Developing translational tools for measuring pain in neonates

Maria Mercedes Cobo Andrade

Oriel College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Hilary 2022

Abstract

Despite the high burden of pain experienced by hospitalised neonates, there are few analgesics with proven efficacy. In part, this is due to the lack of a gold standard to measure pain in neonates. Assessing a complex experience like pain is challenging and, in the absence of self-report, all indicators of pain are surrogate measures. Clinicians often rely on behavioural and autonomic physiological responses which can be subjective and have limited sensitivity and specificity to detect pain. The pain experience requires the nociceptive signal to be processed in the brain. From a very young age, infants have the basic neural functional connectivity for pain perception. Thus, brain activity may provide a quantitative and objective measure of nociceptive processing in the infant brain.

Noxious-evoked brain activity using electroencephalography (EEG) has been previously characterised following experimental noxious stimuli and heel lances. In this thesis, I assessed the generalisability of a brain-derived surrogate measure of pain across various body locations and stimulus modalities. The morphology and latency of the evoked potentials were characterised following clinically-required injections applied to the thigh, and the efficacy of paracetamol during routine immunisations was evaluated in a pilot study.

The magnitude of the brain activity response to the same stimulus intensity can be highly variable between infants. As a result, large sample sizes are often required in studies of analgesic efficacy to account for inter-individual variability. In the second study, I developed an EEG-based paradigm that can be used to determine the baseline sensitivity of individual infants to noxious stimuli and I demonstrated that this paradigm can help to reduce sample sizes in analgesic clinical trials in infants.

The normal development of the nervous system is vulnerable to alterations by multiple factors, with the potential for long-term structural and functional detrimental outcomes. Immune function and sensitivity to pain are closely related, but the impact of early life inflammation on sensory nervous system development is poorly understood. In the final study, I used electrophysiological measures to investigate this relationship. I demonstrated in a cohort of term neonates that inflammation is associated with increased spinal cord excitability and hyperalgesia.

In summary, this thesis advances our understanding of the processing of nociceptive information in the infant brain and demonstrates how the use of brain-derived measures of pain can improve the evaluation of analgesic interventions.

Acknowledgements

This work would not have been possible without the encouragement and support of a team and community of amazing people. Heartfelt thanks to Rebeccah Slater for the opportunity to pursue this academic endeavour in her lab and for her inspirational mentorship and investment in me, which I appreciate deeply. Thank you to Aomesh Bhatt for his insightful guidance and to Eleri Adams for facilitating the conduction of this research in the Neonatal Unit. Likewise, the generous help from the members of the Paediatric Neuroimaging Group, past and present, proved essential to my progress. In particular, I am thankful to Foteini Andritsou, Ria Evans Fry, Gabi Schmidt Mellado, Sezgi Goksan, Luke Baxter and Kirubin Pillay for their clinical and technical assistance and expertise. And to my unofficial academic supervisor, Caroline Hartley thank you for your help throughout the design, analysis and interpretation of the results presented in this thesis, without which I would not have made it this far. I would also like to thank all parents and infants who took part in the studies and USFQ and Senescyt for their financial support.

Special thanks to Deniz Gursul, Marianne van der Vaart and Miranda Buckle for your remarkable friendship, moral and practical support, inspiring example and encouragement throughout the DPhil and beyond. To the friends I encountered in Oxford and became my bubble during pandemic times - thank you Tanzil, Matt, Rebecca, Miriam, Catherine, Daria and Jacob for the much needed and appreciated laughs, chats and celebrations. Thank you to the spiritual support of the University Catholic Chaplaincy particularly to Fr. Matthew, Fr. Ian and Fr. William and to all the Greyfriars community for their warmth, compassion and humour.

Thank you to my family: Elsa, Nani, Juanpa, Yuri, Moni whose love, encouragement and regular video calls from an ocean of distance brightened the often-grey Oxford days. To my mom and my sister thanks for your unconditional love, advice, and everything you have given me to make it to this point. This achievement is as much yours as it is mine. My immense gratitude to, and love for, my husband, Bernardo Gutierrez, for being the best teammate on this, and every adventure; thank you for believing in me and for being so positive and patient, you are truly my vocation and my Home. Finally, all thanks convey to Christ, the source of all I am and aspire to be.

Publications and Funding

Journal Articles

M. M. Cobo, G. Green, F. Andritsou, L. Baxter, R. E. Fry, A. Grabbe, D. Gursul, A. Hoskin, G. S. Mellado, M. Van Der Vaart, E. Adams, A. Bhatt, F. Denk, C. Hartley, R. Slater, Early life inflammation is associated with spinal cord excitability and nociceptive sensitivity in human infants, Nat. Commun.1–8 (2022).

M. M. Cobo, C. Hartley, D. Gursul, F. Andritsou, M. van der Vaart, G. Schmidt Mellado, L. Baxter, E. P. Duff, M. Buckle, R. Evans Fry, G. Green, A. Hoskin, R. Rogers, E. Adams, F. Moultrie, R. Slater, Quantifying individual noxious-evoked baseline sensitivity to optimise analgesic trials in neonates, Elife 10, 1–24 (2021).

S. Kasser, C. Hartley, H. Rickenbacher, N. Klarer, A. Depoorter, A. N. Datta, **M. M. Cobo**, S. Goksan, A. Hoskin, W. Magerl, E. A. Huhn, G. Green, R. Slater, S. Wellmann, Birth experience in newborn infants is associated with changes in nociceptive sensitivity, Sci. Rep. 9, 4117 (2019).

M. van der Vaart, C. Hartley, L. Baxter, G. Schmidt Mellado, **M. M. Cobo**, R. Evans Fry, E. Adams, S. Fitzgibbon, R. Slater, Premature infants display discriminable behavioural, physiological and brain responses to noxious and non-noxious stimuli, Cereb. Cortex , 1–17 (2021).

G. Schmidt Mellado, K. Pillay, E. Adams, A. Alarcon, F. Andritsou, **M. M. Cobo**, R. Evans Fry, S. Fitzgibbon, F. Moultrie, L. Baxter, R. Slater, The impact of premature extrauterine exposure on infants' stimulus-evoked brain activity across multiple sensory systems, NeuroImage Clin. 33, 102914 (2021).

Conferences

M.M. Cobo. (2021) Improving the measurement of neonatal pain. Speaker at the 11th Annual Oxford Neuroscience Symposium.

M.M. Cobo, C. Hartley, S. Goksan, A. Hoskin, and R. Slater. (2019) Noxious-evoked responses after experimental stimulation of the hand, foot and thigh. Poster at the 12th International Symposium on Pediatric Pain, Basel, Switzerland.

Funding

Secretariat for Higher Education, Science, Technology, and Innovation of Ecuador (SENESCYT), Excellence Universities Scholarship. (2017). Doctoral training scholarship, Ecuador.

Contents

List of Figures

List of Tables

List of Abbreviations

ACC . . . . . Anterior Cingulate Cortex
ANOVA . . . . Analysis of Variance
BNFc . . . . . British National Formulary for Children
BOLD . . . . . Blood Oxygen Level-Dependent
CNS . . . . . Central Nervous System
COX . . . . . Cyclooxygenase
CT . . . . . C-Tactile
DC . . . . . Direct Current
DRG . . . . . Dorsal Root Ganglion
DTaP . . . . . Diphtheria, Tetanus, acellular Pertussis
E . . . . . Embryonic Day
ECG . . . . . Electrocardiography
EEG . . . . . Electroencephalography
EMG . . . . . Electromyography
EONI . . . . . Early Onset Neonatal Infection
EPSP . . . . . Excitatory post-synaptic potential
ERP . . . . . Event-Related Potential
fMRI . . . . . Functional Magnetic Resonance Imaging
GA . . . . . Gestational Age
GABA . . . . . Gamma-Aminobutyric Acid
HDU . . . . . High Dependency Unit
HepB . . . . . Hepatitis B
Hib . . . . . Haemophilus influenzae type b
HIE . . . . . Hypoxic Ischaemic Encephalopathy
IASP . . . . . International Association for the Study of Pain
IPSP . . . . . Inhibitory post-synaptic potential
IPV . . . . . Polio
IQR . . . . . Interquartile range
IV . . . . . Intravenous
IVH . . . . . Intraventricular Haemorrhage

KC . . . . . Kangaroo care
MenB . . . . . Meningococcal group B
MHC . . . . . Major Histocompatibility Complex
NCF . . . . . Nucleus Cuneiformis
NFCS . . . . . Neonatal Facial Coding System
NICU . . . . . Neonatal Intensive Care Unit
NIRS . . . . . Near Infra-Red Spectroscopy
NK . . . . . Natural killer
PAG . . . . . Periaqueductal Grey
PC . . . . . Principal Component
PCA . . . . . Principal Component Analysis
PCV . . . . . Pneumococcal
PICC . . . . . Peripherally inserted central catheter
PIL . . . . . Patient Information Leaflet
PIPP . . . . . Premature Infant Pain Profile
PIPP-R . . . . . Premature Infant Pain Profile - Revised
PMA . . . . . Postmenstrual Age
PNA . . . . . Postnatal Age
PSP . . . . . Post-synaptic potential
RMS . . . . . Root Mean Square
ROP . . . . . Retinopathy of prematurity
RVM . . . . . Rostroventral Medulla
SSC . . . . . Skin-to-skin contact
STT . . . . . Spinothalamic Tract

1 Introduction

1.1 Neonatal pain

1.1.1 History of neonatal pain

At the core of the Hippocratic tradition in medicine is the moral principle of *primum non nocere*, "above all, do no harm". While therapeutic interventions are performed to treat patients and improve their health, they can often cause pain and discomfort. In adult medicine pain can only be inflicted with the consent of the patient, provided that they understand and accept that pain is necessary as part of a beneficial treatment [1]. Paediatric practice is more complex as young children may not be competent to consent to necessary diagnostic tests and treatment. Nonetheless, to the extent of their understanding, they can make their views known to their parents and caregivers. As newborn infants have no such understanding and are unable to communicate verbally they are particularly vulnerable to unnecessary or unacceptable levels of pain during medical procedures.

Accounts of pain and disease in infants and children date back to ancient writings with authors arguing on philosophical and physiological grounds [2]. Contrary to the perception that in previous centuries childhood welfare was characterised by neglect and abuse, the idea that infants were particularly sensitive to pain was present before the 20th century. For example, the Hippocratic Aphorisms suggested that pain was in part determined by previous experiences: “Those who are used to bearing an accustomed pain, even if they be weak and old, bear it more easily

than the young and strong who are unaccustomed" [3]. Moreover, the physiological immaturity of infants was thought to be related to more pain sensitivity as the surgeon Felix Wurtz described in *"The Children's book"* in the 17th-century [4]. A review published by The Journal of Pain in 1992 explores these and other views of ancient writers who were concerned about pain in infancy and childhood [2].

Neonatology was formally acknowledged as a discipline in medicine in the 1950s [5]; yet, it is surprising that in the late 20th century the standard practice failed to adequately treat neonatal pain [6]. Newborn infants would undergo surgery without analgesia, under the sole administration of muscle relaxants [7]. Circumcisions were performed without appropriate sedation or analgesia and analgesics were not routinely used during the postoperative period [6]. Awake intubation was recommended as stated in [8] "Small neonates can be intubated awake without muscle relaxation". These practices would not be considered appropriate in older children or adults. Incorrect assumptions about infant pain and over-caution in the use of analgesics are some possible explanations to understand the denial and undertreatment of infant pain in clinical practice. Some of these are discussed below.

- **Infant's nervous systems are too immature to experience pain.** While several studies reported physiological and behavioural changes during painful procedures like heel lances and pinpricks in various body locations [9–11], these were often interpreted as mere reflexes with no cortical involvement and therefore not an indication that infants feel pain [12]. The work of Flechsig in 1920 suggesting that complete myelinization is necessary for pain perception [13] contributed to perpetuating this argument that was widely used as a justification of the undertreatment of pain in infants.

- **Pain relieving drugs and anaesthetics might be harmful to sick newborn infants.** This was an important argument that even now has not been completely solved. Caution on the introduction of new treatments and medications is always justified. A survey of the members of the Association of Paediatric

Anaesthetists in the UK showed that although most people considered that babies were able to perceive pain, they thought the dangers of prescribing opioids outweigh the potential benefit [14]. However, available evidence from initial reports on the pharmacokinetics and haemodynamic effects of fentanyl, ketamine, and halothane in preterm infants suggested these drugs to be safe and effective [15–17].

- **Infants have no memory of pain**. It was thought that even if infants may experience some discomfort this would have no long-lasting effects as they would not be able to recall the experience [7]. It is now widely accepted that the effects of pain experienced early in life can have long term detrimental consequences as described in Section 1.1.3.

Standard clinical practice concerning the provision of analgesia was challenged in 1987 when two key papers were published by Anand who conducted his DPhil research on pain in neonates supported by a Rhodes Scholarship in Jesus College, University of Oxford. The first one, a placebo-controlled randomised trial of fentanyl anaesthesia in preterm newborns undergoing surgical ligation of patent ductus arteriosus (PDA), reported a significant reduction in the stress responses and fewer postoperative complications in the fentanyl group (13% of infants in the fentanyl group required increased respiratory support compared with 50% in the non-fentanyl group, [18]). Later that year, Anand and Hickey published a review of the available evidence for pain perception in human newborns including physiological, neurological, metabolic and behavioural aspects of pain processing [19]. Anand and colleagues also conducted a systematic study where different metabolites associated with stress were measured in infants just before and up to 24 hours post-surgery [20]. Results demonstrated that when limited pain relief was provided, levels of adrenaline were elevated and this correlated with high concentrations of glucose up to six hours after the surgery. Authors suggested that this prolonged endocrine imbalance could be particularly detrimental in infants born prematurely due to their reduced capacity to produce insulin compared with term neonates [20].

At about the same time that Anand was completing his DPhil in Oxford, one family's experience was unfolding in the United States [21]. Jeffrey Lawson was born in February 1985, at 25–26 weeks gestational age. He was transferred to the Washington National Children's Hospital where he underwent heart surgery for ligation of a patent ductus arteriosus receiving only pancuronium, a paralysing agent, as was standard practice at that time. He later went into shock and died several weeks later. When his mother enquired about the use of anaesthetics, the anaesthetist informed her that "Jeffrey was too sick to tolerate powerful anaesthetics" and that "it had never been demonstrated to her that premature babies feel pain" [21].

Anand's publications and Jeffrey Lawson case made it into the mainstream media revealing that while these perceptions and practices were commonplace among medical professionals, the public and many parents, were completely unaware [22]. Scientific evidence and public concern led to a drastic change in clinical practice and drove the beginning of a new area of scientific research on infant pain. After 1987, the American Society of Anesthesiologists and the American Academy of Pediatrics Committee published a statement acknowledging the increasing body of evidence that demonstrates that neonates, including those born prematurely, show physiological responses following surgical procedures and the importance of the use of anaesthetic agents [23].

Since then many researchers have investigated the epidemiology and neurophysiology of pain in infants and new challenges have arisen regarding the measurement and management of pain in nonsurgical settings, such as during routine procedures on the neonatal unit. In the next section, I provide an overview of the epidemiology and treatment of painful procedures in neonates.

1.1.2 Epidemiology of neonatal pain

It is estimated that 15 million infants are born prematurely (before 37 weeks gestational age) worldwide every year [24]. Medical and technological advances

have increased the survival rates of premature and sick infants; however, prolonged hospitalisation and neonatal care can negatively affect neurodevelopment and result in life-long consequences [25]. Exposure to painful procedures without adequate analgesia, particularly during such a critical period of neurodevelopment, can contribute to these detrimental outcomes.

Over the last 25 years, numerous studies have recorded the number of invasive painful procedures infants are exposed to during their hospitalisation in the neonatal unit [26–34]. Barker and Rutter quantified the burden of pain from invasive procedures in one hospital in the UK where 54 infants received 3283 invasive procedures, an average of 61 procedures per infant during their total neonatal intensive care unit (NICU) stay [26]. A prospective study in the Netherlands recorded data from 151 infants who received a range of 0 to 53 painful procedures per day and a mean of 14 procedures per infant per day [28]. The first multicentre European study was conducted in 13 NICUs in France in 2008 by Carbajal and colleagues who reported that infants received an average of 10 painful procedures per day. These patterns are similar outside of Europe. In four studies in Canada, China, South India and Kenya, authors report two to six daily painful procedures per infant [27, 31–33]. It is important to notice that the number of multiple attempts when procedures failed was collected in some studies but not always reported.

Gestational age (GA) and severity of illness were associated with the frequency of daily painful procedures [26, 30, 33]. Preterm infants with the lowest GA, infants requiring respiratory support and with the highest severity of illness scores received the greatest number of painful procedures on the first days of hospitalisation [35].

Across studies, heel lance and suctioning were the most frequent invasive painful procedures performed in infants [26, 27, 30, 32, 36]. Nasal and endotracheal suctioning had the highest percentages in preterm ventilated infants and in neonates who underwent surgical procedures [33, 34, 37]. Other procedures such as peripheral

venous cannulation, line insertion, endotracheal intubation and intramuscular or subcutaneous injections were also commonly reported in these studies [30].

Regarding the use of pain management interventions in the NICUs, pharmacological and non-pharmacological interventions were rarely used before invasive procedures to prevent pain in infants [35]. The procedural and postoperative pain management administration was highly variable within and between countries. For instance, French neonatal units reported the use of analgesia for a median of 20.8% of the total of painful procedures during the study period [29]. In Canada, only 30% of painful procedures were performed with the use of analgesia during the study period in 2007, compared with 7% reported in the Canadian units 10 years earlier [27]. In contrast, both Kyololo and colleagues and Chen and colleagues reported that from the total of 404 to 10,633 painful procedures, none were performed with the use of analgesics or non-pharmacological interventions in Kenya and China [31, 33]. Despite the evidence on the benefits of interventions such as non-nutritive sucking, swaddling, facilitated tucking and skin-to-skin contact [38, 39], these are underused in clinical practice. Moreover, some units do not have neonatal pain management guidelines [31, 33] in place; for those with pain management protocols, their use is inconsistent or solely based on the assessment made by the physician or nurse [35].

The evidence demonstrates that hospitalised newborn infants, particularly those born prematurely are exposed to numerous invasive painful procedures inconsistently and inadequately managed in neonatal units across the world. This could be explained by the challenges associated with pain assessment in infants which translates to limited clinical tools and a lack of licensed analgesic treatments for this vulnerable population. Currently, there is conflicting evidence regarding analgesic efficacy and safety for some of the most commonly used pharmacological agents. In addition to the ethical responsibility to provide pain relief, the detrimental long-term consequences of inadequately treated pain in infants makes effective analgesic treatment a critical need in neonatal care.

1.1.3 Consequences of pain in early life

Infants undergoing painful procedures display immediate signs of physiological instability and distress including increased heart rate, decreased oxygen saturation and respiratory rate, and changes in facial and motor activity [40–42]. The long term consequences of pain experienced in early life have been investigated in several preclinical and clinical studies which have shown associations between infant pain and altered neurodevelopmental and cognitive functions as well as altered somatosensory and pain processing in later life [43–45].

Exposure to pain in early life can negatively impact neurological development resulting in poor cognition and motor function at eight and 18 months of age [46]. At eight years old, children born preterm had reduced amygdala and thalamus volumes which were predicted by the frequency of neonatal invasive procedures [47]. Neonatal pain has also been associated with slower corticospinal tract development [48], reduced cortical thickness in various brain regions [49] and altered white matter structure [50]. In children who were born preterm and experienced early life pain, altered brain structures have been associated with poor cognitive outcomes such as reduced IQ, language and attention deficits, and poorer visual-motor and behavioural outcomes [47, 51–53]. These cognitive deficits related to deviant brain development can persist into adulthood [54, 55].

Studies in rodents have demonstrated similar impairments to cognitive function and neurodevelopment associated with early life pain. Rodents are commonly used as models of neonatal pain with repetitive needle prick applied at different time points during the early postnatal period to simulate the experience of preterm infants in the NICU [45]. Adult mice exposed to repetitive acute pain during the first week of life presented poorer spatial memory compared to controls [56]. Similarly, inflammatory pain on the first day of life significantly impaired hippocampal-dependent memory in adult rats and suppressed the expression of glucocorticoid receptor mRNA in the hippocampus [57].

Somatosensory function and pain processing are also affected by early life pain [43, 45, 58]. As described above, increased procedural pain exposure during the neonatal period can lead to changes in brain structure and connectivity, these include regions relevant for pain processing [59, 60]. Hohmeister and colleagues investigated the cerebral processing of pain in children born prematurely using functional magnetic resonance imaging (fMRI) and identified increased activation in the somatosensory cortex, anterior cingulate and insula during a thermal painful stimulus [61]. Similarly, brain activity in response to noxious stimuli was enhanced in preterm infants compared to controls born at term [62]. Pain sensitivity can be altered by pain in early life with various studies reporting different patterns. For instance, prematurely born children at school age showed thermal and mechanical hyposensitivity in previously injured areas [63] and elevated heat pain thresholds [64]. In contrast, prior pain has also been associated with higher pain sensitivity [65], hyperalgesia [66] and pain catastrophising behaviour [67] in children and adolescents. This could suggest the involvement of different mechanisms possibly related to the type of pain experienced and the time window of early life pain experience [44]. Studies in animal models allow further exploration of these effects and provide support to the clinical findings. Following repeated needle prick stimuli, adolescent and adult rats demonstrated reduced thermal sensitivity and peripheral hyposensitivity [68, 69]. However, after a secondary acute noxious injury animals showed hyperalgesia and enhanced dorsal horn neurons responses [70–72].

The long-term effects of neonatal pain on behavioural responses to subsequent pain exposure have not been clearly characterised. While some studies showed lower pain-related behaviours and pain scores [73, 74], others reported exaggerated facial grimacing and elevated pain scores [75, 76]. These conflicting observations could be a result of the limitations of behaviours and facial expressions discussed in Section 1.3.1

Exposure to pain in early life is developmentally unexpected and evidence from numerous studies show the detrimental neurodevelopmental consequences affecting

the cognitive and pain processing functions in the long term. Understanding the development of the immature nervous system and the maturation-related plasticity of the nociceptive system is essential to evaluate the acute and long term effects of early life injury and to identify safe and effective analgesic interventions. The following section outlines the distinction between pain and nociception - integral to the discussion of pain management in infants. This is followed by an overview of the development of nociceptive pathways.

1.1.4 Distinction between nociception and pain

Pain is a complex unpleasant sensory experience that serves the primary function of protecting organisms from actual or potential harm. It promotes recovery, facilitates avoidance of future damage and can help to warn others of potentially damaging inputs. However, when it results from a maladaptation and altered functioning of the pain system it can lead to chronic pathological pain conditions [77]. In order to discuss the assessment and treatment of pain, it is important to first review its definition. The Taxonomy Task Force of the International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" [78]. This definition highlights the subjective and multidimensional nature of pain encompassing an emotional and a sensory component.

Pain is a personal subjective experience that can be a symptom of disease and result from actual tissue injury, but it can also be experienced in the absence of tissue damage. The current gold standard to assess pain and guide its treatment is self-report which allows the description of various aspects of pain such as location, quality, duration, intensity and unpleasantness [79]. While the IASP definition notes clarify that: "Verbal description is only one of several behaviours to express pain" and that "inability to communicate does not negate the possibility that a human or a nonhuman animal experiences pain"; it is evident that pain assessment

in non-verbal infants can not be determined directly and all indicators of pain are necessarily surrogate measures.

Based on the somatosensory mechanism, pain can be classified into three categories: nociceptive, neuropathic and nociplastic [80]. Nociceptive pain is the perception of harmful actual or potential damage to non-neural tissue and it is caused by the activation of specialized sensory neurons called nociceptors. Neuropathic pain is caused by an injury or condition of the somatosensory nervous system, and nociplastic pain is due to an altered pain processing system despite no evidence of actual tissue injury or lesion of the somatosensory nervous system. Pain can also be classified by time into acute - pain lasting ≤ 3 months, or chronic - pain lasting or recurring for ≥ 3 months [80]. As described in the following chapters, the investigations presented in this thesis study acute nociceptive pain elicited by medically required skin breaking procedures such as blood tests and vaccinations; and mild experimental stimuli. Thus, understanding how nociceptive inputs are transmitted from the periphery, processed at different levels of the nervous system, and the behavioural manifestations of this process can aid in the development of translational tools for the assessment and treatment of infant pain.

Nociception is defined as "the neural process of encoding noxious stimuli" [78] and this sensory mechanism begins with the detection of the noxious stimuli by nociceptors in the skin [81]. Noxious stimuli are transduced and transmitted from the periphery to the spinal cord where motor reflexes are generated - a rapid withdraw of the limb helps to protect the body from further damage [82]. Second-order neurons in the dorsal horn ascend to the brainstem where autonomic and metabolic responses are produced including facial expressions and cardiovascular, respiratory and hormonal changes to maintain the body's homeostasis [83, 84]. The neural signals are transmitted to the thalamus and reach the brain where pain perception is generated through the activation of a range of areas associated with the sensory, emotional, and cognitive aspects of pain [85]. Therefore, while nociception always

occurs following an acute nociceptive stimulus, nociception does not necessarily lead to pain. While the study of motor reflexes and physiological responses are important to gain insights into the nociceptive behaviours of the human infant, measuring the neural signals transmitted to the cerebral cortex may provide the closest estimate of pain in newborn infants. Importantly, pain-related cortical activity in adults from haemodynamic and electrophysiological analysis show concordance with verbal pain reports, demonstrating the central importance in pain perception and modulation [86].

The mechanisms of nociceptive pain alter with age as the pain system and all the body systems mature throughout infancy, childhood and later development. The developmental plasticity of the nociceptive pathways is likely to influence the adaptive ability of the somatosensory nervous system and possibly drives the structural and functional changes in the system observed following tissue injury in early life [87]. The fundamental biological mechanisms and developmental trajectories of nociceptive pathways are discussed in the following section.

1.2 Overview of the development of nociceptive pathways

1.2.1 The development of peripheral nociceptive pathways

Nociception is initiated via the activation of nociceptors of the peripheral nervous system. Mechanical, thermal and chemical stimuli activate the free nerve ending of specialised primary sensory neurons found in the skin, joints, muscles, vessels, fascia, and viscera [88]. These receptors transform the nociceptive stimuli into electrical signals in dorsal root ganglion (DRG) neurons [89]. There are two major classes of nociceptors that innervate the skin that are specialised to detect multiple stimulus modalities and can be categorised according to their threshold sensitivity and signal conduction velocity. The first includes thinly myelinated Aδ-fibres medium in

diameter that rapidly conduct action potentials at a velocity of 12-30 m/s [90]. There are two subtypes of Aδ nociceptors; both activated by mechanical stimuli but differentiated by their thermal response thresholds [91]. The second class of nociceptor include unmyelinated, small diameter C fibres that transmit information from noxious mechanical, heat, and chemical stimuli at a slow velocity of 0.5-2 m/s [90]. Aδ nociceptors mediate the sharp localised sensation of "first pain" and C-fibres mediate the delayed diffuse dull "second pain" sensation evoked by noxious stimuli [91].

The cell bodies of nociceptors are located in the DRG where transcription and protein synthesis occur. Here, the primary afferent axons are organised into dorsal root bundles which project to the superficial laminae of the spinal dorsal horn and make connections with second-order neurons. For these connections to be possible, activated Aδ and C nociceptors release a range of neurotransmitters including glutamate, substance P, calcitonin gene related peptide and ATP [91]. The postsynaptic neuron activates calcium-dependent signalling pathways and protein kinase signalling cascades that will increase the excitability of the dorsal horn neurons and facilitate the signal transmission from the spinal cord to the thalamus [92].

Primary sensory neurons originate from multipotent neural crest cells in the dorsal neural tube. Around embryonic day (E) 12 GAP-43, a nerve proliferation protein marker, is first detectable in the skin of rodent limbs and terminates innervating the tip of the toes at E19 [93]. A and C fibres innervate the skin at E13-14 but A fibres dominate over small fibre innervation until later foetal stages. Both A and C fibres initially hyperinnervate the skin surface but near birth, these retract subepidermically [94]. Some of the nociceptive C fibres functional features such as the expression of the neural growth factor receptor TrkA and selective binding of plant lectin isolectin B4 (IB4) are present at an early embryonic age [95]. Moreover, from the first postnatal days, noxious chemical, mechanical and

thermal stimuli elicit activity characteristic of mature C fibres, can be recorded from polymodal nociceptors [96].

Spinal cord circuits generate reflex responses triggered by motor neurons that result in the contraction of flexor muscles and movement away from a stimulus. In the neonate, this circuitry is functional from early gestational stages and is called the "withdrawal reflex" [97]. While spinal reflexes should not be interpreted as pain perception, these indicate sufficiently developed peripheral nociceptive mechanisms. In early life, withdrawal reflexes thresholds are lower and the cutaneous receptive fields of the dorsal horn larger than those of older animals [97]. After birth, as the neonate develops, the thresholds increase and the receptive fields gradually reduce. This is observed in animal and human neonates who exhibit exaggerated spinal reflexes to both noxious and tactile stimulation [95, 98] which refine and decline in duration, magnitude and latency as age increases [95, 99]. This developmental pattern is possibly linked to the late maturation of interneural pathways such as those from the descending inhibitory pain system [95].

Somatosensory signals are processed in the dorsal horn of the spinal cord before being transferred to the brain via the thalamus. Second-order projection neurons in lamina I of the spinal dorsal horn are responsible for the ascending nociceptive transmission which is modulated by a network of inhibitory and excitatory interneurons within the dorsal horn [100]. During development, A fibres penetrate the dorsal horn at E15-17 in the rat [101]. In rodents, A fibres transiently innervate the dorsal horn beyond their final locations, occupying laminae I-IV. Over the first three postnatal weeks, their axons progressively withdraw to laminae III-IV [101, 102]. Moreover, C fibres penetrate the dorsal horn after A fibres and grow specifically to laminae I and II [103]. Thus, during a considerable postnatal period, laminae I and II are occupied by both A and C fibre terminals which has been suggested to have a functional effect in the nociceptive processing in early life [95].

1.2.2 The development of central nociceptive pathways

Noxious input is transmitted from the spinal cord dorsal horn to higher-level structures in the central nervous system (CNS) through multiple tracts. One of the most studied in the context of pain and nociception is the spinothalamic tract (STT) [104, 105]. In primates, dorsal horn neurons project to sites related to autonomic functions in the spinal cord and homeostatic sites in the brainstem. Lamina I neurons also project by way of the crossed lateral STT to the thalamus where regions including the ventral posteromedial nucleus transmit modality-specific sensory representations of the stimuli [105]. Neurons from deeper laminae target the central lateral thalamic nucleus and more diffusely the amygdala and hypothalamus and could play a role in motor and alertness components of pain [106].

Retrograde neuron tracers have been used to characterise STT at different stages of development in the mouse. Davidson and collaborators demonstrated that STT neurons develop mid-gestation in mice and by E18 are similar to adult SST neurons in number and distribution. Before birth axon terminals of the SST neurons are present in the ventrobasal complex of the thalamus displaying cellular structural features required for synaptic transmission [104]. While neurons of the STT continue to increase in size over the first postnatal week, the functional transmission of nociceptive inputs to the thalamus and other supraspinal regions is not observed until later in the postnatal period. Following hindpaw formalin-induced injury, rats expressed Fos protein (a marker of neural activity) in the ventrolateral medulla by postnatal day 3 and in the thalamus, hypothalamus and periaqueductal grey (PAG) regions by postnatal day 14 [107].

The perception of pain, including the sensory and unpleasant emotional aspects require neural activation of the cortical and subcortical regions of the brain. This process depends on functional thalamocortical and cortico-cortical connections which are formed in the subplate - a transient developmental structure involved in cortical circuit development [108–110]. Thalamocortical fibres are well developed in

the human brain by mid-gestation when they accumulate in the superficial subplate. Migration of thalamocortical fibres to the cortical plate occurs as early as 25-weeks gestation, and synaptogenesis begins from 26 to 28 weeks of gestation from the deepest region of the cortical plate [108, 111]. From this age, somatosensory evoked potentials have been recorded in premature neonates from the visual auditory and frontal cortices [112, 113]. Between 31 and 34 weeks of gestation, the cortical plate differentiates into six defined layers and there is a rapid development of primary sulci and gyri [108, 114]. Figure 1.1 shows the developmental trajectory of the early cortical folding in the human infant constructed from magnetic resonance imaging (MRI) of the preterm newborn brain [114]. Cortical lamination leads to thalamocortical terminals synapses in layer IV [108] which represent an anatomical substrate for a transition from endogenous spontaneous processing to sensory-driven neural activation [115, 116]. In the late preterm period, the subplate begins to gradually resolve (main white matter tracts are in place by term age) and it is no longer detectable between 36 weeks of gestation and the first postnatal weeks [114, 117]. Other developmental processes such as gliogenesis, myelination, neurogenesis and apoptosis also occur throughout the late gestational period and continue after birth where activity- and sensory-dependent processes are key to refine the neural circuit formation [80].

The cortical processing of noxious information differs from other sensory modalities in the sense that there is no dedicated "pain" cortex. Neuroimaging studies have identified patterns of activity in adults in multiple brain areas following noxious stimulation including primary and secondary somatosensory cortices (S1, S2), insula, anterior cingulate cortex (ACC), amygdala, prefrontal cortex, brainstem and cerebellum [86, 118]. Different areas within this network of brain regions are involved in different aspects of the pain experience. The primary and secondary somatosensory cortices are thought to play a role in the discrimination of the location and intensity of a noxious stimulus (sensory discrimination) [119], while the ACC, insula and amygdala are associated with the motivational, affective and

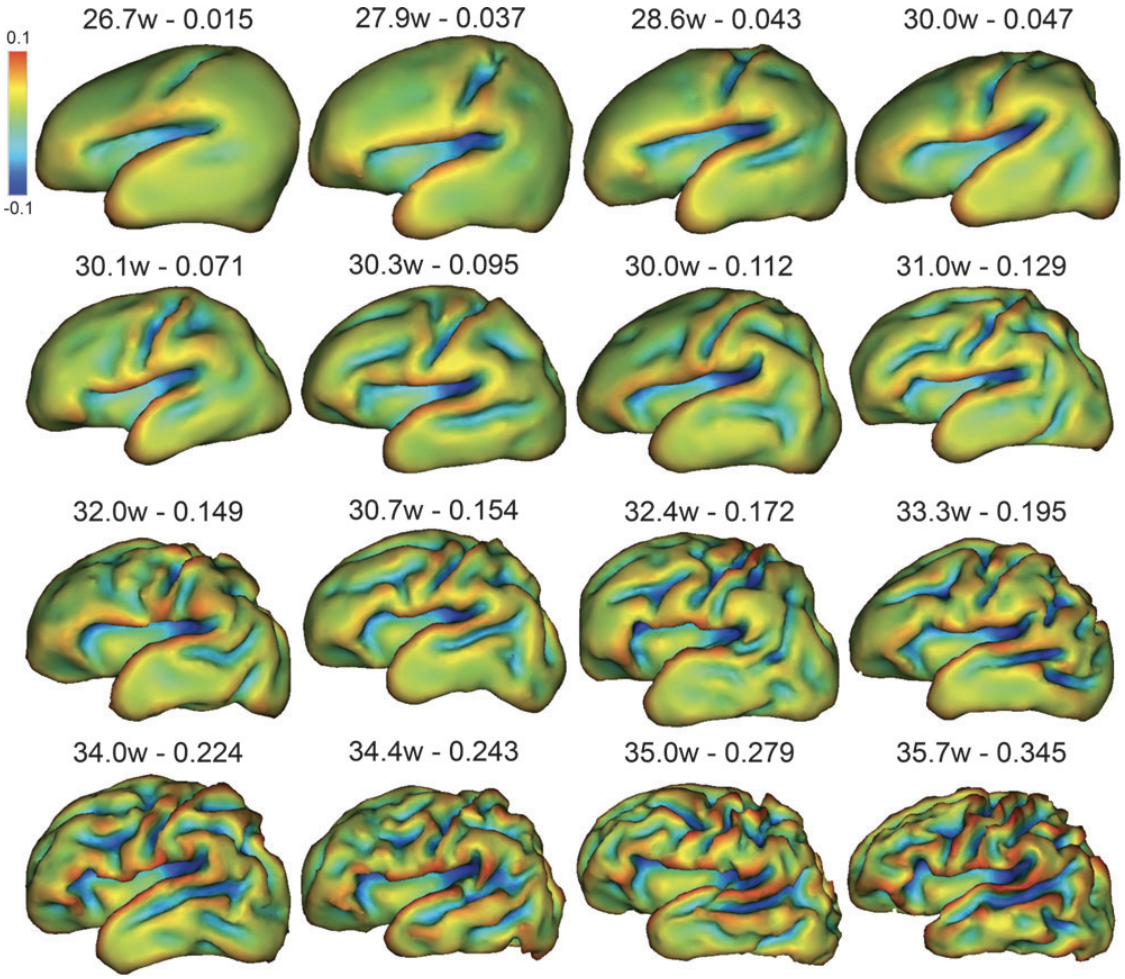

Figure 1.1. Cortical folding process in the human brain. Representative images of the early cortical folding patterns between 30 and 35 weeks gestational age (in weeks, left numbers) and sulcation index (right numbers). The colours outline the surface curvature. The surfaces are not displayed with the same spatial scale. Figure reprinted from Cerebral Cortex [114], with permission from Oxford University Press.

cognitive dimensions of pain perception [120]. However, these areas show significant modulation depending on the context of the stimulus (e.g., degree of attention, expectation, emotional status) [121], and a fixed set of brain areas involved in pain perception has not been consistently identified across neuroimaging studies [122–124]. Thus, it has been proposed that pain perception arises from a dynamic network of brain activity comprising multiple regions that are not unique to pain and that produce a constantly adjusting pain signature [125, 126]. The use of non-invasive brain imaging techniques has been fundamental to gaining insight into human brain development. Analysis of near infra-red spectroscopy (NIRS), fMRI

and electroencephalogram (EEG) recordings from newborn infants show patterns of noxious-evoked brain activity similar to adults [127–129]. These techniques and their applications to assess pain in infants are discussed in detail in Section 1.3.3.

