001; Grunau et al. 2005). Moreover, the timing of the last invasive and/or noninvasive procedure appears to impact how the infant responds to pain/stress (Grunau et al. 2000; Holsti et al. 2005; Holsti et al. 2006; Johnston et al. 1999; Porter, Wolf, Miller 1998). Greater illness severity is also associated with dampened responses to invasive procedures (Evans et al. 2005; Johnston and Stevens 1996; Valeri et al. 2012; Williams et al. 2009).

Sleep/awake state matters for pain/stress behaviors, such that full-term (Grunau & Craig 1987) and preterm infants who are awake during heel lance procedure are more likely to display facial change in response to the stimulus (Slater et al. 2009; Stevens, Johnston, Horton 1994). Therefore, there appear to be a large number of factors that lead to either increased or decreased behavioral expression in response to invasive procedures, particularly in infants born very preterm, undergoing neonatal intensive care.

### 1.8.2 Physiological Responses to Invasive Procedures

Many studies have also examined the physiological responses to a heel lance procedure in infants born preterm. Similar to behavioral responses, there are several factors that may influence physiological responses to pain/stress. GA was found to be significantly associated with oxygen saturation and/or heart rate, such that oxygen saturation levels were lower and heart rate was higher in lower GA infants (Gibbins et al. 2008; Grunau et al. 2001). Greater exposure to invasive procedures in the NICU was associated with greater heart rate variability during heel lance procedures (Grunau et al. 2001). Although exposure to analgesia was found to reduce heart
rate variability, steroid exposure increased heart rate variability in infants born preterm (Grunau et al. 2001). Greater illness severity on the other hand, has been associated with both raising and lowering heart rate during invasive procedures (Morison et al. 2003; Valeri et al. 2012). Given that physiological responses are affected by clinical conditions such as infection, autonomic measures can be even more variable than facial responses and finger splaying in response to pain/stress in hospitalized neonates (Grunau et al. 2000; Holsti et al. 2005; Stevens et al. 2007). Therefore physiological responses have often been measured together with behavioral responses in multidimensional scales to assess pain/stress in infants born preterm.

1.9 Cortical Responses to Invasive Procedures in the NICU
The use of neuroimaging at the bedside may also assist clinicians in identifying pain/stress responses in infants born very preterm. Using near-infrared spectroscopy, changes in cerebral oxygenation have been detected over the prefrontal and somatosensory cortex in response to noxious stimulation (routine heel lance) in infants between the ages of 25 and 45 weeks PMA (Bartocci et al. 2006; Ozawa et al. 2011; Slater et al. 2006). Responses within the somatosensory cortex were significantly greater in infants that were awake (Slater et al. 2006). Moreover, the strength of signal increased with PMA (Bartocci et al. 2006; Slater et al. 2006), and the latency to respond decreased with PMA (Slater et al. 2006). Neuroactivity in response to stimuli was also measured within the somatosensory cortex using electroencephalography (EEG) in infants between the ages of 28-45 weeks PMA (Fabrizi et al. 2011; Slater et al. 2010a). Prior to 35 weeks PMA, both touch (tapping of a tendon hammer again the heel) and routine heel lance lead to nonspecific neuronal bursts on EEG (Fabrizi et al. 2011). However, for infants between 35–37 weeks PMA, touch and heel lance evoked characteristic somatosensory potentials that differed both in timing and morphology for the two modalities of stimulation (Fabrizi et al.
By 40 weeks PMA, infants born very preterm demonstrated larger evoked potentials on EEG following a routine heel lance compared to infants born full-term, but not for non-noxious (light tapping of a rubber bung) stimuli (Slater et al. 2010a). This difference in responding could not be explained by the infants’ age at birth or presence of brain injury (Slater et al. 2010a). Therefore, given that prior to 35 weeks PMA, the brain responds similarly to both invasive and non-invasive stimulation, and by 40 weeks PMA, neural responses to skin-breaks are greater for infants born very preterm, it is important that we consider the long-term impact of repeated invasive procedures on the developing brain.

### 1.10 Vulnerability of the Developing Brain

Over the course of their NICU stay, infants born very preterm are repeatedly exposed to invasive procedures during a critical period of brain development, which is characterized by cell proliferation, migration, axonal growth, neuronal differentiation and synaptogenesis (Kostovic and Judas 2010). Repeated exposure to invasive procedures in the NICU appears to disrupt the maturation of the developing brain in infants born very preterm (Brummelte et al. 2012; Doesburg et al. 2013; Ranger et al. 2014; Zwicker et al. 2013). Two cell populations are particularly vulnerable to injury in the premature brain: subplate neurons and preoligodendrocytes (Back and Miller 2014; Volpe 2009).

Subplate neurons are among the first cells generated in the mammalian cerebral cortex, and are the first cortical neurons to receive excitatory synaptic inputs from thalamic axons, establishing a temporary link between thalamic axons and their final target in the cerebral cortex (Kostovic et al. 2002; Kostovic and Judas 2002; McQuillen and Ferriero 2005). Subplate neurons are particularly vulnerable to excitotoxic death, as was demonstrated by the selective ablation of
subplate neurons after the administration of glutamate agonist kainite into embryonic (embryonic day 42) and newborn kittens (Ghosh et al. 1990; Ghosh and Shatz 1992). Glutamate n-methyl-D-aspartate (NMDA) receptors are more active during early life because of the developmentally delayed expression of NR2A receptor subunits, relative to NR2B in the developmental course (Monyer et al. 1994; Sheng et al. 1994). Therefore, repeated excitation of subplate neurons can lead to an excessive release of glutamate, influx of calcium and apoptosis, (Deng et al. 2003; McDonald and Johnston 1990; Qu et al. 2003; Talos et al. 2006), thereby disrupting neuronal migration, synapse formation and dendritic pruning (Gambrill and Barria 2011; Luthi et al. 2001; Zhang, Peterson, Liu 2013).

Preoligodendrocytes, are cells that ensheath axons prior to differentiating into myelin-producing oligodendrocytes (Volpe 2009). There are four stages of oligodendroglial maturation, which include: 1) the oligodendroglial progenitor, 2) the pre-oligodendrocyte (or late oligodendroglial progenitor), 3) the immature oligodendrocyte, and 4) the mature myelin-producing oligodendrocyte. At 28 weeks GA preoligodendrocytes account for 90% of the total oligodendroglial population (Back et al. 2001). Between 28–40 weeks of gestation, preoligodendrocytes begin to differentiate into immature oligodendrocytes. Immature oligodendrocytes account for approximately 30% of the total oligodendrocyte population during the later premature period, and about 50% by term-equivalent age (Back et al. 2001). Disruption of the preoligodendrocytes and/or immature oligodendrocytes can lead to the arrest of their development, and alterations in myelination in preterm infants (Buser et al. 2012). Fewer cortical connections or poorer myelination of white matter tracts may underlie the cognitive, motor and behavior problems frequently observed in children born very preterm (Ball et al. 2015; Bora et al. 2014; Chau et al. 2013; Counsell et al. 2008; Doesburg et al. 2011; Estep et al. 2014; Mullen
et al. 2011; Thompson et al. 2014).

Both subplate neurons, preoligodendrocytes and immature oligodendrocytes are vulnerable to inflammation, and oxidative stress, which involves the overproduction of reactive oxygen, nitrogen species, and cytokines secreted by microglia (Adams et al. 2010; Back et al. 1998; Back et al. 2005; Buntinx et al. 2004; Chau et al. 2009; Chau et al. 2012; Dean et al. 2011; Glass et al. 2008; Haynes et al. 2003; McQuillen et al. 2003; Pang, Cai, Rhodes 2005; Sizonenko et al. 2003; Sizonenko et al. 2005; Wikstrom et al. 2008; Zwicker et al. 2013). Repeated exposure to pain/stress may lead to neuroinflammation or oxidative stress within the CNS, thereby disrupting the development of the subplate neurons or preoligodendrocytes, which may lead to long-term alterations in brain microstructure (Brummelte et al. 2012; Doesburg et al. 2013; Ranger et al. 2013; Zwicker et al. 2013), and adverse neurodevelopmental and behavioral outcomes in children very preterm.

1.11 Inflammation and Invasive Procedures in the NICU

Tissue damage results in localized inflammation, hyperalgesia and allodynia (Fitzgerald and Beggs 2001). Inflammation associated with tissue damage results in the leak of intracellular contents into the extracellular fluid, the co-release of ions (hydrogen, potassium), amines (5-hydroxytryptamine, histamine), kinins (bradykinin), prostanoids (prostaglandin), purines (ATP), nitric oxide, cytokines (tumor necrosis factor-α, interleukin-1 and interleukin-6), growth factors (leukemia inhibitory factor, nerve growth factor), and further recruitment of inflammatory cells (Woolf and Costigan 1999). These may act directly with peripheral nociceptors, or act indirectly to sensitize nociceptors and alter their responses to subsequent stimuli (Woolf and Costigan 1999), increasing the likelihood of nociceptive neurons, A-delta and C-fibres firing an action
potential.

In adult rats, intense stimulation of peripheral C-fibres activates microglia within the spinal cord (Hathway et al., 2009). Microglial activation in the dorsal horn of the spinal cord results in the release of cytokines and growth factors, which excite nociceptive dorsal horn neurons, thereby contributing to the development of central sensitization and hyperalgesia (Trang et al., 2011). In preterm neonates C-fibre evoked activity is not observed until approximately 28 weeks PMA (Jennings and Fitzgerald 1998), and tissue injury-induced activation of spinal microglia is reduced in neonates compared to adults (Moss et al., 2007; Costigan et al., 2009). However, neonatal tissue injuries may ‘prime’ the immune system so that it becomes more easily activated in later life. A neonatal skin incision in the hindpaw of a rat pup led to enhanced microglial reactivity when the same hindpaw was re-incised during adulthood (Beggs et al., 2012). Therefore, microglial activity may be enhanced by early exposure to invasive procedures.

Both subplate neurons and preoligodendrocytes are vulnerable to neuroinflammatory responses (Adams et al. 2010; Beggs et al. 2012; Buntinx et al. 2004; Chau et al. 2009; Chau et al. 2012; Dean et al. 2011; Glass et al. 2008; Pang, Cai, Rhodes 2005; Grunau et al. 2013; Strunk et al. 2014; Wikstrom et al. 2008; Zwicker et al. 2013). In animal models, inflammatory pain leads to increased cell death in the neonatal rat brain (Anand et al. 2007; Rovnaghi et al. 2008), and in preterm neonates inflammation/infection has been associated with alterations to the developing brain microstructure and function (Adams et al. 2010; Chau et al. 2009; Chau et al. 2012; Ellison et al. 2005; Glass et al. 2008; Strunk et al. 2014; Wikstrom et al. 2008; Zwicker et al. 2013). Taken together, these studies suggest that inflammatory factors may play a role in effects of pain/stress on the CNS.
1.12 Oxidative Stress and Invasive Procedures in the NICU

Oxidative stress is another mechanism through which repeated exposure to invasive procedures may interfere with cortical maturation. Both cerebral oxygenation and cerebral blood volume are affected with changes in oxygen saturation (Pryds 1991; Yamamoto et al. 2003). Infants born preterm are known to exhibit lower mean arterial blood pressure and poorer cerebral perfusion (Menke et al. 1997; Pryds et al. 1989; Pryds 1991). Oxidative stress results from the production of reactive species or oxidants, and is a sequela of cerebral ischemia (shortage of oxygenated blood/reperfusion; Traystman, Kirsch, Koehler 1991). Previously it has been shown that exposure to invasive procedures leads to reduced oxygen saturation (Bauer et al. 2004; Gonsalves and Mercer 1993), and increased heart rate, indicative of increased energy expenditure and oxygen consumption (Bauer et al. 2004). A recent study examined markers of adenosine triphosphate (ATP) utilization and oxidative stress (uric acid and malondialdehyde concentration) in the plasma of preterm before and after a tape removal procedure during discontinuation of an indwelling central arterial or venous catheter. Slater et al. (2012) compared the results of preterm infants that underwent this tissue-damaging procedure to preterm infants that did not undergo this procedure. Although the markers for uric acid significantly decreased over time in the control group, these values remained stable for the infants that underwent the tape removal (Slater et al. 2012). Malondialdehyde levels, however, decreased over time for the control neonates, but increased for the preterm infants that underwent the tape removal (Slater et al. 2012). Moreover, their concentrations of malondialdehyde correlated with their pain scores on the Premature Infant Pain Profile (Slater et al. 2012). Taking these results together, it would appear that there is a relationship between exposure to invasive procedures in the NICU and oxidative stress in infants born very preterm. Given the selective vulnerability of subplate neurons and preoligodendrocytes to oxidative stress (Back et al. 1998; Baud et al. 2004;
McQuillen et al. 2003; Sizonenko et al. 2003; Sizonenko et al. 2005), it is possible that repeated exposure to invasive procedures may lead to disturbances in cortical connections and myelination in infants born very preterm (Brummelte et al. 2012; Zwicker et al. 2013).

1.13 Brain Injury in Infants Born Very Preterm

The immaturity of the very preterm brain leaves infants vulnerable to brain injuries (i.e. periventricular leukomalacia [PVL], intraventricular hemorrhage [IVH], white matter injury [WMI], ventriculomegaly). Although cystic PVL was previously the major form of WMI in preterm infants, major advances in perinatal care (e.g. use of antenatal corticosteroids and exogenous surfactant therapy) has led to a marked decline in the incidence of cystic PVL (<5% of cases; Counsell et al. 2003; Groenendaal et al. 2010; Hamrick et al. 2004; Inder et al. 2003; Maalouf et al. 2001; Miller et al. 2003). Advances in neuroimaging (e.g. Magnetic Resonance Imaging [MRI] versus ultrasound) have led to the identification of diffuse WMI, which is now the pattern of brain injury most frequently observed in infants born very preterm (Back and Miller 2014). The extent of diffuse WMI is still difficult to define using conventional neuroimaging; however, it is often identified on MRI scans as multifocal lesions, which are seen in approximately, 37% of infants born very preterm (Chau et al. 2009; Miller et al. 2005).

Advances in MRI techniques have allowed us to also obtain quantifiable measurements of brain development in vivo (Duerden, Taylor, Miller 2013). Techniques, such as Diffusion Tensor Imaging (DTI), allow us to extract information from the scans at a microstructural level, thereby expanding our interpretation of scans beyond visible injuries to measure dysmaturation.

1.13.1 Diffusion Tensor Imaging

DTI is an MRI sequence that allows for the robust and noninvasive capture of maturation-
dependent changes of water diffusivity in the cortex of premature newborns, reflecting cortical microstructure. Specifically, DTI describes an ellipsoid space, where the size, shape, and orientation are given by eigenvalues ($\lambda_1$, $\lambda_2$ and $\lambda_3$; Mukherjee et al. 2002). Fractional anisotropy (FA) reflects the variance of $\lambda_1$, $\lambda_2$ and $\lambda_3$, and thereby describes overall directionality of the water diffusion. $\lambda_1$ corresponds to axial diffusion (Song et al. 2002). This is the preferred diffusion direction because water readily diffuses along white matter tracks and radial glia of the developing cortical gray matter. In contrast, $\lambda_2$ and $\lambda_3$ correspond to radial diffusion (Song et al. 2002). In the cerebral cortex, FA decreases between 25 and 40 weeks PMA, corresponding with neuronal maturation, synaptogenesis and the disappearance of the radial glia (Deipolyi et al. 2005; Jespersen et al. 2012; Kroenke et al. 2007; McKinstry et al. 2002; Sizonenko et al. 2007). In the white matter, FA increases with maturation, corresponding to the maturation of the oligodendrocyte lineage and early events of myelination (Drobyshevsky et al. 2005; Huppi et al. 1998; Partridge et al. 2004; Song et al. 2002). Parameters obtained through DTI are highly sensitive, which allow for the quantitative measure of change in brain microstructure over time that are relevant to brain injury and dysmaturation in infants born preterm.

DTI has been shown to be particularly useful for detecting and quantifying microstructural changes associated with brain injuries in children born very preterm. Focal brain injuries can affect overall brain development (Chau et al. 2009; Dubois et al. 2008b; Inder et al. 1999), and lead to moderate to severe neurodevelopmental disability (Inder et al. 2005; Miller et al. 2005; Woodward et al. 2006). However, DTI measures of brain microstructure have been shown to be better predictors of outcomes in children born very preterm seen at 18 months corrected age (CA), than white matter injuries observable on MRI (Chau et al. 2013). Using DTI imaging, our group has found widespread alterations in microstructure that have been associated with the
infant’s clinical condition (Bonifacio et al. 2010; Chau et al. 2009; Chau et al. 2013; Deipolyi et al. 2005) and interventions (Brummelte et al. 2012; Zwicker et al. 2013) during neonatal care. Our group is using DTI to study the relationship between repeated exposure to invasive procedures in the NICU and brain development in children born very preterm.

**1.14 Invasive Procedures in the NICU and Stress**

Exposure to invasive procedures activates the sympathetic adrenomedullary system, and the hypothalamic-pituitary adrenal (HPA) axis. However, in the NICU, stress from the invasive procedures cannot be differentiated from stress due to factors such as illness and maternal separation. Therefore, measures of stress hormones in the NICU are not specific to pain and may even be downregulated in the youngest, sickest infants due to repeated stimuli (Bolt et al. 2002; Grunau et al. 2005; Scott and Watterberg 1995).

**1.14.1 Stress Systems**

The sympathetic adrenomedullary system is part of the sympathetic division of the autonomic nervous system, which increases circulating epinephrine to facilitate rapid mobilization of metabolic resources and activate the fight/flight response. Epinephrine produced by the adrenal medulla, and norepinephrine produced by the postganglionic sympathetic neurons act together to activate metabolic resources, increasing heart rate, dilating blood vessels in the muscles, and constricting blood vessels in the skin and gut to ensure adequate blood supply to vital organs and muscles (Gunnar and Quevedo 2007; Tsigos and Chrousos 2002). Epinephrine also stimulates glycogenesis of the liver resulting in and increase in serum glucose, while norepinephrine promotes vigilance, arousal, and attention, and activates the HPA system (Gunnar and Quevedo 2007).
Stress triggers a cascade of events activating the HPA axis, which results in the production of glucocorticoids by the adrenal cortex. Activation of the hypothalamus leads to the co-release of corticotropin-releasing factor (CRF) and arginine vasopressin (Francis et al. 1999b; Gunnar and Quevedo 2007; Heim and Nemeroff 2001; Peters 1998), which in turn stimulates the synthesis and release of adrenocorticotropin from the anterior pituitary. This influences the release of glucocorticoids (cortisol in humans) from the adrenal cortex into the general circulation (Francis et al. 1999b; Gunnar and Quevedo 2007; Heim and Nemeroff 2001; Peters 1998). Cortisol binds with glucocorticoid receptors in the hypothalamus, hippocampus and other brain regions to inhibit further production of cortisol (Francis et al. 1999b; Gunnar and Quevedo 2007). Importantly, glucocorticoids regulate the transcription of genes (Chrousos 2009). Given the widespread actions of cortisol on multiple systems, repeated exposure to invasive procedures could lead to the suppression of growth, dampening of the immune system, and changes to the developing brain.

1.15 Neonatal Stress and Growth

Although approximately 80% of preterm infants born very preterm are born an appropriate weight for their GA, many preterm infants develop persistent growth deficits postnatally (Steward and Pridham 2002; Wilson et al. 1997). By discharge from the NICU the majority of preterm infants are considered growth restricted, that is, <10th percentile for their PMA (Steward and Pridham 2002; Wilson et al. 1997). Cortisol inhibits growth hormone secretion (Tsigos and Chrousos 2002). Growth hormones stimulate the production of insulin-like growth factor 1 (IGF-1) from the liver, which is important for the promotion of cell growth, and multiplication and inhibition of apoptosis in cells throughout the body. IGF-1 is lower in preterm infants (Cutfield
et al. 2004), and is associated with lower body weight, length and head circumference at birth (Lo et al. 2002). Moreover, poorer postnatal growth has been associated with lower IGF-1 levels (Ahmad et al. 2007). Postnatal growth failure in the NICU is associated with increased incidence of cerebral palsy and neurodevelopmental impairment, after accounting for prenatal growth, systemic illness, and brain injury (Ehrenkranz et al. 2006). Therefore, it is important to identify and ameliorate factors, which may impact postnatal growth in infants born very preterm.

Ongoing pain/stress in the NICU may contribute to suppression of postnatal growth, given that early environmental stressors in animals have been shown to induce slower body weight gain (Bhatnagar et al. 2006; Gamallo, Villanua, Beato 1986). Reduced weight gain has been reported in rat pups exposed to pain for the first 7 days of life (Anand et al. 1999). Invasive procedures, a common stressor in the NICU, have not been examined in relation to postnatal growth in infants born very preterm. However, very preterm infants who received massage therapy for three 15-minute periods over 5 consecutive days did gain more weight during hospitalization, and had higher IGF-1 levels (Field et al. 2008). These relationships could not be attributed to caloric intake (Field et al. 2008). It is possible that massage therapy led to the reduction of stress, and increase in vagal activity (Acolet et al. 1993; Diego, Field, Hernandez-Reif 2005; Hernandez-Reif, Diego, Field 2007), thereby allowing for the secretion of growth hormones, promoting growth in the preterm neonates. However, more studies are needed to identify whether pain and/or stress is one of the factors contributing to the persistent growth deficits in the NICU.

1.16 Neonatal Stress and Immune Function

Another important factor to consider is the effect that ongoing neonatal pain/stress can have on immune function. Inflammatory cytokines, tumor necrosis factor-α, interleukin-1β and
interleukin-6 can activate the HPA axis alone, or in combination with each other (Chrousos 1995; Tsigos et al. 1997). Activation of the HPA axis has profound inhibitory effects on the inflammatory/immune response, as almost all the components of the immune response are inhibited by cortisol production (Chrousos 1995; Elenkov et al. 1999). Therefore, repeated exposure to invasive procedures in the NICU may lead to immune suppression. This is of concern given that infants born preterm are already at increased risk for numerous neonatal complications that are related to inflammation and immune function (e.g. infection, necrotizing enterocolitis, chronic lung disease; Rubens et al. 2014). Infants born earlier and sicker require more medical interventions to ensure their survival. Therefore, there are close relationships between infant GA at birth, illness severity, PMA, mechanical ventilation and invasive procedures. While at this time it is difficult to prevent premature birth (Rubens et al. 2014), reducing pain/stress in the NICU may allow clinicians the opportunity to improve neurodevelopmental outcomes among children born very preterm.

1.17 Neonatal Stress and Brain Development

Glucocorticoids are involved in normal brain maturation and cell survival (Korte 2001; Meaney et al. 1996; Meyer 1983). However, either excess levels or too low levels of glucocorticoids can have deleterious effects on the developing brain. Glucocorticoids increases serotonin (5HT) transporter expression, thereby reducing 5HT availability both in the hippocampus and throughout the brain (Fumagalli et al. 1996; Slotkin et al. 1996). This is important because 5HT acts on ketanserin-sensitive 5HT7 receptor subtypes within the hippocampus to stimulate cyclic adenosine monophosphate (cAMP; Meaney et al. 2000; Yau et al. 1997). cAMP induces the expression of nerve growth factor 1-A (NGF1-A), which binds to the glucocorticoid receptor gene promoter (Meaney et al. 2000). Fewer glucocorticoid receptors within the hippocampus
leads to poorer negative feedback following glucocorticoid expression (Francis et al. 1999b; Gunnar and Quevedo 2007). Rat pups exposed to early in life stress, have fewer hippocampal glucocorticoid receptors, and higher corticotropin releasing factor, adrenocorticotropin and corticosterone production, during adulthood (Meaney et al. 1996). Similarly, rat pups exposed to inflammatory pain on day 1 of life have fewer hippocampal glucocorticoid receptors, and an attenuated stress response during adulthood (Victoria et al. 2013). Results from these animal studies suggest that there is the potential for repeated exposure to invasive procedures in the NICU to lead to poorer glucocorticoid feedback and altered brain maturation in infants born very preterm, which may persist in adulthood.

While very preterm infants are in the NICU, their cortisol levels are frequently lower than expected, considering the amount of pain/stress they are exposed to during hospitalization (Fernandez and Watterberg 2009; Grunau et al. 2005; Peters 1998). Downregulation of glucocorticoids may occur among these physiologically immature neonates due to multiple clinical factors such as illness and infection in this medical context. Greater exposure to invasive procedures in the NICU has been associated with lower cortisol responses to stress at 32 weeks PMA, after statistically adjusting for GA a birth, early illness severity and morphine exposure (Grunau et al. 2005). Furthermore, greater exposure to neonatal pain/stress after accounting for neonatal risk factors related to prematurity, is associated with an altered trajectory of cortisol expression from infancy through age 7 years (Brummelte et al. 2015; Grunau et al. 2005; Grunau et al. 2013; Grunau, Weinberg, Whitfield 2004). It would appear that repeated exposure to stress early in life programs the endocrine stress system to prepare for a stressful postnatal environment.
1.18 Repeated Exposure to Invasive Procedures in the NICU and the Developing Brain

There is a major literature in preterm children describing the neurodevelopmental and behavioral differences between children born very preterm and full-term (e.g. Anderson, Doyle, Victorian Infant Collaborative Study Group 2003; Doyle, Casalaz, Victorian Infant Collaborative Study Group 2001; Doyle and Anderson 2010; Grunau, Whitfield, Fay 2004; Johnson et al. 2009; Loe et al. 2011; Marlow et al. 2005; Marlow et al. 2007; Spittle et al. 2009). It has also been well-established that individuals born preterm demonstrate altered brain maturation (e.g. reduced brain volumes) throughout infancy (Nguyen et al. 2009; Srinivasan et al. 2007; Thompson et al. 2007; Thompson et al. 2011), childhood (Kesler et al. 2004; Kesler et al. 2008; Lax et al. 2013; Lowe et al. 2012; Peterson et al. 2000; Yung et al. 2007), adolescence (Gimenez et al. 2006; Nagy, Lagercrantz, Hutton 2011; Nosarti et al. 2002; Nosarti et al. 2008; Nosarti et al. 2011), and young adulthood (Aanes et al. 2015; Bjuland et al. 2014; Lawrence et al. 2014; Nosarti et al. 2014), in comparison to individuals born full-term. Perinatal and neonatal risk factors such as postnatal infection have been identified as being linked to altered brain development (Chau et al. 2009; Chau et al. 2012; Glass et al. 2008; Leviton et al. 2010; Miller et al. 2005; Shah et al. 2008). Recent studies by Brummelte et al. (2012), and Smith et al. (2012) have demonstrated for the first time in very preterm infants that repeated exposure to invasive procedures in the NICU was associated with altered brain development from early in life to term-equivalent age, above and beyond known risk factors related to prematurity (Brummelte et al. 2012; Smith et al. 2011). These studies are supported by animal models, which have demonstrated that both inflammatory pain and repeated injections increase cell death in the neonatal rat brain (Anand et al. 2007; Duhrs en et al. 2013; Rovnaghi et al. 2008). Associations between the number of invasive procedures in the NICU and brain development also appear to extend beyond the relationships early life (Doesburg et al. 2013; Ranger et al. 2013). At 7 years of age, higher numbers of
invasive procedures in the NICU were associated with thinner cortical gray matter in 21 out of 66 cerebral regions assessed, predominately affecting the frontal and parietal lobes (Ranger et al. 2013). Moreover, among infants born <28 weeks GA, greater exposure to neonatal pain/stress was also associated with alterations in spontaneous neuromagnetic activity (Doesburg et al. 2013). Therefore, it appears that repeated exposure to invasive procedures in the NICU may be altering brain development and may account for some of the neurodevelopmental and behavioral differences observed between infants born preterm versus full-term.