1.2.3 The development of descending pain modulatory pathways

The descending pain modulatory system is an anatomical network that regulates nociceptive processing by producing either facilitation or inhibition of pain. It is a complex system where cortical and subcortical brain regions integrate contextual, emotional and cognitive factors to directly or indirectly amplify or decrease nociceptive transmission [85, 130, 131]. As a result, the same noxious stimuli may produce different pain experiences in different individuals, and within the same individual, the response to an identical stimulus may change in different contexts. Endogenous pain modulation observations in humans date back to the Second World War when H. K. Beecher noted that severely wounded soldiers reported no to moderate pain during combat situations and did not want pain relief medication [132]. Early evidence from animal models demonstrated that nociceptive reflexes were enhanced following spinal cord transection [133] while Reynolds showed that direct stimulation of the PAG produced profound endogenous analgesia in awake moving rats [134]. It is now known that a variety of brain regions with intricate connectivity play a role in this descending pain modulatory system [85, 135]. The PAG is a midbrain region where cortical and spinal signals converge. The PAG receives input from the ACC, insula, amygdala, prefrontal cortex and hypothalamus and transmits descending outputs to the rostroventral medulla (RVM) which in turn has two types of neuronal projections to the spinal dorsal laminae where nociceptive inputs can be upregulated or downregulated [136, 137]. Moreover, numerous brainstem nuclei including the PAG, RVM and nucleus cuneiformis (NCF) release inhibitory endorphins and enkephalins, which activates μ-opioid receptors found

throughout the descending pain modulatory system [138, 139]. This system is the target of analgesic drugs such as opiates and mediates placebo and nocebo effects [135].

The maturation of this descending system occurs relatively late in development and evidence from animal studies suggest that although the descending anatomical structure is present from birth this is not functional [140, 141]. The RVM receives no nociceptive input in the first days of life [142] and before postnatal day 21, RVM stimulation amplifies nociception and no inhibitory effects are observed [140]. The developmental switch from facilitation to bimodal nociceptive inhibition and facilitation occurs from day 21 and it is likely mediated by μ-opioid receptor maturation in the RVM and Gamma-aminobutyric acid (GABA) signalling regulation [143, 144]. Moreover, in adult animals, RVM activation targets spinal circuits with strong nociceptive C fibre input [145]; however, during the first three weeks of life, the RVM appears to primarily target A fibre afferent inputs in rat pups [146]. The slow postnatal development of C fibre spinal innervation could explain the RVM nociceptive facilitation in early life [146] and is consistent with the spinal cord hyperexcitability observed in premature infants.

1.2.4 The development of neuroimmune signalling

The nervous and immune systems are tightly interlinked, with each system capable of influencing the other to maintain body homeostasis and to respond to injury or pathogen perturbations [147]. Neuroimmune interactions have been extensively described in the peripheral and central nervous system with neural function being affected by elements of the immune system and vice versa [147–149]. For example, immune cells such as macrophages can express neurotransmitter receptors and bind to GABA and acetylcholine to modulate the inflammatory response by reducing the secretion of cytokines during autoimmune diseases or infections [150, 151]. On the other hand, major histocompatibility complex (MCH) proteins - essential in the adaptive immune system to present antigens and activate T cells - are expressed

in neurons from the lateral geniculate nucleus in the thalamus and drive synaptic refinement of retinal inputs during foetal development [149]. Similarly, complement proteins (molecules that signal antibodies and phagocytic cells to destroy pathogens) were shown to be secreted by glial cells in the CNS [152] and are critical in synaptic maturation and refinement of the neuronal circuitry [153, 154].

Microglia are the primary immune cells of the central nervous system (CNS). These derive from myeloid-lineage cells that migrate to the CNS during early embryonic stages [155]. During development, microglia play an important role in refining immature neuronal circuits and clearing excessive synaptic structures [156]. While the complete molecular pathways underlying this process in different CNS regions is not known, microglia are likely involved in the development and maturation of the nociceptive system including dorsal horn circuits where considerable postnatal refinement of afferent input and local connections occurs [95]. Stressors during critical periods of postnatal development can lead to aberrant pruning and contribute to neurodevelopmental disorders [157]. Unexpected immune activation in early life can therefore have a direct impact on the microglia-mediated neural circuit refinement, "priming" the system and affecting basal nociceptive function and behavioural responses to noxious stimuli in later life.

To promote recovery from infection and injury, animals and humans present patterns of "sickness behaviour" such as lethargy, depression, anorexia, reduced grooming and hyperalgesia. In human adult models of endotoxemia, inflammatory processes following administration of lipopolysaccharide (LPS) were associated with hyperalgesia and hypersensitivity as well as increased neural activation during painful stimuli [158, 159]. Animal models have provided evidence of some of the mechanisms involved in inflammation-induced hyperalgesia. Direct bacterial activation of nociceptors can occur through N-formyl peptides [160] and indirectly by the action of immune-derived proteins like cytokines and prostaglandins on receptors expressed by nociceptors [161]. In the CNS, cytokines are primarily

released by microglia and can reduce the threshold of activation of nociceptor terminals [162].

However, little is known about immune-to brain mechanisms and neuroimmune pathways in human infants. Studies conducted in neonatal rat pups have shown that an immune challenge in early life results in increased sensitivity and hyperalgesia to subsequent injury in adolescence and adulthood [163]. In Chapter 5 of this thesis, electrophysiology is used to characterise noxious evoked responses during suspected infection in term infants. Given the inter-species differences in both immune and nociceptive systems, it is important to investigate the effect of inflammation in human infants to advance our understanding of pain perception and neuroimmune interactions during critical periods of plasticity in early postnatal life and the potential of persistent changes in nociceptive pathways.

1.3 Measurement of neonatal pain

The assessment of pain provides the basis for diagnosis, treatment selection and evaluation of treatment effectiveness in clinical practice and clinical research. In non-verbal infants, this assessment necessarily relies on surrogate measures of pain. A measurement, in the context of pain, consists of the quantification of the extent, intensity or degree of pain experienced [164]. Based on the FDA Biomarkers, EndpointS, and other Tools (BEST) classification, measurements can be "made through a report by a clinician, a patient or a non-clinician observer" (clinical outcome assessments), or "characteristics measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic intervention" (biomarkers) [165]. Various surrogate measures of pain have been developed for neonates and are reviewed in this section.

1.3.1 Behavioural measures

In response to a noxious stimulus, the primary method by which infants communicate their distress and discomfort to the care providers is their behaviour. Given that the nociceptive system in infants is functional, although immature, it is reasonable to infer that a newborn infant expresses pain in response to a procedure that older children or adults would consider painful. Surrogate behavioural measures are easy to identify visually and were first analysed in detail by Grunau and collaborators [166, 167]. Brow bulge, eyes squeeze, and nasolabial furrow were the facial features most commonly identified during heel lance and intramuscular injection in term newborns. The duration of facial expressions and latency to cry were associated with the sleep state, with infants in awake-alert state displaying stronger facial expressions and shorter latency to cry compared to sleeping infants [166]. These observations were foundational for the development of pain scales and The Neonatal Facial Coding System (NFCS) was the first scale validated for the clinical assessment of pain in newborns [166, 168]. The NFCS was initially developed and validated by recording facial expressions during painful and non-painful procedures and analysing the video footage to identify the presence or absence of 10 specific facial actions [166]. The feasibility and validity of using the NFCS for clinical assessment in real-time has also been shown [169].

Behavioural measures can be categorised as binary - present or absent, ordinal - greater than or lower than, and according to an interval - duration or frequency over time. Pain scales are tools created by assigning weights or scores to individual measures or a combination of observed features and can be unidimensional (one item or multiple items included from a single domain) or multidimensional (multiple items within multiple domains). It has been argued that the use of measures from multiple domains may better capture the full scope of a complex perception like pain [170]; however, combining measures in total scores require arbitrary weighting factors that may lead to inaccurate assessments.

Since the 1980s more than 40 different multidimensional pain scales have been developed for neonatal care and research [171]. While these include a range of tools designed for the measurement of acute/procedural pain, postoperative pain, prolonged/ongoing pain for premature infants to children of 3 years of age; the scales are not substantially different from each other and most include a set of observed behaviours and components of physiological activity [172]. In response to acute painful procedures, infants can show autonomic responses including changes in heart rate, respiratory rate, blood pressure and oxygen saturation. Although these are not reliable predictors of pain on their own [173], they are commonly found in composite pain scales.

The most commonly used scale in clinical research is the Premature Infant Pain Profile (PIPP) which measures the duration of facial expressions (brow bulge, eyes squeeze and nasolabial furrow), changes in heart rate and oxygen saturation and contextual factors including GA and behavioural state at the time of the stimulus [174]. A revised version of the PIPP score (PIPP-R) was developed to address validity and feasibility issues in all GA groups. In the revised version, the contextual indicators are scored only when the physiological (heart rate and oxygen saturation) or behavioural (brow bulge, eye squeeze and nasolabial furrow) indicators are scored as > 0 [170]. This clinical tool has been validated for acute and procedural pain in term and preterm infants and has been used in research studies and clinical trials [175–179]. PIPP scales require trained observers and the PIPP-R was calculated and used in the investigations presented in this thesis.

Despite the large number of scales available, their use in clinical practice is inconsistent [37] and even those with better reliability and validity parameters have significant limitations. While systematic scoring of facial features has been a useful tool in studying infant pain [171], the use of behaviour as reliable measures of pain is challenging given their limited sensitivity (ability to detect true positive rate) and specificity (ability to detect true negative rate). Facial expressions and physiological

indicators such as increased heart rate observed during painful procedures can also be evoked by non-noxious tactile stimuli [40], and during arousing and distressing events like nappy changes [180, 181]; which may result in high pain scores when an infant is not in pain [182]. On the other hand, these measures are highly influenced by factors like age, illness or sedation which can hamper their ability to manifest external behaviours; potentially resulting in low scores when an infant is in pain. For example, very premature infants display reduced changes in facial expressions compared with term-born infants [183, 184]. Additionally, the PIPP-R scale and other clinical tools are assessed by visual inspection, which is subjective and prone to inter- and intra-rater variability.

The assessment of pain in infants is challenging, and ultimately all the indicators are surrogate measures of the complex pain experience. Nonetheless, every effort should be made to develop robust and developmentally appropriate pain indicators to assess pain in the clinical setting and for use as endpoints in clinical trials to adequately measure analgesic efficacy.

1.3.2 Reflex withdrawal

The study of spinally-mediated reflexes has contributed to the understanding of the sensory mechanisms in neonates [97]. In humans and animals, the reflex that results in the brisk withdrawal of a limb from a noxious stimulus is a useful indicator of spinal-cord excitability and is one of the most commonly used surrogate measures of pain in animal models [185]. In infants, reflex withdrawal can be measured using surface electromyography (EMG) by placing electrodes on the skin overlying muscle to quantify muscle contraction. It has been demonstrated that the latency to the reflex decreases with increasing stimulus intensity [97] and the amplitude of the reflex increases with the stimulus intensity [97, 186]. Reflex withdrawal activity has also been used in studies to characterise mechanical pain thresholds in infants across different development stages [187–189]. Moreover, in term-born neonates, the

amplitude of spinal reflex is strongly correlated with noxious-evoked brain activity and it is observed at low stimulus intensities that do not evoke other behavioural responses [186].

While the EMG reflex withdrawal activity is an objective measure, its utility in the assessment of pain may be particularly limited in younger infants. Numerous studies have shown that reflex withdrawal threshold in newborn infants is very low but increases gradually with age [101, 140, 190]. Newborn rat pups are highly sensitive to tactile stimuli and their reflexes are exaggerated and disorganised. [146, 191]. Similarly, in premature human infants prolonged, non-specific reflexes have been observed, elicited by both tactile and noxious stimuli [99, 189, 192]. These are observed in both limbs ipsilateral and contralateral to the site of the stimulus. During postnatal development, these reflexes get refined and there is a reduction in reflex amplitude and duration. This developmental pattern likely reflects the reorganisation of peripheral inputs, initially dominated by low threshold A fibre inputs, and the maturation of descending nociceptive pathways [140].

It is therefore important to account for the developmental stage and gestational age when assessing and interpreting nociceptive reflex responses. In healthy term neonates, noxious-evoked reflexes are more refined and at higher intensity, predominantly observed ipsilateral to the site of the stimulation [186]. In this thesis, the root mean square (RMS) of the reflex withdrawal is assessed with surface EMG as this metric is a sensitive indicator that allows for the discrimination between noxious and non-noxious stimuli in neonates born at term [193].

1.3.3 Brain-derived measures of pain

The perception of pain is generated at the level of the cerebral cortex; therefore it can be argued that the most appropriate surrogate measure of pain could rely on the detection of cortical brain activity. In the past 15 years, brain-derived

surrogate measures of pain have been developed in infants using techniques like near-infrared spectroscopy (NIRS), electroencephalography (EEG), and functional magnetic resonance imaging (fMRI). An overview of these measures is presented in this section.

Near-infrared spectroscopy

NIRS is a non-invasive optical technique used to measure tissue oxygenation. It measures oxygenated and deoxygenated haemoglobin based on the capacity of biological tissues to absorb near-infrared light (approximately 700–900 nm) [194]. Neural activity is dependent upon the delivery of oxygenated haemoglobin transported from the blood to the tissue which results in changes in the concentration of cerebral oxygenated and deoxygenated haemoglobin [195]. Haemoglobin is a light-absorbing molecule or chromophore and the oxygenated and deoxygenated forms have different optical absorbing properties. By measuring absorption changes at two (or more) wavelengths, changes in the relative concentrations of these molecules can be calculated [196] by NIRS. NIRS devices are portable and comprise a light source coupled via optodes to the scalp and a light detector.

NIRS was the first measure of brain activity used in infants to characterise nociceptive activity at the cortical level. Increased cerebral oxygenation was observed following clinical procedures such as heel lances and venepunctures but not during non-noxious control stimuli [197–199]. Cortical responses were greater in the hemisphere contralateral to the site of stimulation and were associated with infants sleep state [197]. These observations demonstrated that nociceptive signals are transmitted to the cortex and importantly this functional activation of the cortex occurs in infants as young as 25 weeks' gestation [197]. Further research has shown that cortical activity was correlated with behavioural scores although in some infants who did not display facial grimacing cortical responses were present [200]. The magnitude of NIRS cortical activity increases with gestational age and its

potential is exemplified by a study where NIRS signals were observed in sedated infants following chest drain removal [201]. NIRS is an objective measure of cortical brain activity, nonetheless, it has several limitations including a limited spatial resolution and limited range of tissue penetration [202]. Moreover, while it is reasonable to assume that haemodynamic responses correlate with cortical activity this relationship may be influenced by other factors such as illness [203].

Functional magnetic resonance imaging

Functional magnetic resonance (fMRI) is widely used in clinical research to generate high-resolution representations and maps of brain activity. It is also based on haemodynamic processes in the brain and it measures blood oxygen level dependent (BOLD) signals. Following cortical activation, the net increase in blood oxygenation leads to an increase in the BOLD signal [204] due to magnetic differences induced by the changing concentrations of oxyhaemoglobin and deoxyhaemoglobin. Similar to NIRS, an increase in BOLD signal is interpreted as a reflection of the physiological changes associated with increased neuronal activity [205]. While fMRI has a very high spatial resolution that allows the localisation of functional activity, the temporal resolution may be limited by the relatively slow haemodynamic response, with BOLD changes detected few seconds after the onset of neuronal activity [205, 206].

fMRI has provided invaluable insights into the brain structure and has been fundamental for the study of pain neurophysiology. In adults, fMRI studies investigating nociceptive processing have been conducted with different experimental paradigms with a range of noxious stimuli including thermal, mechanical and chemical stimuli [207]. These studies have identified brain regions activated during pain processing including the insula, the primary somatosensory cortex, prefrontal cortex, anterior cingulate cortex, thalamus and amygdala [207–209]. In infants, one study showed functional activations in the somatosensory cortex, supplementary

motor area and frontal cortex following experimental noxious stimulation. In response to brush and von Frey hairs functional brain activity was associated with stimuli intensity a generalised reduced response was observed in sedated infants [210]. Goksan and collaborators compared the brain responses of adults and newborn infants to the same type of experimental noxious stimulus and found that most regions observed in the adult brain were also activated in the infant brain, including brain regions involved in both sensory and affective processing [128] (Figure 1.2). Further investigation using fMRI found that infants with lower noxious-evoked brain activity had greater connectivity between areas related to the descending modulatory system suggesting an inhibitory function at the brain level [129]. While this may appear to contradict the opposite excitatory effect of spinal cord descending modulatory function observed in rodents [140, 141], it is possible that maturation at the spinal and supraspinal levels occur at different rates. This also may be related to inter-species differences between human infants and neonatal rat pups. fMRI has also been used to understand inter-individual variability in pain sensitivity in infants, with a study showing that brain activity during resting state can predict the magnitude of responses to noxious stimuli [211]. Taken together, these results could suggest that the infant pain experience is similar to that of adults but further investigations looking at how these measures correlate with other surrogate indicators of pain would be valuable when assessing infant pain.

Investigating nociception in infants using fMRI presents significant challenges as coupling the recordings with clinical procedures such as heel lancing is inappropriate and impractical in the scanner. Furthermore, the considerable neuroanatomical and developmental differences between the infant and the adult brain limit the applicability of MRI analysis tools that have been designed for use in adult neuroimaging. In recent years, the development of analytical approaches optimised to measure haemodynamic activity in the infant brain [212, 213] has been a significant advance that will facilitate future research in the field.

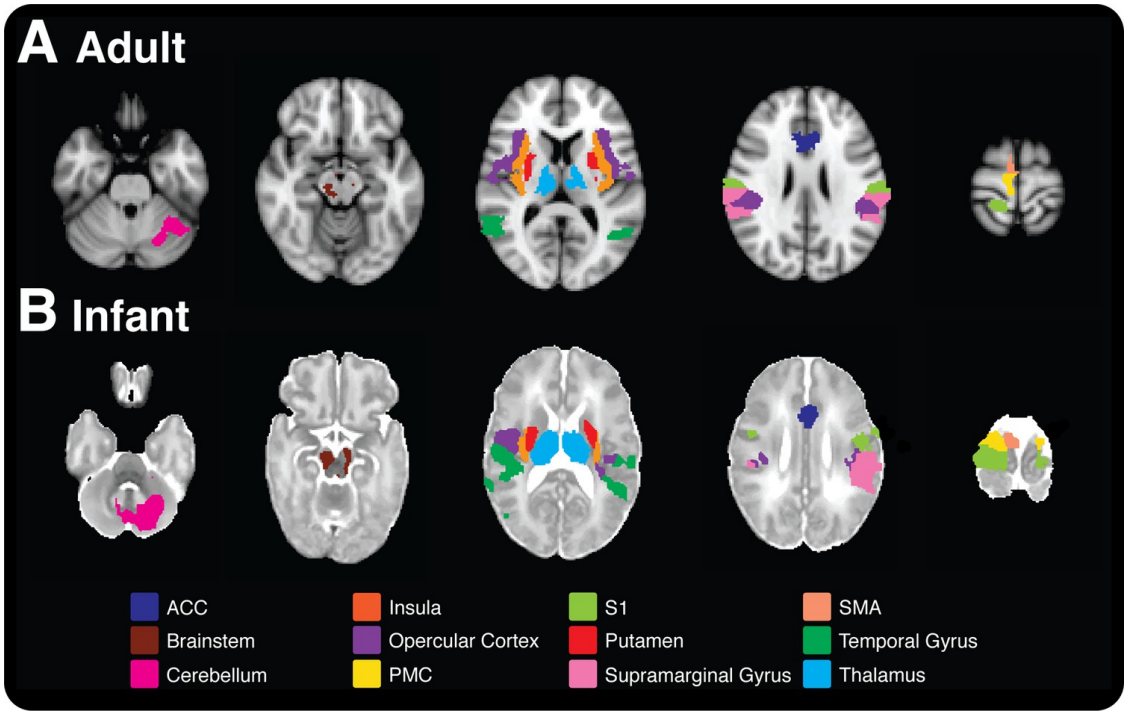

Figure 1.2. Brain regions activated following noxious stimulation in adults and infants. Figure reprinted from Goksan et al. [128], licensed under CC BY 4.0

Electroencephalography

Electroencephalography (EEG) is a technique used to record the synchronised electrical activity generated by populations of neurons in the brain. The main source of the cortical activity recorded with EEG is neural post-synaptic potentials (PSPs) [214]. The resting membrane potential at electrochemical equilibrium is 70 mV on the nerve cell body. Synaptic activity produces a change in the membrane conductance and transmembrane potential. If an action potential travels along a nerve fibre that ends in an excitatory synapse it produces a depolarization (local reduction of the transmembrane potential) and it is called an excitatory post-synaptic potential (EPSP), commonly located in the dendrites. Inhibitory post-synaptic potentials (IPSPs) induce hyperpolarisation typically located on the cell body of the neuron [215]. The combination of EPSPs and IPSPs induces electrical fields - currents of positive sodium and potassium and negative chloride, that flow within and around the neurons with a potential field sufficient to be recorded on the scalp [214].

EEG is only sensitive to large unipolar changes in the signal. Given that it measures the net change in electrical activity, the presence of equal and opposing positive and negative charges would result in a neutral signal. The field potentials can be detected with an exceptionally high temporal resolution, with sampling rates up to 2000 Hertz [214]. However, the spatial resolution of the EEG is low and depends on the number of recording electrodes placed on the scalp. A small number of electrodes will result in a limited capacity to infer the source of the electrical activity because the electrical field's effect diminishes as the distance from the source increases. This means that events producing maximal voltage on a particular electrode will affect adjacent electrodes as well, but to a lesser extent as the potential fades from the point of origin [215]. Similarly, very large amplitude responses originating from a deeper location distant from the cortex could resemble a smaller amplitude response originating near the cortical surface. The distance from the source of neural activity also affects the signal polarisation detected by the EEG which means that it is not possible to determine if the signal derives from excitatory or inhibitory neural activity [214].

Despite these limitations, EEG has a significant advantage over NIRS and fMRI given that it directly measures neural activity related to the processing of nociceptive input and does not rely on changes in cerebral perfusion and haemodynamic processes. Moreover, EEG can be recorded non-invasively by the bedside which means that it can be employed in infants, children and adults. EEG is widely used for the clinical assessment of common conditions like epilepsy and in clinical research for the study of human brain function in health and disease. A widely used application of EEG is the investigation of sensory and cognitive processing associated with specific types of stimulation. Changes in voltages generated in response to specific events or stimuli are known as event-related potentials (ERPs). Characteristic ERP waveforms comprise peaks and troughs which reflect the synchronised neural

activity in the brain in response to an external stimulus [216]. Stimuli can be timed-locked to an EEG recording [217, 218] and applied multiple times to obtain average responses across trials. This increases the signal to noise ratio by reducing unrelated background activity and amplifying the signal of interest [216]. Specific properties of ERPs such as polarity (positive or negative), latency (time in milliseconds (ms) after the stimulation) and topography (scalp distribution) allow, to some extent, the estimation of brain function. For example, the amplitude and latency of successive peaks in the waveform relate to the time course of signal processing in the brain, whereas the scalp voltage distribution can be used to infer the neuroanatomical location of these processes [219].

ERPs following a noxious stimulus were first characterised in adults in the 1960s [220]. The most widely used experimental design involves the application of a laser stimulus that selectively activate thermosensitive Aδ and C nociceptors [221, 222]. Lasers deliver a direct pulse of heat to the superficial epidermal layers of the skin causing a pain sensation described as sharp and pricking at first and dull afterwards. The laser evoked response consists of a waveform with two negative peaks with maximal amplitude at the vertex, at the Cz electrode which is positioned at the midline [223]. The first negative-positive complex occurs 140 - 240 ms after the stimulus and is consistent with input transmitted via Aδ fibres [219, 224, 225]. The second negative-positive complex peaks around 1000 ms and it has been associated with slow-conducting C-fibres [219, 224]. The early peak is generated by the primary and secondary somatosensory cortices, whereas the later peak has been localised to the ACC [226, 227]. It has been suggested that the later evoked response represents the later processing of the nociceptive stimulus and that it may be associated with affective components of pain [223].

In infants, the EEG signal undergoes continuous changes during the preterm period reflecting the maturation and organisation of brain networks occurring during foetal development. Extremely premature infants have EEG patterns with

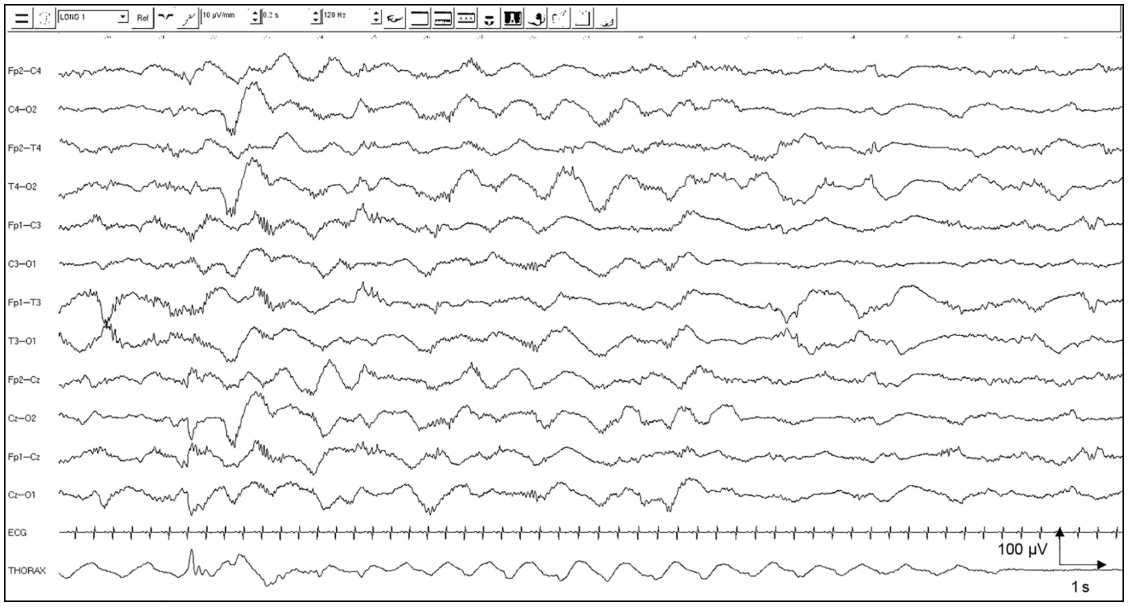

Figure 1.3. EEG during active sleep in 34 week old (PMA) neonate. Continuous activity disrupted by delta brushes. Figure reprinted from the "Handbook of Clinical Neurology" [230], with permission from Elsevier.

infrequent bursts of activity and long periods of slow waves which reduce in duration with age [228, 229]. Between 31 and 33 weeks gestation, the EEG signal starts to be continuous during sleep, and sleep/awake states can be identified [230]. By term age, the EEG is continuous during most states and background activity is more reactive to stimulation [230, 231]. Figures 1.3 and 1.4 show typical EEG activity during active sleep in a premature 34 week (postmenstrual age - PMA) and a full-term neonate respectively. Consistent with these observed trajectories, the patterns of brain activity evoked by sensory stimulation evolve in morphology across the developmental period [232–234]. During early prematurity the neuronal bursts known as delta brushes are commonly present by spontaneous activation or in response to sensory stimulation; these patterns are gradually replaced by adult-like ERPs [233, 235].

Noxious-evoked brain activity recorded using EEG was first characterised in neonates by Slater and collaborators in 2010 [236]. In infants between 35 and 39 weeks' gestation an evoked potential was identified in response to a clinically required heel-lance. The event was time-locked to the EEG recording and Principal

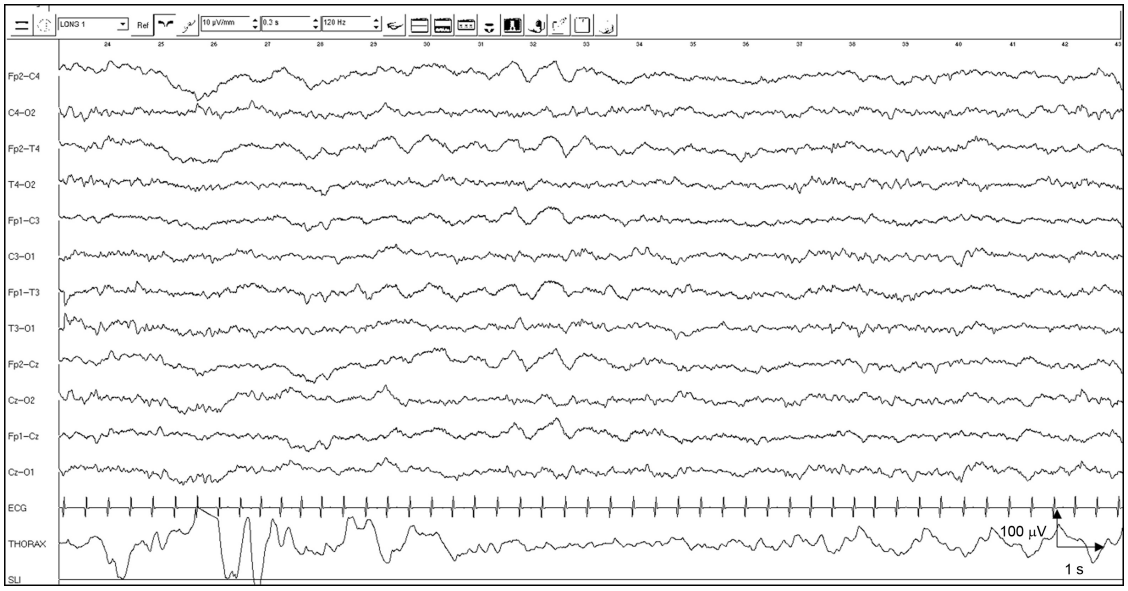

Figure 1.4. EEG during active sleep in a full term neonate. Figure reprinted from the "Handbook of Clinical Neurology" [230], with permission from Elsevier.

Component Analysis was used to decompose the signal and identify the evoked potential consisting of a negative-positive complex occurring between 420 - 560 ms, with maximal amplitude at the vertex (Cz and CPz electrodes). This potential was not present during a non-noxious control stimulus (applied by rotating the heel lance by 90 degrees so that the spring-loaded blade is released into the air and not against the infant's skin) and was observed in single trials. Moreover, an early negative potential occurring between 150 - 260 ms was detected following both the noxious heel lance and the non-noxious control with maximum amplitudes at the vertex [236]. Further research studies have reproduced these findings and authors have suggested that the early potential could be related to the non-noxious arousal components of the stimulus and the late potential to the noxious-specific response [199, 235–237]. These observations are supported by the similarities between these responses and laser-evoked potentials identified in adults and older infants in response to vaccinations and cannulations [219, 238–240].

This pattern of noxious-evoked brain activity is also evoked by experimental noxious stimuli of mild and low intensity in infants. The PinPrick™ experimental noxious stimulator activates Aδ fibres and is has been widely used to assess

mechanical sensitivity in patients [241]. The magnitude of the noxious-evoked brain activity in response to these experimental stimuli correlates with their intensity, suggesting that this response is related to the processing of noxious input [186]. The use of experimental noxious stimuli in infants has an advantage over the single-trial nature of recordings during clinical procedures like heel lances in the sense that it can be applied multiple times to improve the signal to noise ratio. Importantly, the PinPrick™ is not tissue-damaging and there is no evidence of behavioural distress when used with term neonates [186].

Analytical methods were further developed by Hartley and collaborators and a template of noxious-evoked brain activity was derived and validated in neonates aged 34-42 weeks' gestation during noxious stimulation of the foot including heel lances and experimental noxious stimuli [237]. This template can be fitted to new EEG data sets to quantify the magnitude of noxious-evoked brain activity in response to the noxious input. During validation studies, the template has been demonstrated to be specific given that it detects noxious-evoked brain activity in response to noxious but not tactile, visual, or auditory stimuli. The magnitude of the noxious-evoked brain activity quantified with the template is also correlated with changes in facial expressions following heel lancing [237]. The potential of the template to be used in studies of analgesic efficacy has been demonstrated by the reduced magnitudes of noxious-evoked brain activity observed following the application of topical local anaesthetic and after a gentle touch intervention [237, 242].

The template of noxious-evoked brain activity is a tool that can provide an objective surrogate measure of pain in infants [127]. It could also provide a robust systematic approach that if implemented by multiple centres can facilitate the reproducibility and comparability of results across different populations. However, the application of the template is limited to the procedures where it has been previously characterised which do not include all the range of acute procedures

applied to infants on neonatal units. In this thesis, the transferability of the template for the quantification of pain-related brain activity following other clinical procedures is explored (Chapter 3).

1.4 Treatment of infant pain

Improving the treatment of pain is recognised as a neonatal research priority [243, 244] and a major concern amongst parents and healthcare professionals [245]. Although there are many gaps in knowledge regarding the most effective way to relieve pain in neonates, there is a range of non-pharmacological and pharmacological interventions commonly recommended in local and international guidelines.

1.4.1 Non-pharmacological pain-relieving interventions

Non-pharmacological pain-relieving strategies have been introduced over the last few decades including sweet-taste solutions, skin-to-skin contact, breastfeeding and swaddling. Sucrose is the most commonly studied of these interventions, and there is extensive evidence of the effectiveness in reducing behavioural responses following minor painful procedures [246]. However, this widely promoted pain-relieving strategy does not reduce noxious input to the brain as demonstrated in a randomised controlled trial of infants receiving sucrose or placebo before heel lancing [247]. Sucrose did not affect EEG-recorded noxious-evoked brain activity despite a reduction in behavioural pain scores. It is possible that sucrose has an impact on behavioural nociceptive responses by a mechanism of action that is not necessarily mediated by supraspinal cortical regions [248, 249]. While many gaps remain regarding the mechanism of action, appropriate dosing, and soothing versus analgesic effects; recent publications have suggested that sucrose may not mitigate long-term consequences of early life pain, and it may have long-term neurodevelopmental effects with repeated use [56, 250, 251].

Other measures including swaddling and facilitated-tucking of infants are useful to provide comfort to the infants but have shown variable effectiveness in reducing pain [252]. Breastfeeding also reduces behavioural and physiological responses to pain in full-term infants undergoing heel lancing, intramuscular injection, and venepuncture [253]. However, this strategy can be challenging for new mothers and it may not be practical to implement in premature or sick infants.

Skin-to-skin contact (SSC) or kangaroo care (KC) is a comfort measure consisting of ventral skin contact of the newborn with the caregiver's chest. SSC has been shown to reduce behavioural and physiological indicators of infant pain to acute noxious procedures including heel lancing [254–256], venepuncture [257], and intramuscular injection [258], and has been recommended for pain relief during blood sampling [259]. A Cochrane review of 25 studies concluded that SSC is safe and effective in reducing physiological (heart rate) and behavioural (crying time) indicators of pain following clinically-required painful procedures [260]. However, the quality of evidence for an effect on acute pain response was low.

The observed efficacy of KC or breastfeeding could derive from natural maternal tactile contact. Stroking, by repeatedly applying gentle pressure to the skin, can activate CT fibres, a subclass of slow-conducting unmyelinated sensory neurons, mostly found on hairy skin [261–263]. These fibres project to brain regions associated with affective processing such as the insular cortex, prefrontal cortex, superior temporal sulcus, and cingulate cortex [264–267] and are thought to have evolved to promote affiliative behaviours and social touch [268–270]. CT fibres are optimally activated by stroking at a velocity of 3cm/s (optimal range 1-10cm/s) [271–273]. In adults, activation of CT-fibres using gentle brushing or stroking paradigms has been shown to reduce pain. Using EEG, a study has also demonstrated that CT optimal touch reduces noxious-evoked brain activity arising from laser stimulation, in addition to reducing pain ratings [274]. A previous study investigating pre-procedural stroking for pain relief in neonates [242], demonstrated that CT optimal

stroking (at 3cm/s) before an experimental noxious stimulus or clinical heel lance significantly reduced noxious-evoked brain activity in term neonates compared to non- CT-optimal stroking. This non-pharmacological intervention is used in this thesis in Chapter 4 to test a new paradigm that measures baseline nociceptive sensitivity to optimise the power that can be achieved to detect effects in small samples.

Despite guidelines recommending the use of non-pharmacological interventions in the context of minor painful procedures, these simple non-pharmacological strategies are scarcely used in maternity and neonatal units [275, 276] and the mechanisms underpinning the effectiveness of these interventions are still being established.

1.4.2 Pharmacological pain-relieving interventions

Analgesic medications are not licensed for pain management in newborn infants. Studies of appropriate dosing, safety, efficacy, and long-term effects of analgesics given during the neonatal period are lacking or inconclusive. In addition to the challenge of measuring pain in infants, understanding how the body processes a medication is essential to evaluate the optimal dosing strategy [277]. Dose adaptations from older children or adults are generally not appropriate. The study of pharmacokinetics in the neonatal population is complex and developmental aspects of absorption, distribution, metabolism and excretion processes need to be carefully considered [278]. Therefore, the treatment choice, dose and route of administration are usually decided based on expert consensus and individual experience [80].