1.19 Early Exposure to Stress and Cognitive, Motor and Behavioral Outcomes

Given that ongoing exposure to stress early in life is associated with changes in hormones, brain structure and function, we would also expect to find relationships between exposures to neonatal pain/stress and neurodevelopmental outcomes. Early maternal separation in animal models causes significant distress and is commonly used as a neonatal stressor in basic research. Rat pups periodically separated from their mothers demonstrated more anxiety-like behaviors and cognitive impairments as adults, in comparison with pups that either remained with their mother or were briefly handled (Aisa et al. 2007; Kalinichev et al. 2002; Ogawa et al. 1994; Oomen et al. 2010; Wigger and Neumann 1999). Similarly peer-reared rhesus monkeys demonstrated reduced locomotion, fewer interactive behaviors, and increased stereotypical behaviors compared to the mother-reared monkeys (Feng et al. 2011). Therefore, animals models of early life stress have revealed that greater exposure to stress in the first week(s) of life results in poorer cognitive and behavior outcomes.

In recent years, studies have started to explore the potential long-term impact of neonatal pain/stress on neurodevelopmental outcomes of both humans and animals. Rat pups exposed to
either repeated skin-breaking procedures or inflammatory pain in the first week of life, demonstrated poorer cognitive, behavioral and motor outcomes during adulthood (Anand et al. 1999; Bhutta et al. 2001; Negrigo et al. 2011; Rovnaghi et al. 2008). In humans, higher numbers of invasive procedures in the NICU were also associated with poorer cognitive and motor outcomes at 8 and 18 months CA in children born very preterm, independent of early illness severity, morphine and postnatal corticosteroid exposure (Grunau et al. 2009). Moreover, greater exposure to invasive procedures was associated with more internalizing (anxious/depressive) behaviors at 7.5 years of age in non-ventilated children born very preterm, after accounting for neonatal confounders and concurrent parenting stress (Ranger et al. 2014). While previous research, particularly studies using animal models, has given us insight into how repeated exposure to invasive procedures may be lead to adverse outcomes in children born very preterm, more research is required in order for us to understand the mechanisms underlying these relationships.

1.20 Caregivers and Stress Management

Parenting plays a central role in stress regulation and normal brain development in both humans and animals.

1.20.1 Maternal Licking and Grooming Behavior in Rats

Positive parental interaction is important not only for the relationship between the parent and child, but also, for the overall development of the child, particularly in at risk children, such as those born very preterm (Brummelte et al. 2011b; Crnic and Greenberg 1987; Tu et al. 2007). Animal models have given us insight into the mechanisms that underlie the importance of positive maternal interactions. Rat dams exhibit considerable variation in the amount they lick
and groom their pups (Champagne et al. 2003), and these behaviors remain stable across their litters. Variations in pup licking and grooming during the first week of life affected HPA and behavioral responses to stress, and were correlated with hippocampal glucocorticoid receptor expression in adulthood (Caldji et al. 1998; Francis et al. 1999a; Liu et al. 1997; Menard, Champagne, Meaney 2004; van Hasselt et al. 2012; Weaver et al. 2004; Zhang et al. 2006). The adult offspring of low licking and grooming mothers showed reduced hippocampal glucocorticoid receptor expression, poorer glucocorticoid feedback sensitivity, greater corticotrophin releasing factor and greater glucocorticoid production in comparison to pups reared by high licking and grooming mothers (Francis et al. 1999a; Liu et al. 1997). These changes in physiology were related to alterations in behavior as adults. Offspring of low licking and grooming mothers showed greater anxiety-like behavior and deficits in spatial learning and memory (Caldji et al. 1998; Liu et al. 2000; Pena et al. 2014; Starr-Phillips and Beery 2014; van Hasselt et al. 2012). Therefore, variations in maternal behavior are related to differences in HPA axis functioning, as well as alterations in learning and behavior in offspring. Given that the same pathways altered by repeated exposure to early life stress are programmed by maternal behavior, positive maternal behavior may ameliorate some of the negative effects of repeated exposure to early life stress on brain and stress system development, as well as neurodevelopmental and behavioral outcomes.

1.20.2 Maternal Interaction in the NICU

The hospitalization of neonates born very preterm interferes with mother-infant bonding, given that these infants spend prolonged periods in an incubator, often separate from their mother. Kangaroo care, also known as skin-to-skin contact, involves resting a diapered infant on the caregiver's bare chest. The skin-to-skin contact is an efficacious method for reducing pain-
related stress and improving pain regulation in infants born preterm (Pillai Riddell et al. 2011). Parent sensitivity training in the NICU is designed to help parents recognize signs of infant stress and teach them how to proactively soothe their infant without overwhelming them (Milgrom et al. 2010; Milgrom et al. 2013; Rauh et al. 1990). In a randomized control trial comparing mothers who received stress sensitivity training versus standard care, in just a few short sessions with a developmental specialist, mothers in the intervention group demonstrated greater sensitivity to infant cues, and were able to reduce infant stress behavior (Milgrom et al. 2013). Importantly, infants of mothers who received sensitivity training showed increased maturation of the white matter microstructure at term equivalent age (Milgrom et al. 2010), and more advanced communication development at 6 months CA, compared to controls (Milgrom et al. 2013). Participation in this training in the NICU, plus 4 training sessions at home was found to reduce parental concerns about the infant long after discharge from the NICU (Landsem et al. 2014). Therefore, supporting positive parent interactions in the NICU, and involving parents in infant care is important for minimizing neonatal pain/stress and improving outcomes after discharge from the NICU.

1.21 Parenting Stress

Stress in families with preterm infants has been found to be high during infant hospitalization (Glazebrook et al. 2007; Poehlmann and Fiese 2001; Thomas, Renaud, Depaul 2004), and persists well beyond discharge from the NICU (Brummelte et al. 2011a; Garel, Dardennes, Blondel 2007; Singer et al. 2003; Treyvaud et al. 2014). Parenting stress may partly reflect realistic concerns regarding the child’s development (Brummelte et al. 2011a; Docherty, Miles, Holditch-Davis 2002). In infants born very preterm, decreasing cognitive scores between 8 and 18 months CA was associated with higher parenting stress (Brummelte et al. 2011a). However,
other factors, such as lower maternal education, a predictor of premature birth and a standard indicator of socioeconomic status, was also associated with greater parenting stress in mothers of infants born very preterm (Brummelte et al. 2011a; Docherty, Miles, Holditch-Davis 2002; Woodward et al. 2014). Lower parenting stress was associated fewer internalizing behaviors at school age in children born very preterm (Ranger et al. 2014). However, it is important to note that this relationship is bidirectional, such that greater child internalizing may have contributed to higher parenting stress. Lower parenting stress may modulate the adverse effects of repeated invasive procedures on negative reactivity at 8 months CA (Voigt et al. 2013), and cognitive outcomes at 18 months CA (Grunau et al. 2009). Furthermore, the relationship between maternal stress and outcomes may be modulated by maternal behavior. Mothers who reported low concurrent stress were more sensitive when interacting with their child post-discharge (Muller-Nix et al. 2004; Tu et al. 2007). However, among mothers who reported low concurrent stress, maternal behavior buffered the relationship between greater exposure to invasive procedures and poorer focused attention (Tu et al. 2007). It is recognized in the literature that the birth of a preterm child is stressful for parents and families, and that greater parenting stress is associated with poorer outcomes among children born very preterm. Importantly, involving parents in NICU care may lower parenting stress, improve parent-child interactions and contribute to the optimization of neurodevelopmental and behavioral outcomes in children born very preterm.

1.22 Maternal Interaction Post-Discharge from the NICU

The caregiver continues to be important for regulating the HPA axis in premature infants post-discharge. In humans, positive maternal interaction at 18 months CA was associated with better regulation of cortisol levels in children born very preterm (Brummelte et al. 2011b). In neonatal rats, cross-fostering the animals to high licking and grooming mothers, and enriching social
environments post-weaning, reverses the adult phenotype associated with poor quality maternal care (Champagne and Meaney 2007; Francis et al. 1999b). Based on these findings, through sensitive and responsive caregiving, parents of children born very preterm may be able to ameliorate the long-term effects early environmental pain/stress and maternal deprivation on the brain and neurodevelopmental and behavioral outcomes, post-discharge from the NICU.

Children born very preterm are particularly influenced by their environmental context and are more greatly affected by their interactions with their parents than their term-born peers (Brummelte et al. 2011b; Crnic and Greenberg 1987; Erickson et al. 2013; Forcada-Guex et al. 2006; Tu et al. 2007). There is debate as to whether the quality of interaction differs between parents of preterm versus full-term children, given that the differences between these two groups narrows, after accounting for factors such as maternal education, parenting stress and child IQ (Brummelte et al. 2011b; Forcada-Guex et al. 2006; Greenberg and Crnic 1988; Greene, Fox, Lewis 1983; Harrison 1990; Jaekel, Wolke, Chernova 2012; Muller-Nix et al. 2004; Potharst et al. 2012; Rahkonen et al. 2014; Tu et al. 2007). However, specifically among families with children born very preterm, higher quality parent-child interactions post-discharge were associated with better cognitive and behavioral outcomes (Beckwith, Rodning, Cohen 1992; Erickson et al. 2013; Magill-Evans and Harrison 2001; Rahkonen et al. 2014; Spittle et al. 2010; Tu et al. 2007). Therefore, evidence-based interventions focusing on the parent-child relationships may lead to improved outcomes in children born very preterm.

### 1.23 Caregiver Emotional Availability

A relatively new construct used to assess the parent-child relationship is emotional availability (EA). Attachment theory was one of the founding concepts of EA, due to its emphasis on
appropriate responding to infant cues and communications (Ainsworth et al. 1978; Bowlby 1969; Bowlby 1973). In addition to attachment theory, several other influences contributed to the conceptualization of EA. Mahler et al. (1975) first used the term “emotional availability” to describe a mother’s supportive presence and encouragement during child exploration (Mahler, Pine, Bergman 1975). Emde and Easterbrooks (1985) described emotional availability as being an affective barometer of the relationship. Finally, the systems view proposed by Guttman (1991) was important for its description of reciprocity of influence, where each person both contributes to the relationship, and is affected by the other person’s involvement in the relationship (Guttman 1991). Each of the above perspectives contributed to the conceptualization of EA and provided the foundation for the observation scales developed by Biringen and colleagues (Biringen, Robinson, Emde 1998; Biringen et al. 2014; Biringen 2000; Biringen 2008). These scales evaluate the caregiver and child as an interactive dyad, where each person in the relationship is capable of influencing and affecting how the other responds. Thus, the EA scale examines whether a caregiver is supportive of their child, and whether the caregiver possesses an authenticity of affect, appropriate responding (sensitivity/nonhostility/nonintrusiveness), and provision of guidance (structuring), which encourages the child to respond and/or involve the caregiver in their play or general activities (Biringen 2008). The EA scale is comprised of four parent dimensions, caregiver Sensitivity, Structuring, Nonintrusiveness and Nonhostility (Biringen 2008). The descriptions for each of these parent behaviors are listed below, and reflect the individual items addressed in each dimension included in the 4th edition of the EA scales (Biringen 2008).

1.23.1 EA Caregiver Sensitivity

Sensitivity reflects expression of genuineness and appropriateness of affect. For example the
Caregiver may generally be balanced and low-keyed. When appropriate, however, they may be more animated in their behavior, and demonstrate a clear enjoyment of the child. The caregiver should be aware of their child’s signals (i.e. approach, avoidance), and demonstrate a willingness to respond their needs. The caregiver should also be aware of their own timing (e.g. abruptness) while interacting with their child. To be considered sensitive the caregiver must also demonstrate flexibility and creativity during play with the child. Finally, a sensitive caregiver speaks and acts in respectful ways, is present within the interaction, and effectively moves conflicts towards a resolution.

1.23.2 EA Caregiver Structuring

Structuring requires that the caregiver provides guidance, and creates an environment that holds the child’s attention. A caregiver is considered emotionally available in their structuring if they use subtle and varied suggestions, to bring the child’s learning to a higher level. Such scaffolding may include verbal and nonverbal cues, but should not overwhelm the child. Within a given task an emotionally available parent should set appropriate limits, remain committed to those boundaries, and maintain a controlled environment.

1.23.3 EA Caregiver Nonintrusiveness

Child exploration is a stepping-stone to acquiring autonomy. It is important that within the limits the caregiver sets for the child that the parent allow the child space to figure out tasks/challenges, without controlling, manipulating (physically or verbally) or completing the exercise for the child. An emotionally available, nonintrusive, caregiver will wait for the opportune time to enter the interaction and/or offer assistance, as opposed to interrupting the flow verbally or physically (except in cases of emergency). They will also use commands sparingly. Talking and teaching
should be an interactive form of communication between the parent and child.

**1.23.4 EA Caregiver Nonhostility**

Emotionally available nonhostile caregivers will have control over their emotions. They will attempt to avoid being overly negative or stressed when interacting with the child. This includes maintaining control over both verbal and nonverbal expressions. Caregivers will receive lower scores on the EA scale if they mock, ridicule, or express any kind of disrespectful statement or behavior (e.g. boredom, impatience). Hostility also includes using silence and/or leaving as a threat to gain control over their child, not just the use physical or verbal assaultive behaviors. Furthermore, a nonhostile caregiver will play in a manner that is appropriate to the context/materials and is not unnecessarily malevolent.

**1.24 Maternal Emotional Availability in Infants Born Very Preterm**

Emotional Availability is a relatively new construct, and few studies have examined parental EA in families with children born very preterm. When infants were 5 months CA, mothers who reportedly perceived their preterm infant as being more vulnerable, behaved more intrusively, and displayed greater hostility while interacting with their child (Stern et al. 2006). This corresponds with earlier research, using other mother-infant interaction scales, which has shown that mothers of preterm infants may work harder to engage their infants, and display less positive affect during interactions with their infant compared to parents of infants born full-term (Brachfeld, Goldberg, Sloman 1980; Crnic et al. 1983). Higher maternal education and lower maternal anxiety in the NICU were associated with higher maternal sensitivity at 24 months CA, after adjustment for birth weight (Zelkowitz et al. 2009). Although Zelkowitz et al. (2011) reported significant correlations between maternal sensitivity and child cognitive, motor and
behavioral development at 24 months CA, the relationships between concurrent maternal behavior and outcomes were no longer significant after adjustment for GA, neonatal morbidity, maternal education and anxiety (Zelkowitz et al. 2011). Therefore, it is not clear to what extent parental EA may moderate the relationships between maternal and environmental stressors and child neurodevelopmental and behavioral outcomes. It would appear, however, that parental EA is an important factor to consider when examining the etiology of child developmental outcomes.

1.25 Dissertation Overview

Infants born very preterm have the nociceptive circuitry required to perceive pain. However, tactile threshold is lower, descending inhibitory pathways are immature, and neonates become sensitized to repeated tactile and skin-breaking stimulation, leading to greater sensitivity to invasive procedures during this vulnerable period. As part of their life-saving care, infants born very preterm undergo repeated invasive procedures in the NICU. These procedures occur during a period of rapid brain and stress system development, and therefore, repeated exposure to invasive procedures in the NICU could lead to alterations in brain microstructure. Dysmaturation of the cerebral white and gray matter may underlie the persistent differences in cognitive, motor and behavioral outcomes observed between children born very preterm versus full-term. Importantly, positive parental interaction may partially ameliorate the long-term effects of repeated exposure to neonatal stress in the NICU, suggesting opportunities for intervention.

The purpose of this dissertation is to firstly examine whether repeated exposure to invasive procedures during neonatal intensive care is associated with altered brain microstructure, postnatal growth, and neurodevelopment in children born very preterm. A further aim was to examine for the first time the extent that parental emotional availability may either ameliorate or
exacerbate the relationships of invasive procedures with child behavior at 18 months CA. Studies presented in Chapters 2 and 3 include data from a prospective cohort of infants recruited from the NICU at the BC Children’s and Women’s Hospital, born between March 2006 and January 2009, and followed to term equivalent age. Studies presented in Chapters 4 and 5 include data from an earlier prospective cohort of infants recruited from the same NICU, born between February 2001 and July 2004, and followed to age 7 years.

Chapter 2 is the first known study to examine whether exposure to invasive procedures in the NICU is related to postnatal body and head growth in infants born very preterm, after adjusting for age, prematurity, size at birth, and clinical factors. We found that greater exposure to invasive procedures in the NICU was associated with delayed early postnatal body and head growth, independent of medical confounders. This work has uniquely identified repeated exposure to pain/stress as one of the factors underlying early postnatal growth failure in the NICU in infants born very preterm.

Given our finding that repeated exposure to invasive procedures was associated with slower growth in the NICU, using data from the same cohort in Chapter 3 we examined whether postnatal growth indicated by change in weight, length and head circumference between approximately 32 and 40 weeks PMA is related to cortical brain development in infants born very preterm, after controlling for GA, size at birth, sex, PMA, brain injury, and systemic illness. We found that impaired postnatal growth (weight, length, and head circumference) was significantly associated with delayed cortical maturation, even after adjusting for neonatal and medical confounders. However, it was the cortical gray matter, rather than the white matter, which appeared to be most susceptible to impairments in postnatal growth. This study
demonstrated for the first time that postnatal growth, over and above prenatal growth, systemic illness and brain injury was associated with cerebral cortical maturation in children born very preterm.

Previously, in the cohort born between February 2001 and July 2004, our group found that in mothers with lower parenting stress, maternal interactive behavior buffered the relationship between greater pain/stress in the NICU and poorer attention at 8 months CA (Tu et al. 2007). When these same children were seen at 18 months CA, more positive mother-child interactions were associated with better cortisol regulation during cognitive testing, and fewer anxious/depressive behaviors in children born very preterm (Brummelte et al. 2011b). My research extended these findings in the same cohort, by introducing the newer construct of emotional availability (EA) in parent-infant interaction. In Chapter 4 we examined whether parent EA (adjusted for parenting stress), moderates the relationship between pain/stress in the NICU and internalizing behaviors (adjusted for child cognition) in children born very preterm. Furthermore, we also examined the relationship between parent EA and internalizing behavior in healthy full-term controls who were born at the same hospital. Among the very preterm children, exposure to a higher number of invasive procedures (adjusted for confounding neonatal medical factors), greater parent sensitivity and nonhostility was associated with fewer internalizing behaviors at 18 months CA. In contrast, none of the parent factors was a significant predictor of internalizing behavior in children born full-term. Therefore, our group has robustly demonstrated using two different scales to assess parent behavior that positive parent-child interactions can moderate the adverse relationships between greater pain/stress and neurodevelopmental and behavioral outcomes in children born very preterm.
Previously, in the cohort born between March 2006 and January 2009, our group found a relationship between greater exposure to pain/stress and altered white matter maturation from early in life to term-equivalent age, after adjusting for neonatal clinical confounders (Brummelte et al. 2012; Zwicker et al. 2013). Using data from the other cohort of children born very preterm, born February 2001 and July 2004, in Chapter 5 we examined whether the extent of pain/stress during NICU care was associated with maturation of white matter microstructure at age 7 years, and whether pain/stress together with measures of brain microstructure were associated with cognitive outcome at school age in children born very preterm, after accounting for degree of prematurity, systemic illness, medications, and concurrent brain injury. Greater number of invasive procedures during neonatal care was associated with altered white matter microstructure at school age. Moreover, greater exposure to invasive procedures together with altered myelination of the white matter, predicted lower IQ among children born very preterm, even after adjustment for neonatal and medical confounders. The study in Chapter 5, built on our previous work, to now provide evidence from two independent cohorts, at two separate ages, that neonatal pain/stress is associated with altered brain development. Further, we provided the first link between greater pain/stress, altered brain microstructure and poorer cognitive development at school age.

Chapter 6 summarizes the results of this dissertation and discusses these findings within the context of the current theoretical field regarding the effects of pain on brain development, and parents as moderators of the long-term effects of stress in children born very preterm.
CHAPTER 2

NEONATAL PAIN IN RELATION TO POSTNATAL GROWTH IN INFANTS BORN VERY PRETERM

2.1 Introduction

Invasive procedures are an inherent part of life-saving care in the NICU. However, chronic exposure to procedure-related pain/stress in the NICU may have detrimental effects on the growth and development of infants born very preterm. Infants born very preterm have a limited metabolic reserve (Polin, Fox, Abman 2003). Mounting a pain/stress response requires a substantial amount of energy. Greater numbers of invasive procedures in the NICU have been associated with a dampening of pain/stress and cortisol responses, and may lead to exhaustion and downregulation of resources among infants born very preterm (Grunau et al. 2001; Grunau et al. 2005).

Maintaining a consistent weight gain in infants born very preterm poses a major challenge for clinicians. Although approximately 80% of preterm infants are born at an appropriate weight (10th–90th percentile) for their GA and sex, during hospitalization the growth of the majority of preterm infants appears inadequate, such that by NICU discharge many are considered growth restricted (<10th percentile) (Ehrenkranz et al. 1999; Wilson et al. 1997). Prematurity and illness often prevent clinicians from being able to provide the daily-recommended dietary intake for newborns. Therefore, infants born very preterm develop a significant nutrient deficit over the first few weeks of life (Embleton, Pang, Cooke 2001). However, nutritional intake only accounts for approximately 45% of the variance in postnatal growth (Embleton, Pang, Cooke 2001).

Growth patterns can vary depending on GA, birth weight, days of respiratory support, and use of postnatal corticosteroids (Berry, Abrahamowicz, Usher 1997; Billeaud, Piedboeuf, Chessex 1992; Cockerill et al. 2006; Halliday, Ehrenkranz, Doyle 2010). Therefore, there are multiple factors to consider when evaluating predictors of postnatal growth. To the best of our knowledge, the relationship between invasive procedures in the NICU and postnatal growth has not been examined previously.

Growth hormones stimulate the production of IGF-1, a small peptide, which is primarily synthesized in the liver and is a key regulator of growth (Yamini et al. 2010). IGF-1 values are lower in preterm infants compared to infants born full-term (Cutfield et al. 2004), and are positively associated with body weight, length and head circumference at birth (Lo et al. 2002). IGF-1 values gradually increase after preterm birth and are correlated with postnatal weight gain (Kurtoglu et al. 2010; van de Lagemaat et al. 2013). Stress inhibits the production of growth hormones (Tsigos and Chrousos 2002). Reducing stress in the NICU may improve postnatal growth in infants born very preterm. In a randomized control trial after receiving a 3 µg/kg does of fentanyl, mechanically ventilated preterm infants showed increased IGF-1 and moderately lower cortisol values compared to infants that were given a placebo (Guinsburg et al. 1998).

In animal models, repeated exposure to stress is associated with slower body weight gain (Bhatnagar et al. 2006; Gamallo, Villanua, Beato 1986). Anand et al. (1999) exposed rat pups to either skin-breaking procedures or tactile stimulation daily for the first 7 days of life. Pups that underwent the painful procedures weighed less at postnatal day 8 and 15 (Anand et al. 1999). Moreover, repeated exposure to painful procedures was also associated with greater anxiety-like behaviors during adulthood (Anand et al. 1999).
Slower body weight gain and head growth in the NICU were associated with increased incidence of cerebral palsy and neurodevelopmental impairment (Ehrenkranz et al. 2006; Leppanen et al. 2014). Therefore, it is of the utmost importance that we understand the possible relationships between exposure to invasive procedures and postnatal growth, as this research may lead to evidence-based interventions to improve outcomes within this vulnerable population.

Hypothesis

We hypothesized that greater exposure to invasive procedures in the NICU would be associated with poorer growth in the first weeks of life and at term-equivalent age, independent of other neonatal and medical risk factors.

2.2 Methods

2.2.1 Study Overview

Birth weight and birth head circumference (HC) were obtained from the neonatal medical chart review. Postnatal growth was assessed by recording weight and HC at approximately 32 weeks PMA (early weigh-in), and again at approximately 40 weeks PMA (later weigh-in). Age at the early and later weigh-ins were timed to coincide with other study examinations (Adams et al. 2010; Chau et al. 2009) and varied in cases where infants were unstable or were discharged prior to term-equivalent age. Therefore, PMA was included in statistical analyses to control for variability in age at each weigh-in. Variables obtained from chart review were recorded in “windows” based on birth, early and later weigh-ins (Figure 2.1). The early window included measures between birth and 32 weeks PMA (early weigh-in); the later window included measures between 32 (early weigh-in) and 40 weeks PMA (later weigh-in). Finally, we
combined the early and late windows to create one cumulative (early and late) neonatal window, which included measures between birth and 40 weeks PMA (later weigh-in).

**Figure 2.1: Early and Late Neonatal Windows**

<table>
<thead>
<tr>
<th>Early number of invasive procedures, days of ventilation, infection, morphine, hydrocortisone, dexamethasone exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
</tr>
<tr>
<td>Illness severity day 1</td>
</tr>
<tr>
<td>32 weeks (early weigh-in)</td>
</tr>
<tr>
<td>Later number of invasive procedures, days of ventilation, infection, morphine, hydrocortisone, dexamethasone exposure</td>
</tr>
<tr>
<td>40 weeks (later weigh-in)</td>
</tr>
<tr>
<td>Cumulative (early and late) number of invasive procedures, days of ventilation, infection, morphine, hydrocortisone, dexamethasone, exposure</td>
</tr>
</tbody>
</table>

Medical chart review was performed from birth to term-equivalent age or hospital discharge (whichever came first), and variables from chart review were divided into neonatal windows (early: birth to 32 weeks PMA; later: 32 to 40 weeks PMA; cumulative: early and late) to capture NICU events in relation to the ages infants were weighed and measured (i.e. birth, 32 and 40 weeks PMA).

### 2.2.2 Participants

A prospective cohort of 78 very preterm infants (≤32 weeks GA) born between March 2006 and January 2009 were recruited from the NICU at the British Columbia Children’s and Women’s Hospital, as part of a larger ongoing study of neonatal invasive procedures, brain and neurodevelopment (Adams et al. 2010; Chau et al. 2009; Grunau et al. 2005; Grunau et al. 2007; Grunau et al. 2009). Exclusions from the study were: a major congenital malformation or syndrome, antenatal infection, severe brain injury on neonatal ultrasound (i.e. large parenchymal...
hemorrhagic infarction >2 cm) or missing neonatal data (e.g. due to transfer to hospitals outside of the Lower Mainland of British Columbia).

2.2.3 Weight Percentiles
Infants were weighed and HC measured at birth (median GA 27 weeks; interquartile range [IQR] 25.9-29.7), within the first few weeks of life (early weigh-in: median PMA 32 weeks; IQR 30.7-33.6), and again at term-equivalent age or discharge (later weigh-in: median PMA 40 weeks; IQR 38.6-42.6). These time points were chosen to coincide with other study protocol measures and span the neonatal period (Adams et al. 2010; Chau et al. 2009). Values at all 3 time points were converted into percentiles from sex-specific British Columbia population-based data. Growth percentiles are normed for age and sex and provide a more meaningful description of postnatal growth than changes in raw values. For example, a change from the 8th (growth-restricted) to the 15th (appropriate weight for PMA) weight percentile is a more meaningful interpretation of growth than a change from 1500 to 3000 grams.

2.2.4 Neonatal Medical Chart Review
A neonatal research nurse performed medical and nursing chart review from birth to term-equivalent age or discharge (whichever came first). Data included but were not limited to birth weight, GA at birth, illness severity on day 1 (Score for Neonatal Acute Physiology II [SNAP-II] (Richardson et al. 2001), number of invasive procedures (see Appendix Table A.1), days of mechanical ventilation, presence of infection, and exposure to morphine and corticosteroids (hydrocortisone, dexamethasone). Morphine, hydrocortisone and dexamethasone exposure were entered as binary variables reflecting whether or not these drugs were administered. A high score on the SNAP-II indicates greater illness severity. Postnatal infections were identified by positive
culture in the blood, urine, cerebral spinal fluid or if ≥4 white blood cells were found in the tracheal aspirates associated with clinical pneumonia. Given that these data were drawn from a longitudinal study designed to examine long-term effects of neonatal pain on brain and stress system development, data on nutrition were not collected.