The most commonly used drugs to treat pain in newborns are opioids, with fentanyl and morphine used especially for persistent pain [244]. Opiates decrease pain perception by binding to μ- and δ- receptors at the spinal and supraspinal sites of the nervous system and modulating the ascending transmission of nociceptive inputs. However, several opioid receptor signalling pathways in the developing

brain are associated with neural maturation and differentiation and migration [279]. Findings regarding the analgesic efficacy of opioids in infants are inconclusive. There is evidence of pain scores being reduced after the administration of opioids in ventilated infants [279–281], however in other studies with similar demographic population characteristics, no analgesic effect has been reported [176, 282]. A Cochrane systematic review found insufficient evidence to recommend the routine use of opioids in mechanically ventilated infants [283]. There are concerns for adverse events linked to the use of opioids such as respiratory depression and potential long-term neurodevelopmental outcomes related to the use of morphine infusions in preterm infants [279, 283]. A randomised controlled trial investigating the efficacy of morphine during retinopathy of prematurity (ROP) screening and heel lancing used cortical activity as a primary outcome measure. Unfortunately, the trial was stopped earlier due to evidence of cardiorespiratory adverse events [284].

Fentanyl is a preferred opioid for the treatment of pain in clinical practice with an earlier onset of action and a potentially better pharmacological profile [285] than morphine. A recent study investigating the pharmacokinetic population profile concluded that both prenatal and postnatal maturation are important for fentanyl exposure and that the dosing should consider weight and postnatal age (PNA) [286]. However, there are limited studies of the analgesic efficacy of fentanyl and conflicting evidence regarding the safety profile [287, 288].

Paracetamol is the most widely used drug to treat mild to moderate pain or fever [289] in neonates. A variety of paracetamol dosing regimens are used in clinical practice. In term infants, the use of intravenous paracetamol significantly decreases the amount of morphine needed to treat postoperative pain [290]. Similarly, in preterm infants admitted to the NICU the use of paracetamol showed a relevant morphine-sparing effect [291]. This opioid-sparing effect has been studied for continuous pain when multiple doses of analgesics are required (e.g. for postoperative pain); however, this effect has not been studied after a single dose intervention

for procedural pain. A 2016 Cochrane review concluded that there is insufficient evidence to establish the efficacy of paracetamol for neonatal procedural pain [292]. In various studies, paracetamol did not reduce pain as assessed by behavioural pain scores following different acute painful procedures including heel lances, peripherally inserted central catheters (PICCs) and ROP screening [293–297]. A safety concern related to the administration of paracetamol is hepatotoxicity, which depends highly on drug metabolism. Paracetamol is metabolized in the liver via glucuronidation and sulfation; however, in preterm infants glucuronidation of paracetamol is very low and matures during early childhood [298].

To ethically justify the administration of a medication, the benefits of the analgesic effect must outweigh unwanted adverse effects. Unfortunately, there is a lack of evidence of the safety and efficacy of analgesics prescribed to neonates in the NICUs. Further study of both the pharmacokinetic and pharmacodynamic profile of common analgesics is an imperative need in the neonatal population.

1.5 Thesis motivation, aims and structure

Hospitalised neonates undergo frequent essential painful procedures. Although the negative impact of pain experienced in early life is well documented [41, 47, 299, 300], pain is under-diagnosed and under-treated in clinical settings. Assessing a complex experience like pain is challenging and no gold standard analogous to verbal report exists for the evaluation of pain in infants. As a result, there is a paucity of evidence regarding the efficacy and safety of pain-relieving interventions, and neonatologists need to rely on clinical experience and data from older children and adults for the provision of analgesics [301]. The first step to facilitate the design of evidence-based pain management strategies is to develop tools that accurately evaluate pain in neonates.

Clinical pain scales have been developed for use in neonates based on early observations of facial grimacing and changes in cardiorespiratory parameters [244]. However, behavioural measures are not specific to detect pain and their visual assessment is subjective. These scales are used inconsistently in clinical practice [80]; and when used in clinical investigations the evidence of the efficacy of common analgesics has been inconclusive [302, 303]. The pain experience is ultimately generated in the brain, and infants from a very young age have the basic functional connectivity for pain perception [236]. Therefore, brain activity may provide a quantitative and objective measure of nociceptive processing in the infant brain and possibly the best approximation to the infant pain experience. An EEG template of noxious-evoked brain activity has been previously developed and validated in infants to quantify the magnitude of brain activity evoked by noxious stimulation in new datasets. This pattern of brain activity is not evoked by other equally arousing sensory stimuli such as visual, auditory and tactile stimulation [237]. The template has been used to test the analgesic efficacy of topical local anaesthetic [237] and as an endpoint in a clinical trial [304].

However, this template was developed to quantify pain responses to heel lances and experimental noxious stimuli but not to other clinically necessary procedures in the neonatal unit. In order to test pain-relieving interventions in further procedures, the response to these procedures needs to be characterised. Having a tool to measure brain activity that can be reproduced and transferred to multiple acute procedures would be highly advantageous to facilitate systematic analysis between future investigations and to combine evidence from different study populations. Moreover, understanding the distribution of the outcome measure in the population of interest is key to predetermining the number of observations that will be required to statistically demonstrate an effect from an intervention.

The magnitude of the noxious evoked brain activity to the same stimulus intensity can be highly variable between infants. As a result, large sample sizes are

often required to account for between-subject variability. This variability can be related to multiple factors such as gestational age, behavioural state, prior pain and illness. Balancing these factors can aid in the comparison of the outcome between groups; however, this could still require large samples to reach an adequate balance between groups. Infant research is time and resource-intensive and limited samples are usually available [305]. An alternative approach using a measure of brain activity to account for the between-subject variability could increase the power to detect a true effect in small samples and optimise the design of analgesic clinical trials. Additionally, identifying infants with higher sensitivity could be useful for the stratification of patients during clinical research and to drive the treatment of patients that would benefit the most from analgesic interventions.

The aims of this thesis are to (i) assess the generalisability of a brain-derived measure of pain across various body locations and stimulus modalities in neonates, (ii) quantify noxious evoked baseline sensitivity to increase the power to detect true effects from interventions in small samples, and (iii) investigate the effect of early life inflammation in spinal cord excitability and nociceptive sensitivity in neonates.

Chapter 2 provides the general methods followed in this thesis from infant recruitment to the analysis of brain activity, reflex withdrawal and behavioural responses.

Chapter 3 characterises the brain activity evoked by experimental noxious stimulation applied to three body locations and the suitability of the template for the specific assessment of pain responses to immunisations. Additionally, the validated template is used to investigate the effect of paracetamol during routine immunisations in the neonatal unit.

In **Chapter 4**, a paradigm is developed to assess individual baseline sensitivity by measuring brain activity in response to a low-intensity experimental stimulus. Gentle

touch is used as a pain-relieving intervention to investigate whether accounting for this measure of baseline sensitivity reduces the number of observations required to evaluate analgesic efficacy.

In **Chapter 5**, a novel experimental design is used to answer a clinically relevant question related to the interactions between the nociceptive and immune systems in neonates. Electrophysiology is used to investigate the effects of early life inflammation in spinal cord excitability and nociceptive sensitivity.

Chapter 6 presents a summary of the contributions of the investigations of this thesis and discusses the limitations and opportunities for further investigations.

2
General Methods

This chapter describes the General Methods used in the three Results chapters. It includes the participant recruitment process, the stimulation modalities applied to the participants and the data acquisition techniques used to record noxious evoked responses. The general data processing methods are described as well as the standard care comforting techniques and pain-relieving interventions used across the studies. Methods specific to individual studies are included in each Results Chapter.

2.1 Ethics and Recruitment

2.1.1 Ethical approval

Ethical approval was obtained from the South Central - Oxford C Research Ethics Committee (REC) of the National Research Ethics Service (NRES). Studies were conducted under the ethics application titled "Investigating pain in the developing human brain" (reference 12/SC/0447) and performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

2.1.2 Participant recruitment

Research was conducted in the Maternity wards and Neonatal Unit at the Women's Centre in the John Radcliffe Hospital, Oxford University Hospitals National Health Service Foundation Trust, UK. Approximately 6500 infants are born each year at this centre and the level 3 Neonatal Unit provides both intensive care and special care for neonates born in Oxfordshire and referred from across the region.

Medical notes were reviewed and neonates assessed as clinically stable, with no history of neurological problems, intraventricular haemorrhage (IVH) of grade 3 or 4 and not receiving analgesics or sedatives at the time of the study (except from paracetamol as specified in Chapter 3) were eligible for inclusion. Neonates with a history of maternal substance abuse, or whose mother had opioids throughout pregnancy due to medical reasons, or whose parents were unable to provide informed consent (e.g. language barriers, mothers under age 16 or parents with no legal custody of the baby) were not eligible for inclusion. The nurse, doctor or midwife looking after the eligible neonates confirmed verbally the appropriateness to approach parents to discuss research.

Parents were approached and asked if they are interested in hearing about a research study. The study was explained in detail including the data recording methods and if the study involved the use of experimental noxious stimulation, the stimulator and its application were demonstrated to the parents. Likewise, if the study involved the use of gentle touch, the brush stimulator and its application were demonstrated to the parents. It was emphasized that participation was voluntary and that they can withdraw their baby from the study at any time. A Patient Information Leaflet (PIL, Appendix A.1) was given and any questions were answered. Parents were given appropriate time to consider their participation according to the level of complexity of the studies and the arrangements for the clinical procedures (e.g. immunisations could be planned up to one week in advance whereas blood tests could be programmed within a few hours).

Parents gave written informed consent (Consent Form, Appendix A.2) prior to any neonate taking part in the study. The recruitment process and study participation, including all relevant forms, were documented in the medical notes and in the research study records. A good relationship with the parents and clinical staff caring for the neonates was key for the conduction of the research studies

while keeping disruption of standard clinical practices and additional handling of the neonates to a minimum.

I recruited a total of 64 neonates between February 2018 and November 2020 specifically for the investigations presented in this thesis. Additionally, 18 neonates were co-recruited and included in this study and other research projects undertaken by the Paediatric Neuroimaging Group. Previously collected data from 89 neonates meeting the same inclusion and exclusion criteria, and experimental protocols, were added to the data analysis. In total, data from 160 neonates recruited between July 2012 and November 2020 from the John Radcliffe Hospital Oxford were included in the analysis.

2.2 Stimulation techniques

Noxious and non-noxious stimuli were applied to the participants according to the clinical research protocol of each study presented in this thesis. Clinical procedures including heel lances and immunisations were performed by a trained member of clinical staff only when clinically required as part of the neonate's routine care. In all the studies a background period preceded the stimuli recordings and where a heel lance was applied a non-noxious control was used before the heel lance.

2.2.1 Heel lance and control heel lance

Neonates included in Chapter 4 and Chapter 5 were studied only during a clinically-required heel lance. A clinical member of the research team performed the lancing on the medial or lateral plantar surface of the heel with a mechanical BD Quikheel Premier Infant Lancet (Becton, Dickinson and Company, Franklin Lakes, NJ) with a penetration depth of 1.0mm. The foot was not squeezed for 30 seconds to ensure that the evoked response was only a result of the initial stimulus and to isolate the heel lance stimulus to perform PIPP-R scoring. If a second heel lance was required to obtain the necessary blood volumes, and the baby was settled, this was also recorded. Only one dataset was used per infant for the analysis.

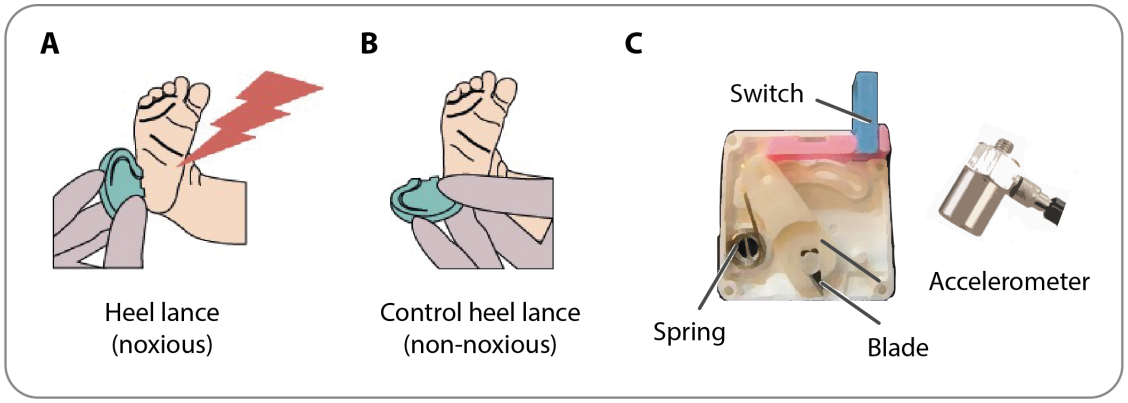

Figure 2.1. Schematic of the heel lance and the non-noxious control. (A) Lancet held against the neonate's heel during the heel lance. **(B)** Lance rotated 90 degrees during the control heel lance (non-noxious control). **(C)** Lancet internal mechanism, figure adapted from [217]. When the switch is depressed the blade is released causing a characteristic vibration that is detected by an accelerometer attached to the surface of the lancet and linked to the electrophysiological recordings.

During each test occasion the heel lance was preceded by a non-noxious control where the mechanical lancet device was rotated 90 degrees and activated so that the response to the non-noxious components of the stimulus (including touch and the sound made when the blade is released) was recorded without the lancet blade being in contact with the neonate's skin (Figure 2.1). Heel lances and non-noxious controls were automatically time-locked to the electrophysiological recordings using an event detection interface [217].

2.2.2 Immunisations

A group of participants included in Chapter 3 received Diphtheria, Tetanus, acellular Pertussis, Polio, *Haemophilus influenzae* type b, Hepatitis B (DTaP/IPV/Hib/HepB), Meningococcal group B (MenB) and Pneumococcal (PCV) routine immunisations at eight weeks postnatal age, DTaP/IPV/Hib/HepB immunisations at 12 weeks postnatal age or DTaP/IPV/Hib/HepB, MenB and PCV at 16 weeks postnatal age [306]. A member of the clinical care team administered the intramuscular immunisations to the anterolateral region in one or both thighs (the meningitis B vaccine is administered on the thigh opposite to the previous immunisations) using a

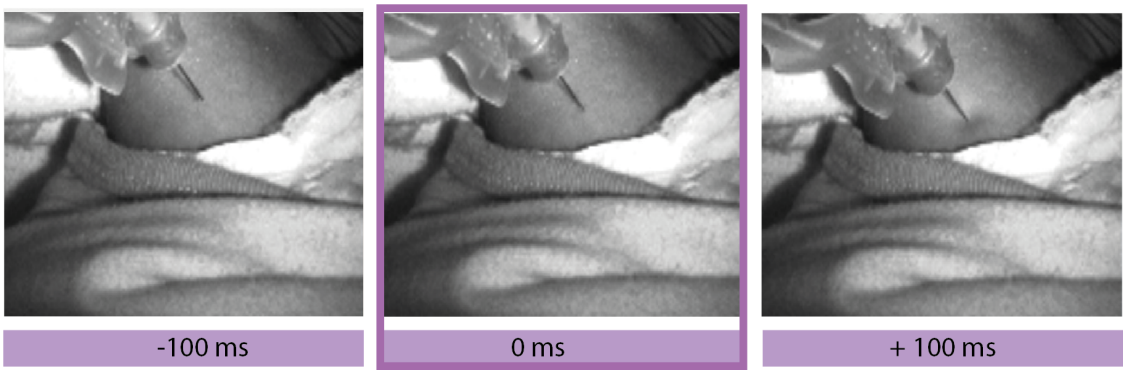

Figure 2.2. Immunisation procedure time-locking. Representative images of the needle and high-speed camera frames with the exact time at which the needle is in contact with the skin (central panel).

25 mm, 25 gauge needle. A minimum inter-stimulus interval of 30 seconds was kept to allow for PIPP-R scoring or until the neonates were settled after immunisations.

A high-speed camera video (220 frames per second; Firefly MV, Point grey research Inc.) was used to time-lock the stimuli with the EEG and EMG recordings. The time of each individual stimulus was identified retrospectively from the video recordings as the first point of contact of the needle with the skin [240, 307], Figure 2.2.

2.2.3 Experimental noxious stimuli

Mechanical acute noxious stimulation was applied using PinPrick™ stimulators, MRC Systems, Germany [308] with a fixed stimulus intensity that applies a force of 128mN. This mechanical stimulator consists of a flat tip needle (with a contact area of 0.2mm diameter) mounted on a plastic cylinder of constant weight [241]. The cylinder moves freely within a guiding tube as it is manually positioned perpendicular to the skin surface. The pinprick activates A-fibre and C-fibre nociceptors in the skin [241, 309] and is commonly used in adults for quantitative sensory testing (QST) of pain and perception thresholds [308, 310]. Pinpricks do not pierce the skin, are safe to use in neonates at this force and the 128mN pinprick does not cause distress or pain-related behavioural responses in term neonates [128, 237]. All

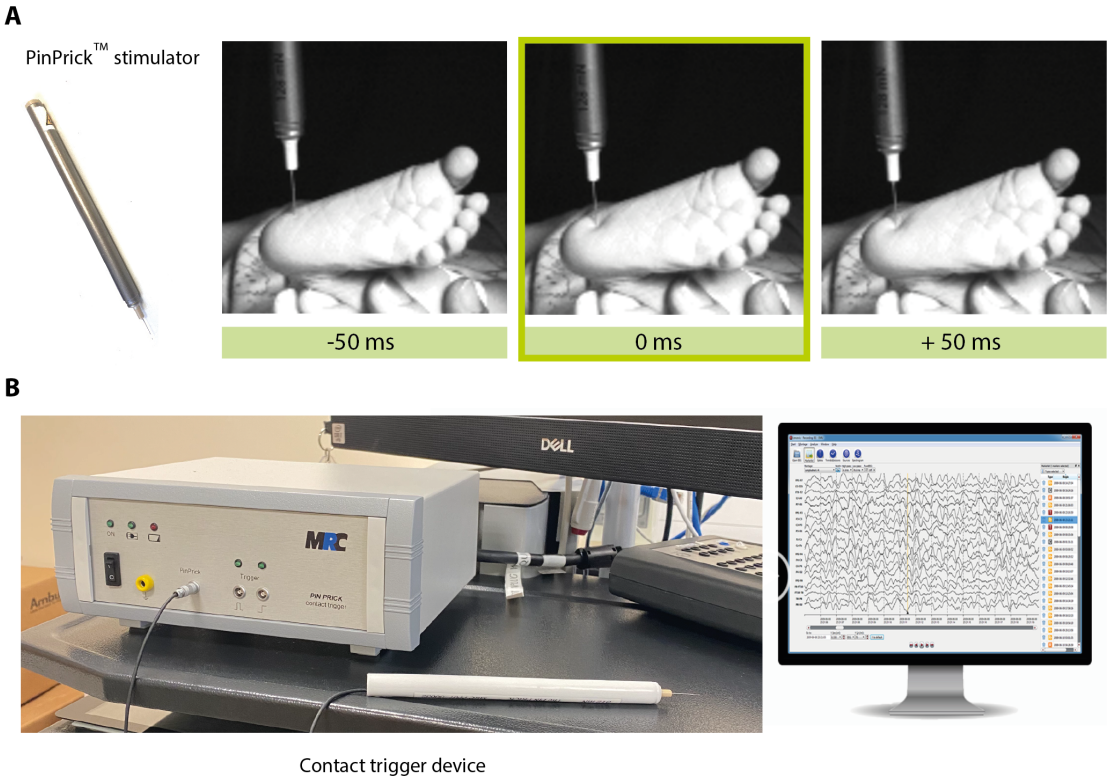

Figure 2.3. Experimental noxious stimulus time-locking. **(A)** Representative images of the PinPrick stimulator and high speed camera frames with the exact time at which the PinPrick stimulus is applied to the neonate's foot. **(B)** Electronic device for automatic detection of the PinPrick stimulation.

stimuli were applied for 1 s in blocks of 10 to 20 stimuli at minimum inter-stimulus intervals of 10 s. The inter-stimulus was extended if the infant became restless.

Experimental noxious stimuli were time-locked to the electrophysiological recordings using two methods. Seven studies in Chapter 3 and nine studies in Chapter 4 used a high-speed camera video (220 frames per second; Firefly MV, Point grey research Inc.) linked to the EEG and EMG recordings. The time of each individual stimulus was identified retrospectively from the video recordings with a manual marker when the pinprick's cylinder was first depressed and the force applied [186], Figure 2.3. The pinprick stimuli used in all the other studies were automatically time-locked to the electrophysiological recordings with a contact trigger electronic device, MCR Systems, Germany [311], Figure 2.3.

2.3 Non-pharmacological and pharmacological interventions

The wellbeing and comfort of the neonates were prioritised during the clinical studies. When non-pharmacological or pharmacological pain-relieving interventions were tested, these were compared with the standard clinical care during acute clinical procedures. The provision of analgesia in neonatal units is mainly dominated by non-pharmacological comfort techniques (such as non-nutritive sucking and swaddling). The interventions used in the studies included in this thesis were based on the current state of equipoise as the evidence of the efficacy of pain-relieving interventions is sparse and contradictory.

2.3.1 Standard care

As deemed clinically appropriate, all neonates received swaddling or non-nutritive sucking (dummy or a gloved finger to suck on). On rare occasions, parents held their babies during the clinical procedures at their request because babies were unsettled. In our local guidelines sucrose is not often used as part of standard clinical care and therefore was not used during the studies.

2.3.2 Paracetamol during immunisations

A subset of neonates in Chapter 3 received oral paracetamol (15mg/kg) for the management of MenB vaccine-related pyrexia according to national clinical guidelines. Our local neonatal unit began administering oral paracetamol to neonates immediately after immunisations in line with the 2015 national clinical guidelines recommending the administration of paracetamol at the time of Meningitis B immunisation due to its antipyretic effect [312]. In October 2018, clinical practice guidelines were updated in our local neonatal unit to administer paracetamol one hour prior to the immunisations where the MenB vaccine was due. This provided an opportunity to study the effect of paracetamol for acute pain management in neonates. Paracetamol was given by a member of the clinical care team and the time of administration was recorded for each participant.

2.3.3 Gentle touch during heel lances

A subset of neonates in Chapter 4 received gentle touch before heel lancing. The gentle touch intervention was provided by a member of the research team using a brush stimulator (SENSELabTM 709 Brush-05, Somedic) [313] designed for QST to apply a standardised force. The gentle touch was applied at a rate of approximately 3 cm/s for 10 seconds as this is the CT-optimal touch velocity which has been reported effective for reducing noxious-evoked brain activity in neonates [242]. The site selected for the gentle touch was the lower leg ipsilateral to the heel receiving the lance and covered approximately 10 cm of hairy skin. A computer visualisation tool was set up using PsychoPy [314] to guide the experimenters to apply the brushing at the standard velocity and the noxious stimuli immediately after the gentle touch.

2.4 Recording techniques and data processing

2.4.1 Electrophysiological recordings

Electrophysiological activity was acquired from DC to 400 Hz using a SynAmps RT 64-channel headbox and amplifiers (Compumedics Neuroscan). Activity was recorded using CURRY scan 7 neuroimaging suite (Compumedics Neuroscan) with a sampling rate of 2000 Hz. All equipment conformed to the electrical safety standard for medical devices, IEC 60601-1. Background activity was recorded and periods of rest were marked before any experimental noxious stimulation was applied.

EEG was used to record electrophysiological brain activity. Non-invasive scalp electrodes were positioned using the international standard 10-20 electrode system [315] as shown in Figure 2.4. EEG channels include Cz, FCz, CPz, C3, C4, T3, T4 and Oz for monitoring the Central (C), Temporal (T), Parietal (P), Frontal (F) and Occipital (O) regions [315]. The EEG output is presented with respect to a reference that was placed at Fz and the common or ground electrode was set at Fpz. The 10-20 system assigns a number to further specify the location in the left (odd numbers) or right (even numbers) hemisphere. Location z is used to indicate

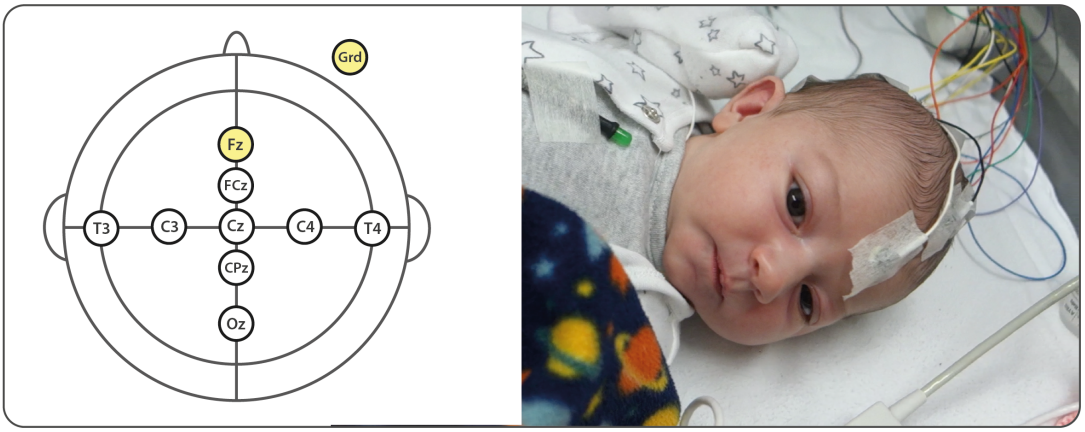

Figure 2.4. EEG electrode placement. The diagram in the left shows the electrode placement scheme used in all infants during EEG studies. Reference electrode was Fz and ground electrode (Grd) FPz. Photograph in the right displayed with parental authorisation.

that the electrode is in the midline or "zero" and Cz is positioned at the vertex, the midcentral point at the top of the head. This electrode placement scheme is common to other studies run in the Paediatric Neuroimaging Group that use visual and auditory stimuli which is why occipital and temporal regions are included. The skin was gently rubbed with a cotton bud with Preparation gel (Nuprep gel, D.O. Weaver and Co.) to clean the surface and minimize the impedances. Disposable Ag/AgCl cup electrodes (Ambu Neuroline) were placed with conductive paste (Elefix EEG paste, Nihon Kohden) to optimise electrode contact with the scalp. Electrodes were held in place using surgical tape (3M Micropore), and electrode leads were tied together to minimize electrical interference.

In Chapters 4 and 5, EMG was used to record reflex muscle activity in response to noxious and non-noxious stimulation. EMG is a technique used to measure spinal cord excitability and is a common surrogate measure of pain in preclinical studies. Its sensitivity varies greatly in infants according to their developmental stage. It has been shown that there is an increase in EMG thresholds to a von Frey hair stimulation with increasing PMA. Moreover, sensitization, resulting in significant decreases in threshold following repeated stimulation, occurs mainly in infants below 35 weeks GA [97]. Newborn rat pups are highly sensitive to tactile stimuli and their

reflexes are exaggerated and disorganised [146, 191]. Similarly, in premature human infants prolonged, non-specific reflexes have been observed, elicited by both tactile and noxious stimuli [189]. This means that reflexes observed in younger infants can be erroneously interpreted as pain-related responses. While the motor reflex activity generated by the spinal cord is not specific to nociception; in term infants the amplitude of spinal reflex is strongly correlated with noxious-evoked brain activity and it is a sensitive indicator that allows for the discrimination between noxious and non-noxious stimuli [99, 193]. Surface electromyography (EMG) was recorded using bipolar EMG electrodes (Ambu Neuroline 700 solid gel surface electrodes) placed on the bicep femoris muscles from both legs. The limb ipsilateral and contralateral to the site of the stimulation was recorded during each study.

2.4.2 Electrophysiological analysis

The analysis of EEG data, either ongoing activity or ERPs requires the extraction of the target signal from contaminating noise and artifacts. One method to achieve this is by applying digital filters post data acquisition. These consist of mathematical operations applied to the discrete, numeric representations of the signal waveforms to highlight or attenuate certain frequencies [316]. In this thesis, EEG activity evoked by each individual stimulus trial was filtered using a 0.5–30 Hz bandpass filter (comprising a high-order low-pass finite impulse response (FIR) and a second-order high-pass Butterworth filter) as well as a 50 Hz notch filter. A notch filter at 50 Hz was used to reduce artefact from power lines [317]. The high pass filter value was chosen to reduce slow drift in the recording which can be caused by factors such as sweating, and 30 Hz was set for the low pass filter as neonates predominately have low frequency brain activity with none expected in the gamma band. An ideal filter (which passes all frequencies above or below a certain value and attenuates all frequencies in the stop band) is not possible so a number of filters exist in the literature which approximate the ideal filter and have different advantages and disadvantages [318]. A Butterworth filter was chosen here as the high pass filter as

they have a flat frequency response and so will not introduce a ripple effect in the low frequencies where the majority of neonatal brain activity lies. A high order filter was used for the low pass filter as it has a high roll-off with a short transition band so better approximates an ideal filter, though may introduce some ripple effects in these higher frequencies. This will have less of an effect as there is very little power at high frequencies in neonatal EEG [319].

Data were epoched from 500 ms before the stimulus and 1000 ms after and were baseline corrected to the pre-stimulus mean. Epochs were rejected if they contained gross movement artefact, or if movement was observed in the baseline period. Event related potentials were analysed at the Cz electrode for all trials, as this is the electrode site at which the maximal evoked response is observed [199, 236, 237].

The magnitude of noxious-evoked brain activity during noxious stimuli was obtained by applying a previously validated template onto each individual trial [237]. The scripts for projecting the template were developed by Dr. Caroline Hartley. The template was projected 400–700 ms after stimulation when the stimulus was applied to the foot, 200-500 ms after stimulation when the stimulus was applied to the hand, and 300–600 ms after stimulation when the stimulus was applied to the thigh – see Chapter 3 Methods - providing a weight representing the magnitude of the noxious-evoked brain activity [237]. Each individual trial was first Woody filtered in the time window of interest to achieve maximum correlation with the template, accounting for individual differences in the latency to the response. A maximum jitter of $\pm$ 50 ms for the experimental noxious stimuli and $\pm$ 100 ms for the heel lance and immunisations was used for the Woody filtering.

The origin and pain-related functions of the noxious-evoked potential fit by the template are difficult to assert given the lack of source localisation studies and comprehensive experimental designs in infant pain research that directly address this question. However, some superficial similarities can be drawn between

the infant's late positive template potential, which is centrally distributed and maximal at Cz [237], and the adult noxious-evoked P2 potential, which is also a late positive potential centrally distributed and maximal at Cz [239, 320–322]. This adult potential originates mainly from the mid-cingulate cortex [320, 322, 323] a brain region with pain-related nocifensive behavioural functions such as avoidance behaviour and body orientation to stimulus [323], which is also known to be active following noxious events in infants [129].

EMG signals were filtered between 10 and 500 Hz, with a notch filter at 50 Hz and harmonics. Epochs were extracted from 2 s before to 4 s after the stimulus and rectified. Individual epochs were rejected due to artefact or movement observed in the baseline period. Ipsilateral and contralateral recordings were evaluated separately for artefacts. The data was split into 250 ms windows and the root mean square (RMS) of the reflex signal was calculated in each window. The average RMS across the first four windows after the stimulus (first second after stimulation) was calculated as the magnitude of the reflex withdrawal. This measure of reflex withdrawal had been used in previous studies [186, 242, 247] and is a robust approach that allows discrimination between a noxious and non-noxious stimulus [193]. EEG and EMG data were processed using MATLAB.

2.4.3 Physiology and behaviour

Physiological data was acquired using a Philips monitor (IntelliVue MX800 patient monitor, Philips). Oxygen saturation was recorded with a pulse oximeter probe placed on the neonate's foot or hand. ECG was acquired from 3 leads placed on the chest to calculate the heart rate. A laptop was connected to the monitor to record data on real time and data was downloaded for analysis using ixTrend software (ixellence GmbH). When the neonate's vital signs were already being monitored by the clinical team, the research laptop was connected to the monitor to record the data. The time of stimulation was manually annotated in the computer recording.

The physiological responses during stimuli were assessed by calculating the changes in heart rate and oxygen saturation. The baseline average heart rate and oxygen saturation were calculated in the 15 second prior to stimulation, and the maximum heart rate and minimum oxygen saturations in the 30 seconds following stimulation. Scores between 0 and 3 were assigned for both metrics according to the PIPP-R score [170]. Physiological data processing was performed using MATLAB.

Facial video was recorded using a handheld camera and a synchronised LED flash activated by the researcher simultaneously with each stimulation was used as a marker for the time of stimulation. The videos were processed and analysed retrospectively by cutting into 45 s clips, which included a 15 s baseline and 30 s after the stimulus. The presence and duration of each facial expression (nasolabial furrow, eye squeeze, and brow bulge) was assessed individually and scores between 0 and 3 were assigned for each feature according to the PIPP-R score system [170], Figure 2.5.

The observer was blinded to the stimulus type when analysing the videos. The final PIPP-R scores were calculated by combining contextual scores (behavioural state and age group) with behavioural and physiological measures according to the Figure 2.5 and documented using a PIPP-R form (see Appendix A.3). The PIPP-R maximum score is 21 with a score of 0 considered no pain, 1-6 mild pain, 7-12 moderate pain and $>$ 13 severe pain [324].

PIPP-R Infant Indicator	Indicator Score			
	0	1	2	3
Gestational Age *	>= 36 weeks'	32 weeks' to 35+6 weeks'	28 weeks to 31+6 weeks	< 28 weeks
Behavioural State *	Active awake: eyes open, facial movements	Quiet awake: eyes open, no facial movements	Active sleep: eyes closed, facial movements	Quiet sleep: eyes closed, no facial movements
Change in Heart Rate (bpm)	0 to 4 beats per minute increase	5 to 14 beats per minute increase	15 to 24 beats per minute increase	>=25 beats per minute increase
Decrease in Oxygen Saturation (%)	0% to 2% decrease	3% to 5% decrease	6% to 8% decrease	>8% decrease
Brow Bulge	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Eye Squeeze	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Nasolabial Furrow	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds

Figure 2.5. Premature Infant Pain Profile-Revised (PIPP-R) scoring system. Contextual, physiological and behavioural parameters in the PIPP-R. * The contextual (postmenstrual age and behavioural state) indicators are scored only when the physiological (heart rate and oxygen saturation) or behavioural (brow bulge, eye squeeze and naso-labial furrow) indicators are scored as > 0. Table adapted from [170].

3

Generalising a brain-derived measure of pain across various body locations and modalities in neonates

3.1 Introduction

Considering the short-term distress and long-term neurodevelopmental impact associated with repeated pain exposure in early life [41, 47, 299, 300], effective pain relief is crucial in neonatal care [325, 326]. Analgesic drugs commonly used in older children and adults are yet to be evaluated in neonates to determine their efficacy and optimal dosing. One of the major barriers to conducting analgesic clinical trials in this population is the lack of objective quantitative measures of pain. Noxious-evoked brain activity is an example of an objective measure to assess the magnitude of responses evoked by noxious stimuli in infants. Neurophysiological measures of brain activity have been previously used in research studies [186, 235, 237, 242, 327] and clinical trials [247, 284] demonstrating their potential as surrogate measures of pain and as objective endpoints to test analgesics.

Given the practical and ethical challenges of the investigation of pain in neonates, the best way to test analgesics is to conduct studies during clinically-required painful procedures. Therefore, it is important to first characterise the patterns of nociceptive brain activity during these clinical procedures under the current standard practice. The development of the template of noxious-evoked brain activity [237] provided

a robust analytical approach to identify the EEG magnitude, morphology and latency of nociceptive brain activity evoked by heel lances and noxious experimental stimulation of the foot. This pattern of brain activity was consistently observed in neonates from 34 to 42 weeks' gestation and its magnitude was significantly reduced when local anaesthetic was applied to the foot before the noxious stimulus [237].

The template serves two functions: one of identification of the EEG noxious evoked potential (which is accomplished through signal decomposition over a previously defined time period following the stimulus) and one of quantification of the intensity of such response. For the latter, an important aspect of the template is that it extracts a single quantifiable parameter which is demonstrably evoked by an acute noxious stimulus, but not by a non-noxious stimulus, and its magnitude is consistent with the intensity of the stimulus [237]. During the initial development and validation of the template, the response evoked by a heel lance was scaled such that the average template magnitude for a cohort of term neonates was a unit vector (i.e a template magnitude of 1 represents the average response to a heel lance in this group) [237]. Therefore, the template serves as a scaling/normalisation tool to quantify and compare the measurements of equivalent stimulus in different test groups.

This is fundamentally similar to a methodology used to quantify stimulus-evoked blood-oxygen-level-dependent (BOLD) signal for fMRI analysis using statistical parametric mapping [328]. The magnitude of the stimulus-evoked signal is quantified by taking a pre-defined unit vector waveform (e.g. double gamma) called the haemodynamic response function (HRF). After convolving the HRF with the stimulus timings, the HRF waveform shape (predicted BOLD response) is fit to the fMRI data. In general, a linear regression is performed to fit the HRF shape to the data, and the magnitude of the stimulus-evoked response is quantified using the linear model regression coefficients. The regression coefficients, or parameter estimates, are the HRF scaling factors that scales with the size of the evoked activity

[328]. Analogously, the EEG template is a unit vector waveform that is referred to as the template. The waveform shape is fit to the EEG data using a linear regression, and the regression coefficients are the scaling factors that scale with the magnitude of the stimulus-evoked signal. In both infant fMRI and EEG pain studies, these temporal waveforms that have been previously derived from relevant infant populations are used [237, 329]. These waveforms are fit to each infant's data, and a linear model scaling factor is used as the measure of noxious-evoked signal magnitude.

This approach provides various advantages including its analytical robustness and applicability across varied data sets. It also eliminates the need to derive sample-specific measures for nociceptive-related brain activity in new datasets, optimising the data usage in sample sizes without sacrificing statistical power and the direct comparability of results across studies [237]. It is also noteworthy that this standardised template analysis approach has been recently used for the study of developmental changes in visual and tactile evoked brain activity [330].