2.2.5 Data Analyses
Normality plots were examined and skewed variables (number of invasive procedures, birth weight percentile, weight and HC percentile at 32 and 40 weeks PMA) were log transformed. Analysis of variance was used to examine whether there were differences between the infants included and excluded in this study. Analysis of variance was also used to examine sex differences in the raw weights and HCs at birth, 32 and 40 weeks PMA, and to examine whether there were differences in the number of early, late and cumulative (early and late) invasive procedures performed on male and female preterm infants. Generalized linear models (GENLIN SPSS 18; IBM, Armonk, NY, USA) were used to examine the independent contributions of: 1) early neonatal variables (birth to 32 weeks PMA) to weight and HC percentile at 32 weeks PMA; 2) early neonatal variables (birth to 32 weeks PMA) to weight and HC percentile a 40 weeks PMA; 3) later neonatal variables (32 to 40 weeks PMA) to weight and HC percentile at 40 weeks PMA; and 4) cumulative (early and late/birth to 40 weeks PMA) variables to weight and HC percentile at 40 weeks PMA. Generalized linear modeling permits examination of potential confounders that are intercorrelated; predictors can be continuous, discrete, dichotomous, or a mix of these, as is the case with our data. This model uses the Wald test to examine the statistical significance of each coefficient B in the model.
2.3 Results

2.3.1 Characteristics of the Cohort

There were no significant differences between the included and excluded infants on birth weight percentile, HC percentile or illness severity on day 1. Characteristics of the included infants are provided in Table 2.1. It is noteworthy that the median number of invasive procedures was more than double in the early neonatal window compared to after 32 weeks PMA. Male and female infants did not differ significantly in raw values of weight or HC at birth, 32 or 40 weeks PMA (each $P > 0.25$). Furthermore, there were no significant differences in the early, late or cumulative (early and late) number of invasive procedures performed on male and female infants (each $P > 0.32$). Sex-specific normative weight and HC percentiles were used in the generalized linear models for all data analyses, and sex was not considered further.
Table 2.1 Infant Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Neonatal Window</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Birth to 32 weeks</td>
</tr>
<tr>
<td>Birth weight (percentile), median (IQR)</td>
<td>34.5 (13.3-57.0)</td>
</tr>
<tr>
<td>Birth HC (percentile), median (IQR)</td>
<td>40.0 (10.0-63.0)</td>
</tr>
<tr>
<td>Sex, (% male)</td>
<td>50.0%</td>
</tr>
<tr>
<td>Illness severity on day 1, median (IQR)</td>
<td>12.0 (5.0-24.3)</td>
</tr>
<tr>
<td>PMA (weeks), median (IQR)</td>
<td>32.1 (30.7-33.6)</td>
</tr>
<tr>
<td>PMA weight (percentile), median (IQR)</td>
<td>9.0 (4.0-16.3)</td>
</tr>
<tr>
<td>PMA HC (percentile), median (IQR)</td>
<td>8.50 (4.0-17.0)</td>
</tr>
<tr>
<td>Number of invasive procedures, median (IQR)</td>
<td>64.5 (35.0-131.5)</td>
</tr>
<tr>
<td>Mechanical ventilation (days), median (IQR)</td>
<td>19.0 (7.0-46.3)</td>
</tr>
<tr>
<td>Morphine exposure, number (%)</td>
<td>47 (60)</td>
</tr>
<tr>
<td>Hydrocortisone exposure, number (%)</td>
<td>11 (14)</td>
</tr>
<tr>
<td>Dexamethasone exposure, number (%)</td>
<td>12 (15)</td>
</tr>
<tr>
<td>Postnatal infection, number (%)</td>
<td>33 (42)</td>
</tr>
</tbody>
</table>

IQR= Interquartile range; HC= Head circumference; PMA= Postmenstrual age

2.3.2 Early (Birth to 32 Weeks PMA) Neonatal Variables in Relation to Weight

Percentile at 32 Weeks PMA

Lower birth weight percentile, greater exposure to invasive procedures and dexamethasone exposure, were independently associated with decreased weight percentile at 32 weeks PMA, after adjusting for the number of days on mechanical ventilation, morphine exposure, hydrocortisone exposure, postnatal infection, illness severity on day 1, and PMA at weigh-in (Table 2.2). As shown in Fig. 2.2, invasive procedures prior to 32 weeks PMA accounted for approximately 21% of the variance in early body growth, and greater exposure to invasive
procedures was related to decreased weight percentiles at 32 weeks PMA, after accounting for multiple medical confounders.

Table 2.2 Early (Birth to 32 Weeks PMA) Neonatal Variables in Relation to

Weight and HC Percentiles at 32 Weeks PMA

<table>
<thead>
<tr>
<th>Early Neonatal variables</th>
<th>Weight Percentile at 32 weeks PMA(^a)</th>
<th>HC Percentile at 32 weeks PMA(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald (\chi^2)</td>
<td>(P)</td>
</tr>
<tr>
<td>Birth weight percentile</td>
<td>124.45</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth HC percentile</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>7.36</td>
<td>0.01</td>
</tr>
<tr>
<td>Days of mechanical ventilation</td>
<td>0.38</td>
<td>0.54</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>0.39</td>
<td>0.53</td>
</tr>
<tr>
<td>Hydrocortisone exposure</td>
<td>0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>Dexamethasone exposure</td>
<td>4.83</td>
<td>0.03</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>0.43</td>
<td>0.51</td>
</tr>
<tr>
<td>Illness severity on day 1</td>
<td>2.06</td>
<td>0.15</td>
</tr>
<tr>
<td>PMA at 32 week weigh-in</td>
<td>0.38</td>
<td>0.55</td>
</tr>
</tbody>
</table>

HC= Head circumference; PMA= Postmenstrual age
Generalized linear models revealed that between birth and 32 weeks PMA: \(^a\)lower birth weight percentile, greater number of invasive procedures, and dexamethasone exposure, independently predicted slower body growth after accounting for the other neonatal factors; \(^b\)lower HC birth percentile, greater neonatal number of invasive procedures and duration of mechanical ventilation, predicted slower head growth, after accounting for the other neonatal factors. Directions of relationships between variables were determined by B values (not shown).
Predicted values of early growth (weight percentile at 32 weeks PMA in relation to early number of invasive procedures from birth to 32 weeks PMA), adjusted for other neonatal variables (i.e. birth weight percentile, illness severity on day 1, PMA at 32-week weigh-in, days of mechanical ventilation, infection, and exposure to morphine and corticosteroids [hydrocortisone, dexamethasone]).

2.3.3 Early (Birth to 32 Weeks PMA) Neonatal Variables In Relation to Weight Percentile at 40 Weeks PMA

Lower birth weight percentile and hydrocortisone exposure, rather than the number of invasive procedures, were independently associated with decreased weight percentile at 40 weeks PMA,
after adjusting for morphine exposure, dexamethasone exposure, infection, illness severity on day 1, and PMA at weigh-in (Table 2.3). There was a trend for duration of mechanical ventilation from birth to 32 weeks PMA to be associated with lower weight percentile at 40 weeks PMA.

### Table 2.3 Early (Birth to 32 Weeks PMA) Neonatal Variables in Relation to Weight and HC Percentile at 40 Weeks PMA

<table>
<thead>
<tr>
<th>Early Neonatal variables</th>
<th>Weight Percentile at 40 weeks PMA $^a$</th>
<th>HC Percentile at 40 weeks PMA $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald $\chi^2$</td>
<td>$P$</td>
</tr>
<tr>
<td>Birth weight percentile</td>
<td>19.91</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth HC percentile</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>0.15</td>
<td>0.70</td>
</tr>
<tr>
<td>Days of mechanical ventilation</td>
<td>3.35</td>
<td>0.07</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>0.26</td>
<td>0.61</td>
</tr>
<tr>
<td>Hydrocortisone exposure</td>
<td>4.80</td>
<td>0.03</td>
</tr>
<tr>
<td>Dexamethasone exposure</td>
<td>0.08</td>
<td>0.76</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>Illness severity on day 1</td>
<td>2.26</td>
<td>0.13</td>
</tr>
<tr>
<td>PMA at 32 week weigh-in</td>
<td>2.10</td>
<td>0.15</td>
</tr>
</tbody>
</table>

HC = Head circumference; PMA = Postmenstrual age

Generalized linear model revealed that between 32 and 40 weeks PMA: $^a$ lower birth weight percentile and hydrocortisone exposure independently predicted slower body growth, after accounting for other neonatal factors; $^b$ lower HC birth percentile and duration of mechanical ventilation predicted slower head growth, after accounting for the other neonatal factors. Directions of relationships between variables were determined by B values (not shown).

### 2.3.4 Later (32 to 40 Weeks PMA) Neonatal Variables in Relation to Weight Percentile at 40 Weeks PMA

Lower weight percentile at 32 weeks PMA and later neonatal infection, rather than the number
of invasive procedures, were independently associated with decreased weight percentile at 40 weeks PMA, after adjusting for the number of days on mechanical ventilation, morphine, hydrocortisone and dexamethasone exposure, and PMA at weigh-in (Table 2.4).

Table 2.4 Later (32 to 40 Weeks PMA) Neonatal Variables in Relation to Weight and HC Percentile at 40 Weeks PMA

<table>
<thead>
<tr>
<th>Later Neonatal variables</th>
<th>Weight Percentile at 40 weeks PMA(^a)</th>
<th>HC Percentile at 40 weeks PMA(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald ( \chi^2 )</td>
<td>( P )</td>
</tr>
<tr>
<td>Weight percentile at 32 weeks</td>
<td>63.58</td>
<td>0.001</td>
</tr>
<tr>
<td>HC percentile at 32 weeks</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>0.05</td>
<td>0.82</td>
</tr>
<tr>
<td>Days of mechanical ventilation</td>
<td>0.76</td>
<td>0.38</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>0.81</td>
<td>0.37</td>
</tr>
<tr>
<td>Hydrocortisone exposure</td>
<td>0.02</td>
<td>0.90</td>
</tr>
<tr>
<td>Dexamethasone exposure</td>
<td>0.39</td>
<td>0.53</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>5.09</td>
<td>0.02</td>
</tr>
<tr>
<td>PMA at 40 week weigh-in</td>
<td>50.54</td>
<td>0.001</td>
</tr>
</tbody>
</table>

HC= Head circumference; PMA= Postmenstrual age

Generalized linear models revealed that between 32 and 40 weeks PMA: \(^a\)lower weight percentile at 32 weeks PMA and neonatal infection independently predicted slower body growth, after accounting for other later neonatal factors; \(^b\)lower HC percentile at 32 weeks PMA and dexamethasone exposure predicted slower head growth, after accounting for other later neonatal factors. Directions of relationships between variables were determined by B values (not shown).

2.3.5 Cumulative (Early and Late/Birth to 40 Weeks PMA) Neonatal Variables in Relation to Weight Percentile at 40 Weeks PMA

Lower birth weight percentile, hydrocortisone exposure and infection, rather than the number of invasive procedures, were independently associated with lower weight percentile at 40 weeks PMA, after adjusting for the number of days on mechanical ventilation, morphine exposure,
dexamethasone exposure, illness severity on day 1, and PMA at weigh-in (Table 2.5).

### Table 2.5 Cumulative (Early and Late/Birth to 40 Weeks PMA) Neonatal Variables in Relation to Weight and HC Percentile at 40 Weeks PMA

<table>
<thead>
<tr>
<th>Cumulative Neonatal Variables</th>
<th>Weight Percentile at 40 weeks PMA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HC Percentile at 40 weeks PMA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald χ²</td>
<td>P</td>
</tr>
<tr>
<td>Birth weight percentile</td>
<td>23.19</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth HC percentile</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neonatal pain (number of skin-breaking procedures)</td>
<td>1.64</td>
<td>0.20</td>
</tr>
<tr>
<td>Days of mechanical ventilation</td>
<td>0.001</td>
<td>0.98</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>1.52</td>
<td>0.22</td>
</tr>
<tr>
<td>Hydrocortisone exposure</td>
<td>4.23</td>
<td>0.04</td>
</tr>
<tr>
<td>Dexamethasone exposure</td>
<td>1.08</td>
<td>0.30</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>4.02</td>
<td>0.05</td>
</tr>
<tr>
<td>Illness severity on day 1</td>
<td>0.94</td>
<td>0.33</td>
</tr>
<tr>
<td>PMA at 40 week weigh-in</td>
<td>19.40</td>
<td>0.001</td>
</tr>
</tbody>
</table>

HC= Head circumference; PMA= Postmenstrual age

Generalized linear models revealed that between birth and 40 weeks PMA: <sup>a</sup>lower birth weight percentile, hydrocortisone exposure and infection independently predicted slower body growth, after accounting for other neonatal factors; <sup>b</sup>lower birth HC percentile and hydrocortisone exposure predicted slower head growth in the NICU, after accounting for other neonatal factors. Directions of relationships between variables were determined by B values (not shown).

#### 2.3.6 Early (Birth to 32 Weeks PMA) Neonatal Variables in Relation to HC Percentile at 32 Weeks PMA

Lower birth HC percentile, greater number of invasive procedures and longer duration of mechanical ventilation, were independently associated with decreased HC percentile at 32 weeks PMA, after adjusting for morphine, dexamethasone and hydrocortisone exposure, illness severity.
on day 1, and PMA at weigh-in (Table 2.2). As shown in Figure 2.3, the number of invasive procedures prior to 32 weeks PMA accounted for approximately 12% of the variance in early head growth, and greater exposure to invasive procedures was related to decreased HC percentiles at 32 weeks PMA, after accounting for multiple medical confounders.
Predicted values of early growth (weight percentile at 32 weeks PMA in relation to early neonatal pain (number of skin-breaking procedures from birth to 32 weeks PMA), adjusted for other neonatal variables (i.e. birth weight percentile, illness severity on day 1, PMA at 32-week weigh-in, days of mechanical ventilation, infection, and exposure to morphine and corticosteroids [hydrocortisone, dexamethasone]).

2.3.7 Early (Birth to 32 Weeks PMA) Neonatal Variables In Relation to HC Percentile at 40 Weeks PMA

Lower birth HC percentile, longer duration of mechanical ventilation and hydrocortisone exposure, rather than the number of invasive procedures, were independently associated with decreased HC percentile at 40 weeks PMA, after adjusting for morphine exposure,
dexamethasone exposure, infection, illness severity on day 1, and PMA at weigh-in (Table 2.3).

2.3.8 Later (32 to 40 Weeks PMA) Neonatal Variables in Relation to HC Percentile at 40 weeks PMA

Lower HC percentile at 32 weeks PMA and dexamethasone exposure, rather than the number of invasive procedures, were independently associated with decreased HC percentile at 40 weeks PMA, after adjusting for the number of days on mechanical ventilation, morphine exposure, hydrocortisone exposure, infection, and PMA at weigh-in (Table 2.4).

2.3.9 Cumulative (Early and Late/Birth to 40 Weeks PMA) Neonatal Variables in Relation to HC Percentile at 40 Weeks PMA

Lower birth HC percentile and hydrocortisone exposure, rather than the number of invasive procedures, were independently associated with lower HC percentile at 40 weeks PMA, after adjusting for the number of days on mechanical ventilation, morphine exposure, dexamethasone exposure, infection, illness severity on day 1, and PMA at weigh-in (Table 2.5).

2.4 Discussion

To our knowledge, this is the first study to examine the relationship between invasive procedures and postnatal growth in the NICU in infants born very preterm. Our results showed that the timing of invasive procedures and other neonatal interventions were important for postnatal body and head growth. Specifically, we demonstrated that greater exposure to invasive procedures was associated with delayed early postnatal body and head growth in the NICU, independent of other medical confounders. In contrast, reduced weight gain at term was associated with later neonatal infection.
Infants that are born earlier and sicker require more interventions to ensure their survival. Therefore, these factors are strongly associated with the number of invasive procedures in the NICU. In order to determine whether the number of invasive procedures in the NICU independently impacted postnatal growth, it was essential to statistically account for the multiple co-occurring factors. Invasive procedures rather than illness severity on day 1 of life was associated with postnatal growth in the NICU. This suggests that the rate at which infants grow in the NICU depends more on adversities encountered during the first weeks of life, as opposed to how sick the infants are at birth. By effectively managing pain/stress, clinicians may have the opportunity to improve postnatal growth in the NICU. However, despite best attempts to manage pain/stress in the NICU, this area of clinical care remains a challenge.

Prior to the 35 weeks PMA infants born very preterm cannot distinguish between nociceptive and mechanical stimulation (Andrews and Fitzgerald 1994; Fabrizi et al. 2011; Fitzgerald, Millard, McIntosh 1989; Grunau et al. 2001; Holsti et al. 2005; Holsti et al. 2006; Walker, Tochiki, Fitzgerald 2009). Therefore, infants born very preterm may experience pain/stress from both routine care and invasive procedures performed in the NICU. The majority of life-saving procedures are performed prior to 32 weeks PMA. While there are potential medical confounders (as in all clinical cohort studies) that can contribute to the number of invasive procedures performed in the NICU, we identified and statistically controlled for the key indicators. Even after accounting for early illness severity and markers of illness through the NICU stay, such as infection and duration of mechanical ventilation, the relationship between greater numbers of invasive procedures and impaired early growth persisted. The amount of energy expended in response to repeated stimulation, may have exhausted infants limited reserves, leading to slower
growth prior to 32 weeks PMA in infants born very preterm.

Lower pain/stress after 32 weeks PMA may have led to increased growth hormone production, and subsequent growth. At term age, variability in growth patterns increases among infants born very preterm (Yumani, Lafeber, van Weissenbruch 2015). For some infants, exposure to fewer invasive procedures after 32 weeks PMA may equate to rapid catch-up growth. However, environmental conditions must be conducive to postnatal growth. Therefore, even if the number of invasive procedures is reduced after 32 weeks PMA, infants born very preterm may continue to grow slowly if they are ill or continue to be exposed other environmental stressors (Yumani, Lafeber, van Weissenbruch 2015). This may explain why we found a relationship between later postnatal infection and slower postnatal body growth.

Unlike body growth, head growth appeared to be preserved after 32 weeks PMA. This is indicative of “brain protection,” as seen in growth-restricted fetuses, such that energy is preferentially directed to head growth (Peleg, Kennedy, Hunter 1998). However, head growth both before and after 32 weeks PMA may be impacted in infants with underdeveloped lungs. We found a positive association between days of mechanical ventilation prior to 32 weeks PMA and later head growth.

Corticosteroids are used to treat bronchopulmonary dysplasia, and consistent with previous reports, both dexamethasone and hydrocortisone exposure were associated with slower growth in the NICU (Doyle LW, Ehrenkranz RA, Halliday HL 2014a; Doyle LW, Ehrenkranz RA, Halliday HL 2014b). The administration of dexamethasone increases 5HT transporter expression, thereby reducing 5HT availability both in the hippocampus and throughout the brain.
This is important because 5HT incites a downstream cascade of molecular events within the hippocampus that induces the expression of NGF1-A, which binds to the glucocorticoid receptor gene promoter (Meaney et al. 2000; Yau et al. 1997). Therefore, exposure to corticosteroids results in fewer glucocorticoid receptors present within the hippocampus, which results in poorer negative feedback following glucocorticoid expression (Francis et al. 1999b; Gunnar and Quevedo 2007). Glucocorticoids (cortisol in humans) inhibit growth hormone secretion (Tsigos and Chrousos 2002), thereby resulting in slower growth. Cortisol is also expressed following neonatal pain/stress exposure. Importantly, even after statistically adjusting for corticosteroid exposure, a known predictor of reduced postnatal growth; neonatal pain/stress was still significantly associated with poorer growth early in the neonatal period.

Size at birth (weight and HC) continues to be a significant predictor of growth throughout infant hospitalization (Bhatnagar et al. 2006; Embleton, Pang, Cooke 2001). Typically infants born an appropriate size for GA require less medical support, and have better the growth trajectories in the NICU.

An important limitation of this study was that we did not have data on neonatal nutrition. Infants in this study were participants from a larger longitudinal study examining invasive procedures in relation to neurodevelopment and stress systems in infants born very preterm (Adams et al. 2010; Chau et al. 2009; Grunau et al. 2005; Grunau et al. 2007; Grunau et al. 2009). The original study was not designed with the intention of examining nutrition, caloric intake, and feeding of preterm infants. Future studies are needed to examine the role of nutrition in the relationship between invasive procedures and early postnatal growth in the NICU. Given that this was a
correlational study, future research is needed to confirm the mechanisms that underlie the relationship between invasive procedures and early postnatal growth in the NICU.

In conclusion, greater exposure to invasive procedures was associated with decreased early postnatal body and head growth in the NICU. Reducing pain/stress in the NICU may not only improve postnatal growth, but may also optimize long-term neurodevelopmental outcomes in infants born very prematurely.
CHAPTER 3
SLOWER POSTNATAL GROWTH IS ASSOCIATED WITH DELAYED CEREBRAL CORTICAL MATURATION IN PRETERM NEWBORNS

3.1 Introduction
Infants born very preterm have reduced brain volumes (Nguyen et al. 2009; Srinivasan et al. 2007; Thompson et al. 2007; Thompson et al. 2011), and poorer cognitive, motor and behavioral outcomes relative to children born full-term (Anderson, Doyle, Victorian Infant Collaborative Study Group 2003; Doyle, Casalaz, Victorian Infant Collaborative Study Group 2001; Doyle and Anderson 2010; Grunau, Whitfield, Fay 2004; Johnson et al. 2009; Loe et al. 2011; Marlow et al. 2005; Marlow et al. 2007; Spittle et al. 2009). As part of their life saving care infants born very preterm are repeatedly exposed to invasive procedures in the NICU. Greater exposure to invasive procedures is associated with poorer early body and head growth (Vinall et al. 2012) and altered white matter microstructure during NICU care and at term-equivalent age (Brummelte et al. 2012; Smith et al. 2011; Zwicker et al. 2013).

Infants born both earlier and smaller require more medical interventions given that they are at higher risk for neonatal comorbidities (Damodaram et al. 2011). Therefore, size at birth is a significant predictor of growth throughout infant hospitalization (Bhatnagar et al. 2006; Embleton, Pang, Cooke 2001; Vinall et al. 2012). Intrauterine growth restriction (IUGR) refers to infants whose birth weights are <10th percentile due to growth failure in utero. Premature IUGR newborns demonstrate a pattern of discordant gyrification relative to preterm infants born an appropriate weight for gestation age (AGA: 10th to 90th percentile, such that their sulcation

index is high relative to cortical surface area (Dubois et al. 2008b). Preterm IUGR infants also demonstrate reduced cortical volumes and altered microstructure, relative to AGA preterm infants (Dubois et al. 2008b; Toft et al. 1995; Tolsa et al. 2004). Cortical gray matter appears to be more greatly impacted by growth restriction relative to white matter (Dubois et al. 2008b; Padilla et al. 2011; Toft et al. 1995; Tolsa et al. 2004). Moreover, abnormal cortical volumes in premature IUGR infants are associated with poorer neurodevelopmental outcomes at term and 18 months CA (Padilla et al. 2011; Tolsa et al. 2004).

The majority of preterm infants are born AGA, and develop persistent growth deficits postnatally (Steward and Pridham 2002; Wilson et al. 1997). Slower postnatal body and head growth in the NICU is associated with increased incidence of cerebral palsy and neurodevelopmental impairment, after accounting for prenatal growth, systemic illness, and brain injury (Ehrenkranz et al. 1999). It is not known whether alterations to brain microstructure mediate the relationship between postnatal growth and neurodevelopmental outcomes. One study has examined the relationship between postnatal growth and white matter microstructure at term (Lepomaki et al. 2013). Weight and length change from birth to term were not associated with white matter microstructure (Lepomaki et al. 2013). However, fast catch-up head growth between birth and term-equivalent age was associated less mature white matter microstructure at term age, after accounting for gestational age and birth weight (Lepomaki et al. 2013).

There are a number of additional factors to consider when evaluating the relationship between neonatal growth and cortical development in infants born very preterm. Systemic illness and medical interventions are important determinants of growth and brain development (Berry, Abrahamowicz, Usher 1997; Billeaud, Piedboeuf, Chessex 1992; Bonifacio et al. 2010;
Brummelte et al. 2012; Chau et al. 2012; Cockerill et al. 2006; Halliday, Ehrenkranz, Doyle 2010; Lodygensky et al. 2005; Vinall et al. 2012; Zwicker et al. 2013). Moreover, focal brain injuries have been found to affect overall brain development, leading to moderate to severe neurodevelopmental disability (Inder et al. 2005; Miller et al. 2005; Woodward et al. 2006). Therefore it is not only important to determine whether postnatal growth impacts brain development, but also whether this relationship exists independent of prematurity, illness severity, brain injury and exposure to medications in the NICU.

Hypothesis

This study examines whether neonatal growth is related to microstructural development of the cerebral cortex in infants born very preterm. We hypothesized that poorer growth in the NICU would be associated with delayed cortical maturation, independent of prenatal growth, systemic illness, and brain injury.

3.2 Methods

3.2.1 Study Overview

Infants born very preterm (between 24 and 32 weeks GA) were studied twice with DTI: scan 1 at a median of 32.1 weeks (IQR: 30.4 to 33.6) and scan 2 at a median of 40.3 weeks (IQR: 38.7 to 42.7). FA and eigenvalues were recorded from 15 anatomically defined cortical regions. Weight, head circumference, and length were recorded at birth and at the time of each scan. Growth between scans was examined in relation to DTI parameters at scans 1 and 2, accounting for GA, birth weight, sex, PMA, neonatal illness (patent ductus arteriosus, days intubated, infection, and necrotizing enterocolitis) and brain injury (WMI, IVH and cerebellar hemorrhage).
3.2.2 Participants

Infants born very preterm (between 24 and 32 weeks GA) were admitted to the NICU at the British Columbia’s Women’s Hospital between March 2006 and January 2009. As in previous studies, infants from this cohort were excluded if they had a congenital malformation or syndrome, antenatal infection, or evidence on ultrasound of a parenchymal hemorrhagic infarction ≥2 cm (Adams et al. 2010; Chau et al. 2009; Papile et al. 1978). After parental informed consent was obtained, 98 infants were included in the present study. A neonatal research nurse performed medical and nursing chart review from birth to term-equivalent age or discharge (whichever came first). Data included but were not limited to GA, sex, birth weight, presence of patent ductus arteriosus, duration of intubation, infection, necrotizing enterocolitis, and corticosteroid (hydrocortisone and/or dexamethasone) exposure. Infants with clinical sepsis (who had negative cultures but were treated with antibiotics for ≥5 days) or with confirmed infections (positive cultures of the blood, urine, or cerebral spinal fluid, or ≥4 white blood cells found in tracheal aspirates associated with clinical pneumonia) were included in this study because these types of infections are associated with abnormal brain maturation (Chau et al. 2012). This approach is also consistent with the study by Stoll et al., which demonstrated that neonatal infections among extremely low birth weight infants are associated with poor neurodevelopmental outcome, even in the absence of positive cultures (Stoll et al. 2004). Infants were classified as having necrotizing enterocolitis if they met either stage 2 (clinical signs and symptoms, and pneumatositis intestinalis on x-ray) or stage 3 (critically ill, clinical signs and symptoms, and pneumatositis intestinalis on x-ray) of Bell’s criteria (Bell et al. 1978). Infants were assessed for neonatal growth (weight, length, and head circumference) at the time of each MRI scan: median of 32 weeks (IQR: 30.4 to 33.6; total range, 27.3 to 40.7) and 40 weeks (IQR: 38.7 to 42.7; total range, 33.4 to 46.4) PMA.
3.2.3 Magnetic Resonance Imaging

Infants were scanned without pharmacological sedation when stable at median 32 (scan 1) and 40 weeks (scan 2) PMA. All newborns were scanned in an MRI-compatible isolette (Lammers Medical Technology) with a specialized neonatal head coil (Advanced Imaging Research). A Siemens 1.5-T Avanto magnet and VB 13A software were used to obtain the following sequences: three-dimensional coronal volumetric T\textsubscript{1}-weighted images (repetition time, 36; echo time, 9.2; field of view, 200 mm; slice thickness, 1 mm; no gap) and axial fast spin echo T\textsubscript{2}-weighted images (repetition time, 4610; echo time, 107; field of view, 160 mm; slice thickness, 4 mm; gap, 0.2 mm). Neuroradiologist K.J.P., blinded to infant medical history, assessed the images for cerebellar hemorrhage and the severity of WMI and IVH (Miller et al. 2005; Papile et al. 1978). Twenty random scans were rescored; intra-observer reliability of $k > 0.9$ was comparable with previous reported scores (Miller et al. 2005). In addition, K.J.P. identified seven subjects with white matter cysts typical of cystic PVL on at least one imaging study. Three neonates had mild PVL, with less than four cysts <2 mm in diameter; four neonates demonstrated cysts >1 cm in diameter. A variable identifying infants with cystic PVL was not included in the statistical models, given the small number in each category. These infants were, however, included in this study and were identified as having moderate to severe WMI.