However, the template of noxious-evoked brain activity was developed for heel lances and it has not been validated in relation to other clinical procedures. Such procedures may include cannulations, peripheral line insertions and injections. When a noxious stimulus is applied to the heel, the characteristic waveform of the infant noxious-evoked brain activity occurs approximately 400 to 700ms after the stimulus [199, 236, 237, 327]. A latency shift in the response is likely to occur when the stimulus is applied to other body locations given the different distances from the periphery to the brain. Location-related ERP latencies have been shown in adults [238, 331, 332] and following tactile stimulation in infants [217, 333]. Accurately characterising the latency of these responses will allow the investigation of changes in brain activity evoked by other clinical stimuli, such as insertion of cannulas in the hand or intramuscular injections administered in the thigh. Moreover, for the template to be generalised for use with other clinical procedures the morphology of

the response also needs to be consistent across these different modalities.

In this chapter, I focus on the characterisation of brain activity latencies related to noxious stimuli applied to different body locations and particularly following immunisations. This is important to validate the template of noxious-evoked brain activity for the quantification of noxious-evoked brain activity in response to clinical procedures that can be applied in different sites of the body. Additionally, I test whether the noxious-evoked response to immunisations is modulated by oral paracetamol in infants. Routine childhood immunisations are administered to infants at 8, 12 and 16 weeks of age [306]. Infants born prematurely are likely to receive these vaccinations during their hospitalisation in the neonatal unit. This provides an opportunity to conduct clinical research studies and assess immunisation pain-related responses in this population. One study has previously recorded cortical activity following immunisations in infants in outpatients clinics [240]. The pattern of activity identified is similar in morphology to the activity previously characterised during heel lances [237]. However, this study included term infants who will have an older postmenstrual age at the time of immunisations and therefore more mature nociceptive circuits compared to prematurely born infants.

Paracetamol is administered to infants during immunisations to manage fever - a symptom related to the Meningitis B (MenB) vaccination [312]; however, this medication is not licensed as an analgesic for clinical use below 2 months of age [334]. The mechanism of action of paracetamol is thought to be related to peripheral and central inhibition of cyclooxygenase (COX) synthesis and interactions with the descending serotonergic pathways and nitric oxide pathway [335]. In adults, paracetamol reduces pain ratings in chronic and postoperative pain conditions [336, 337]. The effect of paracetamol on acute pain has been investigated in adults and it has been shown that paracetamol increases the detection threshold to mechanical and electrical stimulation [338]. Moreover, pain-related activity recorded with EEG was reduced by paracetamol as well as the magnitude of evoked potentials following

thermal and laser noxious stimulation [339–341]. Similarly, MRI studies have shown a reduction in activity in response to thermal stimuli in the prefrontal cortices, thalamus, insula and PAG following paracetamol administration along with reduced pain ratings reported verbally [342].

While numerous studies in the adult literature demonstrate that paracetamol is an effective analgesic, studies in infants investigating the efficacy of paracetamol for pain management show inconsistent results. For example, some studies have demonstrated that paracetamol has an opioid-sparring effect [290, 291] and that the need for post-operative pain-relieving interventions is reduced after paracetamol administration [343]. However, many other studies have found that paracetamol is not effective for procedural pain in infants. These studies show that behavioural and physiological pain responses are not altered by paracetamol during common painful procedures such as heel lancing [295–297] and eye examinations for retinopathy of prematurity screening [294]. Thus, a Cochrane review concluded that there is insufficient evidence to determine the efficacy of paracetamol for procedural pain in infants. It is possible that these studies were underpowered to detect an effect in clinical pain scores or that the behavioural changes measured by these scores do not allow the distinction between pain and distress [200, 303].

During this investigation, there was a change in guidelines in our local Newborn Unit to administer oral paracetamol (15 mg/kg) 1 hour before immunisation administration instead of immediately after the immunisations. This change provided an excellent opportunity to undertake a pilot study to investigate the analgesic efficacy of paracetamol by comparing the responses of infants who received paracetamol before and after the immunisations. In this chapter, I aimed to: (i) characterise the latencies to EEG noxious-evoked brain activity related to different stimuli body locations by using experimental noxious stimulation applied to the hand, foot and thigh of newborn infants; (ii) Investigate the morphology of the pattern of brain activity evoked by immunisations with the use of the validated

template of noxious-evoked brain activity; and (iii) assess the analgesic efficacy of paracetamol during routine immunisations in infants.

I hypothesised that: (i) the latency shift of the noxious-evoked brain activity would be in accordance with the body location where the stimulus was applied; (ii) the morphology of the noxious-evoked brain activity following immunisations would be consistent with the pattern of noxious-evoked brain activity identified in previous studies; and (iii) paracetamol would reduce the magnitude of noxious-evoked brain activity following immunisations.

3.2 Methods

This chapter presents three studies:

- **Study 1** characterises noxious-evoked brain activity when an experimental noxious stimulus is applied to different body locations where clinical painful procedures are commonly applied to neonates. The morphology and latency of the ERPs at each site are investigated.
- **Study 2** validates the template of noxious-evoked brain activity for use in studies of immunisation.
- **Study 3** investigates the effect of paracetamol on brain activity and clinical pain scores during routine neonatal immunisations using the template of noxious-evoked brain activity tested in Study 1 and Study 2.

3.2.1 Participants

A total of 21 healthy neonates born at term (median GA =40.6 weeks, IQR:41-40) were recruited for Study 1 from the Maternity Unit at the John Radcliffe Hospital, Oxford. At the time of the study, the postnatal median age was 2 days (IQR:3-1). Two neonates were not studied due to technical issues and two studies were

suspended due to restlessness. In addition, 32 premature-born neonates (median GA=28 weeks, IQR:26-28) who had reached term age at the time of the study (median PMA=37.4 weeks, IQR:38-37), and who were due routine immunisations as inpatients in the neonatal unit were recruited for studies 2 and 3. The recruitment was conducted according to the methods described in Section 2.1.2, Chapter 2. Ethical approval (reference: 12/SC/0447) and informed written parental consent were obtained for each participant. The studies were carried out in accordance with the standards set by the Declaration of Helsinki and Good Clinical Practice guidelines. The demographic characteristics of the participants included in the final analysis are presented in Table 3.1. The estimate of cumulative prior pain exposure was quantified from each neonate's clinical records as the total number of acute skin-breaking procedures (including heel lances, venepuncture, and intravenous cannulations) and aspirations (oropharyngeal or endotracheal) from time of birth to time of study [99]. These procedures were chosen based on a prospective epidemiology study describing the most commonly performed clinical procedures neonates are exposed to during hospitalisation [29].

3.2.2 Experimental design

In Study 1, mechanical experimental noxious stimuli (non-tissue damaging) were applied to the participants using a 128mN PinPrick (MCR Systems, Heidelberg, Germany). A total of 10–12 experimental noxious stimuli with an inter-stimulus interval of at least 10 s were applied to the neonate's hand (n = 17 neonates), foot (n = 17), and thigh (n = 10). This study was initially designed to validate the noxious-evoked responses during cannulations applied to the hand as part of a collaboration with Sven Wellmann et al. in Switzerland. After seven participants, it was decided that this experimental design would be useful to also validate the template of noxious-evoked brain activity for procedures like injections applied to the thigh, so the third stimulation site was added, and the additional 10 participants received pinpricks on the hand, foot and thigh. Accurate estimates of baseline

Table 3.1. Participant demographics. Values given are median (lower quartile, upper quartile) or number (%). * Indicates missing data for one neonate.

	Study 1	**Study 2 and Study 3 Control Group**	**Study 3 Intervention Group**
Number of neonates	16	15	14
Gestational age (GA) at birth (weeks)	40.6 (40, 41)	27.6 (25.6, 28.8)	27.3 (26.3, 28.3)
Postmenstrual age (PMA) at time of study (weeks)	40.7 (40.1, 41.4)	38 (37.2, 39.4)	37.2 (36.2, 38.1)
Postnatal age (PNA) at time of study (days)	2 (1, 3)	64 (59, 90)	64 (62, 70.8)
Birthweight (g)	3520 (3103, 3786)	1040 (705, 1268)	880 (708, 1031)
Sex			
Male	12 (75)	9 (60)	9 (36)
Female	4 (25)	6 (40)	5 (64)
Mode of delivery			
NVD (normal vaginal delivery)	8 (50)	6 (40)	3 (21.4)
Assisted vaginal ventouse/forceps	4 (25)	0 (0)	1 (7.1)
Emergency C-section	4 (25)	7 (47)	7 (50)
Elective C-section	0 (0)	2 (13)	3 (21.4)
Apgar score at 1 minute	9 (9, 10)	5 (4, 6)*	7 (3, 8)*
Apgar score at 5 minutes	10 (10, 10)	8 (7, 9)*	8 (7, 10)*
Estimated cumulative prior pain exposure	0 (0, 0)	31 (28, 451)	78 (58, 219)

sensitivity were obtained after five to seven individual trials. To account for data rejections, a total of 10 experimental noxious stimuli were applied to each body location. While applying multiple repeated stimulation allows for the increase of the signal-to-noise ratio, too many stimuli would also take too long given the inter-stimulus interval. Moreover, the current protocols approved by ethical bodies include a maximum of 30 pinprick stimuli per test occasion. The order of the stimulus location and the side were randomly selected by the research team before each test occasion (right = 8, left = 9), Figure 3.1.

In Study 2 and Study 3 neonates received routine intramuscular immunisations at 8 weeks, 12 weeks, or 16 weeks' postnatal age. During the study, each neonate received one or three injections into one or both thighs. In line with the National

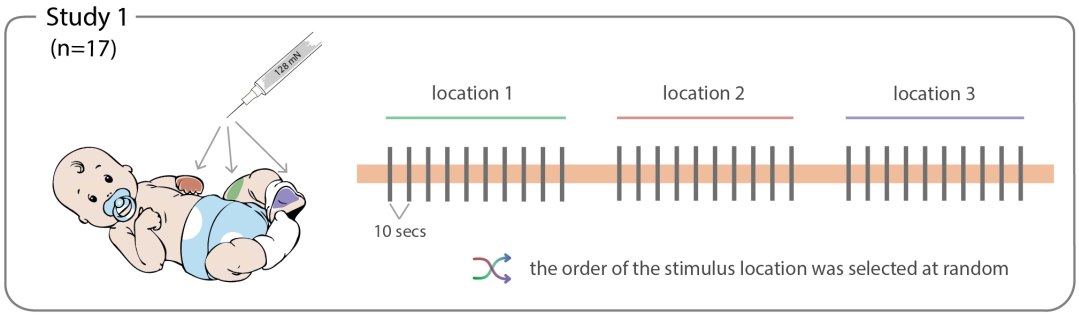

Figure 3.1. Experimental design Study 1, Chapter 3. Schematic representation of the experimental design followed in Study 1 where infants received blocks of noxious experimental stimuli applied to the foot, thigh and hand.

Institute for Health and Care Excellence (NICE) and British National Formulary for Children (BNFc) guidelines, oral paracetamol (15 mg/kg, for neonates born at less than 4 kg) was administered for the management of pyrexia after the administration of the MenB vaccine (immunisations at weeks 8 and 16). A total of 16 neonates were recruited to the study before the clinical practice guidelines were updated in our local neonatal unit to administer paracetamol 1 hour before the MenB vaccine. These participants were included in the validation of the template in Study 2 and as the Control Group in Study 3. Following the guideline change, 16 neonates were opportunistically recruited to Study 3 as the Intervention Group and received paracetamol 1 hour before the immunisations.

On average, neonates in the Intervention Group received the immunisations 79 minutes (min) (range: 65-117 min) after the administration of paracetamol. Comfort techniques including swaddling or non-nutritive sucking were used during the immunisation procedures. The experimental design is shown in Figure 3.2. The details of the stimulation techniques and the intervention are described in Sections 2.2 and 2.3, Chapter 2.

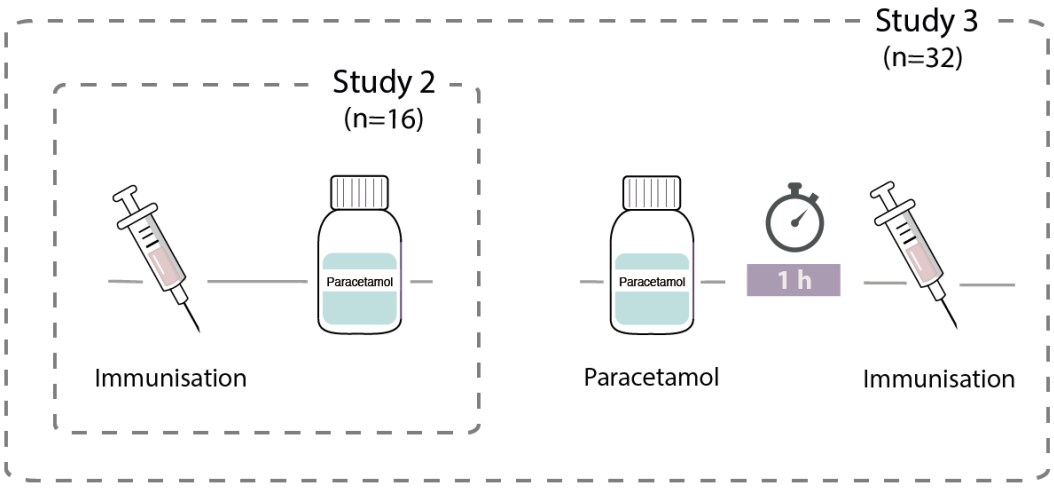

Figure 3.2. Experimental design Studies 2 and 3, Chapter 3. Schematic representation of the experimental design followed in Study 2 (before local guidelines change, paracetamol prescribed after immunisation) and Study 3 where infants received paracetamol before immunisations.

3.2.3 Recording techniques

EEG was recorded continuously for the duration of the experimental noxious stimulation (Study 1) and clinical procedure (Study 2 and Study 3). Recording electrodes were positioned according to the modified international 10–20 electrode placement system at Cz, CPz, C3, C4, Oz, FCz, T3 and T4, with reference at Fz and ground at Fpz. In Study 1, in seven studies, the EEG recordings were linked to a high-speed video camera (Firefly MV, Point Grey Research Inc) to time-lock the experimental stimuli. In the other 10 studies, the stimuli were time-locked to the EEG recordings using a contact trigger device (MRC systems).

In Study 2 and Study 3 needle insertion was time-locked to the EEG recordings using a high-speed video camera and the time of each individual stimulus was identified retrospectively from the video recordings as the first point of contact of the needle with the skin. Due to high-speed camera failure during set-up and accidental deletion of a recording, two neonates were removed from the analysis. Facial expressions and vital signs were also recorded in Study 3 to calculate clinical pain scores. Section 2.4 in Chapter 2 includes a detailed version of recording

techniques.

3.2.4 Data analysis

EEG analysis

EEG signals were bandpass filtered between 0.5 and 30 Hz with a notch filter at 50 Hz. Traces were segmented into 1500 ms epochs including 500 ms before the stimulus and 1000 ms after and were baseline-corrected to the pre-stimulus mean. Noxious-evoked brain activity was analysed at the Cz electrode and individual EEG epochs were visually inspected and removed from the analysis if noise or gross movement artefacts were present.

In Study 1, neonates with less than five trials on any individual locations were removed from the analysis for that location. The final foot, thigh, and hand analysis included 14, 8, and 14 neonates, respectively. Data from all individual trials from all neonates were aligned with respect to the average of the data by Woody-filtering with a maximum shift of ±50 ms in the 0–700 ms interval after the stimulus onset. Individual subjects' average response to the stimulus and the average background activity were calculated from the Woody-filtered data. Epochs from the two experimental conditions (noxious stimulus and background periods marked during the studies) were combined in a single dataset and clusters of time points where the noxious stimulus was significantly different from background activity were identified using the nonparametric statistical analysis described by Maris and Oostenveld [344]. The cluster-based test statistic was calculated from 1000 random permutations of the data, and the threshold for cluster significance was set as the 97.5 percentile of the permuted data. The midpoint of the cluster was identified and the time window for the principal component analysis (PCA) was taken as the 300 ms window about this midpoint, rounding to the nearest 100 ms, for each of

the responses to stimuli applied to the hand, foot, and thigh separately.

PCA was used to decompose the EEG signals into basic waveforms, known as principal components (PC) [235, 236, 345]. The first two PCs accounted for over 73% of the variance in the data across the experimental conditions and were the only components tested. The PC in which the weights were significantly different in response to the experimental noxious stimulus compared with background activity was selected as the noxious-evoked response and was compared with the previously described template of noxious-evoked brain activity [237].

In Study 2 and Study 3, recordings with poor video footage (for which the first point of contact of the needle with the skin was unidentifiable) were rejected from the analysis, and traces with noise or movement artefacts were also rejected. A total of 15 neonates (32 immunisations) were included in the final analysis in Study 2 (also Control Group in Study 3) and 14 neonates (27 immunisations) in the Intervention Group in Study 3.

In Study 2, noxious-evoked brain activity following immunisations was characterised to validate the suitability of the template across different modalities. EEG epochs were Woody-filtered within-subject to achieve maximum correlation with the within-subject average. The same approach used in the analysis of the EEG data in Study 1 was replicated to check that significant noxious-evoked activity following immunisations was observed in the same time window as that observed in response to experimental noxious stimuli applied to the thigh. Clusters of time points where the noxious stimulus was significantly different from background activity were identified using nonparametric cluster analysis, with 1000 random permutations of the data. PCA was applied in the 300-600 ms time window and the weights of the first two PC were compared between the experimental conditions and the waveforms compared against the template of noxious-evoked brain activity.

In Study 3 EEG epochs were first Woody-filtered within-subject to achieve maximum correlation with the within-subject average and to account for within-neonate variation in the latency of the responses due to the use of high-speed videos to mark the events. Each individual trial was then Woody filtered by a maximum shift of ±100 ms in the time window 300 - 600 ms to achieve maximum correlation with the template and account for between-subject latency variation. The template of noxious-evoked brain activity [237] was projected onto each individual trial from 300 to 600 ms after stimulation to calculate the magnitude of the noxious-evoked brain activity for each individual trial.

PIPP-R scoring

Neonates in Study 3 had facial expressions, heart rate and oxygen saturations recorded during a period of background and during immunisations. Video recordings and vital signs signals were clipped into individual trial epochs with 15 seconds before and 30 seconds after each immunisation. PIPP-R scores were calculated as described in 2.4, Chapter 2 by a trained researcher who was blinded to the stimulus type in the epoch. PIPP-R scores were not calculated for eight neonates (four in the Control Group and four in the Intervention Group) due to technical issues with the vital signs monitor during the recording or because the face view was obstructed in the videos. PIPP-R scores from a total of 11 neonates (27 immunisations) in the Control Group and 11 neonates (26 immunisations) in the Intervention Group were included in the final analysis. Behavioural scores were also compared between groups using the facial expressions' score subset from the PIPP-R scores and a total of 14 neonates (31 immunisations) in the Control Group and 13 neonates (26 immunisations) in the Intervention Group were included in this analysis.

3.2.5 Statistical analysis

Statistical analysis was carried out using MATLAB (MathWorks) and R (The R Project for Statistical Computing). A paired t-test was used for comparisons of

the PC weights between background activity and activity evoked by the stimulus of interest. Pearson linear correlation was used to assess the correlation between the previously characterised template of noxious-evoked brain activity and the PC for each independent body location and stimulus modality. The difference in the magnitude of noxious-evoked brain activity between the Intervention and Control Group in Study 3 was assessed using a linear mixed-effects model, with subject set as random effects. The clinical pain score differences between the two groups in Study 3 were assessed with a mixed-effects model for ordinal data with subject set as random effect (using the GLMM adaptive package). Two-sided tests were used for all statistical analyses with a significance level of 0.05.

3.3 Results

The results presented in this chapter have been published in [346, 347].

3.3.1 Study 1: Characterising noxious-evoked responses' morphology and latency when a stimulus is applied to different body sites

A total of 17 term neonates were studied to investigate the ERPs morphology and latency when experimental noxious stimuli are applied to different body locations. A train of experimental noxious stimuli was applied to the foot, hand and thigh and each event was time-locked to the EEG recordings. Previous studies have characterised infant ERPs consisting of negative-positive-negative complexes occurring from approximately 400-700 ms after noxious stimulation of the heel [62, 235–237, 247].

As shown in Figure 3.3A experimental noxious stimuli applied to the foot evoked two clusters of activity significantly different from background activity in the time frames 45-362 ms and 456-654 ms after the stimulus ($p<0.001$ and $p=0.014$ respectively, cluster-corrected nonparametric test). The first cluster is possibly

related to the saliency and arousal induced by the stimuli, as a similar pattern in this time window has been identified following tactile non-noxious stimuli [199, 235–237]. Since this early cluster is not thought to be noxious-specific, it was not analysed further. The second cluster is consistent with previous findings and a PCA applied in the time window 400-700 ms identified a waveform with the same morphology as the previously derived and validated template of noxious-evoked brain activity [237], Figure 3.3B, C. The weights of the second PC (which accounts for 17% of the variance) were significantly higher following the noxious stimulation compared to background brain activity ($p=0.035$, Figure 3.3D) and this component is significantly correlated with the template of noxious-evoked brain activity (Pearson's correlation $r=0.97$, $p<0.001$). The weights of the first PC did not differ between the background and noxious conditions.

Experimental noxious stimuli applied to the hand evokes a cluster of activity that is significantly different from background activity in the time window 298–461 ms as shown in Figure 3.4A ($p=0.01$). PCA was applied in the time window 200–500 ms after the stimulation and the weights of the second PC (which accounts for 31% of the variance) are significantly higher following the experimental noxious stimuli compared with background activity ($p<0.001$, Figure 3.4B, D) This PC is highly correlated with the template of noxious-evoked brain activity (Pearson's correlation $r = 0.97$, $p<0.001$, Figure 3.4C). The weights of the first PC did not differ between the background and noxious conditions.

As shown in Figure 3.5A experimental noxious stimuli applied to the thigh evokes a cluster of activity that is significantly different from background activity in the time window 280–563 ms ($p=0.002$). PCA was applied in the time window 300–600 ms after the stimulation and the second PC (which accounts for 17% of the total variance) is highly correlated with the template of noxious-evoked brain activity (Pearson's correlation $r = 0.98$, $p<0.001$, Figure 3.5C). The weights of this PC are significantly higher following the experimental noxious stimuli compared with

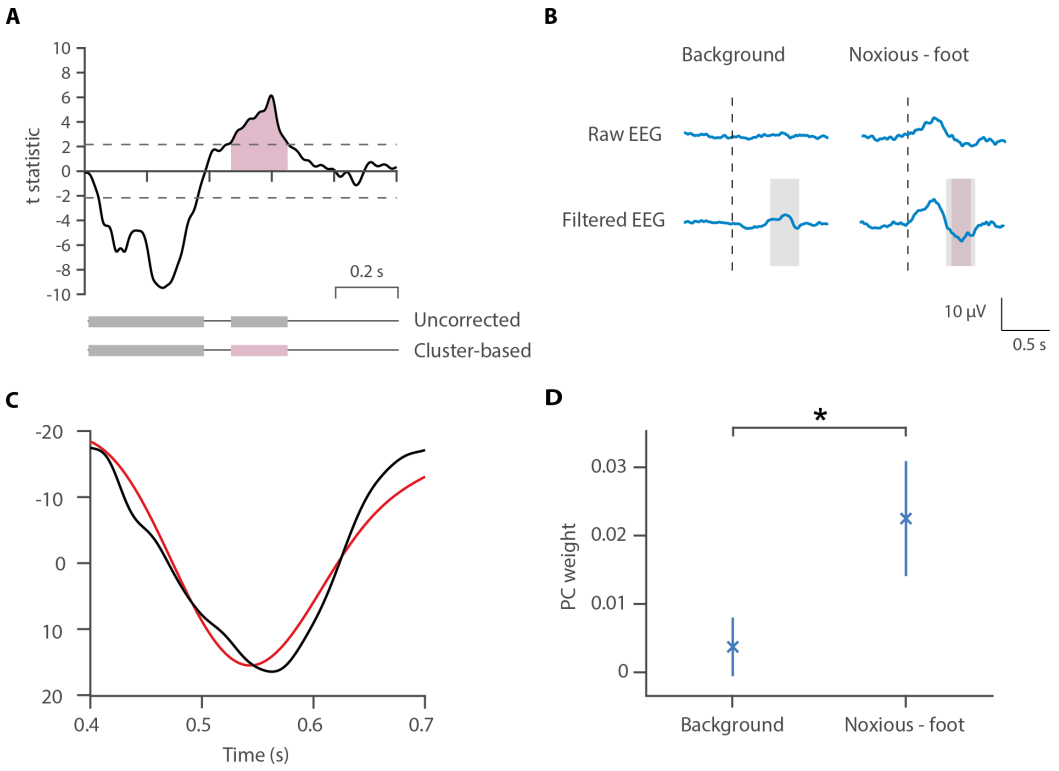

Figure 3.3. Noxious-evoked brain activity during stimulation of the foot. (A) t-Statistics from the comparison of the noxious-evoked brain activity after experimental noxious stimuli of the foot and during background brain activity in 14 neonates. The t-statistic threshold for cluster significance, set as the 97.5 percentile of the permuted data is shown by the dashed lines. The grey bars indicate time periods outside of the t-statistic threshold and the significant time window identified with the cluster analysis is illustrated by the pink shading area. **(B)** Raw average and Woody filtered EEG following experimental noxious stimuli of the foot and during background brain activity. Dashed lines indicate the time point of the stimulation. Pink shading shows the time window of the identified noxious response cluster and grey shading shows the time window where the PCA was performed. **(C)** Principal component analysis (PCA) was conducted in the time window 400–700 ms after the stimulus and the second principal component waveform (black) is significantly correlated with the previously described template of noxious-evoked brain activity (red). **(D)** The second principal component weights are significantly higher to noxious stimulation of the foot compared with the background brain activity (paired t-test, *p<0.05).

background activity ($p<0.001$, Figure 3.5D). There are no significant differences between the weights of the first principal component, which accounts for 57% of the variance ($p=0.41$). Similar to the previous locations, the weights of the first PC did not differ between the background and noxious conditions.

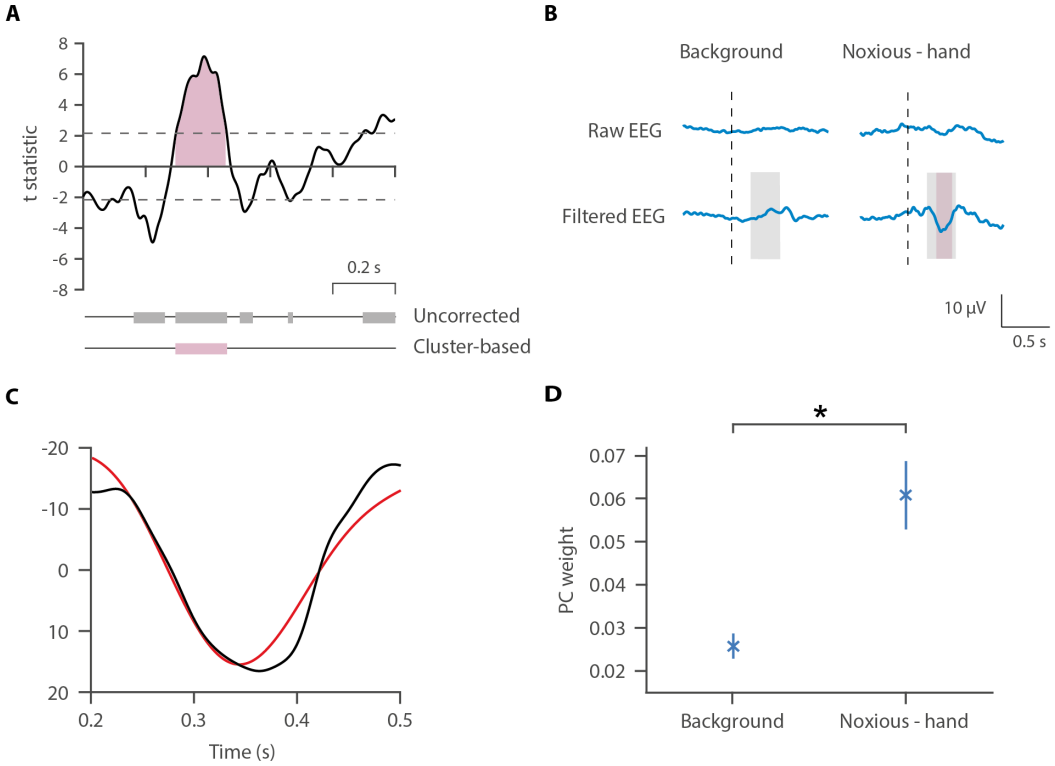

Figure 3.4. Noxious-evoked brain activity during stimulation of the hand. (A) t-Statistics from the comparison of the noxious-evoked brain activity after experimental noxious stimuli of the hand and during background brain activity in 14 neonates. The t-statistic threshold for cluster significance, set as the 97.5 percentile of the permuted data is shown by the dashed lines. The grey bars indicate time periods outside of the t-statistic threshold and the significant time window identified with the cluster analysis is illustrated by the pink shading area. **(B)** Raw average and Woody filtered EEG following experimental noxious stimuli of the hand and during background brain activity. Dashed lines indicate the time point of the stimulation. Pink shading shows the time window of the identified noxious response cluster and grey shading shows the time window where the PCA was performed. **(C)** Principal component analysis (PCA) was conducted in the time window 200–500 ms after the stimulus and the second principal component waveform (black) is significantly correlated with the previously described template of noxious-evoked brain activity (red). **(D)** The second principal component weights are significantly higher to noxious stimulation of the hand compared with the background brain activity (paired t-test, *$p<0.001$).

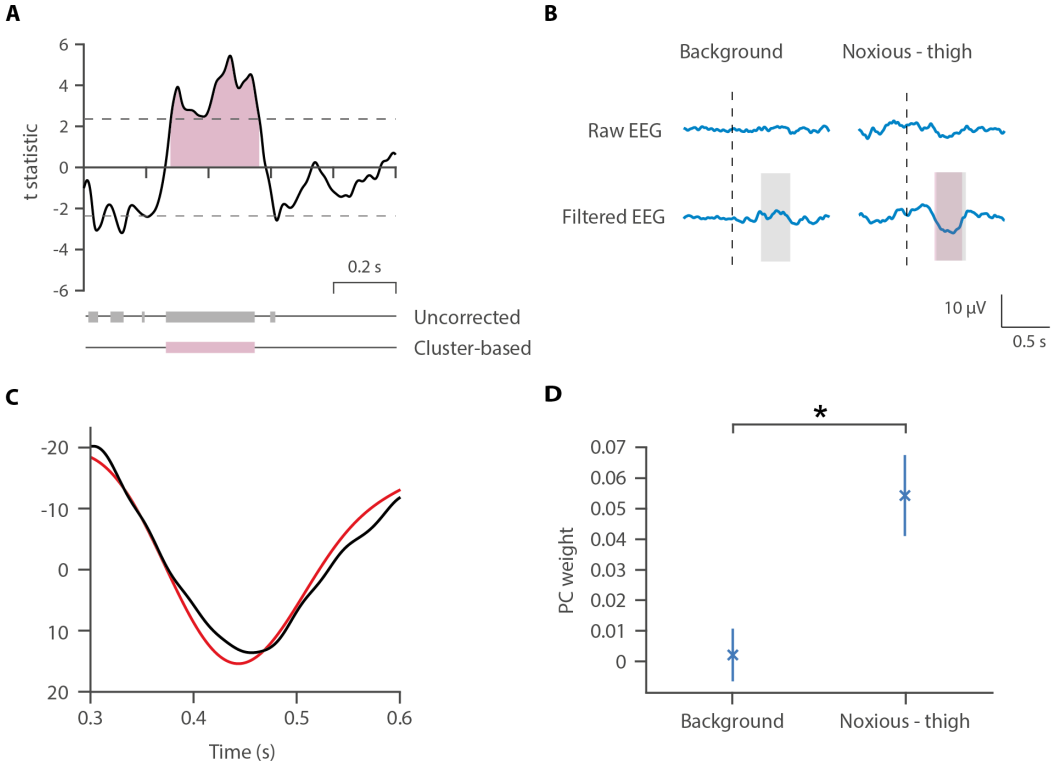

Figure 3.5. Noxious-evoked brain activity during stimulation of the thigh. (A) t-Statistics from the comparison of the noxious-evoked brain activity after experimental noxious stimuli of the thigh and during background brain activity in 14 neonates. The t-statistic threshold for cluster significance, set as the 97.5 percentile of the permuted data is shown by the dashed lines. The grey bars indicate time periods outside of the t-statistic threshold and the significant time window identified with the cluster analysis is illustrated by the pink shading area. **(B)** Raw average and Woody filtered EEG following experimental noxious stimuli of the thigh and during background brain activity. Dashed lines indicate the time point of the stimulation. Pink shading shows the time window of the identified noxious response cluster and grey shading shows the time window where the PCA was performed. **(C)** Principal component analysis (PCA) was conducted in the time window 300–600 ms after the stimulus and the second principal component waveform (black) is significantly correlated with the previously described template of noxious-evoked brain activity (red). **(D)** The second principal component weights are significantly higher to noxious stimulation of the thigh compared with the background brain activity (paired t-test, *p<0.001).

To further evaluate the identified time windows, I tested whether the time window of a given location can be used for the analysis of the response from the other two body locations (e.g. I expected that the foot time window can't be used for the thigh or hand analysis). I performed a PCA across the three time windows and compared the PC weights. As shown in Figure 3.6 there is a significant correlation with the template of noxious-evoked brain activity and significantly higher weights during the noxious stimulation only in the location-specific time frames (i.e 400-700ms after foot stimuli, 300-600 ms after thigh stimuli, and 200-500ms after hand stimuli).

Overall, the EEG waveform morphology after the experimental noxious stimulation of the foot, hand and thigh is consistent with the previously derived template of noxious-evoked brain activity [237] suggesting that the template is suitable to characterise noxious-evoked brain activity during experimental noxious stimuli applied to different body locations. Moreover, the observed latencies are related to the physical distance of the stimulus location from the brain (Figure 3.7)

3.3.2 Study 2: Accounting for different modalities for use in studies of immunisation

Intramuscular immunisations are routinely applied to the thigh. To test the validity of the template in the time frame from 300 to 600 ms to characterise immunisation-evoked brain activity, a study with 16 ex-premature neonates due their childhood routine immunisations was conducted. As shown in Figure 3.8A, a cluster of immunisation-evoked activity significantly different to background is present from 416 to 594 ms (p=0.035, non-parametric cluster analysis) following stimulation. This is consistent with the latency observed in response to experimental noxious stimuli applied to the thigh as shown in section 3.3.1. PCA in the time window 300–600 ms identified the first PC weights (which accounted for 58% of the variance) as significantly higher following immunisation compared with background

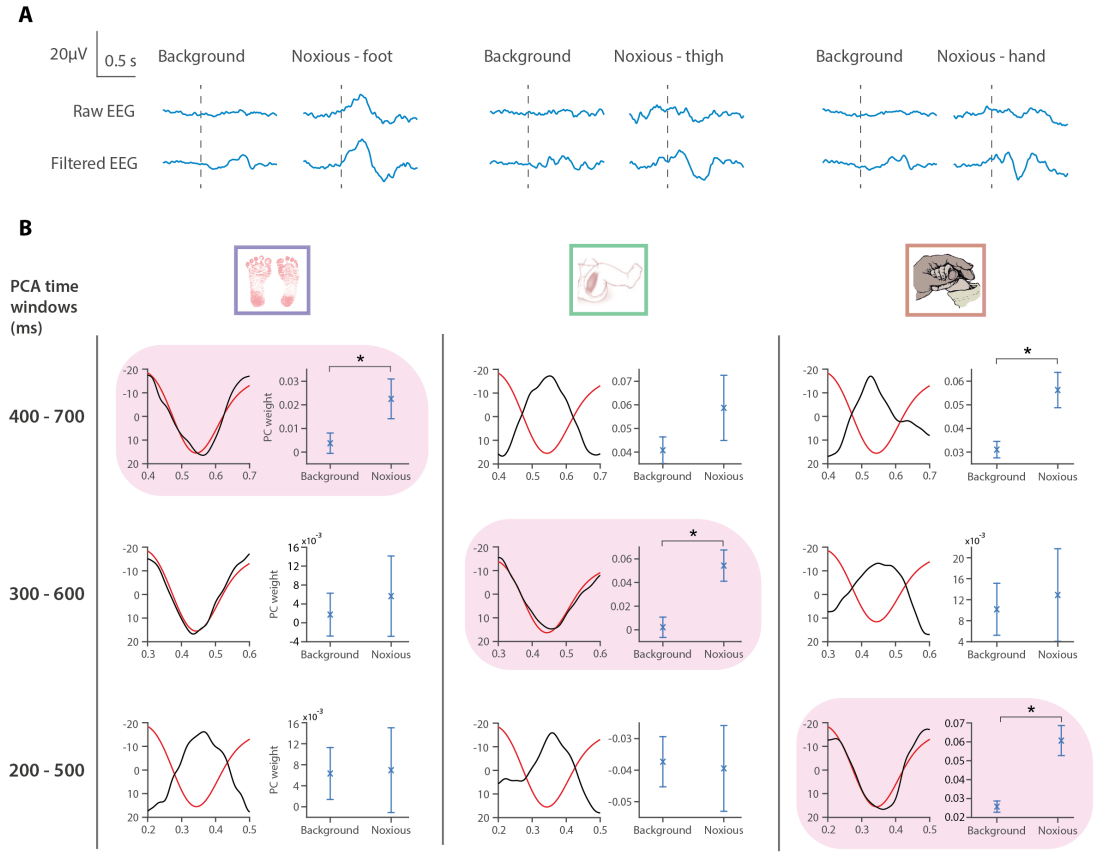

Figure 3.6. Principal component analysis across time windows and body locations. (A) Raw average and Woody filtered EEG following experimental noxious stimuli of the foot, thigh and hand and during background brain activity. **(B)** Principal component waveform and weights across three time windows comparing noxious-evoked bran activity and background activity. The second principal component waveform is shown in black and the previously described template of noxious-evoked brain activity is shown in red. The pink shading area indicates the combinations of stimuli location and PCA time window where the waveform is significantly correlated with the template of noxious-evoked brain activity and the noxious stimuli PC weights are higher than background activity (*p<0.05).

activity (p=0.004, 3.8B) and this PC is highly correlated with the template of noxious-evoked brain activity (r = 0.97, p<0.001, Figure 3.8C). These results are also consistent with a study in 1- and 2-month-old term-born neonates where ERPs with a similar waveform and latency were observed following immunisation [240]. The weights of the second PC did not differ between the background and noxious conditions. The template of noxious-evoked brain activity [237] is therefore considered suitable to characterise responses to immunisations and was used in the analysis presented in the following section.

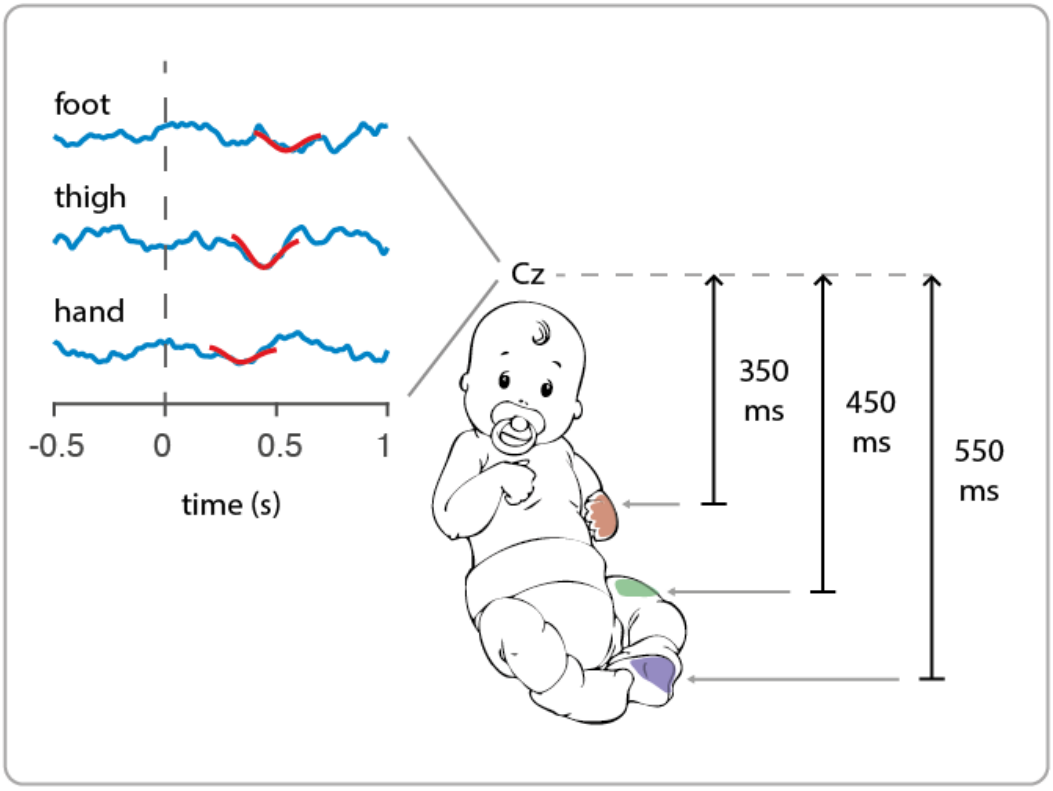

Figure 3.7. Schematic representation of the latency of the brain activity evoked by stimuli applied across different locations. Representative EEG traces of one individual participant with stimuli applied to the foot, thigh and hand. The template of noxious-evoked brain activity applied to the derived time windows is shown overlaid in red.

3.3.3 Study 3: Investigating the effect of paracetamol during immunisations

Effect of paracetamol on immunisation-evoked brain activity

The validated template of noxious-evoked brain activity was used to investigate the analgesic effect of paracetamol during immunisations. A group of 16 neonates who received paracetamol 1 hr before immunisations (Intervention Group) were studied following a change in the local guidelines. Before the guideline change, 16 neonates who did not receive paracetamol before immunisations (Control Group) were studied (these data was also used for the validation of the template presented in section 3.3.2). Every neonate received up to three immunisations and the average

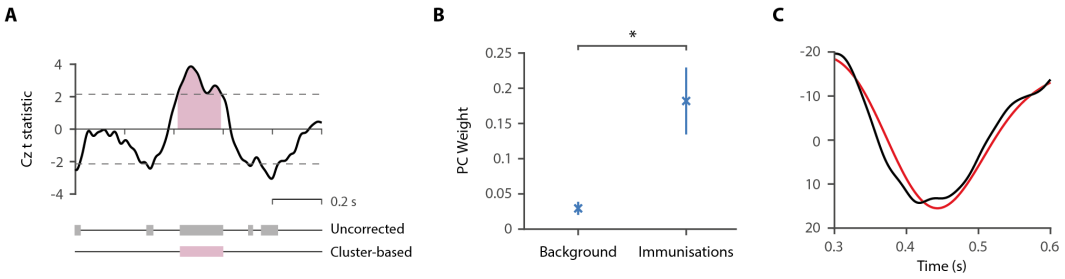

Figure 3.8. Validation of the template for use in immunisation studies.(A) t-Statistics from the comparison of the noxious-evoked brain activity to immunisations and during background activity in 15 neonates. Dashed lines indicate the t-statistic threshold for cluster significance, set as the 97.5 percentile of the permuted data. The grey bars show time frames outside of the t-statistic threshold and the significant time window identified with the cluster analysis is shown in pink. **(B)** Principal component analysis was conducted in the time window 300-600 ms following immunisations and the first principal component weights are higher following immunisations compared with background activity (paired t-test, *p<0.01). **(C)** The first principal component waveform (black) is highly correlated with the template of noxious-evoked brain activity (red).

individual responses are shown in Figure 3.9. The magnitude of noxious-evoked brain activity following immunisation is significantly lower in the neonates who received paracetamol prior to vaccination compared to the Control Group (linear mixed-effects regression model with subject and number of immunisations set as random effects, Control Group mean 0.88 [SD 0.58] (n = 15); Intervention Group mean 0.40 [SD 0.30] (n = 14), t = 3.61, p<0.001, Figure 3.10).

Effect of paracetamol on clinical pain scores during immunisations

PIPP-R scores following immunisations were compared between the Control Group and Intervention Group. There are no significant differences between groups (mixed-effects model for ordinal data with subject set as random effect, p=0.30) and the median scores in both groups fall into the 7-12 range, representing mild to moderate pain [174] (median PIPP-R score Control Group = 11, median PIPP-R score Intervention Group = 12, Figure 3.11A). A similar trend is observed when the behavioural component of the PIPP-R score (facial expressions) is analysed - as

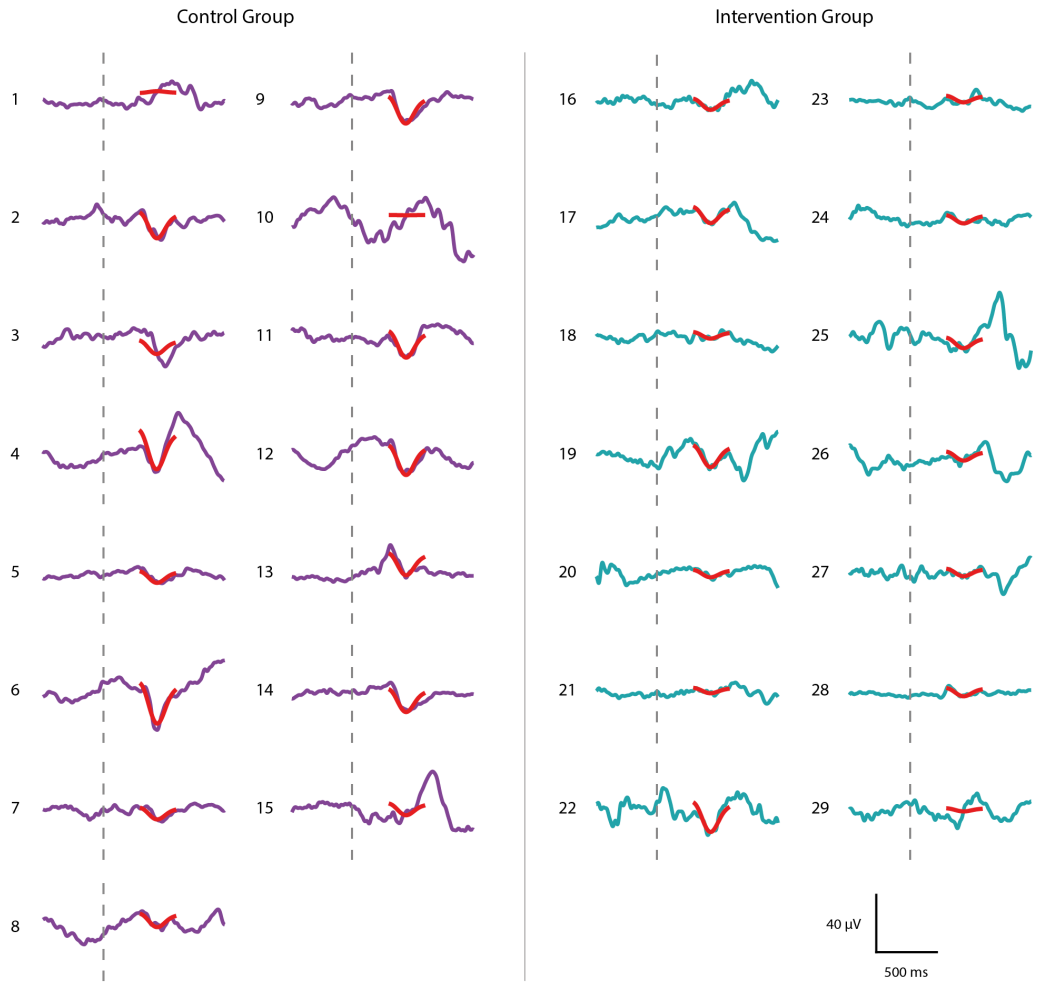

Figure 3.9. Noxious-evoked brain activity following immunisations in individual neonates. Average noxious-evoked brain activity in individual neonates following immunisations. N=29 neonates included in the analysis. Neonates in the Intervention Group (EEG traces in teal) received paracetamol approximately 1 hr before the immunisations. The grey dashed lines indicate the point of stimulation and the template of noxious-evoked brain activity is overlaid in red.

shown in Figure 3.11B the subscores do not differ between groups (mixed-effects model p=0.16). Paracetamol did not have an effect on the global PIPP-R score or any of the individual subcomponents of this scale.

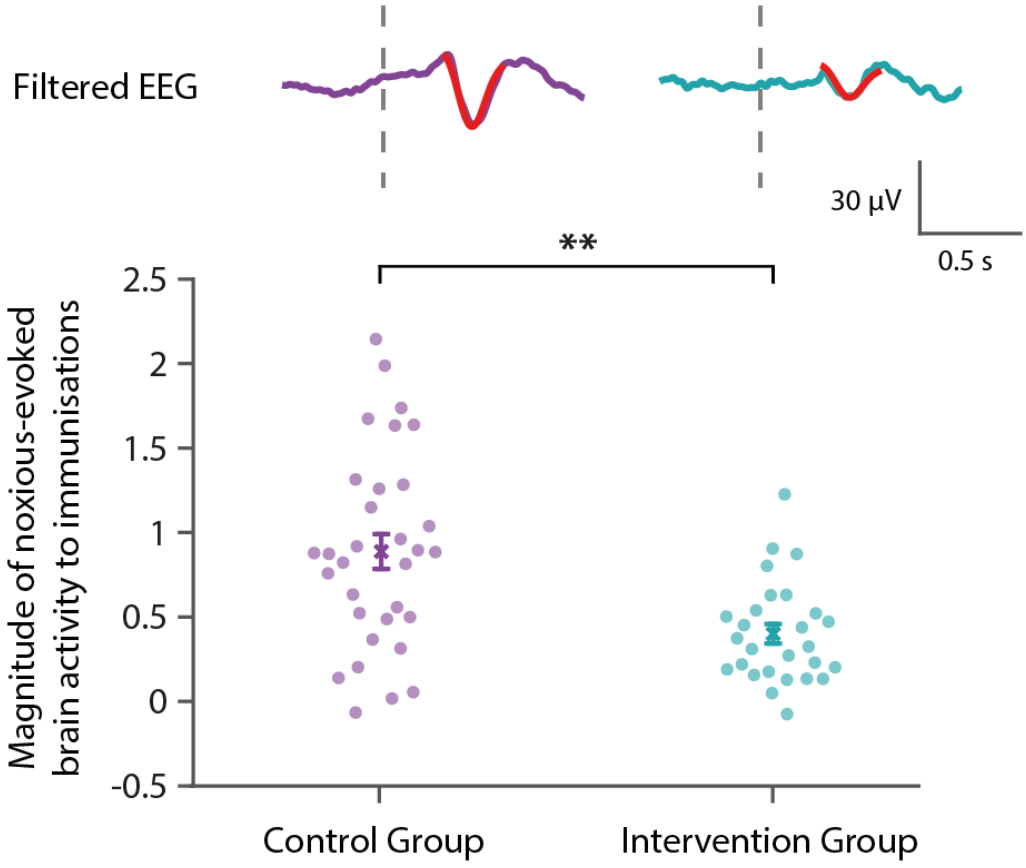

Figure 3.10. Paracetamol significantly reduces noxious-evoked brain activity following immunisation. Top: average woody filtered EEG following immunisations in the Control Group (purple) and Intervention Group (teal). The grey dashed lines indicate the point of stimulation and the template of noxious-evoked brain activity is overlaid in red. Bottom: Magnitude of the noxious-evoked brain activity following immunisations in the two groups (Control Group n = 15, Intervention Group n = 14), error bars indicate mean ± standard error of the mean (linear mixed-effects regression model, **p<0.001). Dots indicate individual immunisations, n=59.

3.4 Discussion

In this chapter EEG recordings from 31 neonates were used to validate the template of noxious-evoked brain activity across different body locations and stimulus modalities. The ERP morphology in each independent site and stimulus modality is correlated with the template of noxious-evoked brain activity [237], demonstrating the transferability of the template to characterise acute pain in a wider range

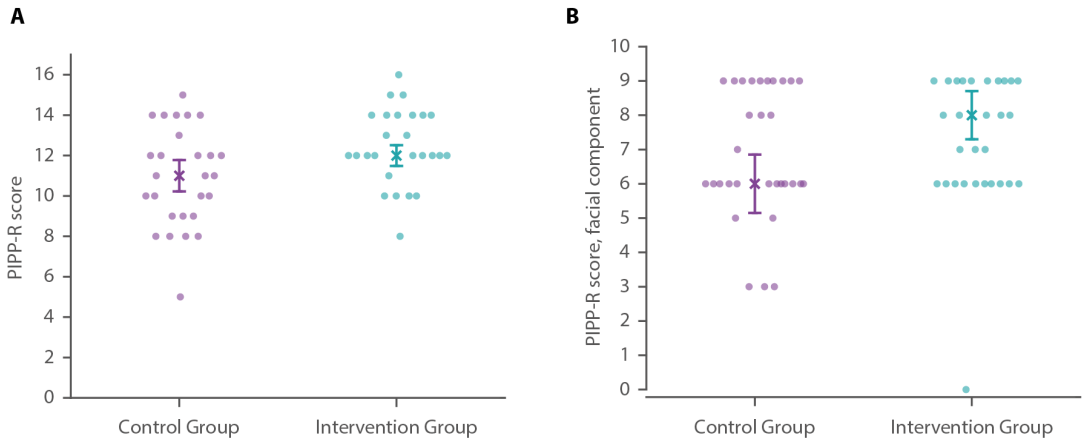

Figure 3.11. PIPP-R scores and behavioural scores are not different between Control and Intervention groups. A) PIPP-R scores during immunisations in the Control Group (purple, n = 11) and Intervention Group (teal, n = 11). **B)** Behavioural scores (facial expression component of the PIPP-R scores) during immunisations in the Control Group (n = 14) and Intervention Group (n = 13). Individual immunisations, n=60. In A and B, error bars indicate median ± standard error of the median. Dots represent individual immunisations, n=53.

of stimuli location and modalities including experimental noxious stimulation of the hand and thigh, and during immunisations. The latency of the potentials evoked by the noxious stimuli is related to the physical distance from the peripheral input site to the brain (i.e the shorter latency potential is observed after noxious stimulation of the hand, followed by the thigh and the longest latency is observed after noxious stimulation applied to the foot). The validated template of noxious-evoked brain activity was subsequently used to investigate analgesic efficacy by conducting an opportunistic pilot study investigating the efficacy of paracetamol during immunisations applied to 29 ex-premature neonates. The results showed a significant effect of paracetamol to reduce noxious-evoked brain activity and randomised clinical trials that include other pain-related measures are warranted to assess the benefit of paracetamol administration before immunisation.

The practical and ethical challenges associated with data collection in neonatal clinical research usually result in a low number of participants and data points for the analysis related to the question of interest [305]. Typically, studies analysing

neurophysiological data characterise the ERPs in a subset of the total sample and evaluate the noxious-evoked brain activity excluding this data subset, limiting the use of all available data [62, 235]. Having an independently derived and validated template can maximise the use of new data collected because it can be directly applied to the new data to obtain the magnitude of noxious-evoked brain activity [237]. This is particularly relevant for the design of clinical trials where all the relevant endpoints and the analytical methodology need to be predefined in the protocol. Moreover, the use of the template of noxious-evoked brain activity provides a standardised approach to facilitate comparisons and meta-analysis.

An ethically appropriate experimental design for neonatal pain investigations is to couple the EEG recordings with clinically required procedures; however, there are practical limitations associated with the single-trial nature of this approach. An alternative approach is to use a mild noxious experimental stimulus. In Study 1 the experimental stimulus used was a mechanical experimental noxious stimulus which is known to activate Aδ-fibres and it does not evoke behavioural changes in term neonates at a force of 128mN [186]. The advantage of using noxious experimental stimuli is that these can be applied multiple times and average the responses within individual neonates, increasing the signal-to-noise ratio. In practical terms, this approach allowed more flexibility to plan and conduct the studies around the availability and convenience of the parents and not around clinically-required procedures that are very time-sensitive. Moreover, recruitment was not limited by infants requiring a clinical procedure on the day but I was able to approach parents from a larger number of eligible neonates compared with studies that rely on a clinical procedure.

EEG has a high temporal resolution which allowed it to differentiate the ERPs at different locations in the millisecond range despite the different methods used to time-lock the events of interest. The latency shift observed during stimulation of

different body sites has been previously observed in infants following touch of the heel, of the shoulder [217], and of the face [333]. Our results are also concordant with studies in adults demonstrating evoked potentials in different time windows following experimental noxious stimulation at different sites reflecting physical distance and peripheral conduction velocity of the afferents mediating nociceptive evoked potentials [331, 332, 348].

The initial identification of the noxious-evoked waveform was done without using Principal Component Analysis (PCA) or Independent Component Analysis (ICA), as the ERP is a signal visible in the raw and group averaged trace. In order to define a fixed template waveform that could be fit to the noxious-evoked waveform and scaled to quantify the magnitude of the evoked signal, the evoked signal was decomposed into a linear combination of components. This could be done using either PCA or ICA (and potentially other methods). PCA is a decomposition method where correlated variables from the signal are converted into uncorrelated variables called principal components (PCs). On the other hand, ICA decomposes the signal into independent components (ICs) assuming that this signal is a linear mixture of true (brain-activity driven) signal and artefactual signals which are independent from each other and instantaneously mixed [349]. From prior experience, PCA decomposes the evoked signal in such a way as to reliably return a component with the waveform of interest. While PCA has not been systematically or formally compared to ICA or other approaches, using PCA to decompose the signal has proved to be a reliable and reproducible method. Given the short time frame (less than 2s), limited spatial information (single channel), and low stimulus trial numbers and low subject numbers inherent to pain studies in newborn infants, decomposition methods that rely on large quantities of data for reliable decompositions are less suitable.

When an experimental noxious stimulus is applied to the thigh an ERP is expected from 300 - 600 ms after the stimulus onset. This time window was

validated across modalities in Study 2 with immunisation-evoked brain responses showing a consistent waveform and significantly different PC weights compared with background activity in the same time frame identified in Study 1. Another study looking at EEG brain responses in 1- and 2-month-old term-born neonates during immunisations recorded an ERP with a similar waveform and a mean positive peak latency of 395 ± 17 milliseconds [240]. Interestingly, the mean magnitude of the immunisation-evoked brain activity in the ex-preterm neonates studied here (obtained by applying the template to the EEG responses, Control Group mean=0.88) is lower than the predefined mean response to a heel lance (mean =1 [237]). A higher magnitude of noxious-evoked brain activity in response to immunisations would have been expected to be consistent with pain severity scales estimated based on normalised clinical pain scores [350] where pain intensity during immunisation is higher than heel lance. However, this inconsistency could be related to a limitation of the approach used in this study where brain activity was measured in response to the first contact of the needle to the skin whereas clinical pain scores are likely to characterise other parts of the immunisation process such as the needle entry into the muscle and the fluid injection. Moreover, although having similar PMA at the time of the study, the maturational differences between the term-born neonates receiving heel lances and the ex-preterm neonates are likely reflected in the EEG and higher cortical responses to heel lances in ex-preterm neonates have been shown in a previous study [62]. Further analytical considerations may be needed to account for these differences.

In Study 3, results show that oral paracetamol administered 1 hr before immunisations significantly reduced the magnitude of the noxious-evoked brain activity during immunisation compared with neonates who did not receive paracetamol before immunisation. However, no differences in behavioural pain scores were observed between the groups. No other studies have used EEG derived measures to assess the effect of paracetamol in neonates and previous work assessing the efficacy of paracetamol (some administering higher doses of oral paracetamol - up

to 40mg/kg) on procedural pain, show similar results based on the evaluation of the change of behavioural clinical pain scores [294–297]. In these studies, a major limitation is the use of behavioural outcome measures which are subjective and may fail to discriminate between pain and distress [303, 351]. Behavioural and physiological pain scores, while widely used, rely primary on motor or autonomic processes that may not be directly linked to pain experience. Given that pain is a perception that involves cerebral activity, in the absence of verbal communication measuring changes in brain activity evoked by noxious input may serve as a better endpoint to evaluate pain and to assess the efficacy of analgesics interventions in both neonatal care and clinical trials.

Given the small sample size of the study presented in this chapter, the lack of randomisation and blinding, and potential confirmation bias [352]; the benefit of paracetamol for immunisations needs to be further assessed in a prospective adequately powered study. Nevertheless, this study suggests paracetamol may be an effective analgesic for acute pain in infants and that this warrants further investigation. Moreover, small studies in adults demonstrate that candidate drugs can modulate pain-related neural activity in the absence of verbally reported analgesia, and these brain-derived measures are recognised as a valuable approach to obtain objective evidence related to potential analgesic efficacy in early proof of concept studies [353].

In summary, the findings presented in this chapter demonstrate the reproducibility of the methods developed by Hartley and colleagues for the characterisation of noxious-evoked responses in response to a variety of stimulus modalities that can be applied across a range of stimulus sites across the body. This represents a step towards the development of translational tools for pain assessment in neonates. Some important limitations yet to be addressed include the evaluation of pain responses across a wider age range of participants, and the development of methods that can be used during other common conditions and procedures where reliable

pain assessment tools are required. Further studies should give careful consideration to the advantages and potential limitations of incorporating other measures to create a multi-modal pain assessment approach.

4

Quantifying noxious-evoked baseline sensitivity in neonates to optimise analgesic trials

4.1 Introduction

Participants in clinical trials risk exposure to potential adverse effects, and therefore every effort should be made to minimise the sample size necessary to demonstrate efficacy [354]. However, as factors such as age [99, 235, 355], prior pain experience [62, 356], stress [357], sex [198, 327], illness [201], and behavioural state [197] influence noxious-evoked responses, large sample sizes are often required to account for between-subject variability [279, 281, 284, 358–360]. In adult studies, cross-over trial designs are often used to minimise sample sizes by reducing between-subject variability [361]. However, this approach may not be appropriate when studying pain in neonates as painful medical procedures can only be performed when clinically necessary and within-subject variables that influence pain can change dramatically across sequential test occasions.

One approach used to balance demographic characteristics or other prognostic factors across treatment groups in clinical trials is to stratify neonates across treatment arms and to adjust for these factors in the statistical analysis [362]. While this can improve comparability across groups for recognised factors, many unknown variables likely influence pain sensitivity, and a more nuanced approach to account for individual differences in noxious-evoked sensitivity could be more

effective in reducing sample sizes. In analgesic studies performed in adults, individual pain thresholds can be identified by applying graded increments of experimental stimulus intensity until pain is reported by the participants. This can be used to stratify treatment groups [363–365] or statistically correct for variability in baseline pain thresholds [366, 367]. In neonates, application of graded non-noxious stimuli such as von Frey hairs has previously been used to identify limb reflex withdrawal thresholds [97, 187, 188, 192], but these have not been used as a baseline measure in analgesic clinical trials.

As described in the previous chapter, in the absence of a validated objective biomarker of pain [368], EEG-derived measures of brain activity may provide a valuable surrogate marker of pain by measuring the noxious-evoked activation of the cortex. In adults, brain activity during painful procedures is strongly correlated with verbal reports [369]; and in neonates, a template of noxious-evoked brain activity that discriminates between noxious and non-noxious procedures has been previously characterised and validated [237].

In this chapter, I aimed to develop an experimental paradigm that assesses individual baseline sensitivity in neonates by measuring noxious-evoked brain activity in response to a low-intensity experimental noxious stimulus and to investigate whether accounting for this measure of noxious-evoked baseline sensitivity substantially reduces the sample sizes required in neonatal studies of analgesic efficacy. I hypothesised that: (i) noxious-evoked brain activity in response to a mild experimental noxious stimulus would be strongly correlated with the response to a clinically required procedure, and therefore could be used as a measure of baseline sensitivity; and that (ii) accounting for the variance explained by the baseline sensitivity could increase the power to detect a true effect in small samples in studies of analgesic efficacy.

The term 'noxious-evoked baseline sensitivity' is used to refer to individual neonate's noxious-evoked baseline brain activity. This will be related to multiple neural and non-neural factors including nociceptive processing, arousal, attention, signal-to-noise ratio of the EEG recording, and differences in head size. In contrast to studies in adults which have shown similar patterns of activity evoked by both painful and non-painful stimuli [348], it has been previously shown that the pattern of brain activity in neonates that I analyse here is not evoked by visual, auditory, and tactile stimuli which evoke similar levels of physiological arousal [237].

In this chapter, the noxious-evoked sensitivity paradigm is developed and tested across three observational studies using clinical, experimental, and simulated data (47 neonates). The paradigm of baseline sensitivity was tested using gentle brushing as a pain relief intervention of known effect. Current evidence suggests that gentle stroking of the skin at a rate of 3 cm/s optimally activates C-tactile fibres involved in the detection of pleasant touch [271–273] and a previous study demonstrated the efficacy of gentle touch to reduce brain-derived measures following a clinically required heel lance in newborn infants [242]. I provide evidence of the efficacy of gentle touch, substantiating the need for randomised controlled trials for this intervention.

4.2 Methods

This chapter presents three studies:

- **Study 1** is a retrospective study to investigate whether the magnitude of noxious-evoked brain activity in response to an experimental stimulus correlates with the magnitude of brain activity evoked by the clinical procedure and thus its potential to be used as a measure of baseline sensitivity.
- **Study 2** uses simulated data to evaluate the statistical power that can be achieved by including baseline sensitivity as a covariate when analysing the effect of an intervention in small samples.

- **Study 3** is a prospective study conducted to test this novel paradigm using a non-pharmacological pain-relieving intervention of known efficacy – gentle touch – prior to heel lancing.

4.2.1 Participants

A total of 47 neonates were included in three studies. In Study 1, the relationship between responses to experimental noxious stimuli and clinically required heel lance was investigated in nine neonates born at term (median GA = 40.7 weeks, IQR:40-41) using unpublished data previously collected for other studies. In Study 2, the potential value of the statistical relationship identified in Study 1 was investigated using computational simulations. In Study 3, brain activity and reflex withdrawal responses from 38 neonates born between 35 and 42 weeks' gestation were recorded during clinically-required heel lance to test the paradigm with gentle touch as a pain-relief intervention. The participants were recruited from the Maternity Unit and Newborn Care Unit at the John Radcliffe Hospital, Oxford. The details of the recruitment methods are presented in Chapter 2. Participant demographics are shown in Table 4.1. The estimate of cumulative prior pain exposure was quantified from the medical records to reflect the most commonly performed acute painful procedures as explained in Section 3.2.1. Parents provided written informed consent and all studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines under the ethics approval: reference 12/ SC/0447.

4.2.2 Experimental design

Study 1

In Study 1, I retrospectively searched all the data previously collected (and that has not been previously published) to identify any term neonates who had received a clinically required heel lance and experimental noxious stimuli on the same test occasion. I identified nine neonates studied between 2014 and 2015 (age range 39–42 weeks' gestational age) who had all received experimental noxious stimuli at a force of 64 mN. The magnitude of the noxious-evoked brain activity was characterised by

projecting the template of noxious-evoked brain activity [237] in response to each stimulus. The mean response to the experimental noxious stimulus in each neonate was correlated with their responses to the heel lance using a Pearson correlation test.

Study 2

In Study 2, I simulated data sets to investigate potential differences in the power achieved by accounting for individual baseline sensitivity at different sample sizes (for a given effect size and significance level). Each simulation consisted of a Control Group and an Intervention Group. Given a sample size of N per group, N individual baseline sensitivity levels for each group were simulated by generating N uniform random numbers within the range of expected sensitivities. The minimum expected sensitivity was set as the minimum response to the experimental noxious stimuli in the data collected in Study 1, and the maximum expected response was set from multiplying the maximum of data collected in Study 1 by 3 (the expected increase in range from changing to an experimental noxious stimulus with a force of 128 mN from previously published data – Study 2 in [237], as using a force of 128 mN is expected to increase the signal-to-noise ratio). Responses to the clinical stimulus were then simulated from these randomly generated individual baseline sensitivities with parameters from the line of best fit in Study 1 [346]. The standard deviation of residuals was set to be 0.37 in most simulations as this is the standard deviation of the residuals from Study 1 but was varied for the simulations in Figure 4.4D. Finally, the responses to the clinical stimulus in the Intervention Group were reduced by a proportion according to the intervention effect. For most simulations, the intervention effect was set at 40% as this is considered a clinically meaningful effect size [274, 370]. However, varying levels of intervention effects were also investigated. The responses in the Control Group and the Intervention Group were compared with and without accounting for baseline sensitivity (see 'Statistical analysis', Section 4.2.6). For each value of N, 1000 Control and Intervention groups were simulated and the percentage of simulations where the group comparisons had $p<0.05$, that is, the power, was calculated. For simulations where the intervention

effect or the noise level was varied, the minimum number of neonates required for a power of 95% was calculated by increasing the group size by 1 (note all data was resimulated with each new sample size – so each simulated data set was fully independent). The power was calculated at each step until a power of 95% was achieved.

Study 3

In Study 3, the noxious-evoked baseline sensitivity paradigm was tested using a gentle touch intervention of known effect in reducing the noxious-evoked brain activity following a clinically required heel lance [242]. The sample size required was obtained from the data simulated in Study 2. Assuming a 40% reduction in the magnitude of the brain activity in the Intervention Group compared with the Control Group, a sample size of 32 neonates (16/group) would be sufficient to achieve a power of 95%, when a neonate's baseline sensitivity is taken into account. Neonates were recruited in parallel to either the Control or Intervention Group and the allocation of participants to the experimental groups was not randomised. A total of 40 neonates were prospectively recruited to the study. EEG and EMG activities were recorded in response to experimental noxious stimuli (128 mN intensity) prior to a clinically required heel lance. The Intervention Group (n = 22) received gentle touch at an approximate rate of 3 cm/s for 10 s before heel lancing and the Control Group (n = 18) did not receive the gentle touch (all neonates received comfort measures including swaddling, non-nutritive sucking, or were held by a parent). Gentle touch was not applied prior to the experimental stimuli.

The gentle touch stimulus was provided by a brush stimulator applied at a rate of approximately 3 cm/s for 10 s prior to heel lancing, across approximately 10 cm of the neonate's lower leg ipsilateral to the heel receiving the lance (see Chapter 2, Section 2.3.3). There was an inter-stimulus interval of approximately 1 s between the end of the brush stimulation and the heel lance.

Table 4.1. Participant demographics. Values given are median (lower quartile, upper quartile) or number (%). * Indicates missing data for one neonate.

	Study 1	**Study 3 Control Group**	**Study 3 Intervention Group**
Number of neonates	9	18	20
Gestational age (GA) at birth (weeks)	40.7 (40.3, 41)	40 (37.1, 40.7)	39.1 (37.1, 40.6)
Postmenstrual age (PMA) at time of study (weeks)	41 (40.9, 41.7)	40.5 (37.6, 40.9)	39.5 (37.8, 41.1)
Postnatal age (PNA) at time of study (days)	2 (2, 4)	1.5 (1, 3.5)	4 (2, 5)
Birthweight (g)	4140 (3725, 4320)	3675 (3021, 3881)	3390 (3051, 3908)
Sex			
Male	4 (44)	11 (61)	9 (45)
Female	5 (56)	7 (39)	11 (55)
Mode of delivery			
NVD (normal vaginal delivery)	2 (22)	6 (33)	7 (35)
Assisted vaginal ventouse/forceps	6 (67)	3 (17)	4 (20)
Emergency C-section	0 (0)	6 (33)	5 (25)
Elective C-section	1 (11)	3 (17)	4 (20)
Apgar score at 1 min	9 (8, 10)	9 (8, 10)	9 (7, 10)*
Apgar score at 5 min	10 (10, 10)	10 (10, 10)	10 (9, 10)
Estimated cumulative prior pain exposure	4 (1, 4)*	2 (2, 6)	5 (3, 8)*

4.2.3 Stimulation techniques/clinical procedures

Experimental noxious stimuli of 64 mN intensity (Study 1) and 128 mN intensity (Study 3) were applied to the plantar surface of the heel following the technique explained in Chapter 2. Stimuli were applied in trains of 10–20 trials, with a minimum inter-stimulus interval of 10 s (the inter-stimulus interval was increased if necessary, to allow the neonate to settle). The 64 mN pinprick stimuli were time-locked to the EEG and EMG recordings using a high-speed video camera that was linked to the recordings at the time of acquisition. The time of each individual stimulus was identified retrospectively from the video recordings with a manual marker when the pinprick's barrel was first depressed [186]. The 128 mN pinprick was automatically time-locked to the EEG and EMG recordings using a contact trigger device (MRC Systems).

Heel lances were applied only when necessary for blood sampling as part of the neonate's clinical care. Heel lances were automatically time-locked to the EEG and EMG recordings using an event detection interface as explained in Chapter 2.

Both the experimental Pinprick and the clinical heel lance induce acute somatic nociceptive pain driven by the activation of A-delta fibres. The magnitude of the noxious-evoked brain activity in response to the experimental stimuli correlates with their intensity, suggesting that this response is related to the processing of noxious input [186]. An appropriate stimulus to assess baseline sensitivity (which will likely account for the maximum variance in the data) is one with sensory modality matched to the subsequent clinically-required procedure. The responses to both types of stimuli share common characteristics given that: (i) they are both applied to the same body site, (ii) represent activity that is maximally evoked at the same electrode site and (iii) account for individual differences in somatosensory arousal. While in pain-related studies it is most appropriate to use noxious-evoked baseline sensitivity to adjust for inter-individual variability; if the interest was to conduct a vision-related study it would be more appropriate to adjust for inter-individual variability using a visual stimulus.

4.2.4 Recording techniques

Electrophysiological activity was recorded from eight locations on the scalp (Cz, CPz, C3, C4, Oz, FCz, T3, T4), with reference at Fz and ground at Fpz according to the modified international 10–20 system. Additionally, in Study 3, surface EMG was recorded from the limb ipsilateral to the site of stimulation. Bipolar EMG electrodes were placed on the bicep femoris muscle. These techniques are detailed in General Methods (Chapter 2) of this thesis.

4.2.5 Data analysis

EEG signals were filtered from 0.5 to 30 Hz with a notch filter at 50 Hz. Epochs were extracted 500 ms before the stimulus and 1000 ms after and were baseline-corrected to the pre-stimulus mean. Epochs were rejected if they contained gross movement artefacts. Noxious-evoked brain activity was analysed at the Cz electrode for all trials (as this is the electrode site at which the maximal evoked response is observed; [237]). The template of noxious-evoked brain activity was projected onto each individual trial in the time 400-700 ms window after the stimulus providing a weight indicating the magnitude of the noxious-evoked brain activity [237]. Each individual trial was first Woody filtered in the time window of interest to achieve maximum correlation with the template, accounting for individual differences in the latency to the response. A maximum jitter of ±50 ms for the experimental noxious stimuli and ±100 ms for the heel lance was used for the Woody filtering. EMG signals were filtered 10–500 Hz, with a notch filter at 50 Hz and harmonics, and rectified. Epochs were extracted from 2 s prior to 4 s after the stimulus. Individual epochs were rejected due to movement artefacts in the baseline period. The data was split into 250 ms windows and the root mean square (RMS) of the reflex signal was calculated in each window. The average RMS across the first four windows after the stimulus (first second after stimulation) was calculated as the magnitude of the reflex withdrawal.