3.2.4 Diffusion Tensor Imaging

DTI was acquired with a multirepetition, single-shot echo planar sequence with 12 gradient directions (repetition time, 4900; echo time, 104; field of view, 160 mm; slice thickness, 3 mm; no gap), three averages of two diffusion weightings of 600 and 700 s/mm\textsuperscript{2} (b values), and an image without diffusion weighting, resulting in an in plane resolution of 1.3 mm. DTI parameters of FA and $\lambda_1$, $\lambda_2$ and $\lambda_3$ were collected bilaterally in 15 regions of interest by two
observers. Eight regions of interest in the cortical gray matter were identified by J.V. in 95 neonates (precentral gyrus, postcentral gyrus, secondary somatosensory cortex, superior frontal gyrus, dorsolateral prefrontal cortex, ventrolateral prefrontal cortex, anterior insula, and occipital gray matter; Figure 3.1), and seven regions of interest in the white matter were identified by V.C. in 97 neonates (anterior, middle, and posterior subcortical white matter, genu, and splenium of the corpus callosum; posterior limb of the internal capsule; and optic radiations), as described previously (Chau et al. 2009). Values from regions of interest on a degraded diffusion tensor image were not measured (7% of regions of interest).

Figure 3.1 Regions of Interest in the Cortical Gray Matter

(A and B) Diffusion tensor image-encoded anisotropy color maps of an infant born at 26.29 weeks gestation and scanned at 30 weeks postmenstrual age. The images demonstrate the relatively high FA of the cerebral cortex typical for this age. The color convention used to display the predominant diffusion direction has red representing right-left, green representing anterior-posterior, and blue representing superior-inferior anatomical directions (56, 58). Eight cerebral cortical regions of interest were examined, and values of each region were averaged bilaterally: (a) precentral gyrus, (b) postcentral gyrus, (c) secondary somatosensory cortex, (d) superior frontal gyrus, (e) dorsolateral prefrontal cortex, (f) ventrolateral prefrontal cortex, (g) anterior insula, and (h) occipital gray matter.
3.2.5 Reliability of the Cortical Gray Matter Regions of Interest

Many considerations were given to the size and placement of the region of interest voxel boxes in the cerebral cortex. First, it was important to determine the size of voxel box that could fit within the thin layer of cortical gray matter, which is about 2 mm thick in the newborn (Dubois et al. 2008b). We observed that 2 x 3 voxel boxes could fit within the boundaries of the cerebral cortex and surrounding structures.

Second, replication of the regions of interest over time was complicated by the fact that there is a marked change in the complexity of the cortex, with increasing sulcation and gyration between 32 and 40 weeks (Dubois et al. 2008a; Kroenke et al. 2007). However, we found that 2 x 3 voxel boxes could be reliably placed at the height of the gyrus in the cortical gray matter for both the first and second scans. Intra-rater reliability was calculated on 20% of the regions of interest in the cortical gray matter, by Bland Altman analyses (Bland and Altman 1986), and values were compared with those previously published in the literature (Adams et al. 2010): Scan 1 showed an FA mean difference of 0.001 (limits of agreement, −0.001 to 0.003), and scan 2 showed an FA mean difference of −0.002 (limits of agreement, −0.004 to 0.000).

Finally, reduction of cortical diffusion takes place according to an inside-out laminar gradient (Jespersen et al. 2012), thereby introducing the possibility for partial averaging within the measured regions of cortex. To address this issue, we considered whether the values of the top three voxels of the 2 x 3 voxel box compared favorably with the bottom three voxels of the 2 x 3 voxel box. FA mean differences for the top three and bottom three voxel boxes across all regions of interest were minor: At scan 1, the mean difference was 0.007 for all regions of interest, with
a mean difference range of −0.010 to 0.020 across individual regions of interest; at scan 2, the mean difference was 0.006 for all regions of interest, with a mean difference range of −0.010 to 0.020 across individual regions of interest. Given that there were no systematic differences between using 1 x 3 versus 2 x 3 voxel boxes, and that use of 2 x 3 voxel boxes improved reliability, we proceeded to use 2 x 3 voxel boxes to extract data from the regions of interest within the cerebral cortex.

3.2.6 Reliability of the White Matter Regions of Interest

On the basis of the repeated analysis of 20% of the regions of interest in the white matter (Chau et al. 2012) by Bland Altman analyses, intra-rater reliability was considered high: FA mean difference of 0.001 (limits of agreement, −0.018 to 0.017).

3.2.7 Data Analyses

Statistical analysis was performed with R version 2.13 (R Development Core Team 2011). Normality plots were examined, and skewed variables (DTI parameters [FA and λ_1, λ_2 and λ_3]) and growth measures (change in weight [gram], head circumference [centimeter], and length [centimeter] between scan 1 and scan 2) were log-transformed. t tests were also used to examine whether there were differences in the cortical gray matter FA values for infants that were growth restricted (<10th weight percentile) compared to those with an appropriate weight for their PMA and sex in the NICU at scan 1 and scan 2, and whether these differences were affected by the exclusion of infants born small for their GA and sex. Then, linear mixed effects models (LMEMs) were used to examine longitudinal associations between change in weight and DTI parameters between scan 1 and scan 2 in the cortical gray matter and white matter. Included in the LMEMs were terms for multiple regions of interest (8 cortical gray matter regions or 7 white
matter regions) and interaction terms for region of interest and postmenstrual age. Splines (values produced by three smooth polynomial segments) were used to account for the nonlinearities between postmenstrual age and FA values (Figure 3.2), and growth over time relative to birth weight. The independent variables entered in the initial model were GA, birth weight, and sex. If weight change was a significant predictor of FA in the basic model (step 1), we extended the model to include brain injury (step 2: WMI, IVH, and cerebellar hemorrhage) and systemic illness (step 3: patent ductus arteriosus, days intubated, infection, and necrotizing enterocolitis). This model was then reapplied while excluding infants who had received postnatal corticosteroids. Steps 1, 2, and 3 were repeated to examine the relationship between weight change and radial ($\lambda_2$ and $\lambda_3$) and axial ($\lambda_1$) diffusion axes. Moreover, if weight change was a significant predictor of FA, results were confirmed by repeating steps 1 to 3 for length change and head circumference change.
FA of the cortical gray matter decreases nonlinearly with increasing PMA. Graphed are the raw, unadjusted FA values from the pre-central gyrus in relation to PMA. FA decreases rapidly in the cortical gray matter until ~36 weeks, when values reached the noise floor. Given the nonlinear relationship between diffusion tensor parameters and PMA, splines (3 smooth polynomial segments) were used to approximate this relationship, and their corresponding values were included in the statistical models to account for the contribution of PMA to cortical maturation.

3.3 Results

3.3.1 Characteristics of the Cohort

The characteristics of the 98 included infants are provided in Table 3.1. Ninety-five newborns had diffusion tensor images of sufficient quality for cortical analyses. In univariate unadjusted analyses, infants with poor postnatal weight gain (n= 27) appeared to have higher cortical gray matter FA values at scan 2 (MRI at median 40.3 weeks postmenstrual age) compared to infants with appropriate weight for their postmenstrual age and sex in the NICU, but the difference was
not statistically significant [ventrolateral cortex: 95% confidence interval (CI)= −0.002 to 0.04; \( P= 0.069 \); Figure 3.3]. The magnitude of this difference was more pronounced and reached statistical significance when infants born small for their gestational age and sex (n= 19) were excluded (ventrolateral cortex: 95% CI= 0.002 to 0.05; \( P= 0.032 \); across all regions of interest: 95% CI= 0.001 to 0.02; \( P= 0.036 \)). Infants born small for their gestational age and sex were therefore included in the longitudinal multivariable models to provide a more conservative estimate of the difference in cortical diffusion tensor imaging parameters related to postnatal growth restriction.
## Table 3.1 Infant Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Birth</th>
<th>Scan 1</th>
<th>Scan 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (male), number (%)</strong></td>
<td>45.0 (45.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age (weeks), median (IQR)</strong></td>
<td>27.4 (26.0-29.6)</td>
<td>32.1 (30.4-33.6)</td>
<td>40.3 (38.7-42.7)</td>
</tr>
<tr>
<td><strong>Weight percentile &lt;10%, number (%)</strong></td>
<td>19.0 (19.4)</td>
<td>54.0 (55.1)</td>
<td>30.0 (30.6)</td>
</tr>
<tr>
<td><strong>Weight (grams), median (IQR)</strong></td>
<td>988 (803-1278)</td>
<td>1310 (1139-1601)</td>
<td>3160 (2543-3685)</td>
</tr>
<tr>
<td><strong>Head circumference (cm), median (IQR)</strong></td>
<td>25.0 (23.5-27.0)*</td>
<td>27.6 (26.0-29.0)</td>
<td>35.0 (33.0-36.8)</td>
</tr>
<tr>
<td><strong>Length (cm), median (IQR)</strong></td>
<td>36.0 (33.5-39.4)*</td>
<td>39.0 (37.0-41.0)</td>
<td>49.0 (45.0-51.5)</td>
</tr>
<tr>
<td><strong>Mild WMI (score), number (%)</strong></td>
<td>-</td>
<td>11 (11.2)</td>
<td>5 (5.1)</td>
</tr>
<tr>
<td><strong>Moderate-severe WMI (score), number (%)</strong></td>
<td>-</td>
<td>20 (20.4)</td>
<td>18 (18.4)</td>
</tr>
<tr>
<td><strong>IVH (Grade 1-2), number (%)</strong></td>
<td>-</td>
<td>36 (37.1)</td>
<td>26 (26.5)</td>
</tr>
<tr>
<td><strong>IVH (Grade 3 or PVHI), number (%)</strong></td>
<td>-</td>
<td>7 (7.1)</td>
<td>6 (6.1)</td>
</tr>
<tr>
<td><strong>Cerebellar hemorrhage, number (%)</strong></td>
<td>-</td>
<td>14 (14.3)</td>
<td>10 (10.2)</td>
</tr>
<tr>
<td><strong>Patent ductus arteriosus, number (%)</strong></td>
<td>-</td>
<td>-</td>
<td>48 (49.0)†</td>
</tr>
<tr>
<td><strong>Days intubated, median (IQR)</strong></td>
<td>-</td>
<td>-</td>
<td>5.5 (1.0-29.8)†</td>
</tr>
<tr>
<td><strong>Postnatal infection, number (%)</strong></td>
<td>-</td>
<td>-</td>
<td>48 (49.0)†</td>
</tr>
<tr>
<td><strong>Necrotizing enterocolitis, number (%)</strong></td>
<td>-</td>
<td>-</td>
<td>9 (9.2)†</td>
</tr>
<tr>
<td><strong>Corticosteroid exposure (hydrocortisone and/or dexamethasone), number (%)</strong></td>
<td>-</td>
<td>-</td>
<td>32 (32.7)†</td>
</tr>
</tbody>
</table>

*12 infants were missing values for head circumference and 14 infants were missing values for length at birth; therefore, birth weight was used in the statistical models as a marker of prenatal growth
†Measured from birth to term-equivalent age or discharge (whichever came first)
IQR= Interquartile range; PVHI= periventricular hemorrhagic infarction; Scan 1= magnetic resonance imaging at ~32 weeks postmenstrual age; Scan 2= magnetic resonance imaging at ~40 weeks postmenstrual age
Figure 3.3 Postnatal Growth Restriction Delays Cortical Gray Matter Maturation

Preterm infants with postnatal growth restriction demonstrated delayed cortical gray matter maturation. Graphed are the raw, unadjusted FA values from the ventrolateral prefrontal cortex of very preterm infants that were growth restricted (<10th weight percentile) at scan 2 (MRI at median 40.3 weeks PMA, versus very preterm infants that were an appropriate weight for their postmenstrual age during neonatal intensive care. (A) Preterm infants that were growth restricted postnatally demonstrated moderately delayed cortical gray matter maturation compared to preterm infants who were an appropriate weight for their PMA. (B) After excluding infants born <10th weight percentile, preterm infants that were growth restricted postnatally demonstrated significantly delayed cortical gray matter maturation compared to preterm infants who were an appropriate weight for their PMA.

3.3.2 Weight Change in Relation to Diffusion Parameters of the Cortical Gray Matter

Longitudinal models revealed that lower GA (effect size= −0.038; SE= 0.011; P< 0.001), birth weight (effect size< −0.001; SE< 0.001; P= 0.016), and slower weight gain [weight at scan 2 (MRI at ~40 weeks PMA) − weight at scan 1 (MRI at ~32 weeks PMA)] (effect size= −0.410; SE= 0.089; P< 0.001) were independently associated with higher FA values in the cortical gray matter, after adjusting for sex, brain injury [WMI, IVH, and cerebellar hemorrhage (brain injury model)], systemic illness [patent ductus arteriosus, days intubated, postnatal infection, and necrotizing enterocolitis (extended model)], and age at scan (Table 3.2). Therefore, neonatal growth was associated with cortical gray matter maturation in the NICU, independent of birth
weight, brain injury, and systemic illness. Change in FA reflected changes in the radial diffusion axes (\(\lambda_2\) and \(\lambda_3\); Table 3.3), but not the axial diffusion axis (\(\lambda_1\); Table 3.4), suggesting a delay in neuronal process formation and/or apoptosis in the cerebral cortices of infants who are born very preterm and have impaired growth.

**Table 3.2 Weight change in relation to mean FA values of 8 regions of interest in the cortical gray matter**

<table>
<thead>
<tr>
<th></th>
<th>Basic Model</th>
<th>Brain Injury Model</th>
<th>Extended Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=95</td>
<td>n=95</td>
<td>n=95</td>
</tr>
<tr>
<td>Effect size</td>
<td>P</td>
<td>Effect size</td>
<td>P</td>
</tr>
<tr>
<td>GA</td>
<td>-0.027</td>
<td>0.007</td>
<td>-0.028</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt;-0.001</td>
<td>0.025</td>
<td>&lt;-0.001</td>
</tr>
<tr>
<td>Male</td>
<td>0.037</td>
<td>0.101</td>
<td>0.034</td>
</tr>
<tr>
<td>Weight change</td>
<td>-0.422</td>
<td>&lt;0.001</td>
<td>-0.424</td>
</tr>
<tr>
<td>WMI</td>
<td>-</td>
<td>-</td>
<td>-0.010</td>
</tr>
<tr>
<td>IVH</td>
<td>-</td>
<td>-</td>
<td>-0.002</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>-</td>
<td>-</td>
<td>-0.009</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Days intubated</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Weight change = weight at scan 2 (DTI at ~40 weeks PMA) - weight at scan 1 (DTI at ~32 weeks PMA)
Table 3.3 Weight Change in Relation to Mean $\lambda_2$ and $\lambda_3$ Values of 8 Regions of Interest in the Cortical Gray Matter

<table>
<thead>
<tr>
<th></th>
<th>Basic Model</th>
<th>Brain Injury Model</th>
<th>Extended Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 95</td>
<td>n= 95</td>
<td>n= 95</td>
</tr>
<tr>
<td>Effect size</td>
<td>$P$</td>
<td>Effect size</td>
<td>$P$</td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
<td>-------------</td>
<td>-----</td>
</tr>
<tr>
<td>GA</td>
<td>0.001</td>
<td>0.703</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>-0.009</td>
<td>0.243</td>
<td>-0.006</td>
</tr>
<tr>
<td>Weight change</td>
<td>0.064</td>
<td>0.056</td>
<td>0.070</td>
</tr>
<tr>
<td>WMI</td>
<td>-</td>
<td>-</td>
<td>0.006</td>
</tr>
<tr>
<td>IVH</td>
<td>-</td>
<td>-</td>
<td>-0.010</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Days intubated</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$\lambda_2$ & $\lambda_3$ = radial diffusion axis; Weight change = weight at scan 2 (DTI at ~40 weeks PMA) - weight at scan 1 (DTI at ~32 weeks PMA)
Table 3.4 Weight Change in Relation to Mean $\lambda_1$ Values of 8 Cortical Regions of Interest in the Cortical Gray Matter

<table>
<thead>
<tr>
<th></th>
<th>Basic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 95</td>
</tr>
<tr>
<td>Effect size</td>
<td>$P$</td>
</tr>
<tr>
<td>GA</td>
<td>-0.012</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>-0.005</td>
</tr>
<tr>
<td>Weight change</td>
<td>0.005</td>
</tr>
</tbody>
</table>

$\lambda_1$: axial diffusion axis; Weight change: weight at scan 2 (DTI at ~40 weeks PMA) - weight at scan 1 (DTI at ~32 weeks PMA)

3.3.3 Weight Change in Relation to FA of the White Matter

Weight change was not significantly associated with FA values in the white matter in the basic statistical model (effect size = −0.035; SE = 0.055; $P = 0.529$; Table 3.5). Therefore, white matter maturation appears to be relatively spared from the effects of postnatal growth restriction. Rather, postnatal infection (effect size = −0.057; SE = 0.020; $P = 0.005$) was independently associated with lower FA values in the white matter after adjusting for gestational age, birth weight, sex, brain injury, systemic illness, weight change, and age at scan.
3.3.4 Weight Change in Relation to Diffusion Parameters of the Cortical Gray Matter Excluding Infants who Received Postnatal Corticosteroids

Neither dexamethasone (effect size, −167.044; SE, 223.072; \(P= 0.454\)) nor hydrocortisone (effect size, −341.346; SE, 245.124; \(P= 0.164\)) was associated with weight change after adjusting for GA, birth weight, sex, brain injury, systemic illness, and age at scan. Nonetheless, as a sensitivity analysis, we examined weight change in relation to FA of the cortical gray matter excluding infants who received postnatal corticosteroids. In newborns who did not receive corticosteroids postnatally, lower GA (effect size −0.034; SE= 0.012; \(P= 0.005\)), birth weight (effect size< −0.001; SE= 0.001; \(P= 0.009\)), and slower weight gain (effect size, −0.512; SE= 0.114; \(P< 0.001\)) between scan 1 and scan 2 were independently associated with higher FA values in the cortical gray matter, in longitudinal models adjusting for gestational age, sex, brain injury, systemic illness, and age at scan (Table 3.6). Given that the relationship between weight change and FA values did not change meaningfully after the exclusion of infants who received postnatal corticosteroids (hydrocortisone and/or dexamethasone), exposed infants were
included in all other longitudinal models.

Table 3.6 Weight Change in Relation to Mean FA Values of 8 Regions of Interest in the Cortical Gray Matter Excluding Infants who Received Postnatal Corticosteroids

<table>
<thead>
<tr>
<th></th>
<th>Basic Model</th>
<th>Brain Injury Model</th>
<th>Extended Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 65*</td>
<td>n= 65*</td>
<td>n= 65*</td>
</tr>
<tr>
<td>Effect size</td>
<td>P</td>
<td>Effect size</td>
<td>P</td>
</tr>
<tr>
<td>GA</td>
<td>-0.022</td>
<td>0.052</td>
<td>-0.024</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt; -0.001</td>
<td>0.035</td>
<td>&lt; -0.001</td>
</tr>
<tr>
<td>Male</td>
<td>0.058</td>
<td>0.030</td>
<td>0.052</td>
</tr>
<tr>
<td>Weight change</td>
<td>-0.541</td>
<td>&lt; 0.001</td>
<td>-0.528</td>
</tr>
<tr>
<td>WMI</td>
<td>-</td>
<td>-</td>
<td>-0.012</td>
</tr>
<tr>
<td>IVH</td>
<td>-</td>
<td>-</td>
<td>-0.014</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>-</td>
<td>-</td>
<td>-0.022</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Days intubated</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*30 infants who had received corticosteroids (dexamethasone and/or hydrocortisone) were excluded from the analyses

Weight change= weight at scan 2 (DTI at ~40 weeks PMA) - weight at scan 1 (DTI at ~32 weeks PMA)

3.3.5 Length Change in Relation to Diffusion Parameters of the Cortical Gray Matter

Longitudinal models revealed that lower GA (effect size= -0.030; SE= 0.010; P= 0.002), confirmed necrotizing enterocolitis (effect size= 0.125; SE= 0.050; P= 0.012), and slower linear growth (effect size= -0.837; SE= 0.177; P< 0.001) between scan 1 and scan 2 were
independently associated with higher FA values in the cortical gray matter, after adjusting for birth weight, sex, brain injury, systemic illness, and age at scan (Table 3.7). Change in FA reflected changes in the radial diffusion axes ($\lambda_2$ and $\lambda_3$: effect size= 0.189; SE= 0.073; $P$= 0.010) and not the axial diffusion axis ($\lambda_1$: effect size= −0.043; SE= 0.062; $P$= 0.488).

Table 3.7 Length Change in Relation to Mean Fractional Anisotropy Values of 8 Regions of Interest in the Cortical Gray Matter

<table>
<thead>
<tr>
<th></th>
<th>Basic Model n= 89*</th>
<th>Brain Injury Model n= 89*</th>
<th>Extended Model n= 89*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effect size</td>
<td>$P$</td>
<td>Effect size</td>
</tr>
<tr>
<td>GA</td>
<td>-0.025</td>
<td>0.009</td>
<td>-0.023</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt;−0.001</td>
<td>0.141</td>
<td>&lt;−0.001</td>
</tr>
<tr>
<td>Male</td>
<td>0.024</td>
<td>0.269</td>
<td>0.021</td>
</tr>
<tr>
<td>Length change</td>
<td>−0.796</td>
<td>&lt;0.001</td>
<td>−0.817</td>
</tr>
<tr>
<td>WMI</td>
<td>−</td>
<td>−</td>
<td>−0.021</td>
</tr>
<tr>
<td>IVH</td>
<td>−</td>
<td>−</td>
<td>−0.008</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>−</td>
<td>−</td>
<td>&lt;−0.001</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Days intubated</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Infection</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Unable to obtain length measurements of 6 infants.
Length change= length at scan 2 (DTI at ~40 weeks PMA) – length at scan 1 (DTI at ~32 weeks PMA)

3.3.6 Head Circumference Change in Relation to Diffusion Parameters of the Cortical Gray Matter

Longitudinal models revealed that lower gestational age (effect size= −0.030; SE= 0.010; $P$=
0.004) and slower head growth (effect size = -1.090; SE = 0.025, \( P < 0.001 \)) between scan 1 and scan 2 were independently associated with higher FA values in the cortical gray matter, after adjusting for GA, birth weight, sex, brain injury, systemic illness, and age at scan (Table 3.8). Change in FA reflected change in the radial diffusion axes (\( \lambda_2 \) and \( \lambda_3 \): effect size = 0.265; SE = 0.098; \( P = 0.007 \)) and not the axial diffusion axis (\( \lambda_1 \): effect size = 0.058; SE = 0.086; \( P = 0.498 \)). Results from these models are consistent with the models above examining the relationship between weight change and length with diffusion parameters, and therefore provide further support for the finding that neonatal growth over and above birth weight, brain injury, and systemic illness predicted cortical gray matter maturation in the NICU.
Table 3.8 Head Circumference Change in Relation to Mean FA Values of 8 Regions of Interest in the Cortical Gray Matter

<table>
<thead>
<tr>
<th></th>
<th>Basic Model n= 94*</th>
<th>Brain Injury Model n= 94*</th>
<th>Extended Model n= 94*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effect size</td>
<td>P</td>
<td>Effect size</td>
</tr>
<tr>
<td>Gestational age</td>
<td>-0.028</td>
<td>0.004</td>
<td>-0.027</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt;0.001</td>
<td>0.180</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>0.008</td>
<td>0.731</td>
<td>0.004</td>
</tr>
<tr>
<td>Head circumference change</td>
<td>-1.030</td>
<td>&lt;0.001</td>
<td>-1.050</td>
</tr>
<tr>
<td>White matter injury</td>
<td>-</td>
<td>-</td>
<td>-0.018</td>
</tr>
<tr>
<td>Intraventricular hemorrhage</td>
<td>-</td>
<td>-</td>
<td>-0.010</td>
</tr>
<tr>
<td>Cerebellar hemorrhage</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Days intubated</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Unable to obtain head circumference measurement of 1 infant.

Head circumference change = head circumference at scan 2 (DTI at ~40 weeks PMA) – head circumference at scan 1 (DTI at ~32 weeks PMA)

3.4 Discussion

This study examined whether neonatal growth is related to microstructural development of the cortical gray and white matter in infants born very preterm. We found that impaired neonatal growth (weight, length, and head circumference) was significantly associated with delayed cortical maturation after accounting for GA, birth weight, sex, PMA, brain injury, and systemic illness. However, consistent with previous studies it was the cortical gray matter, rather than the white matter, which appeared to be most susceptible to impairments in growth (Dubois et al. 2008b; Padilla et al. 2011; Toft et al. 1995; Tolsa et al. 2004).
The finding that the cortical gray matter is more greatly influenced by postnatal growth relative to the white matter, builds on results from previous studies examining the relationship between IUGR and cortical volumes/microstructure early in life (Dubois et al. 2008b; Toft et al. 1995; Tolsa et al. 2004). Animal models of IUGR have demonstrated a transient delay in oligodendrocyte maturation and myelination (Tolcos et al. 2011). Although markers of myelinating oligodendrocytes were reduced in utero, white matter volumes returned to control levels postnatally, and persisted into adulthood (Tolcos et al. 2011). Thus, it has been suggested that the altered neurodevelopment associated with IUGR is likely not due to long-term deficits in myelination. Rather, it is the reduction of cerebral cortical volumes and altered microstructure associated with prenatal growth restriction that have been more pronounced, persistent, and associated with functional impairment (Eixarch et al. 2012; Padilla et al. 2011; Tolsa et al. 2004).

Microstructural integrity of the cortical gray matter can be inferred from diffusion parameters (Deipolyi et al. 2005; Jespersen et al. 2012; Kroenke et al. 2007; McKinstry et al. 2002; Sizonenko et al. 2007). Between 25 and 40 weeks PMA, FA decreases as the developing cortex increases in complexity (Deipolyi et al. 2005; McKinstry et al. 2002), with the arborization of the basal dendrites, formation of thalamocortical and cortico-cortical connections, and disappearance of the radial glia (Kostovic and Rakic 1990; Kostovic and Jovanov-Milosevic 2006; Marin-Padilla 1992; Mrzljak et al. 1988; Sidman and Rakic 1973). We found that higher FA was reflective of change in the radial diffusion axes ($\lambda_2$ and $\lambda_3$) between approximately 32 and 40 weeks PMA, indicative of alterations to the neuronal complexity in infants born very preterm with impaired growth. In a sheep model of premature birth, relative to controls, ischemic
sheep had poorer cortical growth and higher FA values associated with disturbances in the radial
diffusion axis (Dean et al. 2013). The impaired decline in cortical FA was due to altered
maturation of the basal dendritic arbor of cortical neurons, resulting in relatively high anisotropy
compared to controls (Dean et al. 2013).