The magnitude of the noxious-evoked brain activity and the reflex withdrawal was obtained for each individual trial. Each individual neonates' baseline sensitivity was calculated as the mean response to the experimental noxious stimulus. EEG responses were rejected for gross movement artefacts. In Study 3, following removal of neonates whose lances recordings were rejected (n = 5), 118 out of 492 responses to experimental noxious stimuli were rejected from the EEG analysis. Individuals with seven or less responses to the experimental noxious stimuli were not included in the analysis (n = 4) as accurate estimates of baseline sensitivity could not be obtained. This left a total of 31 neonates in Study 3 (Control Group: n = 15;

Intervention Group: n = 16) for the analysis. Similarly, EMG traces with noise and movement artefacts in the baseline period before the stimulus were rejected. Following removal of neonates whose lances recording were rejected (n = 7), 18 out of 459 responses to experimental noxious stimuli were rejected, leaving a total of 33 neonates (Control Group: n = 18; Intervention Group: n = 15) included in the EMG analysis. In Study 3, the individual baseline reflex sensitivity was calculated as the median reflex response to the experimental noxious stimulus.

4.2.6 Statistical analysis

Statistical analysis was performed using MATLAB R2020a (MathWorks). Linear associations were assessed using Pearson correlation tests in Studies 1 and 3. Statistical significance (alpha<0.05) was assessed non-parametrically via permutation testing with 10,000 permutations using the PALM (permutation analysis of linear models) toolbox [371]. Group differences in Studies 2 and 3 were assessed using linear regressions (unpaired two-sample t-tests). When using the paradigm to adjust for baseline sensitivity, I used the following linear regression model: $Y = b_0 + b_1X_1 + b_2X_2$, where Y is the magnitude of the response to the clinical procedure, b_0 is the intercept, X_1 is the intervention, and X_2 is the baseline sensitivity. Without accounting for baseline sensitivity, the model used was $Y = b_0 + b_1X_1$. Statistical significance (alpha<0.05) in Studies 3 comparisons was assessed non-parametrically via permutation testing with 10,000 permutations using PALM. Two-sided tests were used for all statistical analyses with a significance level of 0.05.

4.3 Results

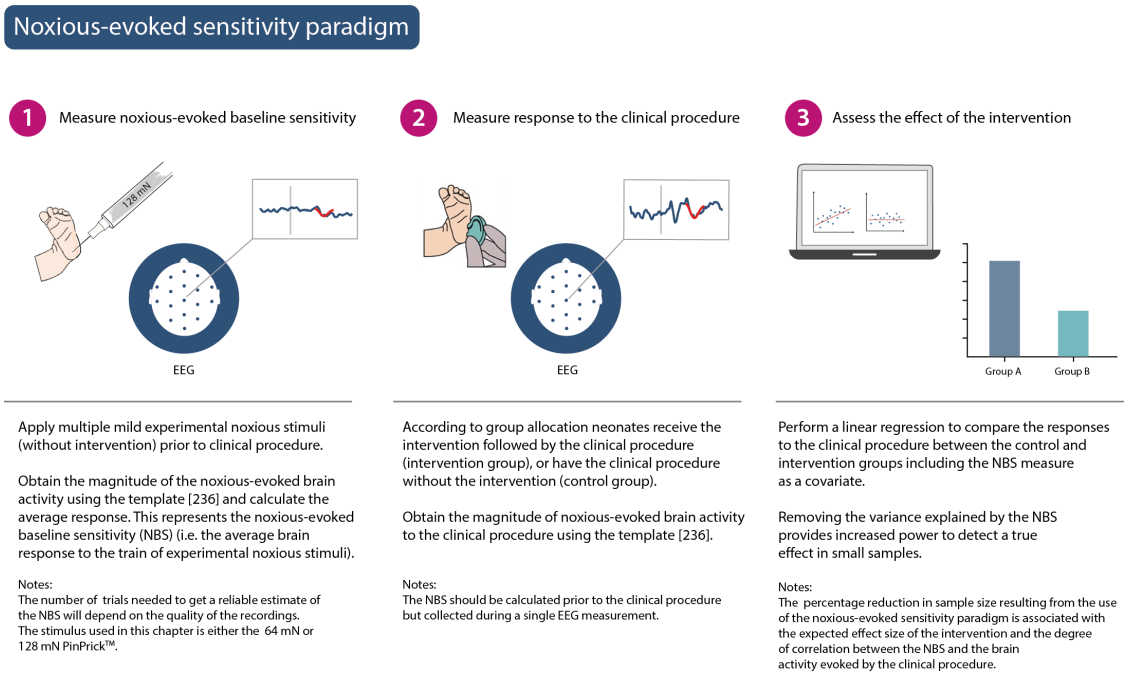

Figure 4.1. A summary of the noxious-evoked sensitivity paradigm. Schematic representation of the noxious-evoked sensitivity paradigm components. A brief description of each step is included with additional explanatory notes.

4.3.1 Study 1: Characterising individual noxious-evoked baseline sensitivity using brain activity in neonates

I hypothesised that a measure of neonatal noxious-evoked baseline sensitivity could be used to account for inter-individual variability in noxious-evoked brain activity in studies of analgesic efficacy and predicted that this would reduce the sample sizes needed in clinical trials. A summary of the proposed paradigm is presented in Figure 4.1. In order to use noxious-evoked brain activity in response to a mild experimental noxious stimulus as a measure of baseline sensitivity, it must be significantly and strongly correlated with the response to the clinically required procedure.

I assessed the feasibility and initial validity of the paradigm by investigating the relationship between individual responses of term neonates to experimental noxious stimuli and a subsequent clinically required heel lance. I retrospectively analysed previously collected EEG data from nine term infants who had received both experimental noxious stimulus and a heel lance on the same test occasion. The

individual raw and filtered EEG traces during both stimulus modalities are shown in Figure 4.2 with the template of noxious-evoked brain activity [237] described in chapter 3 overlaid in red. Overall there is a good fit of the template in the expected time window (by visual inspection) for all the responses with exception of heel lance responses in subjects 2 and 4 where the evoked potential seems to have a shorter latency.

The magnitude of the noxious-evoked brain activity during a controlled mild experimental noxious stimulus applied to the foot (force = 64 mN; magnitude range: 0.15 - 0.62, Figure 4.3) was strongly correlated with the magnitude of the noxious-evoked brain activity during a clinically-required heel lance (range: -0.07 – 2.34) in the same neonates (p=0.0025, Pearson R^2=0.77, Figure 4.3). As shown in Figure 4.3 neonates with larger responses to a mild noxious stimulation of the foot have larger responses to a skin breaking heel lance. In order to use noxious-evoked brain activity in response to a mild experimental noxious stimulus as a measure of baseline sensitivity, a significant and strong correlation with the response to the clinically-required procedure, like the one described here, must be observed. This demonstrates that application of a mild experimental noxious stimulus prior to performing a clinically-required painful procedure could provide a novel measure of neonatal baseline sensitivity, which could be used as a covariate in studies of analgesic efficacy to account for inter-individual variability in pain responses (Figure 4.1).

4.3.2 Study 2: Simulating the effect of accounting for individual noxious-evoked baseline sensitivity

In this study, I first identified in the literature a clinically meaningful effect size that could demonstrate analgesic efficacy. Studies in adults have shown that a 40% reduction in noxious-evoked brain activity is related to significantly lower verbal pain scores [274, 370]. Using the standard deviation of the correlation residuals in Study 4.3.1, Intervention Group and Control group data were simulated across a range of sample sizes including both baseline sensitivity data (responses to a mild

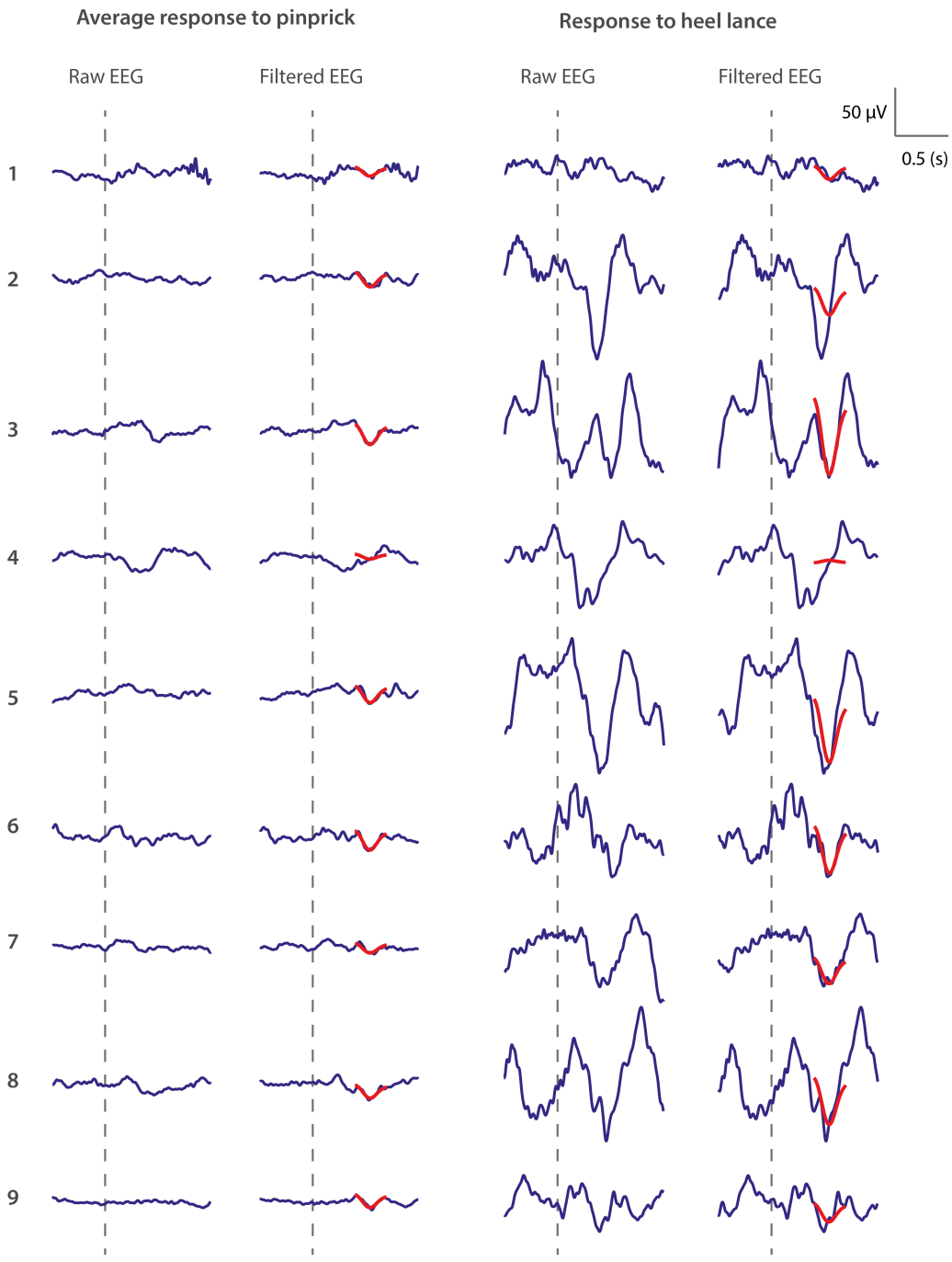

Figure 4.2. Individual responses to experimental noxious stimuli and to a clinically required heel lance. Average raw and filtered EEG data following a train of mild noxious experimental stimuli and during a clinically required heel lance in the nine neonates included in Study 1. The template of noxious-evoked brain activity [237] is overlaid in red and the grey dashed lines indicate the point of stimulation.

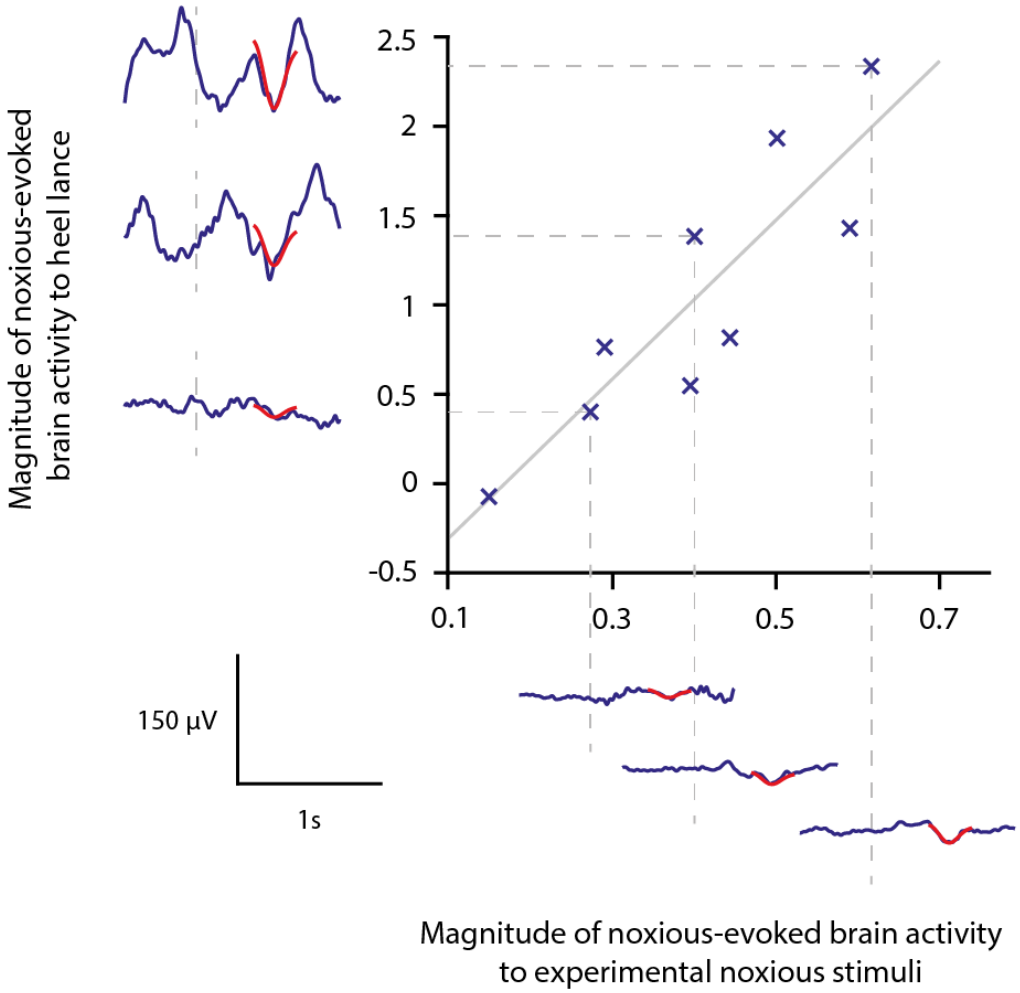

Figure 4.3. Noxious-evoked brain activity during experimental noxious stimuli correlates with the response during a heel lance. The magnitude of noxious-evoked brain activity following mild experimental noxious stimuli and a clinically required heel lance was significantly correlated within-subject (p=0.0025, R^2=0.77, n=9, Pearson correlation test); grey solid line indicates the line of best fit. Dashed lines and their corresponding electroencephalography (EEG) traces indicate three neonates with a range of response magnitudes. The magnitude of the brain activity was quantified using a template of noxious-evoked activity [237], shown overlaid in red.

experimental noxious stimulus) and responses to heel lance.

A linear regression with and without baseline sensitivity as a covariate (see Statistical Analysis, section 4.2.6) was used to compare the simulated Control Group and Intervention Group. As observed in Figure 4.4A, the sample size to observe

a 40% reduction in noxious-evoked brain activity at a significance level of 0.05 is substantially reduced when the baseline sensitivity is accounted for (e.g 76% sample reduction to achieve 95% power to detect a true effect).

As shown in Figure 4.4B, C the level of intervention effect influences the percentage reduction in sample size at a power of 95%. For example, assuming the extremely large intervention effect size of 95% the sample size can be reduced by 27%; a sample of 11 neonates per group would be required without accounting for baseline sensitivity, compared with eight neonates per group when baseline sensitivity is accounted for. By comparison, at the left extreme, assuming an intervention effect size of 5%, the sample size required to achieve 95% power is 5458 neonates per group without accounting for individual baseline sensitivity, compared with 660 neonates per group when baseline sensitivity is accounted for – representing an 88% reduction in sample size (Figure 4.4B, C).

At the 40% intervention effect size, relevant for analgesic trials assessing cortical responses, a sample size of 16 neonates per group (32 neonates in total) would be sufficient to observe a significant intervention effect with 95% power if individual differences in baseline sensitivity are accounted as a covariate in the linear regression analysis. In contrast, a sample size of 66 neonates per group (132 neonates in total) is required to achieve the same power if neonatal baseline sensitivity is not accounted for (Figure 4.4A).

The strength of the initial correlation (calculated using the standard deviation of the correlation residuals) between the brain responses evoked by the experimental noxious stimuli and the clinical procedure also influences the sample size reduction which can be achieved by accounting for individual differences in baseline sensitivity. As shown in Figure 4.4D the reduction in sample size is low when there's a weak correlation between the measures. Conversely, with a strong correlation between measures, a greater reduction in sample size is achieved. For example, with an

assumed intervention effect of 40% and the low standard deviation of the residuals observed in study 1 (SD of residuals =0.37), accounting for baseline sensitivity results in a sample size reduction of approximately 76%, compared to a sample size reduction of 17% when a high noise level (SD of residuals =1.7) is observed in the correlation (Figure 4.4D).

These results using simulated data demonstrate that accounting for individual differences in noxious-evoked baseline sensitivity has the potential to reduce the sample size needed to assess the efficacy of an analgesic intervention in neonates. Importantly, the percentage reduction in sample size that can be achieved by accounting for individual differences in baseline sensitivity is highly dependent on: i) the anticipated effect size of the intervention (Figure 4.4B, C, and ii) the strength of the initial correlation between cortical responses to the experimental noxious stimulation and to the acute clinical procedure 4.4D.

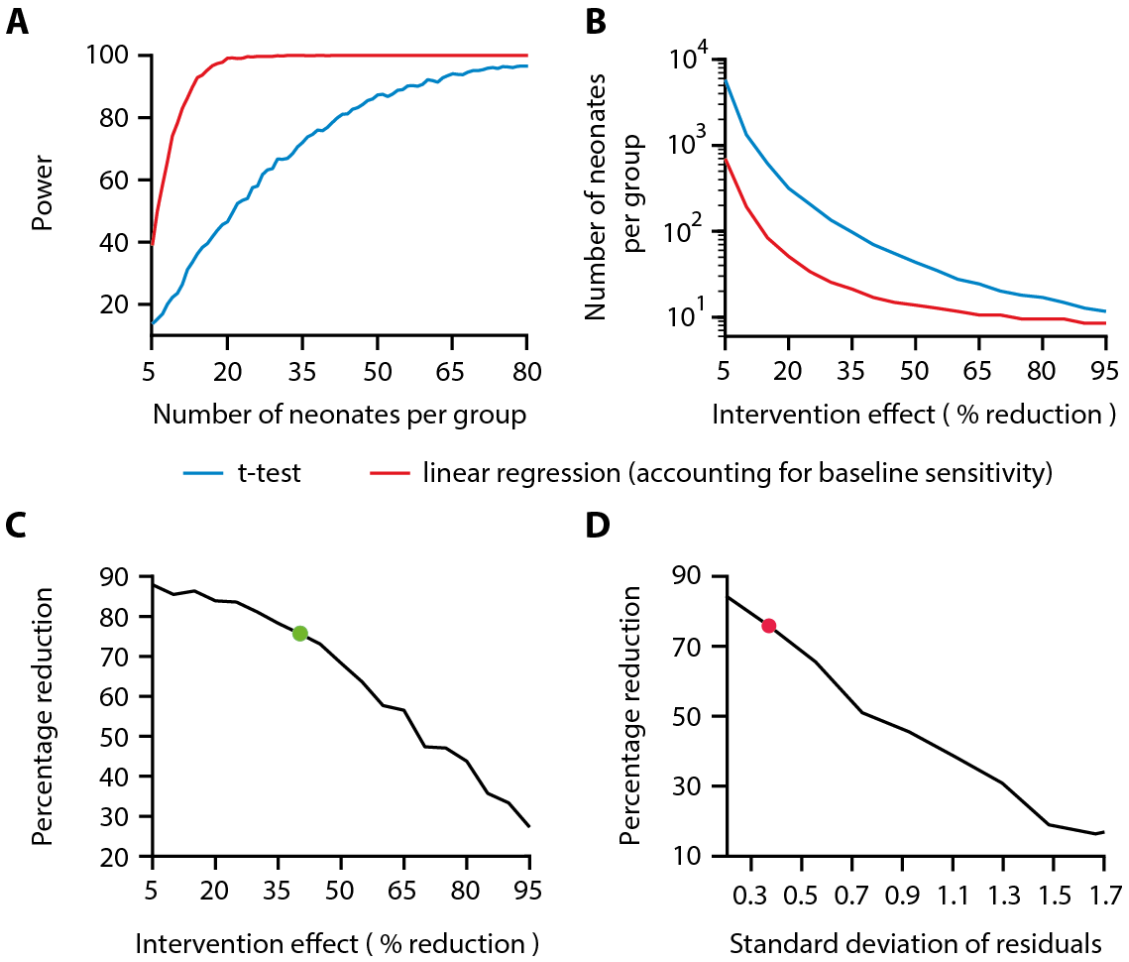

Figure 4.4. Noxious-evoked baseline sensitivity can be used in group comparisons to increase power. I used simulated data to investigate how sample size is altered when the relationship in Study 1 is considered. **(A)** For each sample size, 1000 data sets were simulated with a 40% reduction in the response to a clinically required procedure assumed in the Intervention Group. The power (percentage of significant results, $p<0.05$) to detect a difference between the two groups was calculated for each sample size using a linear regression with (red) and without (blue) accounting for individual differences in baseline sensitivity. **(B)** The number of neonates required to achieve 95% power with different levels of intervention effect. Simulations were run with increasing numbers of neonates until 95% power was achieved. **(C)** Percentage reduction in the number of neonates required per group when individual baseline sensitivity is accounted for compared with not accounting for baseline sensitivity (power=95%). The green marker indicates an intervention effect of 40%. **(D)** The percentage reduction in the number of neonates required per group with different degrees of correlation (standard deviation of residuals) between the responses to experimental noxious stimuli and clinically required procedure (40% intervention effect, 95% power). The red marker indicates the standard deviation of residuals (SD = 0.37) in Study 1.

4.3.3 Study 3: Testing the paradigm using a non-pharmacological pain-relieving intervention

The last study in this chapter was performed to test the effect of using the noxious-evoked baseline sensitivity paradigm and accounting for baseline sensitivity with a non-pharmacological pain-relieving intervention of known effect. Gentle touch intervention (brushing a neonate's leg at a rate of approximately 3cm/s to optimally stimulate C-tactile fibres) prior to a heel lance is an effective non-pharmacological intervention causing a reduction in noxious-evoked brain activity by 40% as demonstrated in a previous study [242]. A power calculation was performed based on the simulated data presented in Study 2, section 4.3.2 and a total of 16 neonates per group would be required to observe a 40% reduction in noxious-evoked brain activity with a two-sample t-test (95% power and a two-sided 5% significance level). A total of 16 neonates were included in the Intervention Group and received gentle brush on the leg ipsilateral to the stimulus site prior to heel lancing. A further 15 neonates were included in the Control Group where the heel lance was performed without gentle brushing. All neonates received mild experimental noxious stimulation prior to a clinically required heel lance to assess their individual baseline sensitivity.

Noxious-evoked brain activity

The individual average noxious-evoked brain activity to experimental noxious stimuli and the noxious-evoked brain activity to a clinically required heel lance are shown in Figure 4.5 for all subjects included in this study. The evoked response to the experimental stimulus was correlated with the response to the clinical procedure in the Control Group (p=0.0013, Pearson R^2=0.65, Figure 4.6A), consistent with the results observed in section 4.3.1 and confirming the necessary strong initial correlation between these measures.

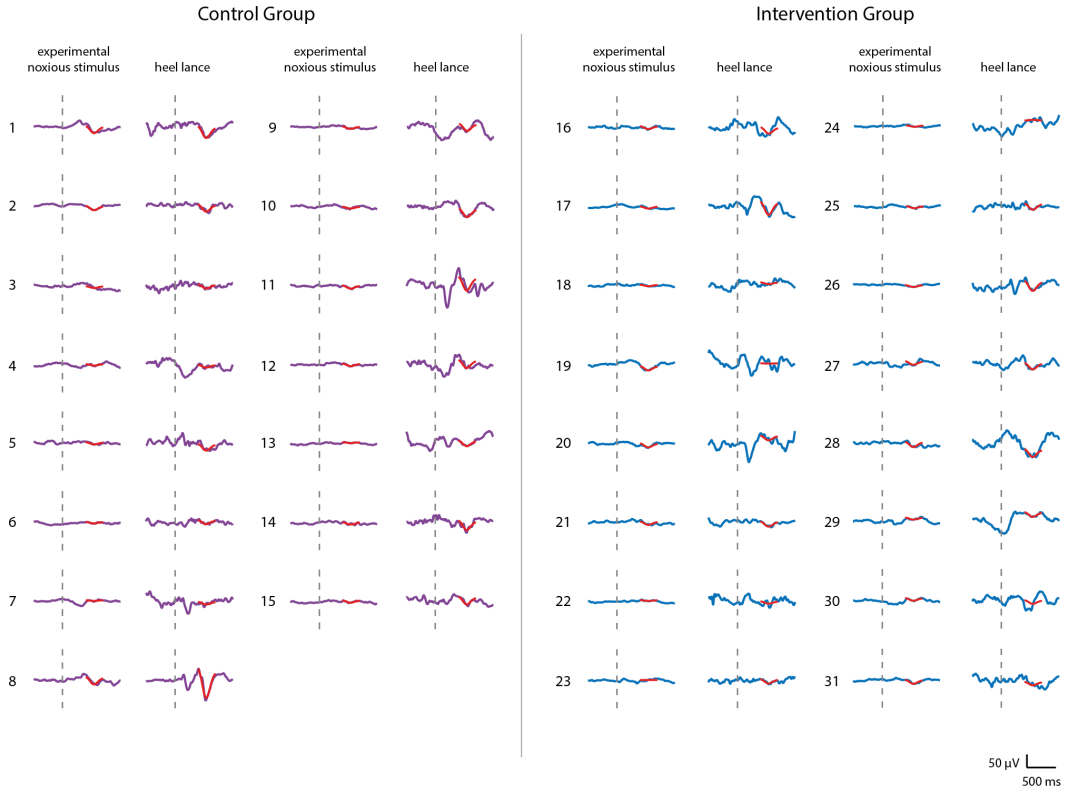

Figure 4.5. Noxious-evoked brain activity in individual neonates in the Control Group and Intervention Group. Average noxious-evoked brain activity to the experimental noxious stimuli and the corresponding brain activity following heel lancing within the 31 neonates in Study 3, section 4.3.3. The template of noxious-evoked brain activity [237] is overlaid in red and the grey dashed lines indicate the point of stimulation. Neonates in the Intervention Group (electroencephalography [EEG] traces in blue) received gentle brushing before the heel lancing.

Consistent with the results reported by Gursul and collaborators [242], the gentle touch intervention resulted in a 39% reduction in the magnitude of the noxious-evoked brain activity. A statistically significant intervention effect was not observed when baseline sensitivity was not accounted for (linear regression, t=1.95, p=0.05, Figure 4.6B). Although a clear trend can be observed and the analysis showed borderline significance, a sample of this size would only provide a power of 40% without accounting for baseline sensitivity (Figure 4.4A). However, when noxious-evoked baseline sensitivity was accounted for as a covariate in the analysis, a significant intervention effect was observed (linear regression, t=2.29, p=0.026).

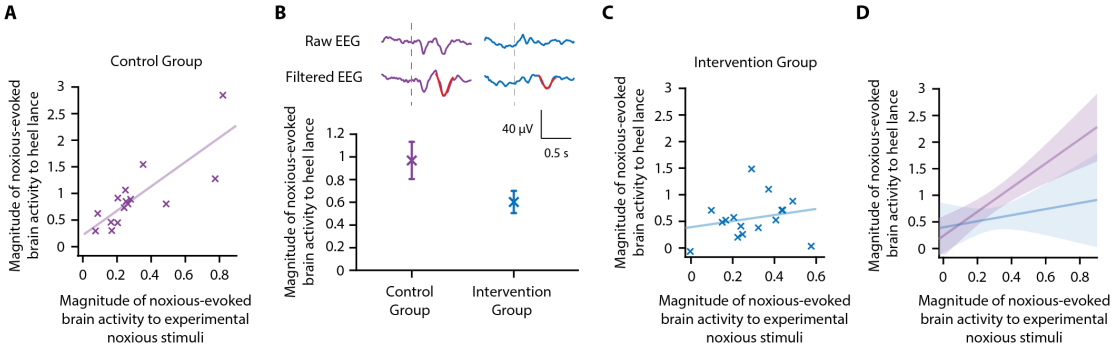

Figure 4.6. Assessment of the efficacy of a gentle touch intervention using individual baseline sensitivity. **(A)** The magnitude of the noxious-evoked brain activity following a mild experimental noxious stimulus compared with the clinically required heel lance for each neonate in the Control Group (n=15). Solid line indicates the line of best fit. **(B)** (Top) Group average raw electroencephalography (EEG) and (Woody) filtered EEG traces in response to the clinically required heel lance; Control Group (purple) and Intervention Group (neonates received gentle touch at a rate of approximately 3 cm/s for 10 s prior to the heel lance, n = 16) (blue). Dashed lines indicate the point of stimulation; the template of noxious-evoked brain activity is shown overlaid in red. (Bottom) Magnitude of the noxious-evoked brain activity following heel lance in the two groups. Error bars indicate mean ± standard error. **(C)** Comparison of the stimulus responses for each neonate in the Intervention Group. Gentle touch was not applied prior to the experimental noxious stimuli so that each neonate's individual baseline sensitivity could be assessed. **(D)** Confidence intervals of the correlations for the two groups shown overlaid: Control Group (purple), Intervention Group (blue), solid lines indicate line of best fit. The effect of the intervention (gentle touch) is demonstrated by the difference between the two groups' confidence intervals and is most evident in neonates who have greater baseline sensitivity (i.e. higher responses to the experimental noxious stimulus).

To further explore these results, I compared the relationship between the magnitude of noxious-evoked brain activity in response to the experimental noxious stimuli and heel lancing within the Control Group and Intervention Group. The strong significant correlation demonstrated in the Control Group was disrupted in the Intervention Group ($p=0.39$, $R^2=0.05$, Figure 4.6C). In particular, reduced noxious-evoked brain activity was observed following the gentle brushing intervention in neonates with high baseline sensitivity (Figure 4.6D), suggesting that the effect of the gentle touch pain-relieving intervention is most prominent in neonates with greater noxious-evoked baseline sensitivity.

Testing the paradigm with other modalities: noxious evoked reflex withdrawal

There is immense value in establishing whether accounting for individual differences in baseline sensitivity can be applied to cortical responses and other pain-related measures in neonates. In response to a noxious stimulation, neonates can manifest a range of physiological responses including facial grimacing, reflex withdrawal, and physiological responses [186, 189, 372]. All infants in Study 3 also had their reflex withdrawal recorded using surface EMG electrodes in response to the experimental noxious stimulation and heel lancing. Figure 4.7 shows the individual average noxious-evoked reflex withdrawal to experimental noxious stimuli and the noxious-evoked reflex withdrawal activity to a clinically required heel lance. As expected, both types of stimuli evoked reflex withdrawal activity, which was distinctively greater in response to the stimulus of greater intensity, in concordance with a previous report showing reflex withdrawal graded with stimulus intensity [186].

In the Control Group, the magnitude of the reflex withdrawal response to experimental noxious stimulation was significantly correlated with the reflex withdrawal evoked by heel lancing ($p=0.009$, $R^2=0.36$, Figure 4.8A). However, this correlation in reflex withdrawal activity was weaker than the relationship in the noxious-evoked brain activity observed in Study 1 (section 4.3.1) and Study 3, limiting its use (Figure 4.4D). With this low correlation level between measures identified within infants, and assuming an intervention effect of 40%, simulated data showed that accounting for baseline sensitivity using noxious-evoked reflex activity provides only 17.3% power to detect a significant difference between the two groups compared with a power of 11.3% without accounting for baseline sensitivity.

A between-group comparison shows that the gentle touch intervention did not significantly reduce the magnitude of the reflex withdrawal activity following heel

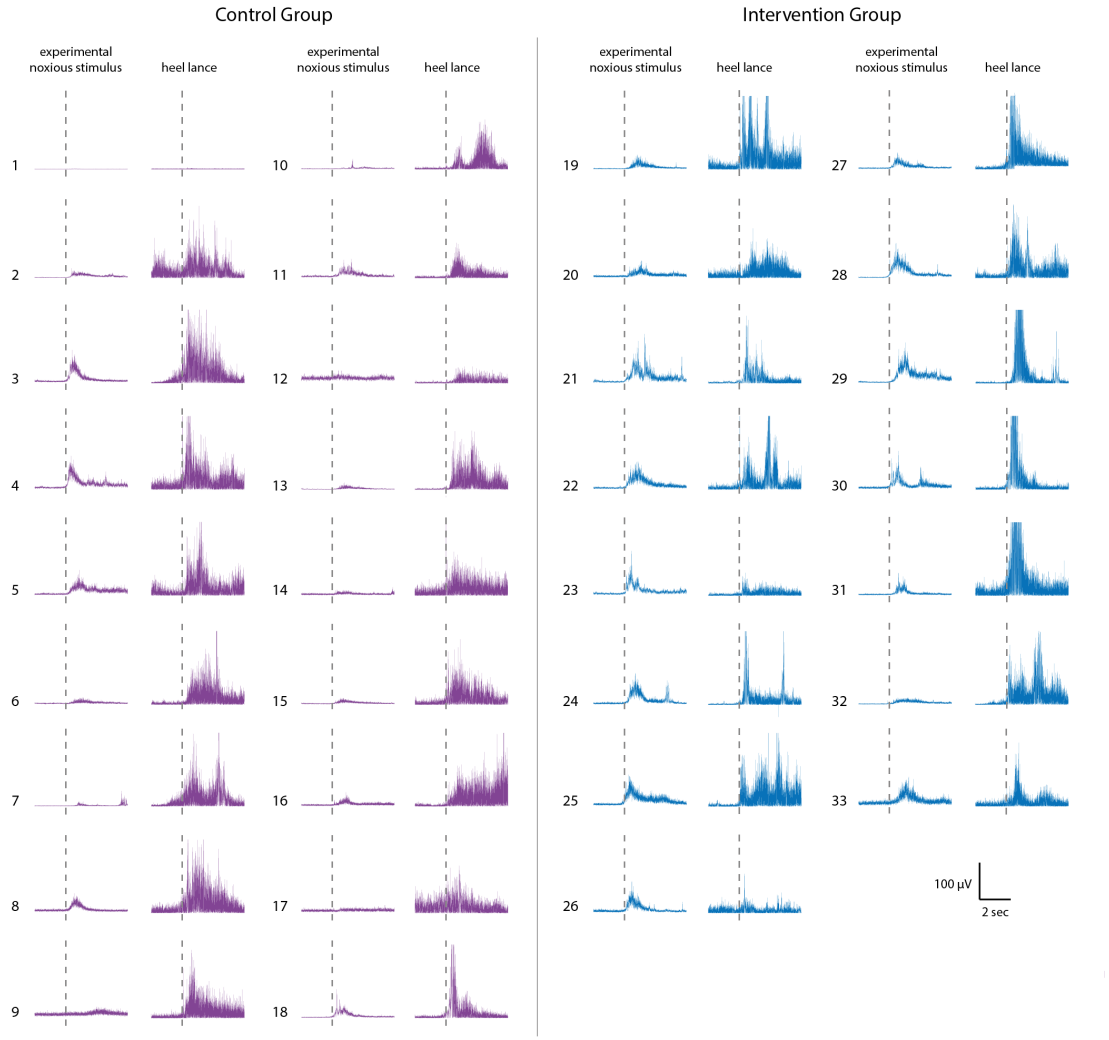

Figure 4.7. Reflex withdrawal activity in individual neonates in the Control Group and Intervention Group Average reflex withdrawal in response to experimental noxious stimulation and reflex withdrawal evoked by heel lancing in the 33 neonates included in Study 3. Neonates in the Intervention Group (EMG traces in blue) received gentle brushing before the heel lancing.

lancing, either when accounting for baseline sensitivity (linear regression, t = -1.43, p=0.17) or without accounting for baseline sensitivity (t = -1.73, p=0.10, Figure 4.8B). While these results and similar observations reported in a previous study [242] could suggest that reflex withdrawal of the stimulated limb is not modulated by gentle touch, the intervention clearly disrupted the correlation between baseline reflex sensitivity and the reflex evoked by heel lancing (p=0.25, R^2=0.1) as shown in Figure 4.8C. The pre-procedural brushing intervention may have caused a change in baseline

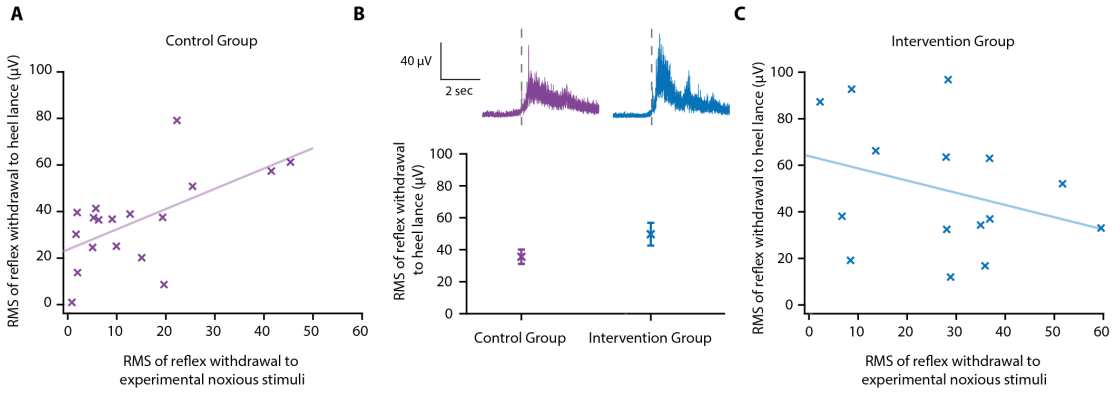

Figure 4.8. Assessment of the effect of gentle touch on reflex withdrawal responses. (A) The magnitude of the reflex withdrawal following a mild experimental noxious stimulus (baseline reflex sensitivity) compared with the clinically required heel lance for each neonate in the Control Group (n = 18). Solid line indicates line of best fit. **(B)** Average electromyography (EMG) traces (top) for neonates in the Control Group (purple) and Intervention Group (blue, n = 15) where neonates were gently brushed at a rate of approximately 3 cm/s for 10 s prior to the heel lance. Dashed lines indicate the point of stimulation. (Bottom) Magnitude of the reflex withdrawal response in the two groups. Error bars indicate the mean of the root mean square (RMS) of the reflex withdrawal ± standard error. **(C)** The magnitude of the reflex withdrawal following a mild experimental noxious stimulus (baseline reflex sensitivity) compared with the clinically required heel lance for each neonate in the Intervention Group (gentle touch).

muscle activity in some individuals resulting in the larger residuals in the Intervention Group (SD of the residuals – Control Group 15.3 mV; Intervention Group 26.2 mV).

4.3.4 Summary of findings

In this chapter, I aimed to develop a paradigm to optimise analgesic trials in neonates using a measure of noxious-evoked baseline sensitivity to account for inter-individual variability in brain responses to painful procedures. The paradigm of noxious-evoked baseline sensitivity was developed, validated and tested across three studies. In Study 1, I demonstrate that there is a correlation between the magnitude of noxious-evoked brain activity in response to an experimental stimulus and the magnitude of brain activity evoked by the clinical procedure and thus the response to a mild stimulus can be used as a measure of baseline sensitivity. In Study 2, I use simulated data to demonstrate the increased statistical power that can be achieved by including baseline sensitivity as a covariate when analysing

the effect of an intervention in a range of sample sizes. In Study 3, I test this novel paradigm using a non-pharmacological pain-relieving intervention of known efficacy - gentle touch- prior to heel lancing. Overall, I demonstrate that a mild experimental noxious stimulus can be used as a measure of baseline sensitivity; and that accounting for noxious-evoked baseline sensitivity could improve the design of analgesic efficacy investigations in limited samples for this patient population.

4.4 Discussion

I demonstrated that accounting for individual differences in noxious-evoked baseline sensitivity significantly reduces the sample size required to assess the efficacy of analgesics in neonates. Noxious-evoked brain activity in response to a low-intensity experimental noxious stimulus can be used in neonates as a marker of baseline sensitivity and is highly correlated with the magnitude of noxious-evoked brain activity produced by clinically required acute painful procedures. Study 1 was a retrospective study presenting previously unpublished data from term neonates studied between 2014 and 2015 who had received both a heel lance and experimental noxious stimuli on the same test occasion (n=9). Whilst this sample size is small this was a feasibility study and this relationship is retested in Study 3.

Using both simulated and experimental data, I demonstrate that the sample size required to observe the effects of analgesic interventions (for a given power and significance level) can be significantly reduced when noxious-evoked baseline sensitivity is accounted for. Importantly, the percentage reduction in sample size is related to the expected effect size of the intervention and the degree of correlation between the baseline sensitivity measure and the brain activity evoked by the clinical procedure.

By testing this paradigm in a prospective clinical study, I reconfirmed the potential efficacy of gentle touch as a non-pharmacological intervention that reduces brain activity evoked by heel lancing [242]. Although this study has a number of

limitations (including lack of randomisation) and only investigates one aspect of the neonatal response to noxious input (namely an EEG-derived noxious-evoked potential), it provides strong evidence to suggest that a randomised clinical trial investigating the efficacy of gentle touch through multi-modal pain assessment measures is warranted.

Minimisation of sample sizes is imperative in clinical research, and particularly in neonatal studies given the inherent ethical, recruitment, and experimental challenges associated with studying this patient population. Considering that inter-individual variability drives increases in sample sizes required to demonstrate efficacy, addressing baseline variability is key. The paradigm I present here likely accounts for multiple factors affecting noxious-evoked baseline sensitivity in neonates including potential effects from prior pain exposure during hospitalisation [73, 373] and prematurity [62]. This provides a robust approach to indirectly control for a vast array of known and unknown demographic and environmental factors that influence noxious-evoked brain activity and result in inter-individual variability in responses, as well as potential experimental confounds which differ between individuals (such as differences in signal-to-noise ratios, head circumference, and skull thickness).

Responses to other modalities such as visual, auditory, or tactile stimuli could be used to obtain a measure of baseline sensitivity, and background resting state brain activity is also predictive of individual noxious-evoked responses [211]. However, the aim of this study was to develop an experimental paradigm that accounts for the maximum variability in responses to acute painful procedures, to maximise the power to detect a true effect of an intervention. Applying an experimental noxious stimulus to obtain a measure of baseline sensitivity optimises the model as it optimally matches the main characteristics of a response that would be evoked by a procedure of clinical interest (e.g. the stimulus can be applied to the same body location, evokes noxious activity as well as other sensory-related brain activity,

and it is measured at the same electrode site). Given the study aim was to reduce sample size in studies investigating acute pain, it is most appropriate to adjust for inter-individual variability using measures of noxious-evoked baseline sensitivity. In contrast, if alternative studies considered other sensory modalities, for example, visual processing, then a better measure of inter-individual baseline variability would be achieved using a visual stimulus.

The experimental noxious stimulus used in these studies provides a practical and ethical paradigm for the assessment of baseline sensitivity in neonates. It is non-tissue damaging in both term and ex-premature neonates, activates Aδ and C fibres [374], does not evoke changes in facial expression or signs of behavioural distress [128, 186, 237], and is acceptable to parents. The application of experimental noxious stimuli provides a reliable measure of baseline sensitivity as the mild stimuli can be repeated and trial averages calculated within individual neonates; an approach that substantially increases the signal-to-noise ratio as compared with responses recorded in response to a single clinical procedure. Moreover, there is no evidence from the analysed data that the experimental noxious stimuli increase the magnitude of the heel lance response given that the responses to heel lance reported here are similar to previous papers where the experimental noxious stimuli were not applied [237], suggesting it is appropriate for use in a clinical setting.

Despite the advantage of using this approach, it cannot be ruled out the potential effects of confirmation bias prior to the data analysis stage[352]. A relatively high number of trials were rejected due to artefacts, which may be more pronounced when there are stimulus-related movements. If these movements are indicative of a more vigorous response to the noxious input, then it is plausible that results are unavoidably biased towards a subset of the population.

The applicability of the noxious-evoked baseline sensitivity paradigm was tested in the context of a pain-relieving intervention that has previously been shown to be

effective in reducing noxious-evoked brain activity – gentle touch [242]. Neonates were gently brushed at a speed of 3 cm/s, which is approximately equivalent to the rate at which parents will naturally stroke their neonates [375] and optimises stimulation of C-tactile fibres [272]. In an independent population of neonates, it was re-confirmed that brushing the skin prior to a clinically required heel lance significantly reduces noxious-evoked brain activity.

CT fibres transmit signals to the dorsal horn of the spinal cord where multiple ascending pathways may project the signals to the thalamus [376–378] and cortical regions involved in the processing of both sensory and affective aspects of CT-optimal touch [265, 379, 380]. The specific neurophysiological mechanisms behind the effects of CT-optimal touch on pain are not fully known but literature suggest that the CT-afferent system can modulate pain through a bottom-up process starting at the spinal cord level. Electrophysiological research in animals shows that tactile stimuli reduce the nociceptive signalling at the spinal cord [381], consistent with the gate theory of pain [79]. Moreover, a specific inhibitory pathway related to CT-afferent input has been characterised in laminae II neurons in the spinal dorsal horn. The activation of laminae II neurons by CT-afferent projections inhibits laminae II neurons from receiving nociceptive input. This prevents nociceptive input from reaching subcortical and cortical brain regions involved in pain processing [382, 383]. Furthermore, there is currently mixed evidence with respect to pain modulation by the CT-system at supraspinal levels. One study showed a significant reduction of laser-evoked potential magnitudes when CT-optimal touch was applied by a romantic partner [274]. On the other hand, a study using fMRI data showed no significant differences in cortical activation between noxious stimulation with and without CT-optimal touch, although participants reported a reduction in pain during the CT-optimal touch test [384]. Taken together it could be possible that pain modulation by the CT-system occurs at both the spinal cord (inhibiting the pain stimulus from reaching ascending pathways and preventing further cortical processing, resulting in pain reduction) and supraspinal downregulating system

(through downregulation of the insula and ACC, both important for the processing of the subjective experience of pain) [385].

The noxious-evoked baseline sensitivity paradigm was used to indirectly account for many factors that influence the magnitude of noxious-evoked brain activity. In addition, a significant difference in reflex withdrawal activity between the control neonates and the neonates who received gentle touch prior to the heel lance was not observed, which is consistent with previous observations [242]. It is possible that either the magnitude of the reflex withdrawal is genuinely not modulated by the brush intervention or that a modulation in reflex activity would only be observed with a larger sample size. Importantly, a significant but weak correlation was observed between the reflex activity in response to the noxious stimuli and in response to heel lancing in the Control Group, suggesting that the paradigm presented here could be useful in future trials where reflex withdrawal activity is used as an outcome measure. As pain perception is a highly complex sensory and subjective emotional experience generated in the brain [78], quantifying noxious-evoked brain activity may represent a better proxy pain measure, and a more sensitive marker of analgesic efficacy, compared with reflex signals generated by the spinal cord.

In addition to minimising sample sizes, assessing baseline sensitivity may also allow for the identification of neonates that would benefit most from analgesic interventions. Neonates with larger magnitudes of noxious-evoked baseline sensitivity had the greatest reduction in response following the intervention. In contrast, neonates with low baseline sensitivity were less likely to demonstrate a benefit of the intervention, as for this clinical procedure the potential reduction in their responses was minimal. This could be due to a floor effect whereby for some neonates noxious-evoked brain responses to heel lance is close to zero and cannot be reduced further. Improving our understanding of inter-individual variability in pain-related responses is pivotal to ensure that for each individual neonate potential

adverse effects of analgesics are carefully weighed against potential benefits.

Although many factors that influence individual variability in responses are accounted for using the noxious-evoked sensitivity paradigm, it does not account for differences in rapidly fluctuating state effects such as differences in attention or sleep state that could vary between the baseline sensitivity testing and the implementation of the clinical procedure. Sleep-wake states start to develop during the third trimester and have an important role in the developing brain [386, 387]. Disturbances in sleep-wake patterns affect the developing central nervous system and preterm infants exposed to routine procedures in the NICU may experience disruption of normal sleep-waking state patterns. Moreover, the use of opioids decreases rapid-eye-movement sleep [388] and is common for very preterm infants to be in light sleep state rather than awake or in deep sleep states. The extrauterine environment plays an important role in the development of sleep architecture with studies showing differential connectivity and brain activity patterns between premature infants and in-utero foetuses [389]. Response patterns induced by noxious events can differ depending on the sleep state of the neonate. For example, studies characterising behavioural responses demonstrated that compared to quiet sleep, neonates during quiet awake state show greater responses to noxious inputs [166]. Similarly, a study showed lower cortical haemodynamic responses to acute noxious procedures in sleeping infants between 25 and 45 PMA [197]. However, there is also evidence suggesting that the sleep state does not influence the EEG magnitude of noxious-evoked brain activity [236] or the proportion of responders [235]. It would be of great value to investigate this question further since changes in the sleep state between the time at which the noxious-evoked baseline sensitivity is measured and the response to the clinical procedure are likely to occur. Sleep state is therefore a potentially important factor to consider for the application of the paradigm.

A recent fMRI study demonstrated that noxious-evoked brain activity can be predicted from a neonate's resting state brain activity as well as the structural

integrity of key white matter pathways [211]. Investigating the role of baseline EEG activity and exploring the neurological differences underlying variability in the noxious-evoked brain activity described here could further improve the utility of the paradigm. In addition, while the paradigm is applicable to many of the most common acute somatic painful procedures which neonates are exposed to including heel lancing, cannulation, and injections, this paradigm may not apply to many types of pain such as visceral pain, post-operative pain, longer procedures, such as retinopathy of prematurity screening, procedures with a slow-rising onset, or chronic pain.

In summary, the assessment of pain in non-verbal neonates is challenging [303] and the wide variability in individual responses to painful procedures complicates the assessment of analgesics. Currently, there is a paucity of evidence regarding the efficacy of pain-relieving interventions used in neonatal practice [298]. Here, I present a paradigm that accounts for individual noxious-evoked baseline sensitivity and demonstrate its utility in terms of sample size reduction. Using this paradigm in clinical trials could optimise resources, maximise the value of collected data, and ultimately expedite the discovery and validation of urgently needed analgesics and pain-relieving interventions for this patient population.

The methodology described in this chapter is appropriate for the aim of the study which was to optimise the statistical analysis for analgesic RCTs in infants. Multiple publications recommend when using a continuous outcome measure in an RCT to measure the baseline score in addition to the post-intervention score and to adjust for baseline at the analysis stage (e.g. with an analysis of covariance ANCOVA) [390–392]. This is the approach used in the baseline sensitivity paradigm presented in this chapter with the novelty of using experimental noxious stimulation to obtain the baseline score in infants.

Alternatively, another method can be to look at responders versus non-responders and stratify the participant cohorts to reduce the variability of the response to an intervention and optimise the sample sizes needed in analgesic clinical trials. Patient stratification biomarkers also have the potential for a personalised approach to treat pain [393], as is frequently used in fields like oncology. However, the development of stratification biomarkers requires an advanced understanding of the pain mechanisms that will support the selection of a sensitive marker that reliably predicts pain reduction to allow the identification of potential treatment responders [368]. The infant pain field is moving towards the understanding of the mechanisms underlying the neurophysiological responses, but we are still at the early stages in relation to the mechanistic understanding of adult brain signals.

The variance observed in the EEG brain activity in response to a noxious stimulus in infants is likely to be related to important biological factors in addition to background noise and other non-biological components. Understanding the variance observed in the infant pain responses and what causes it is also important to elucidate potential mechanisms and therapeutic targets. This approach is used in the following chapter to evaluate the interaction between inflammation and pain.

5

The effect of early life inflammation in spinal cord excitability and nociceptive sensitivity in human infants - a translational study

5.1 Introduction

Our nociceptive and immune systems are intimately intertwined, working together to protect us from injury or disease [394]. In infants and children, both systems undergo extensive fine-tuning and maturation [95, 395]. Evidence suggests that the first three months of postnatal life are a critical period where B, natural killer (NK), and dendritic cells (DCs) development occurs, concomitant with changes in cell populations composition [396]. From birth, neutrophils gradually reduce while CD4+ and CD8+ T cells increase in proportions and B cells increase in abundance from 1 month onward [396]. As described in Chapter 1, nociceptive neural circuitries undergo significant changes during the early neonatal period where pruning and shaping of inhibitory and excitatory pathways are guided by sensory experience [95].

Adverse events during this delicate developmental process can have deleterious and long-lasting consequences. For example, deficiencies in the exposure to certain microorganisms and their products can pre-dispose us to immune-mediated diseases

[396] like asthma and type-1 diabetes [397], while repeated painful procedures in neonates may negatively affect nociceptor sensitivity [62] and have been associated with altered behavioural, motor and cognitive neurodevelopment [51, 300].

How the development of the nervous and immune systems influences each other is much less clear, even though it is a highly relevant clinical question. Proinflammatory cytokines and other inflammatory molecules such as histamine and prostaglandins are released by immune cells following injury; these molecules can directly activate nociceptors to generate action potentials and induce sensitisation and increased nociceptive sensitivity (hyperalgesia) [398, 399]. For example, following repeated inflammatory tissue injury, rat pups exhibit decreased mechanical withdrawal thresholds during graded von Frey hair stimulation [70] and increased frequency in nociceptive behaviours and hyperalgesia when exposed to a subsequent inflammatory injury as adults [400, 401]. Studies in animal models also suggest a sensitisation mechanism involving increased input in spinal neuronal circuits and segmental changes in nociceptive primary afferent axons in adults exposed to tissue injury during the neonatal period [402].

Infections, and their inflammatory cascade are also important modulators of the early nociceptive system. Neonatal infection, particularly in low birth weight and premature infants, is an important cause of morbidity and mortality in the UK and worldwide [403]. Suspected infection is very prevalent in neonates [404], affecting up to 13% of live births [405], yet the consequences that such early-life inflammation may have on human nociceptive circuitry is not known [406].

Evidence from the pre-clinical literature suggests that neonatal inflammation might have very serious consequences [44]. There are, for instance, reports that experimental activation of the neonatal immune system in rat pups, via lipopolysaccharide (LPS) administration, causes long-term increased pain sensitivity [163, 406–411]. Long term programming of the inflammatory pain response appears to

occur through changes at the peripheral and central neuroimmune system [411]. At the peripheral level, neonatal infection in the first postnatal week in rats elevated plasma cytokines and increased excitability within dorsal horn networks [410, 412]. Centrally, LPS exposure enhanced immunoreactivity in structures like the prefrontal cortex, midbrain periaqueductal gray (PAG) and amygdala, and also interfered within the descending inhibitory pathways [410, 412, 413]. However, studies investigating how the acute phase of early life inflammation impacts pain sensitivity are scarce.

Despite extensive characterization in experimental animal models [414], further investigation of neuroimmune pathways and immune-to-brain mechanisms in human neonates is needed, as very little is known about how activation of the neonatal immune system modulates human nociceptive development. Moreover, given the many known inter-species differences in both immune and nociceptive systems [415] it is of vital importance to generate data in human neonates, which can be relevantly back-translated into future rodent models.

Here, I have generated such human neonatal data using a novel experimental design and prospectively studying well-matched cohorts of newborn infants (with similar perinatal histories), who can be differentiated based on the C-reactive protein (CRP) levels in their blood (Figure 5.2). I studied neonates who presented with risk factors for suspected early onset neonatal infection (see [416] for guidelines that were in place during the study period) and received prophylactic antibiotic treatment (Table 5.1). They were grouped according to the level of CRP in their blood or other laboratory or clinical evidence of infection within 24 hours from the start of antibiotic treatment. The 'Neonatal Inflammation Group' presented with CRP $>$ 10 mg/l or evidence of infection, whereas the 'Neonatal Control Group' presented with CRP $<$ 10 mg/l and no evidence of infection. The threshold of 10 mg/l was selected because in the local unit, prophylactic antibiotic treatment was discontinued 36-hours after the first dose if the CRP was below 10 mg/l and there

were no other clinical or laboratory signs of infection (Figure 5.2) [416].

I hypothesised that neonatal inflammation causes increased spinal cord excitability and increased cortical brain activity in response to (i) a noxious heel lance, which was medically-required to establish the level of CRP in the blood, and (ii) in response to non-noxious tactile stimulation to the heel. I tested both these hypotheses by comparing the magnitude of spinal cord mediated reflex withdrawal activity assessed using EMG and stimulus-evoked changes in brain activity assessed using EEG in the Neonatal Inflammation Group and the Neonatal Control Group. These hypotheses were tested in the primary study (Study 1), and an additional cohort of neonates was recruited for Study 2 to assess a potentially sustained increased pain sensitivity after inflammation was treated.

5.2 Methods

This chapter is composed by two studies:

- **Study 1** is a prospective study testing two hypotheses. The principal hypothesis was that neonatal inflammation causes increased spinal cord excitability and hyperalgesia in response to noxious stimulation. The second hypothesis was that neonatal inflammation causes increased spinal cord excitability and arousal related brain activity in response to tactile stimulation.
- **Study 2** is an exploratory study investigating whether hyperalgesia is maintained after inflammation was reduced by antibiotic treatment in an independent cohort of neonates.

Spinally cord-mediated reflex withdrawal activity was assessed using electromyography (EMG) and stimulus-evoked changes in brain activity assessed using electroencephalography (EEG) applying the methods and tools described and validated in the previous chapters.

5.2.1 Participants

A total of 65 term neonates were recruited for Study 1 from the Newborn Care Unit and Maternity wards of the John Radcliffe Hospital (Oxford University Hospitals NHS Foundation Trust, Oxford, United Kingdom) following the methods described in Section 2. The study profile is shown in Figure 5.1. Data acquisition was completed in 61 studies. Participants in the Neonatal Control Group (n=38) and Neonatal Inflammation Group (n=23) were born between 36 and 42 weeks' gestation and were less than 72 hours old at the time of study.

The follow up exploratory study included 20 neonates (2 from the Study 1 cohort and 18 from an independent sample) recruited from the Newborn Care Unit and Maternity wards of the John Radcliffe Hospital. Participants in the Neonatal Antibiotic Treatment Group (n=12) and Neonatal Antibiotic Control Group (n=8) were born between 36 and 42 weeks' gestation and were between 4 and 6 days old at the time of study. The recruitment and data collection of Study 2 were suspended because of the COVID-19 pandemic restrictions. The detailed demographic characteristics of the participants included in the final analysis are presented in Table 5.1.

Neonates with neurological conditions, receiving analgesics or with a history of maternal substance abuse were not eligible to take part in the study. Cumulative prior pain exposure was quantified as the total number of acute tissue-damaging procedures performed including heel lances, injections, and intravenous cannulation [417]. Ethical approval was obtained from the National Research Ethics Service (reference: 12/SC/0447) and parental written informed consent was obtained before each participant was studied. The study was conducted in accordance with the standards set by the Declaration of Helsinki and Good Clinical Practice guidelines.

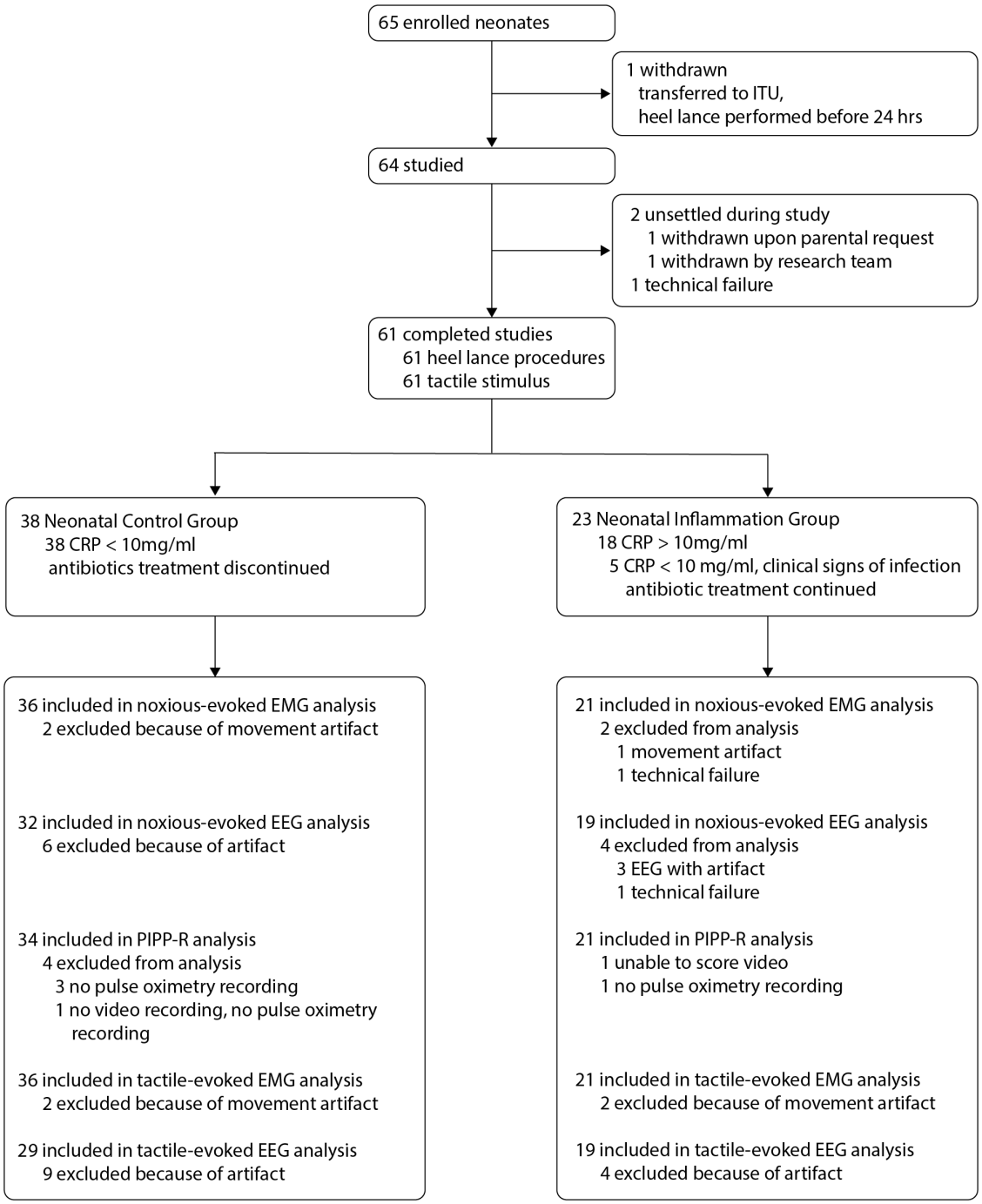

Figure 5.1. Study 1 profile.

5.2.2 Experimental design

All neonates in Study 1 presented with risk factors or clinical indicators of early onset neonatal infection, required a blood test to quantify C-reactive protein (CRP) levels and had commenced antibiotics 18-24 hours before the study (see [416] for

Table 5.1. Participant demographics. Values given are median (lower quartile, upper quartile) or number (%). * Data not documented in clinical notes for one neonate.

	Neonatal Control Group	**Neonatal Inflammation Group**	**Neonatal Antibiotic Control Group**	**Neonatal Antibiotic Treatment Group**
Number of neonates	38	23	13	12
Gestational age (GA) at birth (weeks)	40.6 (40, 41)	27.6 (25.6, 28.8)	27.3 (26.3, 28.3)	40.4 (39.9, 40.7)
Postmenstrual age (PMA) at time of study (weeks)	40.1 (38, 40.9)	40.1 (39.4, 41)	39.1 (37.4, 41.8)	41.1 (40.5, 41.4)
Postnatal age (PNA) at time of study (days)	1 (1, 2)	1 (1, 1)	5 (5, 5)	5 (4, 5)
C-reactive protein at time of study (mg/l)	2.6 (1.6, 4.8)	21.2 (11.1, 45.1)	-	4 (3.1, 6.9)
Birthweight (g)	3590 (3261, 4035)	3818 (3405, 4158)	3232.5 (2420, 4593)	3803 (3704, 3950)
Sex				
Male	23 (61)	13 (57)	2 (25)	8 (67)
Female	15 (39)	10 (43)	6 (75)	4 (33)
Mode of delivery				
Normal vaginal delivery	17 (45)	6 (26.1)	5 (62.5)	3 (25)
Assisted breech vaginal	0 (0)	1 (4.3)	0 (0)	1 (8.3)
Assisted vaginal ventouse/forceps	10 (26)	10 (43.5)	0 (0)	2 (16.7)
Emergency C-section	8 (21)	4 (17.4)	3 (37.5)	6 (50)
Elective C-section	3 (8)	2 (8.7)	0 (0)	0 (0)
Apgar score at 1 min	9 (7, 10)	9 (7, 9)	9 (8, 10)	9 (9, 9)
Apgar score at 5 min	10 (9, 10)	10 (10, 10)	10 (10, 10)	10 (10, 10)
Apgar score at 10 min	10 (10, 10)*)	10 (10, 10)	10 (10, 10)	10 (10, 10)
Respiratory support at time of study				
Self-ventilating in air (SVIA)	35 (92)	21 (91)	8 (100)	11 (91.7)
Low flow (LFT)	1 (3)	0 (0)	0 (0)	0 (0)
High flow (HFT)	2 (5)	2 (9)	0 (0)	1 (8.3)
Estimated cumulative prior pain exposure	2 (1, 4)	2 (1.5, 4)	0 (0, 3)	8 (4, 10)

guidelines that were in place during the study period).

Clinicians and researchers including myself were blinded to the participants' clinical status at the time of the study and neonates were assigned to the Neonatal Control Group or Neonatal Inflammation Group after the study was completed. The diagnosis was based on clinical assessment, CRP levels within 24 hours from presentation of risk factors, other laboratory results and the clinical decision to continue with intravenous antibiotics to complete a minimum of a 5-day course (Figure 5.2). Participants were grouped into the Neonatal Inflammation Group: CRP $>$ 10 mg/l or other laboratory or clinical evidence of infection and the Neonatal Control group: CRP $<$ 10 mg/l and clinically asymptomatic. The threshold of 10

mg/l was selected because in the local unit, prophylactic antibiotic treatment was discontinued 36-hours after the first dose if the CRP was below 10 mg/l and there were no other clinical or laboratory signs of infection (Figure 5.2).

All neonates in Study 2 were prescribed with a 5-day regimen of antibiotics due to suspected infection and were studied during a blood test required to quantify CRP levels 84 - 96 hours after the first dose of antibiotics. Participants with CRP $<$ 10 mg/l at the time of study were included in the "Neonatal Antibiotic Treatment Group" as they previously had raised CRP levels. Age-matched neonates with no previous evidence of infection or antibiotic treatment who required a blood test for routine newborn screening (newborn blood spot test and serum bilirubin [SBR]) were included in the "Neonatal Antibiotic Control Group".

All neonates in Study 1 and Study 2 received a tactile (non-noxious control heel lance) stimulus followed by a clinically required heel lance as described in Chapter 2. An overview of the experimental design of Study 1 is presented in Figure 5.2. The same data acquisition set up used in Study 1 was used for Study 2. If a second heel lance was required to obtain sufficient volume of blood sample this was also recorded after consulting with the parents.

5.2.3 Recording techniques

Participants' electrophysiological activity was acquired as described in Section 2.4 in Chapter 2. EMG was recorded using bipolar electrodes placed on the bicep femoris muscles from both legs. EEG was recorded from eight electrode sites (Cz, CPz, C3, C4, Oz, FCz, T3, T4), according to the modified international 10-20 system with reference at Fz and ground at Fpz.

Oxygen saturation and heart rate were acquired with a pulse oximeter and ECG using a patient monitor and vital signs were continuously downloaded from the

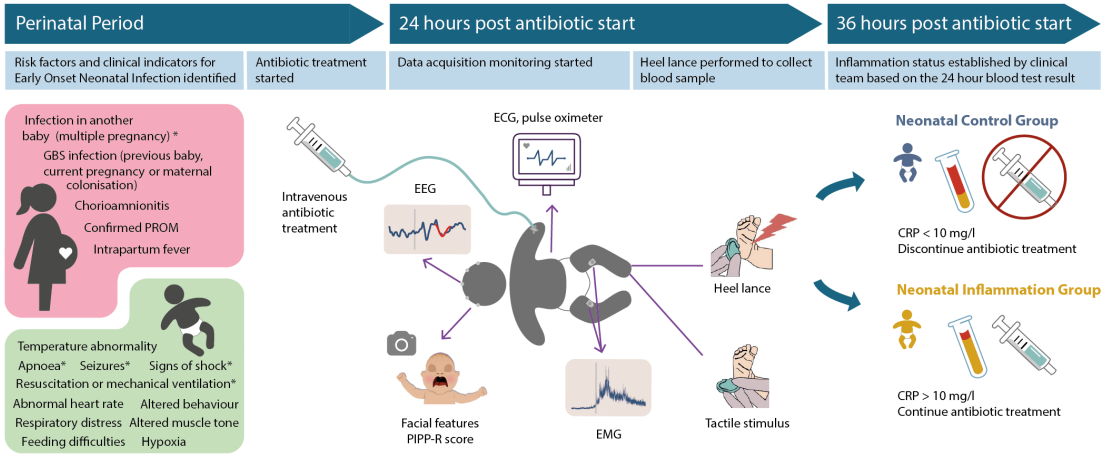

Figure 5.2. Study 1 Experimental design.*Red flag risk factors and clinical indicators for Early Onset Neonatal Infection (EONI). Antibiotic treatment was started if any red flag or two or more non-red-flag risk factors or clinical indicators were identified. See [14] for the complete National Institute for Health and Care Excellence (NICE) EONI screening guidelines in place during the study period. Abbreviations: GBS: Group B *Streptococcus*, PROM: prelabour rupture of membranes, EEG: electroencephalography, PIPP-R: Premature Infant Pain Profile-Revised, EMG: electromyography, ECG: electrocardiogram, CRP: C-reactive protein.

monitor. Facial expressions were also recorded in Study 1 and Study 2 to calculate clinical pain scores. Physiological and behavioural data acquisition are described in detail in Chapter 2.

5.2.4 Data analysis

EMG signals were filtered, epoched and analysed as detailed in Chapter 2. Individual epochs were rejected due to movement artefact in the baseline period. The data was split into 250 ms windows and the root mean square (RMS) of the reflex signal was calculated in each window [186]. The average RMS across the four windows in the first second after stimulation was calculated as the magnitude of the reflex withdrawal. Following artifact removal, a total of 57 participants were included in the final noxious and tactile evoked EMG data analysis (2/38 traces with movement artifact were rejected from the Neonatal Control Group and 2/23 traces – 1 with artifact and 1 due to technical failure - were rejected from the Neonatal Inflammation Group, Study 1, Figure 5.1).

EEG signals were filtered, epoched and baseline corrected as described in Chapter 2. Epochs were rejected if they contained gross movement artefact. Event related potentials were analysed at the Cz electrode for all trials. The magnitude of noxious-evoked brain activity during the heel lance was obtained by projecting the template of noxious-evoked brain activity onto each individual trial 400-700 ms after stimulation [20]. Each individual trial was first Woody filtered to the template with a maximum jitter of ± 100 ms in the time window of interest to account for individual differences in the latency to the response. Following rejections, a total of 51 participants were included in the noxious-evoked EEG data analysis (6/38 traces with artifact were rejected from the Neonatal Control Group and 4/23 traces – 3 with artifact and 1 due to technical failure - were rejected from the Neonatal Inflammation Group, Study 1, Figure 5.1). Study 2 EEG data was processed and analysed using the same methodology and 20 participants (Neonatal Antibiotic Treatment Group: n=12, Neonatal Antibiotic Control Group: n=8) were included in the final noxious-evoked EEG data analysis.

An early evoked potential that is common to both noxious and tactile stimulation has been previously identified during a heel lance and a non-noxious control [199, 235–237]. To characterise this early component in the EEG responses I used data from the Neonatal Control and Neonatal Inflammation Groups (Study 1) recorded during background, heel lance and non-noxious control stimuli. EEG signals were filtered from 0.5 – 30 Hz with a notch filter at 50 Hz. Data were epoched from 500 ms before the stimulus to 1000 ms after and were baseline corrected to the pre-stimulus mean. Epochs were rejected if they contained gross movement artefact (I included the same noxious-evoked EEG epochs from 51 neonates described earlier and tactile-evoked traces from 49 participants - 9/38 tactile-evoked epochs with artifact were rejected from the Neonatal Control Group and 4/23 tactile-evoked traces with artifacts were rejected from the Neonatal Inflammation Group - Figure 5.1). Event related potentials were analysed at the Cz electrode for all trials.

Heel lance and tactile epochs from all individual trials were combined and aligned with respect to the average of the data by Woody-filtering with a maximum shift of ± 50 ms in the 0–700 ms interval after the stimulus onset. Clusters of timepoints where the combined noxious and tactile stimuli were significantly different from background were identified using the non-parametric statistical analysis described by Maris and Oostenveld [344]. The cluster-based test statistic was calculated from 1000 random permutations of the data, and the threshold for cluster significance was set as the 97.5 percentile of the permuted data.

5.2.5 Statistical analysis

A power calculation was performed to determine the sample size required to test the effect of neonatal inflammation on spinal cord excitability and brain activity in response to a noxious procedure (principal hypothesis of Study 1).

Sample size calculation and statistical analysis

Power calculations were performed in G*Power v3.1 [418]. The outcome measures of this study were the noxious-evoked reflex withdrawal and brain activity in response to a heel lance in the Neonatal Inflammation Group and Neonatal Control Group. A 70% increase in the Neonatal Inflammation Group was considered to be clinically significant as this effect has been reported for inflammation induced hyperalgesia in adults [419]. The mean (SD) brain activity evoked by a heel lance in a cohort of healthy term infants is 0.72 (0.69) (data published in [346] and [242]). A sample size of 56 neonates (2:1 Neonatal Control Group to Neonatal Inflammation Group allocation ratio) would be required to observe a 70% increase in noxious-evoked brain activity with a two-sample t-test (80% power and a one-sided 5% significance level). Accounting for 15% of losses due to technical failures or clinical ineligibility (it was expected that after enrolment some infants would require a recannulation for antibiotic administration and therefore precluding the need to perform a heel

lance), a total sample size of 65 neonates would be required. I also calculated the sample size that would be required to achieve adequate power to observe a significant difference in reflex withdrawal activity between the two groups. The mean (SD) RMS of the reflex withdrawal in a cohort of healthy term infants is 23.3 (17.7) (data published in [346] and [242]). Assigning the same assumptions as for the noxious-evoked brain activity a lower sample size of 36 neonates would be required for this measure. Therefore, a sample size of 65 ensured that adequate power could be achieved for both outcome measures. Sample size calculations were not performed for the exploratory study as no hypotheses were being tested.