Lower GA and birth weight were associated with higher FA. However, cortical maturation was
more strongly predicted by postnatal growth. This finding was supported by Smart et al., who
used a rat model to demonstrate that nutritional deprivation during both gestation and neonatal
periods had a greater influence on cortical development as opposed to deprivation during either
one of these periods alone; however, the damage to the forebrain was largely determined by
nutritional deprivation in the postnatal period (Smart et al. 1973). Studies of neonatal rats
deprived of adequate postnatal nutrition have also provided evidence for altered neuronal activity
(Mourek et al. 1967; Seidler, Bell, Slotkin 1990; Villescas et al. 1981). By 20 to 24 weeks GA, a
large proportion of neurons have been produced in the ventricular and subventricular zones
(Kostovic and Jovanov-Milosevic 2006). These precursor cells are vulnerable to nutrient
insufficiency (Inder et al. 1999). Moreover, the nutritional demand of rapid brain growth,
synaptogenesis, and sensory-driven activity between 24 to 42 weeks gestation leaves the
neonatal cortex particularly vulnerable to nutritional insult (Georgieff 2007).

Substantial energy is also required to mount a response invasive/painful procedures in the NICU.
Infants that are born earlier and sicker often require more invasive procedures, however, for
these infants nutritional intakes are usually less, and the deficits in postnatal growth are greater
(Embleton, Pang, Cooke 2001; Vinall et al. 2012). Importantly, stress inhibits the production of
growth hormones, a regulator of IGF-1 (Tsigos and Chrousos 2002). IGF-1 levels positively
correlate with total brain, gray matter, unmyelinated white matter and cerebellar volumes at term, after adjustment for GA, mean protein and caloric intakes, gender, brain injury, and steroid exposure (Hansen-Pupp et al. 2011; Hansen-Pupp et al. 2013). Moreover, IGF-1 levels during infant hospitalization predict neurodevelopmental outcomes at 2 years of age (Hansen-Pupp et al. 2013).

We did not have data on neonatal nutrition, caloric intake, and feeding because the infants in this study were participants from a larger longitudinal study examining as part of a larger ongoing study of neonatal invasive procedures, brain and neurodevelopment (Adams et al. 2010; Chau et al. 2009; Grunau et al. 2005; Grunau et al. 2007; Grunau et al. 2009). The nutrition protocols in place during this study included: 1) starting parenteral nutrition upon admission to the NICU, and 2) encouraging the use of breast milk and early trophic feeds. The standard fluid intake was 150 ml/kg per day, with a goal of 120 calories/kg per day. Weight was measured daily unless the patient was too unstable. Fluid and caloric intake were assessed daily and adjusted to optimize nutrition and growth. Postnatal growth is affected by a multitude of factors, which include fluid management, nutritional and caloric intake, catabolic stressors associated with severity of illness, and endocrine, genetic, and environmental factors, including procedural pain/stress. The consistency in findings across measures of weight, length, and head circumference supports the hypothesis that the alterations in cortical development reflect growth rather than fluid management alone. Our study was able to account for several medical confounders, which were likely to affect both growth and brain development, although residual confounding remains possible. Future studies are needed to examine the specific roles of systemic illness and nutrition, and to determine the optimal postnatal growth for cortical maturation in the NICU.
The results of this study have important clinical implications. Neonatal growth over and above birth weight, brain injury, and systemic illness correlated with cortical gray matter maturation in the NICU. Therefore, by diagnosing, treating, and preventing poor postnatal growth, clinicians may have the opportunity to optimize conditions for cortical development to proceed normally in infants born very preterm.
CHAPTER 4

PARENT BEHAVIORS MODERATE THE RELATIONSHIP BETWEEN NEONATAL INVASIVE PROCEDURES AND INTERNALIZING BEHAVIORS AT 18 MONTHS CORRECTED AGE IN CHILDREN BORN VERY PREMATURELY

4.1 Introduction

Infants born very preterm are repeatedly exposed to invasive procedures in the NICU. Greater exposure to invasive procedures has been associated with slower postnatal growth (Vinall et al. 2012), and slower growth in the NICU is associated with altered cortical development between approximately 32 and 40 weeks PMA (Vinall et al. 2013b). Neonatal rats exposed to 2 to 4 heel pokes over the first 7 days of life, grew more slowly and had increased anxiety-like behaviors during adulthood compared to rats exposed to tactile stimulation (Anand et al. 1999). Greater internalizing (anxious/depressive) behaviors in preterm children compared to full-term controls have been reported as early 2 years CA, persist to late adolescence, and appear to be independent of cognitive ability (Aarnoudse-Moens et al. 2009; Anderson, Doyle, Victorian Infant Collaborative Study Group 2003; Bhutta et al. 2002; Grunau, Whitfield, Fay 2004; Loe et al. 2011; Spittle et al. 2009). A recent study by our group has demonstrated that among non-ventilated children born very preterm, greater exposure to invasive procedures in the NICU was associated with higher reported internalizing behavior at 7 years of age (Ranger et al. 2014).

Experimental animal models have also demonstrated that early stress can permanently reorganize hormonal, physiological and behavioral systems (Matthews 2002; Meaney, Szyf, Seckl 2007; Murgatroyd and Spengler 2011; Pryce and Feldon 2003). Greater exposure to

invasive procedures in the NICU was associated with altered stress hormone (cortisol) regulation in extremely low gestational age (ELGA: 24 to 28 weeks) children (Grunau, Weinberg, Whitfield 2004; Grunau et al. 2007). Cortisol expression among ELGA children, and to a lesser degree very low gestational age (VLGA; 29 to 32 weeks) children, was associated with internalizing behaviors at 18 months CA (Brummelte et al. 2011b). This research emphasizes the importance of managing pain/stress in the NICU, to prevent long-term effects on child behavior.

Parents also play a vital role in the management of stress and development of their infant (Gunnar 1998). However, the birth of a preterm infant is a highly stressful experience for parents (Meyer et al. 1995; Miles, Funk, Carlson 1993; Younger, Kendall, Pickler 1997). One of the most stressful experiences reported by parents of infants in the NICU is seeing their infant in pain (Gale et al. 2004; Miles, Funk, Carlson 1993; Miles and Holditch-Davis 1997). Neonatal intensive care-based interventions that increase parental involvement in infant pain management have been found to improve parents’ efficacy in supporting their infant post-discharge from the hospital (Franck et al. 2011). This is important given that parenting stress appears to persist well-beyond discharge from the NICU (Brummelte et al. 2011a; Garel, Dardennes, Blondel 2007; Holditch-Davis et al. 2009; Singer et al. 2003). Among preterm infants exposed to greater numbers of invasive procedures in the NICU, if parents reported having lower parenting stress, infants showed less negative reactivity at 12 months CA, compared to infants whose parents reported having higher parenting stress (Voigt et al. 2013). While parenting stress may be reflective of realistic parental concerns with their child's development (Brummelte et al. 2011a), higher parenting stress is predictive of child internalizing behavior (Zelkowitz et al. 2011), and is associated with decreased parent emotional availability at 2 years CA in preterm children.
(Zelkowitz et al. 2009). Importantly, parent support to promote sensitive and responsive interactions during hospitalization appears to improve white matter maturation in infants born preterm (Milgrom et al. 2010). Greater maturation of the white matter at term age has been associated with better social-emotional outcomes at age 5 in children born preterm (Rogers et al. 2012). Although more positive parent interaction was found to buffer the relationship between invasive procedures in the NICU and poorer focused attention at 8 months CA (Tu et al. 2007), the extent that parental behavior moderates the relationship between invasive procedures in the NICU and internalizing behavior remains unknown.

Therefore, we examined whether the number of invasive procedures in the NICU (adjusted for neonatal and medical confounders) is related to parent report of internalizing behaviors at 18 months CA, and whether parental EA (adjusted for parenting stress), moderates the relationship between invasive procedures and internalizing behaviors (adjusted for child cognition) in children born very preterm. Further, we examined the relationship between parent EA and internalizing in children born full term.

**Hypothesis**

We hypothesized that greater parent EA would be associated with fewer internalizing behaviors at 18 months CA in children born very preterm exposed to greater numbers of invasive procedures.

**4.2 Methods**

**4.2.1 Study Overview**

Very preterm infants were recruited from the NICU at the B.C. Children’s & Women’s
Hospitals. Full-term infants were born at the B.C. Women’s Hospital and were contacted through their pediatricians. Written consent was obtained from a parent. At 18 months CA, the children and their parent(s) returned to the center for the study visit. The Bayley Scales of Infant Development II was administered (Bayley 1993), followed by the videotaped semi-structured parent–child teaching session, later scored for parent EA (Biringen 2008). We examined whether parental behavior adjusted for parenting stress, parent’s years of education, number of children in the home, and parent age moderated the relationship between invasive procedures (adjusted for GA, illness severity on day 1, days on mechanical ventilation, and total morphine exposure) and internalizing behaviors (adjusted for gender and child cognition) at 18 months CA in children born very preterm.

4.2.2 Participants

Ninety-six infants born very preterm (≤32 weeks GA) and 49 full-term control infants born at the B.C. Children’s & Women’s Hospitals between February 2001 and July 2004 were recruited as part of a larger ongoing study of the effects of neonatal pain on the neurodevelopment of infants born very preterm (Brummelte et al. 2011b; Grunau et al. 2005; Grunau et al. 2007; Tu et al. 2007). Infants were excluded if they were born small or large for GA; if they had a major congenital anomaly, major neurosensory impairment (legally blind, non-ambulatory cerebral palsy, sensory-neural hearing impairment), or severe brain injury evident on neonatal ultrasound (PVL and/or grade 3 or 4 IVH); or if the mother reported use of illicit drugs during pregnancy. All full-term infants in our study were born healthy, and none was under observation for medical complications. Ninety-four mothers and 2 fathers of children born very preterm, and 47 mothers and 2 fathers of children born full term participated in the study at 18 months CA.
4.2.3 Demographics

Parent information was obtained by questionnaire. Because parent’s years of education is the most important socioeconomic status (SES) indicator in relation to child development (Bohm et al. 2002; Resnick et al. 1990), we used parent’s years of education as the index of SES for statistical analysis.

4.2.4 Neonatal Medical Chart Review

A neonatal research nurse carried out medical and nursing chart review from birth to term-equivalent age, as described previously (Brummelte et al. 2011b; Grunau et al. 2007). Data included but were not limited to GA, gender, illness severity on day 1 (SNAP-II; Richardson et al. 2001), number of invasive procedures (see Appendix Table A.1), days of mechanical ventilation, and cumulative morphine exposure adjusted for weight.

4.2.5 Cognitive Development

At 18 months CA, child development was assessed with the Bayley Scales of Infant Development II (Bayley 1993). We used the Bayley Mental Development Index (MDI) to adjust our statistical models for child cognitive function. The Bayley MDI measures language, memory and problem-solving abilities in infants and toddlers aged 1 to 42 months. The Bayley MDI is a standardized score for overall cognitive development, with a mean of 100 and standard deviation of 15.

4.2.6 Parenting Stress

Parent’s completed the Parenting Stress Index III (PSI; Abidin 1995), which includes 120 items rated on a 6-point Likert scale from 1 (strongly agree) to 6 (strongly disagree). The PSI yields a
Total Score and 2 domain scores: Child Domain (concern about the child) and Parent Domain (concern about their own parenting ability). Given that the Child Domain reflects parent’s concerns about the child’s behavior, including internalizing behaviors, we only included the Parent Domain in the statistical analysis because our focus was on how parental factors may influence child behavior.

4.2.7 Child Internalizing Behavior

Parent’s rated their child’s behavior with the Child Behavior Checklist for children ages 1. to 5 years (CBCL; Achenbach and Rescorla 2000), a widely used questionnaire for identifying problem behaviors in children. Ninety-nine items are rated on a Likert scale ranging from 0 (not true) to 2 (very true or often true). Seven syndrome scales (Emotionally Reactive [e.g. moody, whining], Anxious/Depressed [e.g. nervous, sad], Somatic Complaints [e.g. does not eat well, stomachaches], Withdrawn [e.g. avoids eye contact, unresponsive to affection], Sleep Problems [e.g. nightmares, wakes often], Attention Problems [e.g. cannot concentrate, cannot sit still], and Aggressive Behavior [e.g. hits others, easily frustrated]) are empirically derived and form 2 broad domains, Internalizing and Externalizing Problems. The Internalizing scale encompasses the Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn Behaviors, whereas the Externalizing scale includes Attention Problems and Aggressive Behaviors. However, only the Internalizing domain was used, given that Internalizing, not Externalizing problems are associated with prematurity (Aarnoudse-Moens et al. 2009; Grunau, Whitfield, Fay 2004). Reliability for the Internalizing subscale is high (test–retest Pearson r = 0.90; Cronbach’s alpha 0.92; Achenbach and Rescorla 2000).
4.2.8 Emotional Availability

The primary caregiver participated in a 5-min videotaped semi-structured teaching scenario with their child. This involved the caregiver trying to teach her child to perform tasks of varying difficulty. The easier and more familiar task involved stacking or nesting colored cups of varying sizes. The novel and more difficult task involved sorting plastic pigs and cows into separate containers. Parent behavior during this interaction was later scored from videotape using the Emotional Availability Scale IV (Biringen 2008).

The EA scale captures 4 dimensions of parent behavior: Sensitivity (appropriateness/authenticity of affect), Structuring (provision of guidance), Nonintrusiveness (no overstimulation/overprotection), and Nonhostility (nonthreatening/non-frightening; Biringen 2008). Each EA dimension has 7 subscales, which are summed to provide a total score for the dimension. Scores range from 7 to 29, and higher scores denote emotionally available parenting. According to the clinical cutoffs for the EA scale, parents with scores ranging from 7 to 17 are considered to be Nonoptimally EA, 18 to 25 Inconsistently EA, and 26 to 29 Optimally EA (Biringen 2008). An average score, in the mid range, falls within the Inconsistent category. This represents a parent that has adequate EA, but may have moments during the interaction where they are less emotionally available to their child. Trained coders assessed EA from videotape: 1 primary coder and 2 reliability coders blinded to all other information about the participants. Inter-rater reliability assessed with intraclass correlation coefficients was 0.89, 0.86, 0.89, and 0.87 for Sensitivity, Structuring, Nonintrusiveness, and Nonhostility, respectively.

4.2.9 Data Analyses

Predictive Analytics Software (PASW) Statistics 18.0.3 (IBM) was used. Normality plots were
examined and the number of invasive procedures was log transformed. Demographic characteristics of the preterm and full-term groups, and comparisons between infants included and excluded in this study were examined by t-tests or chi-square tests, when appropriate. Univariate analysis of variance (ANOVA) was performed to examine group (preterm and full term; ELGA and VLGA) by gender differences on EA and Internalizing scores at 18 months. Pearson correlations were used to examine associations among measures for both the preterm and full-term groups. Multivariate analyses were performed using GENLIN. For each EA dimension (Sensitivity, Structuring, Nonintrusiveness, Nonhostility), GENLIN modeling was used to examine whether parent EA adjusted for Parenting Stress, parent’s years of education, number of children in the home, and parent age moderated the relationship between the number of invasive procedures (adjusted for illness severity on day 1, days on mechanical ventilation, and total morphine exposure) and Internalizing behaviors (adjusted for gender and Bayley MDI) at 18 months CA in children born very preterm. In addition, we included a variable in our analysis to account for prematurity at birth (ELGA or VLGA), given that ELGA children exhibited greater associations between an altered pattern of cortisol expression and internalizing behaviors at 18 months CA relative to VLGA children (Brummelte et al. 2011b). An interaction term between EA and the number of invasive procedures was included in each of the models. Post hoc t-tests were used to further explore statistically significant interactions. The univariate ANOVAs, GENLIN models, and post hoc t-tests were repeated excluding the 3 mothers in our sample who reported drinking alcohol during their pregnancy. Finally, to better understand the etiology of the highly prevalent internalizing behaviors seen in children born prematurely relative to full-term control children, GENLIN models for each EA dimension (Sensitivity, Structuring, Nonintrusiveness, Nonhostility) were used to examine whether parent EA adjusted for parenting Stress, parent’s years of education, number of children in the home, and parent age
was associated with Internalizing behaviors.

4.3 Results

4.3.1 Characteristics of the Cohort

Of the families we contacted, 120 of 159 (75%) of the parents of children born very preterm and 57 of 71 (80%) of the parents of children born full term returned for the 18-month follow-up. After exclusions, we included 96 very preterm and 49 full-term children in this study. Importantly, the 96 children born very preterm that were included in the present study did not differ in GA, birth weight, or sex from the original sample of infants recruited from the NICU at the B.C. Children’s & Women’s Hospitals between February 2001 and July 2004 (all \( P > .05 \)). Similarly, the 49 full-term control children did not differ in GA or sex from the original sample of full-term infants recruited at birth (all \( P > .05 \)). The full-term control children included in this study had a lower mean birth weight than the full-term infants in the original sample (\( t_{96} = -2.26, P = .03 \)). This difference, however, was no longer significant after the 6 children born large for their GA were removed from the analysis (\( t_{90} = -1.35, P = .18 \)). As expected, GA and birth weight differed between infants born very preterm versus full term. The only significant difference between parents of preterm versus full-term infants was parent’s years of education; parents of infants born very preterm had fewer years of education than parents of infants born full term. Children born very preterm had lower Bayley MDI cognitive scores and demonstrated more Internalizing behaviors at 18 months CA than children born full term. Characteristics of the sample are listed in Table 4.1.
Table 4.1 Characteristics of the Cohort

<table>
<thead>
<tr>
<th>Neonatal characteristics</th>
<th>Preterm</th>
<th>Full-term</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neonatal characteristics</strong></td>
<td>n= 96</td>
<td>n= 49</td>
<td></td>
</tr>
<tr>
<td>GA (weeks), median (IQR)</td>
<td>29.4 (26.6-31.3)*</td>
<td>40.0 (39.4-40.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth weight (grams), median (IQR)</td>
<td>1222 (813-1641)</td>
<td>3475 (3240-3678)</td>
<td>0.001</td>
</tr>
<tr>
<td>Gender (male), number (%)</td>
<td>47 (49)</td>
<td>21 (43)</td>
<td>0.49</td>
</tr>
<tr>
<td>Illness severity on day 1 (score), median (IQR)</td>
<td>9 (5-19)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of invasive procedures, median (IQR)</td>
<td>87 (49-176)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mechanical ventilation (days), median (IQR)</td>
<td>3 (0-18)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total morphine exposure (mg/kg), median (IQR)</td>
<td>0.10 (0.00-1.28)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parent characteristics at 18 month visit</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (IQR)</td>
<td>36 (31-39)</td>
<td>37 (33-40)</td>
<td>0.07</td>
</tr>
<tr>
<td>Marital status (married), number (%)</td>
<td>93 (97)</td>
<td>46 (94)</td>
<td>0.50</td>
</tr>
<tr>
<td>Ethnicity (Caucasian), number (%)</td>
<td>73 (76)</td>
<td>37 (76)</td>
<td>1.00</td>
</tr>
<tr>
<td>Parent education (years), median (IQR)</td>
<td>15 (13-17)</td>
<td>18 (15-19)</td>
<td>0.001</td>
</tr>
<tr>
<td>Children at home (number), median (IQR)</td>
<td>2 (1-3)</td>
<td>2 (1-2)</td>
<td>0.18</td>
</tr>
<tr>
<td>PSI Parenting stress (score), median (IQR)</td>
<td>109 (97-128)</td>
<td>115 (92-122)</td>
<td>0.24</td>
</tr>
<tr>
<td>EA Sensitivity (score), median (IQR)</td>
<td>19 (16-22)</td>
<td>21 (15-23)</td>
<td>0.72</td>
</tr>
<tr>
<td>EA Structuring (score), median (IQR)</td>
<td>20 (17-22)</td>
<td>19 (16-24)</td>
<td>0.37</td>
</tr>
<tr>
<td>EA Nonintrusiveness (score), median (IQR)</td>
<td>19 (16-22)</td>
<td>20 (16-22)</td>
<td>0.62</td>
</tr>
<tr>
<td>EA Nonhostility (score), median (IQR)</td>
<td>21 (19-24)</td>
<td>22 (19-24)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Child Characteristics</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bayley MDI (score), median (IQR)</td>
<td>91 (78-101)</td>
<td>97 (87-107)</td>
<td>0.01</td>
</tr>
<tr>
<td>CBCL Internalizing (t-score), median (IQR)</td>
<td>45 (41-51)</td>
<td>41 (33-49)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*44 children (46%) were born EGLA, 52 children (54%) were born VLGA
CBCL, Child Behavior Checklist (Achenbach and Rescorla 2000); EA, Emotional Availability (Biringen 2000); IQR, interquartile range; Bayley MDI, Bayley Mental Development Index (Bayley 1993); PSI, Parenting Stress Index (Abidin 1995).
4.3.2 Parent and Child Behavior: Group by Gender Analyses

Assumptions were met for the ANOVAs: Levene’s test for equality of variances was nonsignificant, and skewness was between -1 and 1 for the variables Internalizing, Sensitivity, Structuring, and Nonintrusiveness for the preterm, full-term, ELGA, and VLGA groups. Children born preterm demonstrated significantly more Internalizing behaviors at 18 months CA than children born full term ($F[1,141]= 5.88, P= .02$); gender was not significant, and there was no group-by-gender interaction ($P= .76$). Internalizing behaviors, however, did not differ between ELGA and VLGA children ($P= .39$). Internalizing behavior was not correlated with the Bayley MDI for the preterm ($r= -0.19, P= .07$) or full-term children ($r= 0.06, P= .67$). Parent EA (Sensitivity, Structuring, Nonintrusiveness, Nonhostility) did not differ significantly by group (preterm, full term) or by gender (all $P>.36$). However, parents of children born ELGA children provided less Structure than parents of VLGA children ($F[1,92]= 4.68, P= .03$).

4.3.3 Correlations Among Neonatal Variables

Among the infants born very preterm, lower GA at birth was correlated with higher illness severity on day 1, greater number of invasive procedures, more days of mechanical ventilation, and more total morphine exposure (Table 4.2). Given that all correlations were $r< 0.80$, multicollinearity among the neonatal predictors was not considered to be problematic (Katz 2011).
Table 4.2 Pearson Correlations Among the Neonatal Variables of the Preterm Infants

<table>
<thead>
<tr>
<th></th>
<th>Illness severity on day 1</th>
<th># of invasive procedures</th>
<th>Days on mechanical ventilation</th>
<th>Total morphine exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA group</td>
<td>-0.59***</td>
<td>-0.77***</td>
<td>-0.64***</td>
<td>-0.39***</td>
</tr>
<tr>
<td>Illness severity on day 1</td>
<td>-</td>
<td>0.58***</td>
<td>0.51***</td>
<td>0.28***</td>
</tr>
<tr>
<td># of invasive procedures</td>
<td>-</td>
<td>-</td>
<td>0.80***</td>
<td>0.52***</td>
</tr>
<tr>
<td>Days on mechanical ventilation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.77***</td>
</tr>
</tbody>
</table>

*** P < 0.001

GA group (24-28 weeks GA or 29-32 weeks GA)

4.3.4 Correlations Among Parent Variables

The correlations among the 4 EA dimensions for parents of preterm children ranged from $r=0.47$ to $r=0.80$, and for parents of full-term children ranged from $r=0.62$ to $r=0.88$ (Table 4.3).

Each EA dimension was entered in a separate multivariate model. In the preterm group, more years of education was associated with higher parent age, Sensitivity, and Nonhostility. In the full-term group, more years of education was associated with higher parent age and lower Parenting Stress. Unlike the preterm group, higher parent age among parents of full-term children was associated with greater parent Sensitivity, Nonintrusiveness, and Nonhostility (Table 4.3).
Table 4.3 Pearson Correlations Among the Parent Variables for Preterm and Full-Term Groups

<table>
<thead>
<tr>
<th></th>
<th>Preterm</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Full-term</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Structuring</td>
<td>Non-intrusiveness</td>
<td>Non-hostility</td>
<td>Parenting Stress</td>
<td>Parent’s years of education</td>
<td># of children in the home</td>
<td>Parent age</td>
<td>Sensitivity</td>
<td>Structuring</td>
</tr>
<tr>
<td>Preterm</td>
<td>0.70***</td>
<td>0.70***</td>
<td>0.80***</td>
<td>-0.05</td>
<td>0.21*</td>
<td>0.41</td>
<td>-0.02</td>
<td></td>
<td></td>
<td>0.78***</td>
</tr>
<tr>
<td>Structuring</td>
<td>-</td>
<td>0.37***</td>
<td>0.47***</td>
<td>0.30</td>
<td>0.07</td>
<td>0.05</td>
<td>-0.02</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Nonintrusiveness</td>
<td>-</td>
<td>-</td>
<td>0.52***</td>
<td>-0.17</td>
<td>0.11</td>
<td>0.05</td>
<td>0.12</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Nonhostility</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.04</td>
<td>0.25*</td>
<td>-0.05</td>
<td>0.003</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Parenting Stress</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Parent’s years of education</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td># of children in the home</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001
### 4.3.5 Invasive Procedures, EA Sensitivity, and Internalizing Behavior

In the GENLIN models, there was a significant interaction between parent Sensitivity and the number of invasive procedures in relation to Internalizing behavior at 18 months CA in children born very preterm \((B = 1.30, P = .05; \text{Table 4.4})\), after adjusting for neonatal medical confounders (GA group [ELGA or VLGA], illness severity on day 1, days of mechanical ventilation, cumulative morphine exposure), concurrent environmental stressors (Parenting Stress, parent’s years of education, number of children in the home), parent age, gender, and Bayley MDI. In order to understand this 2-way interaction, Internalizing scores were plotted by number of invasive procedures separately for subgroups of parent EA behavior: Nonoptimal Sensitivity \((n = 37)\), Inconsistent Sensitivity \((n = 54)\), and Optimal Sensitivity \((n = 5)\) (Figure 4.1). Post hoc \(t\) tests revealed significant differences in Internalizing behavior between Nonoptimal and Inconsistently Sensitive parents; among preterm children exposed to a higher number of invasive procedures, greater parent sensitivity was associated with lower internalizing behaviors at 18 months CA \((t[44] = 2.32, P = .03; \text{Figure 4.1})\). Higher Parenting Stress \((B = 0.15, P = .001)\), fewer years of education \((B = -0.87, P = .008)\), and fewer children in the home \((B = -2.46, P = .004)\) were independently associated with more Internalizing behaviors at 18 months CA in children born very preterm.
Table 4.4 Greater Parent Sensitivity and Nonhostility were Associated with Fewer Internalizing Behaviors at 18 months CA among Preterm Children Exposed to a Higher Number of Invasive Procedures

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Child Internalizing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity† Model</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>EA†</td>
<td>-2.32</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>-19.08</td>
</tr>
<tr>
<td>EA† x number of invasive procedures</td>
<td>1.30</td>
</tr>
<tr>
<td>Days on mechanical ventilation</td>
<td>-0.06</td>
</tr>
<tr>
<td>Total morphine exposure</td>
<td>0.08</td>
</tr>
<tr>
<td>Illness severity on day 1</td>
<td>-0.004</td>
</tr>
<tr>
<td>GA group</td>
<td>0.12</td>
</tr>
<tr>
<td>Gender</td>
<td>-2.85</td>
</tr>
<tr>
<td>Parenting stress</td>
<td>0.15</td>
</tr>
<tr>
<td>Parent’s years of education</td>
<td>-0.87</td>
</tr>
<tr>
<td># of children in the home</td>
<td>-2.46</td>
</tr>
<tr>
<td>Parent age</td>
<td>-0.13</td>
</tr>
<tr>
<td>Bayley MDI</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

Adjusted R²: 0.26* 0.23* 0.22* 0.31*

†The GENLIN was repeated four times: each time a different EA variable (Sensitivity, Structuring, Nonintrusiveness, Nonhostility) was entered into the model.

*Computed using linear regression.

Parent Sensitivity and Nonhostility moderated the relationship between the number of invasive procedures and Internalizing in children born very preterm at 18 months CA after adjusting for neonatal medical confounders (GA group [24-28 weeks GA or 29-32 weeks GA]), illness severity on day 1, days of mechanical ventilation, cumulative morphine exposure), concurrent environmental stressors (Parenting Stress, parent’s years of education, number of children in the home), parent age, child gender and cognition (Bayley MDI). Higher Parenting Stress, fewer maternal years of education and fewer children in the home were independently associated with greater Internalizing at 18 months corrected age in children born very preterm.
4.3.6 Invasive Procedures, EA Structuring, and Internalizing Behavior

In the GENLIN models, the interaction between parent Structuring and invasive procedures in relation to Internalizing behavior at 18 months CA in children born very preterm was not significant ($B=0.74$, $P=.32$; Table 4.4), after adjusting for neonatal medical confounders (GA group, illness severity on day 1, days of mechanical ventilation, cumulative morphine exposure), concurrent environmental stressors (Parenting Stress, parent’s years of education, number of children in the home), parent age, gender, and Bayley MDI. However, higher Parenting Stress ($B=0.15$, $P=.001$), fewer years of education ($B=-0.81$, $P=.01$), and fewer children in the home.
(B = -2.29, \( P = .009 \)) were independently associated with more Internalizing behaviors at 18 months CA in children born very preterm.

### 4.3.7 Invasive Procedures, EA Nonintrusiveness, and Internalizing Behavior

In the GENLIN models, the interaction between parent Nonintrusiveness and neonatal pain in relation to Internalizing at 18 months CA in children born very preterm was not significant (B = -0.19, \( P = .75 \); Table 4.4), after adjusting for neonatal medical confounders (GA group, illness severity on day 1, days of mechanical ventilation, cumulative morphine exposure), concurrent environmental stressors (Parenting Stress, parent’s years of education, number of children in the home), parent age, gender, and Bayley MDI. However, higher Parenting Stress (B= 0.14, \( P = .001 \)), fewer years of education (B= -0.74, \( P = .03 \)), and fewer children in the home (B= -2.17, \( P = .01 \)) were independently associated with more Internalizing behaviors at 18 months CA in children born very preterm.

### 4.3.8 Invasive Procedures, EA Nonhostility, and Internalizing Behavior

In the GENLIN models, there was a significant interaction between parent Nonhostility and neonatal pain in relation to Internalizing behavior at 18 months CA in children born very preterm (B= 2.32, \( P = .006 \); Table 4.4), after adjusting for neonatal medical confounders (GA group, illness severity on day 1, days of mechanical ventilation, cumulative morphine exposure), concurrent environmental stressors (Parenting Stress, parent’s years of education, number of children in the home), parent age, gender, and Bayley MDI. In order to understand this 2-way interaction, Internalizing scores were plotted by number of skin-breaking procedures, separately for subgroups of parent EA behavior: Nonoptimal Nonhostile (n= 16), Inconsistent Nonhostile (n= 75), and Optimal Nonhostile (n= 5) (Figure 4.2). Although the relationship between
Nonoptimal and Inconsistently Nonhostile parenting and neonatal pain (Figure 4.1) was similar to the Nonoptimal and Inconsistently Sensitive parents whose infants received a high number of skin-breaking procedures (Figure 4.2), the group size was limited ($t[45] = 0.57, P = .57$).

Consistent with the analyses above, higher Parenting Stress ($B = 0.16$, $P = .001$), fewer years of education ($B = -0.82$, $P = .01$), and fewer children in the home ($B = -2.25$, $P = .007$) were independently associated with more Internalizing behaviors at 18 months CA in children born very preterm.

**Figure 4.2 Parental Nonhostility Moderates the Relationship Between Invasive Procedures in the NICU and Internalizing Behavior in Children Born Very Preterm**

Predicted values of Internalizing behaviors ($t$-score) in relation to number of invasive procedures from birth to term-equivalent age adjusted for gestational age (GA) group (24 to 28 weeks GA or 29 to 32 weeks GA), illness severity on day 1, days of mechanical ventilation, total morphine exposure, Parenting Stress, parent’s years of education, number of children in the home, parent age, child gender, and cognition. Differences in Internalizing scores were between nonoptimal and inconsistently nonhostile parents, whose infants received a high number of invasive procedures. Among preterm children exposed to a higher number of invasive procedures, greater parent nonhostility appears to lower Internalizing behaviors at 18 months corrected age.
4.3.9 Exclusion of Mothers Who Reported Drinking Alcohol During their Pregnancy

The univariate ANOVAs, GENLIN models, and post hoc t-tests were repeated excluding the 3 mothers in our sample who reported drinking alcohol during their pregnancy, and the results of our models remained unchanged.

4.3.10 Parent Behavior and Stress in Relation to Internalizing Behavior in Children Born Full-Term

There were no significant associations between parent EA (Sensitivity, Structuring, Nonintrusiveness, Nonhostility) or stress (Parenting Stress, parent’s years of education, number of children in the home), and Internalizing behavior (adjusted for GA, parent age, gender, and Bayley MDI) at 18 months in children born full-term.

4.4 Discussion

In this study, we examined whether parent emotional availability moderated the relationship between invasive procedures in the NICU and internalizing behavior at 18 months CA in children born very preterm. We found that among children born very preterm exposed to a higher number of invasive procedures (adjusted for confounding neonatal medical factors), greater parent sensitivity and nonhostility were associated with lower internalizing behaviors at 18 months CA. In addition, lower parenting stress, more years of education, and more children in the home were also independently associated with fewer internalizing behaviors in children born very preterm at 18 months CA. In contrast, none of the parent factors were a significant predictor of internalizing behavior in children born full term. Despite the similarities in parenting stress and parent behavior, children born very preterm were more influenced by interactions with their
parents compared to their term-born peers, consistent with previous findings from our group and others (Brummelte et al. 2011b; Crnic and Greenberg 1987; Tu et al. 2007).

Both in humans and animals, repeated exposure to invasive procedures is associated with increased anxious/depressive behaviors (Anand et al. 1999; Ranger et al. 2014). Repeated exposure to invasive procedures in the NICU is associated with the reprogramming of the HPA axis. At 32 weeks PMA, greater exposure to invasive procedures is associated with lower cortisol responses, independent GA, early illness severity and morphine exposure (Grunau et al. 2005). However, at 8 and 18 months CA, greater number of invasive procedures in the NICU was associated with higher levels of cortisol (Grunau, Weinberg, Whitfield 2004; Grunau et al. 2007). Among infants born ELGA, there is evidence for a shift from low basal cortisol levels at 3 months to relatively high levels at 8 and 18 months CA, which suggests a biological “resetting” of endocrine stress systems (Grunau et al. 2007). Cortisol levels at 18 months CA were associated with internalizing behaviors among both ELGA and VLGA children (Brummelte et al. 2011b).

Parental behavior also plays an important role in the programming stress response (Ahnert et al. 2004; Coplan et al. 1996; De Bellis 2005; Gunnar et al. 1996; Meaney and Szyf 2005; Pryce and Feldon 2003). In rats, high licking and grooming by the dam results in the hypomethylation of the NGFI-A transcription factor, thereby permitting binding of NGFI-A to the glucocorticoid receptor promoter (Weaver et al. 2004; Weaver et al. 2007). The adult offspring of high licking and grooming mothers show increased hippocampal glucocorticoid receptor expression, better glucocorticoid feedback sensitivity, less corticotrophin releasing factor and less glucocorticoid production compared to pups reared by low licking and grooming mothers (Francis et al. 1999a;
Liu et al. 1997). Rats with higher licking and grooming mothers have fewer anxiety-like behavior during adulthood, indicated by fewer startle responses, increased open-field exploration, greater social interaction and shorter latencies to eat in a novel environment (Caldji et al. 1998; Pena et al. 2014; Starr-Phillips and Beery 2014; van Hasselt et al. 2012). Similarly, in humans, sensitive maternal behavior was associated with lower cortisol levels, and fewer internalizing behaviors at 18 months CA in children born very preterm (Brummelte et al. 2011b). Among the preterm infants exposed to higher numbers of invasive procedures, greater maternal sensitivity and nonhostility were associated with fewer internalizing behaviors at 18 months CA.

Parent’s concern regarding their own parenting ability (parenting stress) was associated with child internalizing behaviors at 18 months CA. Parenting stress may interfere with how the parent interacts with their child. Mothers who reported higher stress in the NICU and/or higher concurrent stress were less sensitive/emotionally available when interacting with their child post-discharge (Muller-Nix et al. 2004; Tu et al. 2007; Zelkowitz et al. 2009). Higher parenting stress may also reflect trait anxiety, which has also been shown to be associated with increased internalizing in children born very preterm at 18 months CA, independent of maternal education and neonatal morbidity (Zelkowitz et al. 2011).

Fewer years of education, an indicator of lower SES, was associated with greater internalizing behavior at 18 months CA in children born very preterm. Importantly, the relationship between parent’s years of education and internalizing behavior remained significant after accounting for child cognition, which is associated with both parent’s years of education and child interactive behavior (Lowe, Erickson, MacLean 2010). Parents with fewer years of education were less sensitive and more hostile compared to parents with more years of education. Previous studies
have shown that the relationship between socioeconomic risk and behavior in preterm infants and children was mediated by maternal behavior (Candelaria, Teti, Black 2011; Linver, Brooks-Gunn, Kohen 2002). NICU-based programs designed to enhance the quality of interaction between low SES mothers and their infants have lead to improved home environments and better infant temperament at 4 and 8 months CA (Parker et al. 1992). It is noteworthy, however, that the level of parent’s education was relatively high in our cohort, with parents of preterm infants having a median of 15 years education, indicative of some postsecondary college attendance.

Fewer children in the home was also associated with greater internalizing behavior in children born very preterm. First-time parents may underestimate the personal impact of the birth of the infant (Evans, Whittingham, Boyd 2012); a life-altering change in combination with an unexpected preterm birth appears to increase the risk for a negative transition to parenthood (Harwood, McLean, Durkin 2007). Realistic expectations of parenthood may improve parent attachment/responsiveness and lessen parent psychological symptoms (Evans, Whittingham, Boyd 2012), factors that are in turn associated with fewer internalizing behaviors (Gravener et al. 2012; O’Connor et al. 2011). Moreover, siblings can also play an important role in behavioral development; support from siblings can be a buffer to feelings of loneliness and depression in contexts of decreased parental and/or peer support (East and Rook 1992; Milevsky and Levitt 2005). Adult siblings of children born preterm have retrospectively described their relationships with their brother or sister as both positive and protective (Gaal et al. 2010).

Given the correlational nature of this study and the bidirectional nature of parent–child interaction, it is important to note that greater child internalizing behavior may contribute to lower parent emotional availability. Parent sensitivity and nonhostility appear to moderate the relationship between neonatal pain and internalizing behavior in children born very
preterm, who are more sensitive to their environment than full-term control children. Future research is needed to determine whether early or concurrent emotional availability training can effectively prevent or reduce the long-term effects of neonatal pain on internalizing behavior in children born very preterm. NICU-based or follow-up programs designed to facilitate parent emotional availability may be able to effectively prevent or reduce internalizing behavior in children born very preterm. However, with limited resources for training, the results from this study suggest that parents who have fewer years of education, are highly stressed, or are first-time parents may be a priority for support. Helping parents to appropriately regulate pain/stress in their infant may also help to improve infant interactions, thereby reducing and/or preventing the development of internalizing behaviors in their preterm child.

Invasive procedures, inherent to life-saving care in the NICU, may contribute to the development of internalizing behavior in children born very preterm, consistent with the animal literature on early stress. Interventions focused on improving parent-child interaction and reducing parenting stress may help to ameliorate the negative long-term effects of invasive procedures on child behavior.
CHAPTER 5

INVASIVE PROCEDURES IN PRETERM CHILDREN: BRAIN AND COGNITIVE DEVELOPMENT AT SCHOOL AGE

5.1 Introduction

Advances in neonatal medical care have greatly improved the chances of survival for infants born very preterm. However, cognitive impairment appears to have increased among children with birth weights ≤800 grams (Doyle et al. 2011; Moore et al. 2012; Synnes et al. 2010). Even in the absence of major disability (e.g., blindness, nonambulatory cerebral palsy, developmental delay), cognitive problems and school difficulties are common among children born very preterm (Grunau, Whitfield, Davis 2002; Grunau, Whitfield, Fay 2004; Grunau et al. 2009; Johnson et al. 2009; Larroque et al. 2008; Saigal and Doyle 2008).

Infants born very preterm are repeatedly exposed to invasive life-saving procedures during a sensitive and rapid period of brain development (Kostovic and Jovanov-Milosevic 2006; Volpe 2009). Two cell populations are particularly vulnerable to injury in the premature brain: subplate neurons and preoligodendrocytes (Back and Miller 2014; Volpe 2009). Subplate neurons and preoligodendrocytes are vulnerable to excitotoxicity, reactive oxygen, nitrogen species and cytokines (Back et al. 1998; Back et al. 2005; Buntinx et al. 2004; Ghosh et al. 1990; Ghosh and Shatz 1992; Haynes et al. 2003; McQuillen and Ferriero 2005; Pang, Cai, Rhodes 2005). Greater exposure to invasive procedures has been shown to be both directly and indirectly associated with altered brain development in the NICU and at term-equivalent age (Brummelte et al. 2012; Smith et al. 2011; Vinall et al. 2012; Vinall et al. 2013b; Zwicker et al. 2013).

These findings are supported by evidence from animal models that have demonstrated both inflammatory pain and repeated injections increase apoptosis in the neonatal rat brain (Anand et al. 2007; Duhrs et al. 2013). Moreover, greater exposure to invasive procedures in the NICU was associated with poorer cognitive outcomes at 8 and 18 months CA in children born very preterm (Grunau et al. 2009).

Recent studies of our group have shown that the impact of invasive procedures on the brain and neurodevelopmental outcomes may extend beyond the neonatal period. At 7 years of age, greater numbers of invasive procedures in the NICU were associated with thinner cortical gray matter in 21 out of 66 cerebral regions assessed, predominately affecting the frontal and parietal lobes (Ranger et al. 2013). Moreover, among infants born ELGA, greater exposure to invasive procedures were also associated with alterations in spontaneous neuromagnetic activity (Doesburg et al. 2013). Lower synchronization of oscillatory activity was associated with poorer visual perceptual ability at school age (Doesburg et al. 2013). Therefore, repeated exposure to invasive procedures in the NICU appears to be associated with long-term alterations to cortical volumes and brain function. However, we still do not know from these studies how the brain microstructure underlying changes in volume and function is altered by repeated exposure to invasive procedures early in life.

The current study examined whether the number of invasive procedures during neonatal care was associated with white matter microstructure at age 7 years, and whether the number of invasive procedures together with measures of brain microstructure predicted cognitive outcome at school age in children born very preterm.
5.2 Methods

5.2.1 Study Overview

Children born very preterm recruited from the NICU of the BC Children’s & Women’s Hospitals. Neonatal data were acquired from medical chart review performed from birth to term-equivalent age. At a median age of 7.6 years (interquartile range, 7.5–7.7), children underwent MRI and cognitive testing. T₁- and T₂-weighted images were assessed for the severity of brain injury. Magnetic resonance diffusion tensor sequences were used to measure FA, an index of white matter maturation, from 7 anatomically defined white matter regions. Multivariate modeling was used to examine relationships between invasive procedures, brain microstructure, and cognition, adjusting for GA, small for gestational age (SGA: <10th weight percentile at birth), illness severity on day 1, days on mechanical ventilation, infection, gender, age at scan, brain injury, surgery, morphine, fentanyl, corticosteroids and midazolam.

5.2.2 Participants

Fifty children born very preterm (≤32 weeks GA) recruited from the NICU of the BC Children’s & Women’s Hospitals between February 2001 and July 2004 underwent MRI at median age 7.6 years (interquartile range [IQR], 7.5–7.7) as part of an ongoing study on the effects of neonatal pain on neurodevelopment of children born very preterm (Doesburg et al. 2013; Grunau et al. 2007; Grunau et al. 2009; Ranger et al. 2013; Ranger et al. 2014; Vinall et al. 2013a). Children were excluded if they had a major congenital anomaly, major neurosensory impairment (legally blind, nonambulatory cerebral palsy, sensori-neural hearing impairment), or severe brain injury evident on neonatal ultrasound (PVL or grade 3 or 4 IVH).
5.2.3 Neonatal Medical Chart Review

Neonatal data were acquired from medical chart review performed from birth to term-equivalent age or discharge (whichever came first) by a neonatal research nurse. We defined the number of invasive procedures as every attempt at a procedure as listed in Appendix Table A.1, from birth to term-equivalent age, adjusted for clinical confounders (e.g. illness severity on day 1 [SNAP-II; Richardson et al. 2001], days of mechanical ventilation, confirmed infection, morphine exposure).

5.2.4 Magnetic Resonance Imaging

Children were scanned at a median age of 7.6 years (IQR, 7.5–7.7). A Siemens 1.5 Tesla Avanto magnet, standard 12-channel head coil, and VB 16 software were used to obtain the following sequences: 3-dimensional T1-weighted spoiled gradient recalled acquisition (repetition time [ms] 18/echo time [ms] 9.2/field of view [mm] 256/slice thickness [mm] 1/gap [mm] 0/matrix 256 x 256) and T2-weighted images axial fast spin echo (4030/90/220/3/1/512 x 354) and axial fluid attenuation inversion recovery (8900/87/220/5/1/256 x 154). Neuroradiologist K.J.P., blinded to the child’s medical history, assessed these images for brain injury (i.e. evidence of cerebellar hemorrhage, ventriculomegaly, or moderate to severe WMI, as described previously; Miller et al. 2005).

5.2.5 Diffusion Tensor Imaging

DTI was acquired with a multirepetition, single-shot echo planar sequence with 12 gradient directions (7800/82/256/2/0/128 x 128), 3 averages of diffusion weighting 700 (b value). DTI parameters of FA, $\lambda_1$, $\lambda_2$, and $\lambda_3$ were obtained from 7 bilateral regions of interest in the white matter (Figure 5.1), consistent with our neonatal studies (Brummelte et al. 2012; Chau et al.
Intrarater reliability, based on the repeated analysis of a random 20% of regions of interest, was comparable with previously published findings (FA mean difference of -0.002 [Bland–Altman limits of agreement, -0.011 to 0.007]; Brummelte et al. 2012; Chau et al. 2009).

**Figure 5.1 Regions of Interest in the White Matter**

A) Superior white matter: (a) anterior, (b) middle, and (c) posterior subcortical white matter. B) white matter tracts: (d) genu of the corpus callosum, (e) posterior limb of the internal capsule, (f) splenium of the corpus callosum, and (g) optic radiations.

### 5.2.6 Cognitive Testing

At age 7 years, IQ was measured by using the standardized Wechsler Intelligence Scale for Children– 4th Edition (WISC-IV; Wechsler 2003), which includes 4 index scores that make up the Full Scale IQ (FSIQ): Verbal Comprehension, Perceptual Reasoning, Working Memory, and Processing Speed.
5.2.7 Data Analyses

Statistical analyses were performed by using Stata 9.2 (Stata Corp, College Station, TX). Normality plots were examined, and skewed variables (number of invasive procedures, days on mechanical ventilation, morphine exposure, FA values, and age at scan) were log transformed. IQ, GA, birth weight, and illness severity on day 1 of the included and excluded preterm infants were compared by using t-tests. Demographic characteristics of the preterm infants exposed to lower and higher numbers of invasive procedures were compared by using t-tests or $\chi^2$, when appropriate. Multivariate analyses were adjusted for confounders: GA, size at birth (small for gestational age versus appropriate for gestational age), illness severity on day 1, days of mechanical ventilation, morphine exposure, infection, gender, age at scan, and concurrent brain injury. A generalized estimating equation was used to examine whether the number of invasive procedures was associated with FA at age 7 years in an initial pain model. This model was repeated for the axial and radial axes. The pain model was extended to include variables for surgery and fentanyl exposure (surgery model), and corticosteroids and midazolam (steroid model). The regression coefficients for these models are reported as effect sizes. FA values were then grouped a priori into superior white matter (anterior, middle, and posterior subcortical white matter) and white matter tracts (genu and splenium of the corpus callosum, posterior limb of the internal capsule, optic radiations), and group means were used for analysis. Generalized linear modeling was used to examine whether the number of invasive procedures interacted with FA values from either the superior white matter or white matter tracts to predict FSIQ.
5.3 Results

5.3.1 Characteristics of the Cohort

Of the 131 eligible children contacted for the 7-year follow-up, 22 refused to participate and 7 withdrew, so that 102/131 (78%) were seen at school age. One child diagnosed with autism was excluded, leaving 101 children in this study. Of the 101 who returned for follow-up (psychometric assessment) at median age 7.6 years (IQR, 7.5–7.8), 58 (57%) parents and children consented/assented to an MRI. Research scans were available only on weekdays after 4 PM, and booking limitations affected study consents for MRI. Scans were not completed for 3 of the participants, and 3 were of poor quality because of motion artifact. Moreover, 2 children were missing either neonatal or follow-up data. Therefore, data from 50 children born very preterm were included in the current study. Importantly, the FSIQ of the children included (n=50) did not differ from that of the other 51 children who returned for 7-year follow-up (95% confidence interval [CI]: -7.18 – 3.86, P=.55). Moreover, children included in the current study did not differ in GA (95% CI: -1.40 – 0.44, P = .30), birth weight (95% CI: -187.33 – 147.52, P=.81), or early illness severity (95% CI: -2.81 – 5.73, P = .50) from the children who returned for follow-up or from the 81 infants in the original sample (95% CI: -1.10 – 0.63, P= .59; CI: -134.99 – 194.17, P=.72; and 95% CI: -2.50 – 5.73, P=.44; respectively).

Among the 50 children with imaging data at age 7 years, exposure to higher numbers of invasive procedures (median 122; IQR, 81 – 210) was associated with lower GA, higher illness severity on day 1, more days on mechanical ventilation, and a greater exposure to surgery, infection, dexamethasone, and morphine compared with children exposed to lower numbers of procedures (median 46; IQR, 30 – 55) (Table 5.1). Among the 101 children born very preterm who returned for follow-up at 7 years, exposure to higher numbers of invasive procedures (median 127; IQR,
87 – 200) were also associated with increased exposure to midazolam and fentanyl and a significantly lower FSIQ compared with children exposed to lower numbers of procedures (median 43; IQR, 32 – 52) (Table 5.2).
### Table 5.1 Characteristics of Children with Magnetic Resonance Imaging at Age 7 Years

<table>
<thead>
<tr>
<th>Neonatal Characteristics</th>
<th>n=50</th>
<th>n=25</th>
<th>n=25</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks), median (IQR)</td>
<td>29.8 (28.1-31.9)</td>
<td>31.4 (29.7-32.3)</td>
<td>28.4 (26.9-30.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Small for gestational age, number (%)</td>
<td>6 (12)</td>
<td>2 (8)</td>
<td>4 (16)</td>
<td>0.13</td>
</tr>
<tr>
<td>Illness severity on day 1, median (IQR)</td>
<td>8 (0-18)</td>
<td>0 (0-9)</td>
<td>14 (5-23)</td>
<td>0.01</td>
</tr>
<tr>
<td>Invasive Procedures, median (IQR)</td>
<td>74 (46-124)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Surgery, number (%)</td>
<td>8 (16)</td>
<td>0 (0)</td>
<td>8 (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infection, number (%)</td>
<td>11 (22)</td>
<td>1 (4)</td>
<td>10 (40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical ventilation (days), median (IQR)</td>
<td>2 (0-8)</td>
<td>0 (0-1)</td>
<td>7 (3-20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dexamethasone or hydrocortisone, number exposed (%)</td>
<td>5 (10)</td>
<td>0 (0)</td>
<td>5 (20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Morphine exposure (mg/kg), median (IQR)</td>
<td>0.0 (0.0-0.6)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.5 (0.1-1.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>number exposed (%)</td>
<td>24 (48)</td>
<td>4 (16)</td>
<td>20 (80)</td>
<td></td>
</tr>
<tr>
<td>Midazolam exposure (mg/kg), median (IQR)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>number exposed (%)</td>
<td>3 (6)</td>
<td>0 (0)</td>
<td>3 (12)</td>
<td></td>
</tr>
<tr>
<td>Fentanyl exposure (µg/kg), median (IQR)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-3.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>number exposed (%)</td>
<td>6 (12)</td>
<td>0 (0)</td>
<td>6 (24)</td>
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</table>
## Child Characteristics

<table>
<thead>
<tr>
<th>Child Characteristics</th>
<th>Lower # of Invasive Procedures</th>
<th>Higher # of Invasive Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median 46</td>
<td>Median 122</td>
</tr>
<tr>
<td></td>
<td>IQR 30-55</td>
<td>IQR 81-210</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>n=50</th>
<th>n=25</th>
<th>n=25</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male), number (%)</td>
<td>21 (42)</td>
<td>6 (24)</td>
<td>15 (60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at scan (years), median (IQR)</td>
<td>7.6 (7.5-7.7)</td>
<td>7.6 (7.5-7.6)</td>
<td>7.6 (7.5-7.7)</td>
<td>0.82</td>
</tr>
<tr>
<td>Moderate to severe brain injury, number (%)</td>
<td>6 (12)</td>
<td>4 (16)</td>
<td>2 (8)</td>
<td>0.13</td>
</tr>
<tr>
<td>WISC-IV FSIQ, median (IQR)</td>
<td>102 (91-110)</td>
<td>103 (92-110)</td>
<td>95 (85-112)</td>
<td>0.30</td>
</tr>
<tr>
<td>WISC-IV VCI, median (IQR)</td>
<td>98 (93-109)</td>
<td>99 (94-109)</td>
<td>98 (89-109)</td>
<td>0.56</td>
</tr>
<tr>
<td>WISC-IV PRI, median (IQR)</td>
<td>104 (94-113)</td>
<td>104 (98-112)</td>
<td>100 (91-119)</td>
<td>0.68</td>
</tr>
<tr>
<td>WISC-IV WRMI, median (IQR)</td>
<td>98 (91-110)</td>
<td>97 (91-109)</td>
<td>99 (88-115)</td>
<td>0.80</td>
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<tr>
<td>WISC-IV PSI, median (IQR)</td>
<td>94 (86-108)*</td>
<td>100 (88-115)</td>
<td>91 (83-105)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

FSIQ, full scale intelligence quotient; IQR, interquartile range; PRI, perceptual reasoning index; PRSI, processing speed index; VCI, verbal comprehension index; WISC-IV, Weschler Intelligence Scale IV (Weschler 2003); WRMI, working memory index.

*2 children did not complete the PRSI.
Table 5.2 Characteristics of All the Children that Returned for Follow-Up at Age 7 Years

<table>
<thead>
<tr>
<th>Neonatal Characteristics</th>
<th>Lower # of Invasive Procedures</th>
<th>Higher # of Invasive Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=101</td>
<td>Median 43 IQR 32-52</td>
<td>Median 127 IQR 87-200</td>
</tr>
<tr>
<td>Gestational age (weeks), median (IQR)</td>
<td>29.9 (27.5-31.7)</td>
<td>31.6 (29.9-32.4)</td>
</tr>
<tr>
<td>Small for gestational age, number (%)</td>
<td>10 (10)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>Illness severity on day 1, median (IQR)</td>
<td>9 (0-19)</td>
<td>5 (0-9)</td>
</tr>
<tr>
<td>Invasive Procedures, median (IQR)</td>
<td>73 (43-129)</td>
<td>-</td>
</tr>
<tr>
<td>Surgery, number (%)</td>
<td>17 (17)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Infection, number (%)</td>
<td>24 (24)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Mechanical ventilation (days), median (IQR)</td>
<td>2 (0-10)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Dexamethasone or hydrocortisone, number exposed (%)</td>
<td>8 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Morphine exposure (mg/kg), median (IQR)</td>
<td>0.0 (0.0-0.6)</td>
<td>0.0 (0.0-0.0)</td>
</tr>
<tr>
<td>number exposed (%)</td>
<td>49 (49)</td>
<td>6 (12)</td>
</tr>
<tr>
<td>Midazolam exposure (mg/kg) median(IQR)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-0.0)</td>
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<tr>
<td>number exposed (%)</td>
<td>8 (8)</td>
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</tr>
<tr>
<td>Fentanyl exposure (µg/kg), median (IQR), number exposed (%)</td>
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<td>0.0 (0.0-0.0)</td>
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<tr>
<td></td>
<td>12 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Child Characteristics</td>
<td>Lower # of Invasive Procedures</td>
<td>Higher # of Invasive Procedures</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td></td>
<td>n=101</td>
<td>n=49</td>
</tr>
<tr>
<td>Gender (male), number (%)</td>
<td>50 (50)</td>
<td>19 (39)</td>
</tr>
<tr>
<td>Age at follow-up (years), median (IQR)</td>
<td>7.6 (7.5-7.8)</td>
<td>7.6 (7.5-7.7)</td>
</tr>
<tr>
<td>WISC-IV FSIQ, median (IQR)</td>
<td>100 (91-110)</td>
<td>104 (94-114)</td>
</tr>
<tr>
<td>WISC-IV VCI, median (IQR)</td>
<td>98 (93-108)</td>
<td>99 (95-114)</td>
</tr>
<tr>
<td>WISC-IV PRI, median (IQR)</td>
<td>100 (92-116)</td>
<td>104 (98-117)</td>
</tr>
<tr>
<td>WISC-IV WRMI, median (IQR)</td>
<td>97 (88-110)</td>
<td>99 (91-110)</td>
</tr>
<tr>
<td>WISC-IV PRSI, median (IQR)</td>
<td>94 (85-106)</td>
<td>100 (90-113)</td>
</tr>
</tbody>
</table>

FSIQ, full scale intelligence quotient; IQR, interquartile range; PRI, perceptual reasoning index; PRSI, processing speed index; VCI, verbal comprehension index; WISC-IV, Weschler Intelligence Scale IV; WRMI, working memory index.

a1 child was missing neonatal data; 2 children did not have neonatal infection data.

b4 children did not complete the FSIQ; 2 children did not complete the VCI; 1 child did not complete the PRI; 3 children did not complete the WRMI; 6 children did not complete the PRSI.

5.3.2 Number of Invasive Procedures in Relation to White Matter Microstructure at Age 7 Years

Children born very preterm exposed to a greater number of invasive procedures in the NICU had lower FA values at age 7 years (effect size= -0.02, P= .01; CI: -0.04 – -0.005) after adjusting for confounders (GA, birth weight, illness severity on day 1, days of mechanical ventilation, morphine exposure, infection, gender, age at scan, and concurrent brain injury; pain model, Table 5.3). Infants who received the lowest number of invasive procedures (i.e. 10 invasive procedures) had 7% higher FA values than infants who underwent the highest number of invasive procedures (i.e. 267 invasive procedures). The relationship between the number of
invasive procedures and FA of the white matter was driven by the radial diffusion axes ($\lambda_2$ and $\lambda_3$: effect size= 0.05; CI: 0.01 – 0.09; $P$= .01), such that greater numbers of invasive procedures from birth to term-equivalent age were associated with higher radial diffusion values. In contrast, the number of invasive procedures was not associated with the axial diffusion axis ($\lambda_1$: effect size= 20.05; CI: -0.15 – 0.06; $P$= .38). Neither adjustment for surgery and fentanyl nor corticosteroids and midazolam significantly changed the results of the pain model (surgery and steroid models, Table 5.3).
Table 5.3 Higher Numbers of Invasive Procedures was Associated with Lower Fractional Anisotropy at Age 7 Years

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Fractional anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pain model n=50</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>Effect Size</td>
</tr>
<tr>
<td>Gestational age</td>
<td>-0.001</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-0.003</td>
</tr>
<tr>
<td>Illness severity</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>-0.003</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>0.009</td>
</tr>
<tr>
<td>Gender</td>
<td>-0.002</td>
</tr>
<tr>
<td>Age at scan</td>
<td>-0.19</td>
</tr>
<tr>
<td>Brain injury</td>
<td>-0.009</td>
</tr>
<tr>
<td>Surgery</td>
<td>-</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>0.008</td>
</tr>
<tr>
<td>Fentanyl exposure</td>
<td>-</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>-</td>
</tr>
<tr>
<td>Midazolam</td>
<td>-</td>
</tr>
</tbody>
</table>

5.3.3 Number of Invasive Procedures Interacts with the Superior White Matter to Predict FSIQ at Age 7 Years

The interaction between number of invasive procedures and FA values of the superior white matter was significantly associated with FSIQ (B= 412.18; $P$= .02; CI: 55.59 – 768.77; adjusted
\( R^2 = 0.22; \) Table 5.4, such that greater numbers of invasive procedures (adjusted for confounders) and lower FA of the superior white matter were associated with lower FSIQ at age 7.5 years in children born very preterm (Figure 5.2). To assist with the interpretation of this interaction, post hoc analyses were conducted. We used a cutoff of FSIQ < 100 versus FSIQ > 100, because children with major impairments had been excluded. Among children exposed to either higher or lower numbers of invasive procedures (median split), we examined whether a change in FA from the 75th percentile to the 25th percentile corresponded with a decrease in FSIQ > 2.60 (i.e. beyond the SE of measurement). Specifically, among the children with lower FSIQ (<100), exposed to higher numbers of invasive procedures (.74 invasive procedures), a change in FA in the posterior subcortical white matter from the 75th percentile (0.67) to the 25th percentile (0.58) corresponded to a 13.1 point decrease in FSIQ. In contrast, a change in FA from the 75th percentile to the 25th percentile for children exposed to lower numbers of invasive procedures (<74 invasive procedures) corresponded to a non-significant 0.86 point change in FSIQ, less than the SE of measurement for FSIQ.
Table 5.4 Higher Number of Invasive Procedures and Lower Fractional Anisotropy of the Superior White Matter Predicts Lower IQ

<table>
<thead>
<tr>
<th></th>
<th>Full Scale IQ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n=50</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td><strong>P</strong></td>
<td></td>
</tr>
<tr>
<td>Number of invasive procedures x fractional anisotropy</td>
<td>412.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Fractional anisotropy</td>
<td>-735.63</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>83.35</td>
<td>0.005</td>
</tr>
<tr>
<td>Gestational age</td>
<td>0.88</td>
<td>0.57</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-1.64</td>
<td>0.80</td>
</tr>
<tr>
<td>Illness severity</td>
<td>-0.50</td>
<td>0.03</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>-5.22</td>
<td>0.54</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>1.75</td>
<td>0.84</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>6.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Gender</td>
<td>0.37</td>
<td>0.93</td>
</tr>
<tr>
<td>Age at scan</td>
<td>-168.10</td>
<td>0.12</td>
</tr>
<tr>
<td>Brain injury</td>
<td>-2.41</td>
<td>0.67</td>
</tr>
</tbody>
</table>
5.3.4 Interaction Between Number of Invasive Procedures and White Matter Tracts in Relation to FSIQ

The interaction between the number of invasive procedures and FA values of the white matter tracts was not associated with FSIQ ($B = -304.22; \, P = .46; \, CI: -1106.38 – 497.94$).

5.3.5 Interaction between Number of Invasive Procedures and Superior White Matter in relation to the WISC-IV Indices

The interaction between the number of invasive procedures and fractional anisotropy of the superior white matter to predict FSIQ, was driven by the Verbal ($B = 402.41, \, P = 0.05, \, CI: -3.40 –$
808.21) and Working Memory ($B=352.98$, $P=0.04$, CI: 24.86–681.11) (Table 5.5) components of the FSIQ, whereas Perceptual Reasoning ($B=296.20$, $P=0.19$, CI: -148.49–740.90) and Processing Speed ($B=266.48$, $P=0.29$, CI: -226.85–759.80) were not significantly associated with the interaction between the number of invasive procedures and FA of the superior white matter.

**Table 5.5 Higher Number of Invasive Procedures and Lower Fractional Anisotropy of the Superior White Matter Predicted Lower Verbal Comprehension and Working Memory**

<table>
<thead>
<tr>
<th></th>
<th>Verbal Comprehension</th>
<th>Working Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Index</td>
<td>Index</td>
</tr>
<tr>
<td></td>
<td>n=50</td>
<td>n=50</td>
</tr>
<tr>
<td>Number of invasive procedures x fractional anisotropy</td>
<td>402.41 0.05</td>
<td>352.98 0.04</td>
</tr>
<tr>
<td>Fractional anisotropy</td>
<td>-675.44 0.09</td>
<td>-638.96 0.04</td>
</tr>
<tr>
<td>Number of invasive procedures</td>
<td>69.93 0.04</td>
<td>76.30 0.005</td>
</tr>
<tr>
<td>Gestational age</td>
<td>-0.88 0.62</td>
<td>1.57 0.27</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>2.81 0.70</td>
<td>-7.14 0.23</td>
</tr>
<tr>
<td>Illness severity</td>
<td>-0.51 0.05</td>
<td>-0.31 0.15</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>0.007 1.00</td>
<td>1.47 0.85</td>
</tr>
<tr>
<td>Morphine exposure</td>
<td>-6.68 0.51</td>
<td>-5.91 0.47</td>
</tr>
<tr>
<td>Postnatal infection</td>
<td>3.65 0.54</td>
<td>6.43 0.18</td>
</tr>
<tr>
<td>Gender</td>
<td>4.28 0.35</td>
<td>-3.62 0.33</td>
</tr>
<tr>
<td>Age at scan</td>
<td>-175.89 0.16</td>
<td>-54.46 0.59</td>
</tr>
<tr>
<td>Brain injury</td>
<td>-8.87 0.17</td>
<td>2.08 0.69</td>
</tr>
</tbody>
</table>
5.4 Discussion

Greater numbers of invasive procedures during neonatal care were associated with altered white matter microstructure at school age in children born very preterm, after accounting for degree of prematurity, systemic illness, medications, and concurrent brain injury. Specifically, in 7-year-olds without severe brain injuries or major neurosensory impairment, a higher number of invasive procedures during NICU care was associated with an increase in radial diffusion values at age 7 years, which in animals models has been related to reduced myelation as opposed to axonal loss (Song et al. 2002; Griffith et al. 2012; Lodygensky et al. 2010). Greater numbers of invasive procedures and reduced myelination of the superior white matter were associated with lower IQ in children born very preterm at school age. The relationship between the number of invasive procedures, brain microstructure and IQ was driven by 2 frontoparietal functions, verbal comprehension and working memory, which share common neural substrates (Glascher et al. 2009).

Preoligodendrocytes are cells that differentiate into myelin-producing oligodendrocytes. These cells are abundant in the preterm brain (Back et al. 2001), and are particularly vulnerable to injury (Back and Miller 2014; Volpe 2009). Oxidative stress results from the production of reactive species or oxidants, is a sequela of cerebral ischemia (shortage of oxygenated blood/reperfusion) (Traystman, Kirsch, Koehler 1991). Reactive oxygen, nitrogen species, and cytokines arrest the development of the preoligodendrocytes (Back et al. 1998; Back et al. 2005; Buntinx et al. 2004; Haynes et al. 2003; Pang, Cai, Rhodes 2005), and lead to altered myelination in preterm infants (Buser et al. 2012).

Oxygen saturation decreases following exposure to invasive procedures (Bauer et al. 2004;
Gonsalves and Mercer 1993; Mainous and Looney 2007). Preterm infants who underwent tape removal during a discontinuation of an indwelling central arterial or venous catheter, had markers of adenosine triphosphate (ATP) utilization and oxidative stress (uric acid and malondialdehyde concentration) in their plasma (Slater et al. 2012). The levels of these markers either remained stable or increased, following the tape removal, correlating with their pain intensity score (Slater et al. 2012). Therefore, the mechanisms underlying the relationship we found between repeated exposure to invasive procedures in the NICU and altered myelination of the superior white matter may be similar to those observed in models of cerebral ischemia (Back et al. 1998).

Myelination first occurs within the central white matter tracts, therefore the superior white matter may have been more enriched in progenitor stages of the oligodendrocyte lineage in contrast with the white matter tracts. Alternatively, the superior and central white matter tracts may have been similarly affected, but the latter had greater potential for recovery.

Alternatively, the superior white matter may have been indirectly affected by elevations in stress hormones. Repeated exposure to invasive procedures is associated with the reprogramming of the HPA axis (Grunau, Weinberg, Whitfield 2004; Grunau et al. 2007). Greater numbers of invasive procedures in the NICU are associated with lower stress hormone cortisol responses at 32 weeks PMA and higher levels at 8 and 18 months CA (Grunau, Weinberg, Whitfield 2004; Grunau et al. 2007). Brain regions rich in glucocorticoid receptors (e.g. prefrontal cortex) are particularly vulnerable to the effects of ongoing stress (McEwen 2004; Meaney et al. 1996). Alterations to the cortical gray matter may have led to alterations to the connecting subcortical white matter regions, as it has been shown that cortical gray and adjacent white matter
demonstrate synchronous maturation in very preterm infants (Smyser et al. 2015). This may also explain why greater number of invasive procedures in the NICU was associated with alterations of the superior white matter, rather than the white matter tracts.

The HPA axis is not the only system changed by repeated exposure to invasive procedures in the NICU. Descending modulation of pain requires the activation of the periaqueductal gray and rostroventral medulla, regions responsible for the release of opioids within the spinal cord. Repeated exposure of invasive procedures in the NICU may lead to hyperinnervation of the periaqueductal gray and rostroventral medulla, thereby altering its functional integrity (LaPrairie and Murphy 2010). Inflammatory pain in neonatal rats has been found to increase the adult endogenous opioid tone (Laprairie and Murphy 2009). Therefore, repeated exposure to invasive procedures in the NICU may lead to chronically elevated opiate peptides, affecting the integrity of the subcortical white matter, which connects to the periaqueductal gray, a region rich in opioid receptors.

We previously reported that postnatal infections were significantly associated with 8% lower overall FA in infants born very preterm (Chau et al. 2012). After accounting for clinical confounders, the magnitude of change observed in this study relative to the number of invasive procedures was comparable (i.e. 7% lower FA in infants exposed to higher numbers of invasive procedures). The difference in FA between those exposed to higher and lower numbers of procedures was significantly related to IQ at 7 years in children born very preterm. This study suggests that the number of invasive procedures infants undergo in the NICU may one of the underlying factors explaining the variation in brain volumes, microstructure, and function, which are associated with cognitive outcomes in children and adults born preterm (Allin et al. 2011;

The study sample was limited; therefore, residual confounding for clinical condition associated with invasive procedures may remain. We were also not able to include concurrent factors such as parenting stress and education in the analysis. Furthermore, the post hoc analyses should be interpreted with caution. FA values reach the noise floor in the cortical gray matter by approximately 36 weeks PMA (McKinstry et al. 2002; Vinall et al. 2013b), coinciding with neuronal maturation, synaptogenesis, and the disappearance of radial glial cells (Benders et al. 2014; Deipolyi et al. 2005; Jespersen et al. 2012; Kroenke et al. 2007; McKinstry et al. 2002; Sizonenko et al. 2007). Therefore, we could not examine the long-term relationships between the number of invasive procedures and DTI measures of cortical gray matter on cognitive outcomes in children born very preterm. More studies using alternative methods for quantifying neuronal integrity (e.g. cortical thickness, volumetrics) are needed. The results of this work are a first step in understanding the relationship between the number of invasive procedures in the NICU, brain microstructure, and neurodevelopmental outcomes at school age.

In this cohort of preterm children without severe brain injury or major neurosensory/motor/cognitive impairment, we demonstrated that after accounting for degree of prematurity, systemic illness, medication exposures, and concurrent brain injury, greater numbers of invasive procedures together with alterations in white matter microstructure predicted lower IQ at school age. This research emphasizes the importance of finding ways to manage procedural stress in the NICU in order to optimize brain and neurodevelopmental
outcomes in this vulnerable population.
CHAPTER 6
SUMMARY AND DISCUSSION OF RESEARCH FINDINGS

6.1 Summary of Results

In two prospective cohorts of infants born very preterm we examined the relationships between invasive procedures in the NICU, postnatal growth, brain development, neurodevelopmental and behavior. Moreover, we investigated the extent that positive parental caregiving can moderate the relationship between invasive procedures and internalizing (anxiety/depressive) behaviors in children born very preterm. We found that over and above prematurity, systemic illness, and brain injury, greater exposure to invasive procedures was associated with poorer growth, altered brain development, and poorer cognitive and behavioral outcomes. Importantly, we also found that among children exposed to greater numbers of invasive procedures in the NICU, higher parent sensitivity and nonhostility were related to less internalizing behavior at 18 months CA in children born very preterm.

Postnatal growth is an indicator of how well an infant is thriving (Ehrenkranz et al. 1999). In the first study we examined whether repeated exposure to pain/stress was related to slower growth in the NICU, independent of size at birth, illness severity on day 1, days of mechanical ventilation, infection, PMA, morphine and corticosteroid exposure. Greater exposure to invasive procedures was associated with slower postnatal body and head growth until approximately 32 weeks PMA. However, neither early (prior to approximately 32 weeks PMA) nor later (after approximately 32 weeks PMA) exposure to invasive procedures was associated with weight and head circumference percentiles at term-equivalent age. Similarly, cumulative exposure to invasive procedures was not associated with weight and head circumference percentiles at term-
equivalent age. Therefore, it is only during the first weeks of life when infants require more procedures, and are subject to significant energy and nutrient deficits (Ehrenkranz et al. 1999; Embleton, Pang, Cooke 2001), that we see a relationship between the number of invasive procedures in the NICU and postnatal growth.

Given that greater number of invasive procedures in the NICU is associated with slower postnatal growth, and slower growth in NICU is associated with poorer neurodevelopmental outcomes (Ehrenkranz et al. 2006), it was important to discern whether postnatal body and head growth was associated with cortical development after accounting for clinical confounders associated with growth and brain development (GA, size at birth, sex, WMI, IVH, cerebellar hemorrhage, patent ductus arteriosus, days on mechanical ventilation, postnatal infection, necrotizing enterocolitis). We found that the change in weight, length and head circumference between approximately 32 and 40 weeks PMA was associated with altered maturation of the gray matter, but not the white matter. More specifically, the direction of change within the cortical gray matter suggested that slower postnatal growth was associated with poorer maturation of the basal dendritic arbor of cortical neurons, consistent with findings of Dean et al., (2013). Corticosteroids were not associated with weight change, and excluding the children who received corticosteroids did not change the results. Therefore, by reducing and better managing the number of invasive procedures in the NICU, and reducing calorie and energy deficits, clinicians may have an opportunity to improve postnatal growth and perhaps to optimize cortical brain development in children born very preterm.

We then considered the role of the parent in moderating the long-term effects of repeated exposure to invasive procedures. Repeated exposure to invasive procedures in the NICU is
associated with increased internalizing (anxiety/depressive) behaviors at school age in children born very preterm (Ranger et al. 2014). As early as 2 years CA, children born very preterm show more internalizing behaviors compared to children born full-term (Spittle et al. 2009). Therefore, we explored whether greater exposure to invasive procedures is associated with internalizing behaviors at 18 months CA, and whether parent behavior (adjusted for parenting stress, number of children in the home, years of education, and age) was able to buffer the relationship between invasive procedures (adjusted for GA, illness severity on day 1, morphine exposure, days on mechanical ventilation), and internalizing behavior (adjusted for cognition and gender) in children born very preterm. Children born very preterm demonstrated more internalizing behaviors compared to children born full-term at 18 months CA, consistent with previous findings at age 2 (Brummelte et al. 2011b; Tu et al. 2007). Greater numbers of invasive procedures in the NICU were associated with higher parental report of internalizing behaviors in children born very preterm. Among children exposed to a higher number of invasive procedures, they demonstrated less internalizing behaviors at 18 months CA if their parents were emotionally available (i.e. sensitive and nonhostile). Additionally, higher parenting stress, more children in the home and more years of parent education were independently associated with fewer internalizing behaviors in children born very preterm. Importantly, in children born full-term, parent factors did not predict internalizing behavior. Our findings are consistent with previous findings from our group and others that found children born very preterm are more sensitive to interactions with their parents compared to children born full-term (Brummelte et al. 2011b; Crnic and Greenberg 1987; Tu et al. 2007). The parent is an important moderator of neonatal procedural stress. By helping caregivers, particularly those who are first time parents, concerned about their parenting ability, and/or are low SES, improve their emotional availability toward the child we may be able to reduce the prevalence of anxious/depressive behaviors among children
exposed to a higher number of invasive procedures in the NICU.

The last study examined whether repeated exposure to invasive procedures in the NICU was associated with altered brain development and cognition in children born very preterm at 7 years of age. Previously, our group has shown that greater number of invasive procedures in the NICU were associated with altered brain microstructure in the NICU and at term equivalent age (Brummelte et al. 2012; Zwicker et al. 2013). These results were consistent with Smith et al. (2011), that also found more stressors in the NICU were associated with decreased frontal and parietal brain width, altered diffusion measures and functional connectivity in the temporal lobes. Our group has also found evidence that these relationships persist beyond early life as greater number of invasive procedures in the NICU were associated with reduced cortical volumes and altered function at school age (Doesburg et al. 2013; Ranger et al. 2013). Moreover, greater exposure to invasive procedures in the NICU was associated with poorer cognitive outcomes at 8 and 18 months CA (Grunau et al. 2009). However, it was not known whether repeated exposure to invasive procedures together with alterations in brain microstructure leads to poorer cognitive outcomes in children born very preterm. Therefore, we examined whether greater exposure to invasive procedures was associated with altered superior white matter or white matter tracts, adjusting for GA, SGA, illness severity on day 1, days of mechanical ventilation, postnatal infection, age at scan, brain injury. In separate models the effects of surgery and corticosteroid exposure were also considered. We also examined whether the number of invasive procedures and the integrity of the white matter were associated with IQ at age 7.5 in children born very preterm, after adjusting for clinical confounders. We found that greater numbers of invasive procedures in the NICU was associated with altered superior white matter, but not the white matter tracts, after adjustment for clinical confounders. Neither
surgeries nor steroid exposure changed the results. The direction of change in the superior white matter suggested that the difference between those exposed to higher numbers of invasive procedures and lower numbers of invasive procedures was the amount of myelination in the superior white matter, as opposed to the number or complexity neural cells, compatible with histopathological correlates of MRI abnormalities using animal models (Song et al. 2002; Griffith et al. 2012; Lodygensky et al. 2010). Higher number of invasive procedures and reduced myelination of the superior white matter were associated with lower IQ at 7.5 years of age in children born very preterm. Therefore, the impact of repeated exposure to invasive procedures extends well beyond the NICU stay, and is related to both altered brain development and lower IQ in children at school age, over and above other clinical confounders. It is important that we find ways to alleviate pain/stress that are brain protective in order to improve the lives and outcomes of this vulnerable population.

6.2 Timing of Exposure to Invasive Procedures

The development of the thalamocortical connections in the late fetus and preterm infant provides the necessary framework for sensory-driven organization, and cortical processing of noxious stimuli. Between 24 and 25 weeks PMA, thalamocortical afferents accumulate in the subplate (Kostovic and Rakic 1990; Kostovic and Judas 2002; Kostovic and Judas 2010). These afferents form transient, functional circuits with subplate neurons, before proliferating into the cortical layers between 26 and 28 weeks PMA (Ayoub and Kostovic 2009; Kostovic and Judas 2010). It is at this time that evoked potentials can be recorded from the somatosensory cortex.

Perlman and Volpe (1983) were the first to examine the relationships between pain/stress on brain activity. They used transcutaneous Doppler to measure blood flow in the anterior cerebral
arteries before, during, and 5 minutes after the cessation of routine suctioning in 35 intubated infants, between 26 to 35 weeks PMA (Perlman and Volpe 1983). They found an increase in cerebral flow velocity during the suctioning procedures, which corresponded with an increase in blood pressure (Perlman and Volpe 1983). The results of this early work have since been extended by studies using EEG and near-infrared spectroscopy technology (Bartocci et al. 2006; Fabrizi et al. 2011; Slater et al. 2006; Slater et al. 2010a).

As early as 24 weeks PMA, nociceptive-specific and sensory potentials have been recorded from the somatosensory cortex (Slater et al. 2010a). However, prior to 35-37 PMA, EEG responses touch and heel lance appear as dispersed neuronal bursts, in contrast to the modality-specific, localized, evoked potentials seen at term-equivalent age (Fabrizi et al. 2011). These changes in the EEG recordings correspond to neuronal maturation, synaptogenesis and disappearance of the radial glial cells in the cortex (Benders et al. 2014; Deipolyi et al. 2005; Jespersen et al. 2012; Kroenke et al. 2007; McKinstry et al. 2002; Sizonenko et al. 2007). Therefore, prior to 35 weeks PMA, given the immaturity of thalamocortical and corticocortical connections, infants are less capable of distinguishing between tactile from nociceptive stimulation. They also demonstrate a significant lowering of threshold or "sensitization" to repeated stimulation (Andrews and Fitzgerald 1994; Fabrizi et al. 2011; Fitzgerald, Millard, McIntosh 1989; Holsti et al. 2005; Holsti et al. 2006). Due to the vulnerability of the developing cortical circuitry (Back and Miller 2014) their inability to differentiate between tactile and noxious stimulation (Fabrizi et al. 2011), and sensitization to repeated stimulation (Andrews and Fitzgerald 1994; Fabrizi et al. 2011; Fitzgerald, Millard, McIntosh 1989; Holsti et al. 2005; Holsti et al. 2006), infants <35 weeks PMA may be particularly vulnerable to repeated procedural pain/stress. Grunau, Miller and colleagues have provided the first evidence that repeated exposure to invasive procedures is
associated with altered brain development, and poorer cognitive motor and behavioral outcomes in infants born very preterm, even after accounting for prematurity and systemic illness in the NICU (Brummelte et al. 2012; Doesburg et al. 2013; Grunau et al. 2009; Ranger et al. 2013; Ranger et al. 2014; Vinall et al. 2013a; Zwicker et al. 2013). However, this research highlights that it is not just the quantity and duration of exposure to invasive procedures that is important to the development of infants born very preterm, but also the timing of exposure.

Lower tactile threshold together with sensitization to repeated touch in preterm neonates, may lead to an exhaustion of resources in infants <35 weeks PMA, which is detrimental to the developing cortex. Subplate neurons are vulnerable to nutrient insufficiency, which can lead to focal or widespread white matter injury, as well as reduced cortical gray matter (Inder et al. 1999). Infants born very preterm have a limited metabolic reserve, and in the first weeks of life, they accumulate a significant nutrient and energy deficit (Embleton, Pang, Cooke 2001; Polin, Fox, Abman 2003). We have demonstrated for the first time that greater exposure to invasive procedures early in life (approximately <32 weeks PMA) is associated with slower body and head growth early in the NICU rather than at term-equivalent age. Furthermore, we found that slower growth in the NICU is associated with reduced maturation of the cortical gray matter.

Repeated exposure to invasive procedures in the NICU appears to interfere with cortical maturation. Subplate neurons and preoligodendrocytes are particularly vulnerable to excitotoxicity, oxidative stress, and inflammation (Back and Miller 2014; Volpe 2009), which can result from repeated exposure to invasive procedures (Anand et al. 2007; Brummelte et al. 2012; Duhrsen et al. 2013; Hansson 2006; Slater et al. 2012). Evidence for the disruption of the subplate neurons as a result of early exposure (birth to approximately 32 weeks PMA) to
pain/stress comes from our preliminary work examining the relationship between invasive procedures and altered maturation of the cortical gray matter (Vinall et al. 2014a). Moreover, our group has found that greater exposure to invasive procedures in the NICU was associated with reduced cortical volumes and altered cortical function at 7 years of age (Doesburg et al. 2013; Ranger et al. 2013). Preoligodendrocytes are cells that ensheath axons prior to differentiating into myelin-producing oligodendrocytes (Volpe 2009). Disturbances in myelination as seen with diffuse WMI, the most extensive lesions in preterm neonates, are due to the selective vulnerability of preoligodendrocytes, which account for approximately 90% of the oligodendrocyte population at 28 weeks PMA (Back et al. 2001; Back and Miller 2014; Buser et al. 2012). Previously, our group has shown that greater exposure to invasive procedures prior to 32 weeks PMA was associated with reduced brain maturation in the NICU, and at term-equivalent age (Brummelte et al. 2012). Early exposure to invasive procedures (birth to approximately 32 weeks PMA) rather than later (approximately 32 to 40 weeks PMA) was more detrimental to the developing white matter (Brummelte et al. 2012). Moreover, this relationship appeared to persist beyond term-equivalent age, as currently we found that greater exposure to invasive procedures in the NICU was associated with altered myelination of the superior white matter at age 7 years in children born very preterm (Vinall et al. 2014a). Alterations in myelination as a result of repeated exposure to invasive procedures was related to poorer cognitive outcomes at 7 years of age. This is important given that children born very preterm have more cognitive problems relative to children born full-term (Doyle, Casalaz, Victorian Infant Collaborative Study Group 2001; Grunau, Whitfield, Fay 2004; Johnson et al. 2009; Larroque et al. 2008; Lind et al. 2011; Marlow et al. 2005). This research suggests that the etiology of neurodevelopmental problems in very preterm infants, who escape major brain injury, is at least in part explained by disturbances in brain development through early exposures.
to invasive procedures in the NICU.

6.3 Mechanisms Linking Invasive Procedures, Growth, and Behavior

Exposure to invasive procedures activates the HPA axis results in the release of cortisol, epinephrine and neuroepinephrine, which stimulates a physiological response to nociceptive stimulation (i.e. increased heart rate, oxygen consumption, and blood pressure). This leads to changes in cerebral oxygenation and cerebral blood volume (Pryds 1991; Yamamoto et al. 2003), which can cause episodes of ischemia and/or reperfusion, affecting the preoligodendrocytes (Back et al. 1998; Baud et al. 2004). This physiological response also requires a substantial amount of energy, which is taxing for infants with limited resources (Ranger, Johnston, Anand 2007). The importance of adequate nutrition for optimal brain development is certainly recognized, though it may be difficult to achieve (Embleton, Pang, Cooke 2001; Keunen et al. 2012). Therefore, infants exposed to a higher number of invasive procedures may deplete energy needed to fully support growth and cortical gray matter development.

Ongoing stress in the NICU may suppress the production of growth hormones (Tsigos and Chrousos 2002). IGF-1 values are associated with postnatal weight gain (Kurtoglu et al. 2010; van de Lagemaat et al. 2013), brain volumes (Hansen-Pupp et al. 2011; Hansen-Pupp et al. 2013), and neurodevelopmental outcomes at 2 years (Hansen-Pupp et al. 2013). Greater exposure to invasive procedures early in life may lead to the reduction in IGF-1 values, thereby contributing to slower growth and altered cortical development in infants born very preterm.

Chronic stress also suppresses immune function (Chrousos 1995; Elenkov et al. 1999; Tsigos
and Chrousos 2002). Infants exposed to greater numbers of invasive procedures early in life were more likely to have an infection after 32 weeks PMA (Vinall et al. 2012). The presence of infection after 32 weeks PMA was associated with slower body growth (Vinall et al. 2012).

Neonatal infection leads to systemic inflammation, and is often associated with reduced cerebral blood flow (Keunen et al. 2012). These factors can lead to the activation of microglia and release of free radicals and pro-inflammatory cytokines (Back et al. 2001; Volpe 2009), arresting the development of preoligodendrocytes (Back and Miller 2014; Buser et al. 2012) and altering the development of the white matter (Adams et al. 2010; Chau et al. 2009; Chau et al. 2012; Vinall et al. 2013b; Zwicker et al. 2013).

Programming of the HPA axis occurs during fetal and neonatal development (Matthews 2002). Repeated exposure to invasive procedures in the NICU is associated with dampened cortisol expression in the NICU, heightened cortisol expression during early childhood, and reduced cortisol expression at school age (Brummelte et al. 2015; Grunau, Weinberg, Whitfield 2004; Grunau et al. 2007; Grunau et al. 2013). It would appear that repeated exposure to invasive procedures in the NICU programs the HPA axis for a stressful postnatal environment. However, evidence from animal models suggest that positive parental interaction may be able to prevent and/or ameliorate the effects of invasive procedures on stress system development. Variations in pup licking and grooming during the first week of life affects HPA and behavioral responses to stress, and is correlated with hippocampal glucocorticoid receptor expression in adulthood (Caldji et al. 1998; Francis et al. 1999a; Liu et al. 1997; Menard, Champagne, Meaney 2004; van Hasselt et al. 2012; Weaver et al. 2004; Zhang et al. 2006). In animals models, adult offspring of high licking and grooming mothers had greater hippocampal glucocorticoid receptor expression, better glucocorticoid feedback sensitivity, reduced corticotrophin releasing factor and less
corticosteroid production in comparison to pups reared by low licking and grooming mothers (Francis et al. 1999a; Liu et al. 1997). Offspring of high-licking and grooming dams also demonstrate fewer anxiety-like behaviors as adults (Caldji et al. 1998; Pena et al. 2014; Starr-Phillips and Beery 2014; van Hasselt et al. 2012). Previously, our group has demonstrated that positive maternal interaction was associated with lower cortisol levels at 18 months CA (Brummelte et al. 2011b). Moreover, in the same study lower cortisol expression at 18 months CA was associated with less internalizing behavior (Brummelte et al. 2011b). Among the present reported studies, we found that among children exposed to a higher number of invasive procedures in the NICU, if parents were more sensitive and nonhostile, their children also showed less internalizing behavior at 18 months CA (Vinall et al. 2013a). Therefore, it would appear that positive parental interaction moderates the effect of repeated exposure to invasive procedures on the development of stress-sensitive anxiety or depressive behaviors in children born very preterm.

In summary, repeated exposure to invasive procedures in the NICU is associated with alterations in postnatal growth, brain development and neurodevelopmental outcomes, even after accounting for prematurity, systemic illness and brain injury. However, more research using animal models of prematurity is needed, in order to better understand the mechanisms underlying each of these relationships. In particular, this research highlights the importance of managing procedural pain/stress in the NICU in order to improve longitudinal outcomes in this vulnerable population.

6.4 Pain/Stress Management in the NICU

Although clinicians recognize that there is a physiologic rationale to manage procedural pain,
there are major challenges related to both pharmacological and non-pharmacological interventions.

6.4.1 Morphine

Morphine has been the most commonly used opiate for analgesia in the NICU (Anand 2007). In each of the three studies examining impact of invasive procedures, morphine exposure neither ameliorated nor exacerbated the effects of invasive procedures on postnatal growth, brain microstructure, cognitive or behavioral outcomes (Vinall et al. 2012; Vinall et al. 2013a; Vinall et al. 2014b). Similarly, Grunau and colleagues found no effect of morphine exposure cumulatively from birth to term-equivalent age on white or subcortical gray matter, cortisol levels, or cognitive development (Brummelte et al. 2012; Doesburg et al. 2013; Grunau et al. 2005; Grunau et al. 2007; Grunau et al. 2009). They did, however, find that greater morphine exposure was associated with altered cerebellar maturation early in life and at term-equivalent age (Zwicker et al. 2012), and poorer motor development at 8 months, but not 18 months CA (Grunau et al. 2009). Among ventilated infants born very preterm, greater morphine exposure was associated with increased internalizing behaviors at 7 years of age (Ranger et al. 2014).

The risks and benefits of continuous use of analgesics and anesthetics in very preterm infants are unclear (McPherson and Grunau 2014). In two large randomized controlled trials of effects of morphine ventilated preterm neonates, continuous morphine infusions did not reduce infant pain scores relative to placebo during an acute painful procedure (i.e suctioning) (Anand et al. 2004; Simons et al. 2003b). Moreover, providing morphine as a loading dose prior to continuous infusion also did not lower preterm infant’s pain scores in response to a heel lance procedure (Carbajal et al. 2005). Although there was evidence that morphine reduced pain/stress from
mechanical ventilation, it did not appear to provide adequate analgesia for acute procedural pain among preterm infants (Anand et al. 2004; Simons et al. 2003b).

Results from these two trials also raised concerns regarding the short and long-term effects of morphine. In the short-term continuous morphine infusions lead to longer duration of mechanical ventilation and longer time to reach enteral feeding (Anand et al. 2004). Following a small of subset of these children (N=19), at age 5-7 years children in the morphine-exposed group weighed less, had smaller head circumferences, impaired short-term memory, and according to parent report, they had more difficulty establishing friendships compared to children in the placebo-treated group (Ferguson et al. 2012). Morphine exposed children from the other trial were found to have poorer visual processing at age 5 years, and greater internalizing behaviors according to teacher report at age 8 to 9 years relative to the placebo-treated children (de Graaf et al. 2011). Although in present cohort studies morphine exposure neither ameliorated nor exacerbated the effects of invasive procedures on outcomes, given the lack of efficacy for acute pain management and risk of short and long-term effects on neurodevelopmental outcomes, it is recommended that opiates be used sparingly in the NICU for nonsurgical pain management of ventilated preterm neonates.

6.4.2 Sucrose
Sucrose is the most widely used non-pharmacologic intervention for the treatment of minor procedures in preterm infants (Taddio et al. 2009). However, the BC Children’s and Women’s Hospital is one of the few remaining hospitals in Canada that does not use sucrose for the management of acute procedural pain in preterm infants, due to lack of studies of long-term
effects beyond NICU discharge. At this time very little is known about the mechanisms of action of sucrose in human infants, and whether there are long-term effects of repeated use of sucrose in the NICU on brain, metabolism or neurodevelopmental outcomes (Holsti and Grunau 2010). In preterm infants, administration of 24% sucrose (0.01 to 0.02 g), 2 minutes prior to minor procedures is efficacious in reducing crying, facial grimacing, and motor activity, therefore, it reduces pain scores in infants (Stevens et al. 2013). However, its effectiveness in modifying physiological indices (e.g. heart rate, heart rate variability, oxygen saturation) varies (Stevens et al. 2013), and it has been suggested that sucrose may act as a sedative rather than an analgesic (Fitzgerald 2009; Holsti and Grunau 2010). One study to date has examined the effects of sucrose on neurodevelopmental outcomes at 36 and 40 weeks PMA, and found that greater exposure to sucrose (>10 doses in 24 hours) was associated with poorer attention and motor outcomes (Johnston et al. 2002; Johnston et al. 2007). Infants born very preterm can receive as many as 15 invasive procedures per day in the first few weeks of life (Carbajal et al. 2008; Stevens et al. 2003). The most recent national review of sucrose use revealed that infants in the NICU may receive as many as 24 doses in one day (Taddio et al. 2009). While there is consistent support for the use of sucrose for acute painful procedures (Stevens et al. 2013), much more research is needed with regards to long-term effects, given the potential for high cumulative exposure to sucrose over the course of the NICU stay (Holsti and Grunau 2010; Stevens et al. 2013).

Most relevant to the work presented here was the finding that although sucrose reduces behavioral and sometimes physiological responses (Stevens et al. 2013), it does not dampen EEG responses to invasive procedures (Slater et al. 2010b). Therefore, sucrose does not appear to protect the brain from repeated stimulation. Furthermore, it was recently reported that very
preterm infants given a single dose of oral sucrose, prior to heel lance demonstrated significantly greater adenosine triphosphate (ATP) use and oxidative stress (Asmerom et al. 2013), increasing the likelihood that the intervention given to protect the infant from the adversity may have unintended consequences. This research highlights the importance of evaluating our current pain management strategies in the NICU for the extent that they are brain protective.

6.4.3 Swaddling, Facilitated Tucking, Non-nutritive Sucking, Kangaroo Care
Sucrose reduces behavioral responses by 16% and 28% on pain-assessment scales (Johnston et al. 1997; Stevens et al. 2005). However, this can also be achieved by using other environmentally supportive interventions such as swaddling, facilitated tucking, non-nutritive sucking and kangaroo care (Axelin, Salantera, Lehtonen 2006; Carbajal et al. 1999; Castral et al. 2008; Ferber and Makhoul 2008; Ludington-Hoe, Hosseini, Torowicz 2005; Pillai Riddell et al. 2011). Swaddled infants are securely wrapped in a blanket to prevent the infant's limbs from moving around excessively. Facilitated tucking involves holding the infant, keeping the arms and legs in a flexed position, close to the trunk. For non-nutritive sucking, a pacifier is placed into an infant's mouth to stimulate sucking behavior. During kangaroo care the infant is placed on the caregiver's bare chest for skin-to-skin contact. At the BC Children’s and Women’s Hospital these non-pharmacological interventions are the standard of care for acute painful/stressful procedures in very preterm infants. To the best of our knowledge, no studies to date have examined whether swaddling or non-nutritive sucking influence brain responses or long-term outcomes in infants born very preterm.

One study examined whether sucrose, facilitated tucking, or sucrose together with facilitated tucking influenced heart rate, oxygen saturation, and/or cortical responses following a heel lance
Despite increases in heart rate in all three groups, Gerull et al. (2013) did not find changes in oxygen saturation or near-infrared spectroscopy measures following heel lance. The lack of placebo group in this study makes it difficult to discern whether facilitated tucking and sucrose provided adequate analgesia. No studies to date have looked at whether the use of facilitated tucking in the NICU leads to better longitudinal outcomes in very preterm infants.

Effects of kangaroo care on brain function has been examine previously, however these analyses were not performed in conjunction with an invasive procedure. One study found that 30-min of skin-to-skin contact with mothers lowers infant heart rate and improves peripheral oxygen saturation and cerebral blood flow (Korraa et al. 2014). Another compared preterm infants who underwent 8 weeks of kangaroo care to two cohorts of premature and full-term neonates that did not undergo skin-to-skin intervention (Kaffashi et al. 2013). They found that the kangaroo care group had more complex EEG signaling, indicative of greater brain maturation (Kaffashi et al. 2013). The pattern of signaling at 40 weeks PMA was comparable to that of full-term neonates (Kaffashi et al. 2013). These changes to brain maturation and function may have led to long-term improvements in neurodevelopmental outcomes. Infants that received 1 hour of kangaroo care for 14 consecutive days had attenuated stress responses, more mature autonomic functioning, better organized sleep, more cognitive control, and greater mother–child reciprocity at 10 years of age (Feldman, Rosenthal, Eidelman 2014). Although these studies did not examine directly the extent that kangaroo care protects the brain from aversive stimuli, there is growing evidence of its effectiveness in stress reduction, emphasizing the importance of parental involvement in the management of stress in the NICU.

Therefore, to the best of our knowledge no studies to date have examined whether swaddling,
facilitated tucking, non-nutritive sucking or kangaroo care ameliorates the relationship between repeated exposure to invasive procedures in the NICU and poorer neurodevelopmental outcomes in infants born very preterm. Moreover, there is very little evidence to suggest that these procedures protect the brain from repeated exposure to aversive stimuli. Currently, these pain management strategies are the standard of care at the BC Children’s and Women’s Hospital for acute painful/stressful procedures. Despite the routine management of pain in the NICU, we still find relationships between greater exposure to invasive procedures and altered growth, brain development, and poorer neurodevelopmental and behavioral outcomes in 2 cohorts of children born very preterm (Vinall et al. 2012; Vinall et al. 2013b; Vinall et al. 2014b). Future research is needed to evaluate the extent that current pain management strategies reduce the long-term effects of repeated exposure of invasive procedures on developing pain/stress systems.

6.5 Importance of Parent Involvement in the NICU

Supporting positive parent interactions in the NICU, and involving parents in infant care is important for minimizing neonatal pain/stress and improving outcomes after discharge from the NICU. Just a few short training sessions with a developmental specialist could help to minimize infant stress and improve white matter microstructure at term equivalent age in infants born very preterm (Milgrom et al. 2013). There is mounting evidence that caring for infants in single-family rooms, as opposed to the traditional open-bay model, will improve parent involvement and outcomes of infants born very preterm (Shahheidari and Homer 2012). Therefore, there has been widespread adoption of the single family room model of care. Recently, it was found that very preterm infants in the single family room NICU weighed more at discharge, had a greater rate of weight gain, required fewer medical procedures, had a lower PMA at full enteral feed, less sepsis, showed better attention, less physiologic stress, less hypertonicity, less lethargy, and
less pain (Lester et al. 2014). Differences in weight at discharge, and rate of weight gain were mediated by increased developmental support, whereas differences in stress and pain were mediated by maternal involvement (Lester et al. 2014). However, in private NICU rooms if families are less involved, than at term-equivalent age, infants born very preterm had alterations in brain structure and function, which may have contributed to their lower motor and language scores at 2 years of age (Pineda et al. 2014). Therefore, encouraging parental involvement in the NICU continues to be an important part of infant care, given that positive parent interaction may moderate pain/stress in children born very preterm (Vinall et al. 2013a), thereby improving behavioral outcomes.

6.6 Limitations

There are several limitations to the papers presented in this dissertation. Overall, the goal of this work was to understand the impact of repeated exposure to invasive procedures during neonatal intensive care on brain microstructure, growth, neurodevelopment and behavior of children born very preterm. However, in clinical cohort studies, cause and effect cannot be inferred, given that only associations among variables can be examined. Therefore, it is important that our findings are consistent with the basic animal studies in this field.

As is a limitation with all clinical studies, we could not account for all of the factors that may impact the outcome variables in question. The data for these papers were drawn from longitudinal cohort studies designed to examine long-term effects of invasive procedures on brain and neurodevelopment. Examination of physical growth, for example, was not included in the original aims of these studies. Therefore, data on nutrition were not collected. However, it has been shown previously that birth weight and nutritional intake accounts for approximately
52% of the variance in postnatal growth (Embleton, Pang, Cooke 2001). Therefore, a portion of the unexplained variance in postnatal growth may be accounted for by repeated exposure to invasive procedures. However, there still remains antenatal (e.g. maternal nutrition, smoking) and neonatal stress factors (e.g. noise, light, maternal deprivation) not accounted for by our study, which may also impact postnatal growth, brain and neurodevelopment.

Standard care in the NICU at the BC Children’s and Women’s Hospital for acute painful procedures includes the use of either facilitated tucking, non-nutritive sucking, swaddling and/or kangaroo care, which may have reduced infant stress during procedures. Moreover, morphine was often provided for infants undergoing mechanical ventilation. In our data analyses, we adjusted the statistical models for cumulative morphine exposure, but we could not account for what was happening when the medication was given or how well timed it was to the painful/stressful procedure. Moreover, we could not account for the specific pain management that may or may not have been provided during each procedure. The efficacy of morphine for alleviating procedural pain/stress is likely not the same for every procedure in the NICU (Anand et al. 2004; Carbajal et al. 2005; Simons et al. 2003b). Therefore, it is notable, that in the context of our routine care that we still found an association of invasive procedures with growth, brain, neurodevelopment, and behavior in preterm infants and children, after adjusting for neonatal, clinical, and social confounders.

Pain and stress are difficult to discriminate in infants born very preterm undergoing neonatal intensive care. Preterm infant responses to invasive procedures vary depending on GA, sleep-wake state, illness severity, medications, previous exposures to pain and NICU care (Gibbins et al. 2008; Grunau et al. 2001; Holsti et al. 2005; Holsti et al. 2006; Holsti et al. 2008; Johnston
and Stevens 1996; Johnston et al. 1999; Slater et al. 2009; Stevens, Johnston, Horton 1994; Valeri et al. 2012). Although it is unlikely that every procedure elicits the same amount of pain/stress, the extent of reactivity is not simply a function of the type of procedure. Thus, we counted each procedure and/or attempt at a procedure listed in Appendix Table A.1, as 1 invasive procedure, without assigning any sort of ranking to the various types of procedures included in this list. It was not feasible to measure the pain/stress reactivity of neonates who receive on average 4 to 14 invasive procedures per day, across the NICU stay (Brummelte et al. 2012; Carbajal et al. 2008; Doesburg et al. 2013; Grunau, Weinberg, Whitfield 2004; Johnston et al. 2011; Simons et al. 2003a). Moreover, handling, diaper changes, bathing, and other procedures that are not inherently stressful to full-term newborns, can also induce considerable stress in this fragile population. During the “recovery” phase (first 4 min after the last contact by the technician), infants born very preterm continued to demonstrate stress cues following clustered care (changing the diaper, measuring the abdominal girth, taking the axillary temperature, and cleaning the mouth with gauze and sterile water) (Holsti et al. 2005). Prior to 35 weeks PMA, after repeated stimulation infants show lower threshold and sensitization to procedures (Andrews and Fitzgerald 1994). Consistent with this, our group found that infants between the ages 30 and 32 weeks PMA had heightened facial responses to a heel lance procedure when it was preceded by clustered nursing care (Holsti et al. 2006). Although we did not include procedures such as handling in our measure of cumulative procedural stress, another study, which did include a wider range of stressful procedures in the NICU (e.g. diaper and position changes), reported similar findings as our group, with regards to stress and brain development at term-equivalent age (Smith et al. 2011). Therefore, quantification of the number of invasive procedures in the NICU is a useful measure of neonatal pain/stress and is a modifiable risk factor for altered brain and neurodevelopment in children born very preterm.
There are limitations to manual acquisition of data from DTI. “Region of interest” based analysis of DTI can be limited by reproducibility of voxel sampling between scans, and by the risk of partial averaging. To improve accuracy of our measurements and replication between scans, we compared different sizes and positions for our region of interest voxel boxes, and determined the optimal size and placement for them. Future advances in MRI acquisition and analysis that allow for automatic segmentation and quantification of cortical FA from early in life to term-equivalent age may refine our ability to detect differences in cortical maturation related to growth and outcome (Ball et al. 2013; Brown et al. 2014).

In chapter 5, regions of interest were averaged together based on their structural similarity. If regions of interest had been averaged together either by functionally relatedness or using data driven methods, our results may have differed. More studies are needed to determine whether associations between the number of invasive procedures in the NICU and brain development are global, regionally, or functionally specific, particularly in relation to outcome. In addition, cortical gray matter FA values reach the noise floor by 36 weeks PMA (McKinstry et al. 2002; Vinall et al. 2013b). Therefore, we could not examine the long-term effects of the number of invasive procedures on the DTI measures of cortical gray matter. Using alternative neuroimaging methods, our group has demonstrated that greater exposure to invasive procedures in the NICU is associated with reduced cortical volumes and altered cortical function at 7 years of age (Doesburg et al. 2013; Ranger et al. 2013). Animal models may help us to understand the long-term impact of repeated exposures to invasive procedures on the cerebral microstructure of infants born very preterm.
Clinically important, is the threshold at which exposure to invasive procedures may impact growth, brain and neurodevelopmental outcomes. Previously, Grunau et al. (2001) reported that at 32 weeks PMA, prior exposure to 20 invasive procedures appeared to be the point at which diminished behavioral expressions and autonomic responses to heel lance procedures were evident. With regards to the present studies, we were hesitant to propose a threshold for the number of invasive procedures during neonatal care, given the variability in GA, PMA and the number of invasive procedures. Moreover, it is unlikely that all procedures listed in Table A.1 are equally noxious. Therefore, recommending a cut-off for a number of invasive procedures that would not be deleterious for either brain development or neurodevelopmental outcomes is not possible at this stage of knowledge. Although identifying a threshold for the number of invasive procedures during neonatal care is clinically relevant, more research is required before this kind of recommendation can be made. The focus currently is to find ways to reduce the number of procedures performed in the NICU, and to find ways to manage pain/stress that are neuroprotective.

We emphasized how parents can play a vital role in the regulation of stress and development of their infant. Previously it has been shown that mothers who reported lower concurrent stress relative to mothers who reported higher concurrent stress were more sensitive when interacting with their child post-discharge (Muller- Nix et al. 2004; Tu et al. 2007). However, greater parenting stress was related to declining cognitive development between 8 and 18 months CA (Brummelte et al. 2011a; Docherty, Miles, Holditch-Davis 2002). Therefore, parenting stress may in part reflect realistic concerns about their child. A child that displays more internalizing behavior may also influence their parent’s level of stress and quality of responding. Therefore, it is important to keep in mind that although parental sensitivity/nonhostility appears to moderate
the relationship between the number of invasive procedures and internalizing behavior in children born very preterm, infants exposed to higher number of procedures that are reported as having greater internalizing behavior may contribute to parent’s reduced emotional availability.

6.7 Significance of this Research

More than half of children born very preterm will develop cognitive, motor and/or behavioral problems that persist to at least to early adulthood (Aarnoudse-Moens et al. 2009; Anderson, Doyle, Victorian Infant Collaborative Study Group 2003; Doyle, Casalaz, Victorian Infant Collaborative Study Group 2001; Doyle and Anderson 2010; Grunau, Whitfield, Fay 2004; Johnson et al. 2009; Loe et al. 2011; Marlow et al. 2005; Marlow et al. 2007; Spittle et al. 2009; Synnes et al. 2010). This can affect their quality of life, and cause considerable stress and burden for their families. Support for these individuals also puts a substantial strain on medical, educational and social systems.

A major body of literature has focused on describing the differences between children born preterm and full-term. However, few studies have focused on the mechanisms underlying these differences. This collection of works goes beyond the descriptive literature to show that it is not just the matter of being born very preterm, or the illness course in the NICU that leads to differences between these two groups. These infants undergo repeated exposure to invasive procedures, during a sensitive period of brain and stress system development, when they would normally be developing within the protective intrauterine environment. Greater exposure to invasive procedures in the NICU, over and above adjustment for prematurity, systemic illness, brain injury, and other clinical confounders was related to poorer growth, altered brain
development and poorer neurodevelopment and behavior in children born very preterm. Therefore, by reducing frequency and improving management of invasive procedures in the NICU, clinicians may have the opportunity to improve later outcomes of this population.

This work also points to the important role of the parent for managing stress in the premature infant. Infants born very preterm are sensitive to interactions with their parents. We found that even among children exposed to a higher number of invasive procedures in the NICU, if their parent was more emotionally available they had fewer internalizing behaviors in early childhood. We know from previous work that NICU-based interventions that increase parental involvement in pain/stress management have been found to improve parents’ efficacy in supporting their infant post discharge from the NICU (Franck et al. 2011). Moreover, parent sensitivity training in the NICU has been shown to improve white matter maturation at term (Milgrom et al. 2010). Therefore, including parents in the pain/stress management plan of infants born very preterm should help not only to improve the quality of interaction between the parent and child, but also improve neurobehavioral outcomes within this population.

Altogether, the results of this research provides a foundation for beginning to understand the role of neonatal pain/stress in the etiology of neurodevelopmental and behavioral problems in children born very preterm, and provides insight into the potential for early parent intervention strategies to optimize development in this vulnerable population.
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### Table A.1 Invasive Procedures in the Neonatal Intensive Care Unit

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<thead>
<tr>
<th>Procedure</th>
<th>Description</th>
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<tbody>
<tr>
<td>Injections*</td>
<td>Umbilical artery catheter insertion</td>
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<tr>
<td>Chest tube insertion*</td>
<td>Umbilical venous catheter insertion</td>
</tr>
<tr>
<td>Pleural tap*</td>
<td>Lumbar puncture reservoir tap*</td>
</tr>
<tr>
<td>Peripheral artery line insertion*</td>
<td>Brainz needle insertion*</td>
</tr>
<tr>
<td>Peripherally inserted central line insertion/removal*</td>
<td>Heel poke (including glucometer pokes)*</td>
</tr>
<tr>
<td>Penrose insertion/removal*</td>
<td>Suprapubic bladder tap*</td>
</tr>
<tr>
<td>Abscess drained*</td>
<td>Catheter insertion for urine collection</td>
</tr>
<tr>
<td>Peripheral intravenous sited or re-sited*</td>
<td>Venous blood collection*</td>
</tr>
<tr>
<td>Endotracheal tube prong change or re-taping</td>
<td>Glycerin suppository</td>
</tr>
<tr>
<td>Nasogastric tube insertion</td>
<td>Orogastric tube insertion</td>
</tr>
<tr>
<td>Healon/wydase for intravenous burns</td>
<td>Insuflon device site change*</td>
</tr>
<tr>
<td>Pericentesis*</td>
<td>Endotraceal or nasopharyngeal intubation*</td>
</tr>
<tr>
<td>Eye exam</td>
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*Each attempt was counted
*Skin-breaking procedures