Statistical analysis was performed in MATLAB R2020 (MathWorks) and R (The R Project for Statistical Computing). Group differences in noxious-evoked brain activity, tactile-evoked brain activity, reflex withdrawal and clinical pain scores were assessed using unpaired two-sample t-tests. Statistical significance (one-sided alpha<0.05) was assessed non-parametrically via permutation testing with 10,000 permutations using the PALM (permutation analysis of linear models) toolbox [371]. One-sided tests were used to reflect the directional hypotheses presented in this study. Linear associations between noxious-evoked responses and CRP levels were assessed using Pearson correlation tests and statistical significance (alpha<0.05) was assessed non-parametrically via permutation testing with 10,000 permutations using PALM. The Holm's method [420] was used to correct p-values for multiple comparisons across the principal hypothesis analysis (2 comparisons: noxious-evoked brain activity between groups and noxious-evoked reflex withdrawal between groups). Secondary hypothesis analyses p-values are presented without correction for multiple comparisons as the purpose of the secondary analysis was to support and provide greater insight to the primary results.

5.3 Results

The results presented in this chapter have been published in Nature Communications [421].

5.3.1 Study 1: Effect of inflammation on pain responses during a heel lance procedure

Noxious evoked reflex withdrawal and brain activity responses

The principal hypothesis of this study was that neonatal inflammation causes increased spinal cord excitability and hyperalgesia in response to noxious stimulation. Neonates with similar perinatal histories were differentiated based on the CRP levels in their blood and clinical evidence of infection as presented in detail in Section 5.2.1 and Figure 5.2. Reflex withdrawal activity responses were assessed using EMG and noxious-evoked brain activity assessed using EEG in all participants in the Neonatal Inflammation Group and Neonatal Control Group during a clinically required heel lance procedure.

Newborn infants in the Neonatal Inflammation Group have significantly greater spinal cord mediated reflex withdrawal EMG activity and noxious-evoked EEG brain activity in response to heel lancing (within 24 hours from presentation of risk factors) compared to neonates who do not have raised inflammatory markers (magnitude of noxious-evoked reflex withdrawal activity: Neonatal Inflammation Group: n=21, mean=42.1, SD=30.9; Neonatal Control Group: n=36, mean=30.2, SD=20.6; t-test t=1.74, p=0.048; magnitude of noxious-evoked brain activity: Neonatal Inflammation Group: n=19, mean=1.04, SD=0.68; Neonatal Control Group: n=32, mean=0.64, SD=0.52; t=2.4, p=0.036, p-values corrected for multiple comparisons using Holm's method, Figure 5.3).

Due to the continuous nature of the CRP level variable, I assessed correlations between CRP level and EMG and EEG responses, and as shown in Figure 5.3,

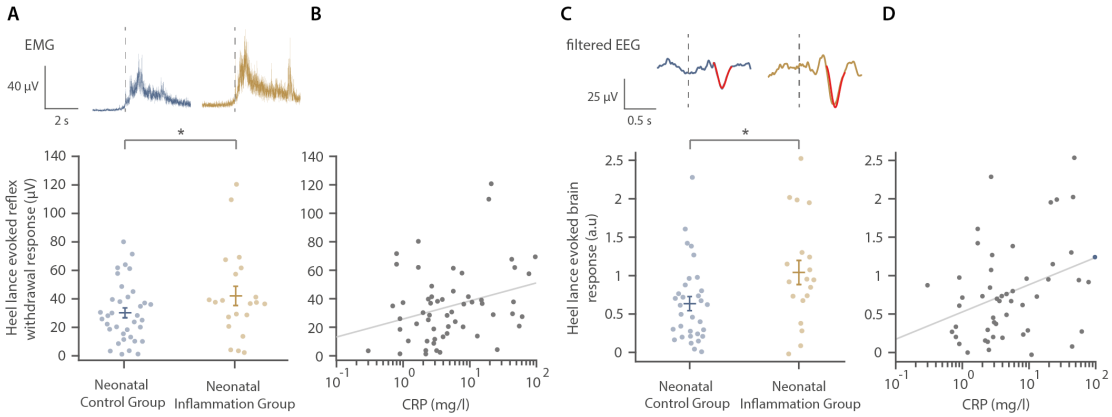

Figure 5.3. Inflammation causes increased spinal cord excitability and hyperalgesia during a noxious procedure in neonates. **(A)** (Top) Average electromyography (EMG) traces during a clinically-required heel lance for participants in the Neonatal Control Group (blue, n=36) and Neonatal Inflammation Group (gold, n=21, CRP >10 mg/ml or presented ongoing clinical signs of infection). (Bottom) Root mean square (RMS) of the reflex withdrawal in the limb ipsilateral to the stimulus site in the two groups. **(B)** Relationship between the CRP level at the time of the study and the magnitude of the reflex withdrawal following a clinically required heel lance (n = 57). **(C)** (Top) Group average Woody filtered electroencephalography (EEG) traces in response to the clinically required heel lance; Neonatal Control Group (n=32) and Neonatal Inflammation Group (n=19). The template of noxious-evoked brain activity [237] is shown overlaid in red. (Bottom) Magnitude of the noxious-evoked brain activity following heel lancing in the two groups. **(D)** Relationship between the CRP level at the time of the study and the magnitude of the noxious-evoked brain activity following a clinically required heel lance (n = 51). In A and C, dashed lines indicate the point of stimulation and error bars indicate mean ± standard error, In B and D, solid line indicates line of best fit. *p<0.05, corrected for multiple comparisons.

both the spinal cord mediated reflex withdrawal activity and noxious-evoked brain activity are significantly correlated with the level of CRP in the blood sample 24 hours post antibiotic start (reflex withdrawal activity: n=57, Pearson correlation r=0.28, p=0.035, noxious-evoked brain activity: n=51, r=0.31, p=0.028). Taken together, the combined evidence of significant correlations with CRP level and significant differences between groups (when CRP level is dichotomised at the clinical decision-making threshold of 10 mg/l), suggest these effects are potentially of clinical value, despite their modest p-values.

Clinical pain scores and physiology

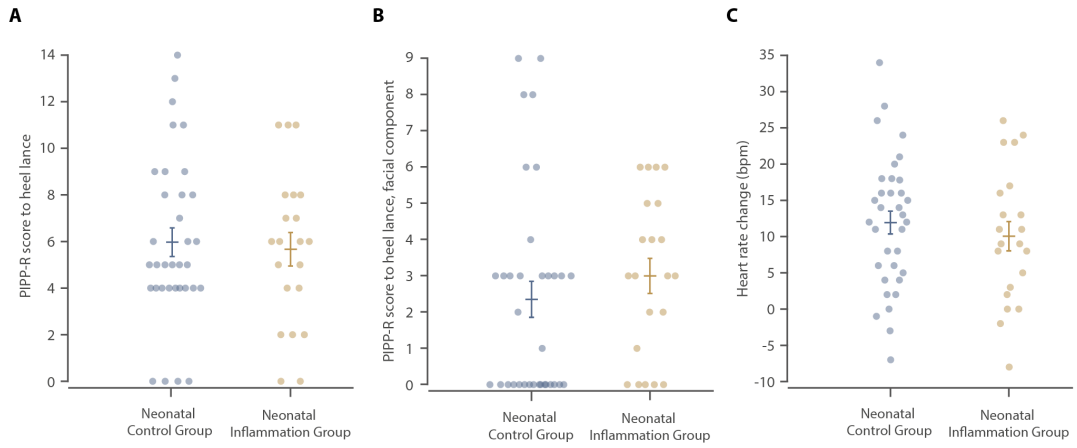

Figure 5.4. PIPP-R scores, behavioural features and changes in physiology during a heel lance are not altered by the presence of inflammation. (A) PIPP-R scores during a clinically required heel lance in the Neonatal Control Group (blue, n = 34) and Neonatal Inflammation Group (gold, n = 21). **(B)** Behavioural scores (facial expression component of the PIPP-R scores) during heel lancing in the Neonatal Control Group (blue, n = 34) and Neonatal Inflammation Group (gold, n = 21). **(C)**Heart rate change (maximum heart 30 seconds post heel lance minus mean heart rate in the 15 seconds baseline period before heel lance) in both groups. Error bars indicate mean ± standard error of the mean.

Clinical pain scores were calculated using the well-validated Premature Infant Pain Profile-Revised (PIPP-R) for all the neonates and scores were compared between the two groups. PIPP-R scores are not significantly altered by the presence of inflammation (PIPP-R score: Neonatal Inflammation Group: n=21, mean=5.7, SD=3.3; Neonatal Control Group: n=34, mean=6, SD=3.6; t=-0.32, p=0.64) as illustrated in Figure 5.4. Figure 5.4 also shows the behavioural component of the PIPP-R score (facial expressions) and the change in heart rate from baseline to 30 seconds post procedure in both groups. Significant differences in facial expression scores (Neonatal Inflammation Group: mean=3 SD=2.2; Neonatal Control Group: mean=2.4, SD=2.9, t=0.88, p=0.2) or heart rate change (Neonatal Inflammation Group: mean=10 SD=9.3; Neonatal Control Group: mean=11.9, SD=9.2, t=-0.7, p=0.77) were not observed between the Neonatal Inflammation Group and Neonatal Control Group.

5.3.2 Study 2: Effect of inflammation on arousal responses following a tactile stimulus

Characterising an early-event potential in response to a heel lance and to a tactile non-noxious control

The second hypothesis of this study was that neonatal inflammation causes increased spinal cord excitability and arousal-related brain activity following tactile stimulation. To test this hypothesis, an early-event potential that was present during both a noxious an a non-noxious stimulus was characterised to quantify the magnitude of the arousal-related EEG response following tactile stimulation (non-noxious control).

Characterisation of the early component of the EEG responses was conducted using data from the Neonatal Control and Neonatal Inflammation Groups recorded during background, heel lance and non-noxious control stimuli. The morphology of the average raw EEG signal in the central electrode (Cz) is shown in Figure 5.5A and is consistent with previous reports showing an early potential during both tactile and noxious-stimuli approximately 250 ms after the stimulus [199, 235–237].

Heel lance and tactile stimulation evoked an activity cluster significantly different from background activity in the time window from 151-272 ms after the stimulus ($p = 0.001$, cluster-corrected non-parametric test, Figure 5.5B). Principal component analysis (PCA) was applied to the EEG epochs in the time window 100 – 350 ms after the stimulus to capture the whole waveform and 94% of the variance was captured by the first four principal components (PCs). The first PC morphology, illustrated in Figure 5.5C, was comparable to that identified in previous studies [199, 235–237] and its weights were significantly higher following the heel lance ($t=5.4$, $p<0.001$) and the tactile stimulus ($t=4.9$, $p<0.001$, p-values corrected for multiple comparisons using Holm's method) compared to background brain activity, Figure 5.5D. The weights of this PC for the tactile-evoked EEG (n=49) represent

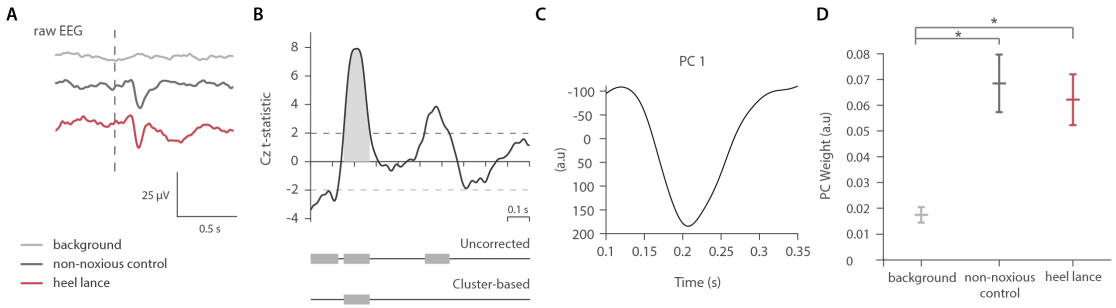

Figure 5.5. Characterisation of the tactile-evoked early potential during a heel lance and a tactile stimulus. (A) Average raw EEG traces during background activity and in response to a tactile stimulus (non-noxious control) and a heel lance showing an evoked potential around 250 ms post stimulus. Dashed line indicates the point of stimulation. **(B)** t-statistics from the comparison of the noxious-evoked brain activity during noxious and tactile stimulation (combined) and during background brain activity in 56 neonates. Dashed lines indicate the t-statistic threshold for cluster significance, set as the 97.5 percentile of the permuted data. The grey bars indicate time periods outside of the t-statistic threshold and the significant time window identified with the cluster analysis is illustrated by the grey shading area. **(C)** Principal component analysis was conducted in the time window 100-350 ms after the stimuli and the characterised waveform with a positive peak was similar to the waveform previously described in the literature [199, 235–237]. **(D)** First principal component weights were significantly higher following tactile stimulus and heel lance compared with background brain activity, error bars indicate mean ± standard error (*$p < 0.001$, corrected for multiple comparisons).

the magnitude of the early potential in response to a tactile stimulus.

Investigating the effect of inflammation on tactile evoked reflex withdrawal and brain activity responses

To test the hypothesis that neonatal inflammation increases spinal cord excitability and arousal, I assessed whether the presence of neonatal inflammation (i) increased spinal cord mediated reflex withdrawal activity and (ii) increased arousal-related brain activity [236] following tactile stimulation. As hypothesised, neonatal inflammation caused increased reflex withdrawal activity to tactile stimulation (magnitude of reflex withdrawal: Neonatal Inflammation Group: n=21, mean=17.9, SD=12.2; Neonatal Control Group: n=36, mean=11.8, SD=12; t=1.85, p=0.037, Figure 5.6A) and evoked significantly greater brain activity following tactile stimulation (magnitude of tactile-evoked brain activity: Inflammation Group,

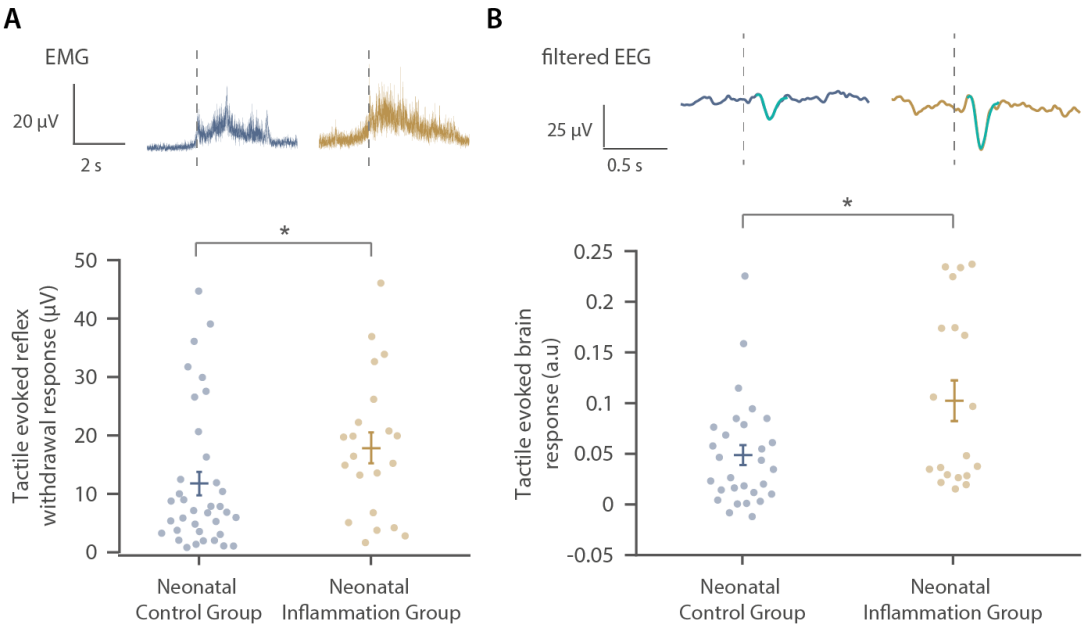

Figure 5.6. Neonatal inflammation increases spinal cord excitability and arousal-related brain activity during a tactile stimulus. **(A)** (Top) Average electromyography (EMG) traces during a tactile stimulus for participants in the Neonatal Control Group (blue, n=36) and Neonatal Inflammation Group (gold, n=21, CRP > 10 mg/l or presented ongoing clinical signs of infection). (Bottom) Root mean square (RMS) of the reflex withdrawal in the limb ipsilateral to the stimulus site in the two groups. **(B)** (Top) Group average (Woody) filtered electroencephalography (EEG) traces in response to a tactile stimulus; Neonatal Control Group (n=29) and Neonatal Inflammation Group (n=19). The evoked potential is shown overlaid in teal. (Bottom) Magnitude of the brain activity following a tactile stimulus in the two groups. Dashed lines indicate the point of stimulation and error bars indicate mean ± standard error,*p<0.05.

n=19, mean=0.1, SD=0.09; Control Group, n=29, mean=0.05, SD=0.05; t=2.66, p=0.007, Figure 5.6B).

5.3.3 Study 3: exploratory study assessing noxious evoked responses in neonates exposed to early life inflammation after antibiotic treatment

Noxious evoked reflex withdrawal and brain activity responses

The primary purpose of the hypothesis-testing study was to examine the acute consequences of neonatal inflammation on spinal cord and cortical sensory systems.

However, I also performed an exploratory hypothesis-generating study to examine whether spinal cord excitability and hyperalgesia are maintained after inflammatory markers are reduced by antibiotic treatment. In an independent sample of neonates who were treated with 5 days of antibiotics (referred to as the Neonatal Antibiotic Treatment Group), the average CRP levels reduced from 26 mg/l (within 24 hours from presentation of risk factors) to 5 mg/l (within 96 hours from presentation of risk factors). Reflex withdrawal and brain activity were recorded in response to a clinically-required heel lance and observations were compared with postnatal age-matched healthy neonates, with no evidence of infection, who were having a blood test for routine newborn screening (Neonatal Antibiotic Control Group).

The noxious-evoked reflex withdrawal responses were similar between the two groups (magnitude of noxious-evoked reflex withdrawal: Neonatal Antibiotic Treatment Group: n=13, mean=31.7, SD=14.8; Neonatal Antibiotic Control Group: n=11, mean=33.9, SD=25.9, Figure 5.7A). However, when the magnitude of the noxious-evoked brain activity was compared between the two groups, the magnitude of the brain activity was higher in the Neonatal Antibiotic Treatment Group, (magnitude of noxious-evoked brain activity: Neonatal Antibiotic Treatment Group: n=12, mean=1.21, SD=1.02; Neonatal Antibiotic Control Group: n=8, mean=0.9, SD=0.59; 34% increase, Figure 5.7B). Given the exploratory nature of this study, no formal hypothesis testing was conducted. The results that I present here are descriptive (non-inferential statistics) and the subsequent discussion of these data descriptions and how they might motivate future studies are presented in Section 5.4.

Clinical pain scores and physiology

There were no differences in clinical pain scores, calculated using the PIPP-R scale, between the two groups (PIPP-R score: Neonatal Antibiotic Control Group: n=12, mean=8.8, SD=3.3; Neonatal Antibiotic Treatment Group: n=10, mean=8, SD=4; Figure 5.8A). Breaking down the PIPP-R score into the facial component (Figure 5.8B, Neonatal Antibiotic Control Group: mean=3.8, SD=3.3; Neonatal

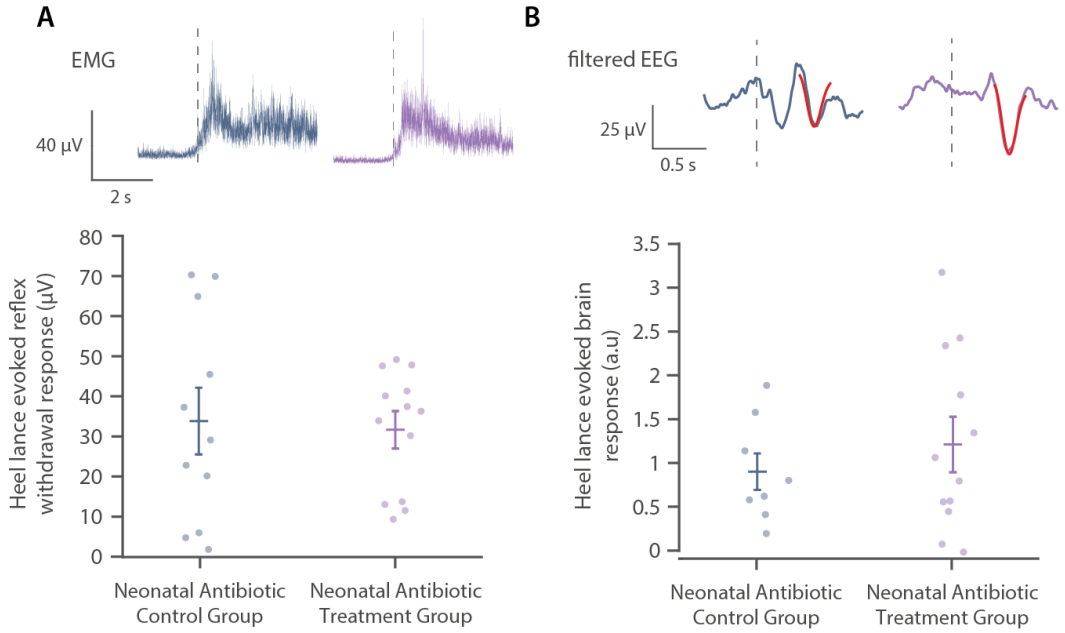

Figure 5.7. Spinal cord excitability and brain activity during a noxious procedure in neonates exposed to early life inflammation after antibiotic treatment. (A) (Top) Average electromyography (EMG) traces during a heel lance for participants in the Neonatal Antibiotic Control Group (blue, n=11) and Neonatal Antibiotic Treatment Group (purple, n=13, CRP $<$ 10 mg/l after antibiotic treatment). (Bottom) Root mean square (RMS) of the reflex withdrawal in the limb ipsilateral to the stimulus site in the two groups. **(B)** (Top) Group average (Woody) filtered EEG traces in response to the clinically required heel lance; Neonatal Antibiotic Control Group (blue, n=8) and Neonatal Antibiotic Treatment Group (purple, n=12). The template of noxious-evoked brain activity [237] is shown overlaid in red. (Bottom) Magnitude of the noxious-evoked brain activity following heel lance in the two groups. Dashed lines indicate the point of stimulation and error bars indicate mean ± standard error.

Antibiotic Treatment Group: mean=3.3, SD=3.2) and the change in heart rate from baseline to 30 seconds after the procedure (Figure 5.8C, Neonatal Antibiotic Control Group: mean=17.5, SD=9.3; Neonatal Antibiotic Treatment Group: mean=21.6, SD=17.1) showed similar trends.

5.3.4 Summary of findings

Overall, the results suggest inflammation-induced increased spinal cord excitability and hyperalgesia during an acute painful procedure and increased arousal to a

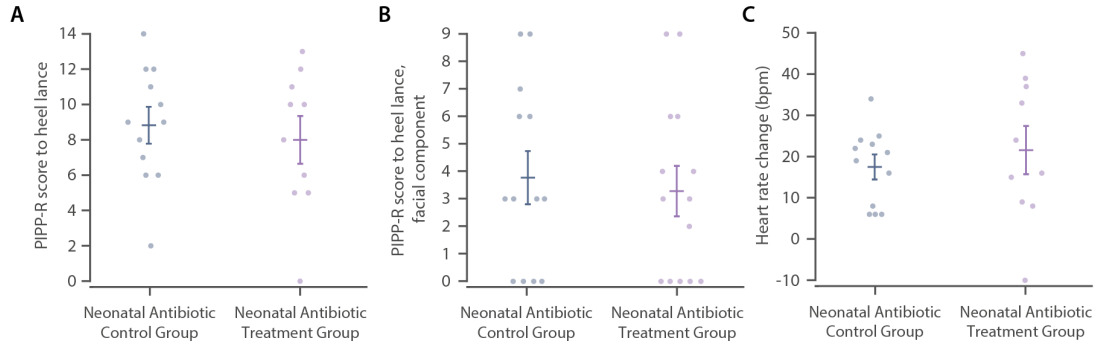

Figure 5.8. PIPP-R scores, behavioural scores and changes in physiology during a noxious procedure in neonates exposed to early life inflammation after antibiotic treatment. **(A)** PIPP-R scores during a clinically required heel lance in the Neonatal Antibiotic Control Group (blue, n = 12) and Neonatal Antibiotic Treatment Group (purple, n = 10). **(B)** Behavioural scores (facial expression component of the PIPP-R scores) during heel lancing in the Neonatal Antibiotic Control Group (blue, n = 13) and Neonatal Antibiotic Treatment Group (purple, n = 21). **(C)**Heart rate change (maximum heart 30 seconds post heel lance minus mean heart rate in the 15 seconds baseline period before heel lance) in both groups. Error bars indicate mean ± standard error of the mean.

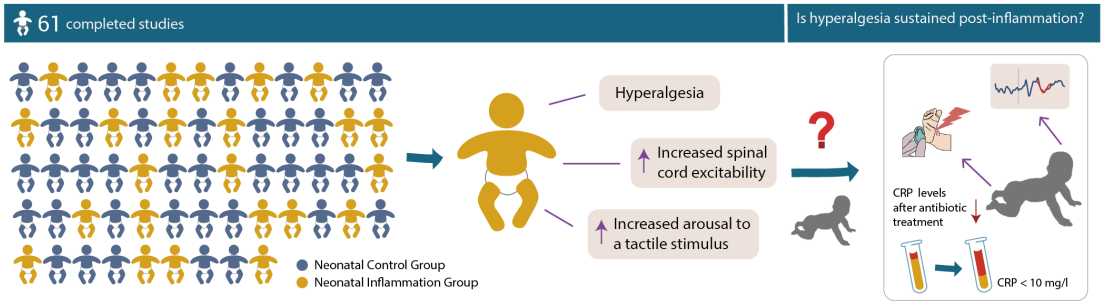

Figure 5.9. Summary of the results of Chapter 5 and sustained hyperalgesia hypothesis representation. A total of 61 neonates (Neonatal Control Group n=38, Neonatal Inflammation Group n=23) were included in the primary study.

tactile stimulus in term neonates. Prospective studies are needed to investigate whether hyperalgesia is maintained after inflammation is reduced and CRP return to normal levels after the antibiotic treatment.

5.4 Discussion

Immune function and sensitivity to pain are closely related, but the impact of early life inflammation on sensory nervous system development is poorly understood

– especially in humans. In this chapter, in term-born infants, I measured brain activity and reflex withdrawal activity (using EEG and EMG) and behavioural and physiological activity (using the PIPP-R score) to assess the impact of suspected early-onset neonatal infection on tactile- and noxious-evoked responses. It has been demonstrated that neonatal inflammation is associated with increased spinal cord excitability and evoked brain activity following both noxious and tactile stimulation. There are early indications that this hyperalgesia could be maintained post-inflammation, supporting pre-clinical reports that early-life immune dysfunction influences pain sensitivity in adults.

Neonates with elevated CRP levels shown increased reflex withdrawal and noxious-evoked brain activity to a clinically required heel lance in the first 72 hours of life compared to well matched controls. These observations are in line with evidence from adult humans and animals, which shows that neuro-immune interactions increase spinal cord excitability and nociceptive sensitivity [158, 400, 411, 422]. However, evidence from neonatal humans and animals is lacking. Pain assessment during the period of neonatal inflammation has not been extensively investigated and studies undertaken in neonatal rat pups report conflicting results [412, 423]. Our specific findings in human infants using CRP as a marker of inflammation could be further explored in rodent models to elucidate the potential mechanisms of these neuro-immune interactions.

Several mechanisms involved in this relationship between inflammation and increased pain sensitivity have been described in adult animal models and human adults, which could potentially play a role in the responses observed in this study. Pro-inflammatory cytokines released during infection increase the excitability of peripheral nerves [161], reaching specific regions of the rodent neonatal dorsal horn circuitry and augmenting nociceptive reflexes [408, 423, 424]. Activated microglia in the peripheral and central nervous system are also an important source of cytokine synthesis and growth factors which enhance dorsal horn neuron activation and

amplify the sensory transmission of the nociceptive signal from the periphery to the brain [424–426].

Observations of enhanced peripheral transmission are supported by adult studies using human experimental endotoxemia and fMRI to describe the neural circuitry involved in the inflammation-induced central pain amplification [158, 427]. Participants in a double blind, randomised controlled fMRI study showed enhanced pain-induced BOLD responses within the posterior insula, dorsolateral prefrontal (DLPFC), anterior midcingulate (aMCC) and somatosensory cortices [158]. Importantly, in addition to the up regulation of activity in regions involved in peripheral transmission, there is evidence of inflammation-induced decreased activity in the ventrolateral prefrontal cortex and the rostral anterior cingulate cortex (rACC); areas involved in descending inhibitory pain regulation [428–430]. Thus, it is plausible that several neuroimmune mechanisms work together to alter pain perception during inflammation; these include peripheral nerve sensitization, modulations in the spinal cord, and functional changes in the brain's pain network.

Given that in the presence of inflammation an increase in reflex withdrawal and noxious-evoked brain activity was observed, an increase in PIPP-R scores may also have been expected. However, this was not the case, as PIPP-R scores were not altered by the presence of inflammation. In the research setting the calculation of PIPP-R scores can be performed retrospectively by assessing the recorded facial videos and physiological ECG and oxygen saturation data. While changes in physiological parameters such as heart rate and oxygen saturation are objective and quantifiable (in contrast with the subjective assessment of facial features), these have shown little correlation with noxious-evoked brain activity in term and preterm infants [431]. Moreover, in the context of the PIPP-R scale, these are limited to a single metric of the difference between the baseline average heart rate and oxygen saturation in the 15 seconds prior to stimulation, and the maximum heart rate and minimum oxygen saturations in the 30 seconds following

stimulation [170]. The physiological data obtained during the studies might be better used by applying other approaches. For example, a study tested 24 different heart rate measures and showed that the maximum change in heart rate in the first 15 seconds after the stimulus was most discriminative between a noxious and non-noxious condition in infants [193]. High-frequency analysis has also been used to obtain heart rate variability indices which show some potential for the assessment of acute and prolonged pain in infants [432, 433].

While it is possible that the study was underpowered to observe an effect on clinical pain scores (given the subjective nature of PIPP-R assessments in contrast to objective EMG/EEG assessments), this observation is consistent with clinical observations in adults and neonates, as well as data from animal studies, which demonstrate that inflammation suppresses behavioural activity, causing listlessness and lethargy [434, 435]. These protective inhibitory behavioural responses to inflammation, often reported to be a key symptom of 'sickness syndrome' [436], likely provide a mechanism whereby available energy can be directed towards fighting pathogens rather than classic exploratory behaviours and social interactions [434]. Increased lethargy and inhibition of behavioural activity will be highly influential on the magnitude of clinical pain scores, as these scores are predominantly based on observing behaviours [437]. This potentially limits the utility of these scores in neonates with pathological conditions, where motor inhibition in response to aversive stimulation may be a critical mechanism to conserve energy. Further research is warranted to establish how the presence of neonatal inflammation impacts clinical pain scores and the degree to which subjective observational assessment of infant pain-related behaviours might influence pain scoring.

In neonates clinical signs of 'sickness syndrome' also include increased irritability and restlessness [438]. This suggests that in the absence of nociceptive input infants display more erratic body movements, which could be mediated by increased spinal cord excitability. In this study, this hypothesis was tested and neonates

with inflammation presented elevated reflex withdrawal and evoked brain activity following a tactile stimulus on the foot. Amplification of muscle reflexes in the presence of inflammation is likely mediated by increased excitability within dorsal horn networks as discussed above [408, 423, 424], as well as potential influences from central brain networks [439]. This evidence that cerebral arousal responses are increased during neonatal inflammation likely underpins clinical observations of restlessness in infants presenting with inflammation.

Inflammation sensitises the pain system in neonates; however, the time-course and distribution of the resultant effects are yet to be studied. The nervous system undergoes considerable postnatal maturation and may be 'primed' by early life inflammation such that it is more easily activated in later life [406, 411]. Given that plasticity of the neuroimmune system occurs during critical developmental periods, an early immune challenge may be associated with the long-term effects observed in rodent adult pain responses [44, 407, 410, 440].

The results from the exploratory study, particularly the brain activity in response to a heel lance following resolution of the initial inflammatory process showed an increased noxious-evoked brain activity in the group with Early Onset Neonatal Infection (EONI). One interpretation of this exploratory finding is that hyperalgesia could outlive the acute inflammatory period that caused it, with neonatal inflammation potentially causing long-term changes in pain sensitivity. The current experimental design does not allow for formal hypothesis testing, but if this trend was to be confirmed in a future study, it would be consistent with observations made by other research groups in pre-clinical models [422, 441]. It would also provide a potential underlying biological mechanism for what has been observed in epidemiological studies, which report that chronic pain is more likely in individuals who have experienced adverse events in childhood or adolescence [417]; this includes physical trauma and serious illness, all presumably associated with significant levels of inflammation. However, the effect observed in the exploratory

study might be no larger than difference due to random chance. Thus, an alternative interpretation of this observation is that early life infection that has been treated with antibiotics results in no long-term consequences in noxious evoked responses. An appropriately powered confirmatory study is warranted to test the hypothesis derived from this exploratory study.

The study design used to test the main hypotheses of this chapter capitalised on the combination of electrophysiological and behavioural monitoring for the evaluation of pain during clinical procedures and the NICE guidelines to diagnose early onset neonatal infection, in place in the local unit. One of the biggest strengths of this approach is the homogeneous baseline characteristics in the Neonatal Inflammation Group and Neonatal Control Group due to their well matched perinatal histories. Moreover, potential confirmation bias was minimised as the investigators were blinded to the inflammation condition and diagnosis at the time of the data collection. The same experimental paradigm and neurophysiological methods used in this study can be applied to investigate whether pain sensitivity is maintained after the inflammation has resolved. Such investigations would involve a longitudinal design with multiple measures taken from the same individuals across a period when routine acute noxious procedures are clinically necessary, for example following up infants with suspected sepsis from birth until the first set of immunisations around eight weeks of postnatal life. Recruitment and follow-up for this type of study may bring new challenges given that parents will be asked to return multiple times to the hospital to receive the routine tests and checks that would otherwise be managed at the community level.

The exploratory study design was limited by the unbalanced number of prior painful procedures between the groups and the missing Neonatal Antibiotic Control Group CRP values. Since neonates in the control group had never presented with risk factors for infection, the number of painful procedures prior to the study occasion was minimum. However, neonates in the Neonatal Antibiotic treatment group

required multiple blood tests as part of the sepsis screening and CRP monitoring and therefore the observed results can be confounded by the prior experience. Furthermore, due to the ethics regulations for this study, it was not possible to request additional laboratory tests (e.g. to measure CRP levels in the control group) to those required by the clinical team. Unfortunately, due to COVID-19 pandemic restrictions the exploratory study was suspended and I was not able to collect more data and address some of these questions. When planning future studies these limitations need to be considered and addressed to facilitate the interpretation of the results and to facilitate an appropriate study design to investigate the question of interest.

A range of inflammation markers have been used in pre-clinical and adult literature, which mostly quantifying pro-inflammatory cytokines closely associated with hyperalgesia [406]. CRP is an acute-phase inflammatory protein which exhibits elevated expression during inflammatory conditions. It has been traditionally used as a marker of infection and more recent evidence links CRP with specific inflammatory processes dependent on the complement pathway, nitric oxide release and the production of cytokines particularly IL-6 and TNF-α [442]. Here, we used CRP as an inflammation marker because it is routinely used during Early Onset Neonatal Infection screening in the clinic and was used to split the participants between the Neonatal Control Group and Neonatal Inflammation Group. Potential mechanisms of the association between CRP and hyperalgesia can be investigated in future studies by back-translating some of these findings to preclinical models used for the study of the immune system and pain processes during early development.

Understanding how pain is perceived and processed in this patient population is not possible without methods to characterise the neonatal responses to painful stimulation. Enhanced pain sensitivity and other detrimental effects of repeated painful procedures in early life are well described in the literature, and specific neurobiological mechanisms underlying injury-induced plasticity in the developing

pain system have been studied in animal models [44]. However, the long-term alterations in sensory and pain processing, following hospitalisation due to prematurity or life-threatening medical conditions, can be caused by multiple factors present in the clinical setting such as physiological stress, handling, illness, medications, invasive procedures and infections [58]. Disentangling the long-term effects of each of these factors is challenging considering the close interactions between multiple organs and systems including the nervous and immune systems as presented in this chapter. The direct effect of inflammation on nociceptive processing in human infants is only beginning to be understood and this first study in human neonates demonstrates increased nociceptive sensitivity at day two of life. These results can be complemented and evaluated with pre-clinical studies to elucidate the mechanisms that underpin the neuroimmune interactions. The principles and methods used here could be potentially applied to investigate the independent and combined effects of exposure to early life pain and infection in this vulnerable population. Evidence from a pre-clinical study points to a potential cumulative effect of infection and early life pain exposure that can lead to an alteration of nociception in rat pups [414].

In conclusion, the results indicate that there is a connection between neonatal inflammation, spinal cord hypersensitivity and cortical nociceptive processing. To my knowledge, this is the first study to demonstrate this in human infants. It thus represents a vital step towards translating what has become a sizable pre-clinical literature on neonatal inflammation and pain - and which, until now, has been unmoored from any clinical data. Future work, involving carefully matched, longitudinal cohorts of neonates, will be required to further elucidate the potential long-term consequences of early-life neuro-immune interactions.

6 Discussion

6.1 Thesis summary

In the absence of self-report, we cannot know whether a non-verbal infant is in pain, and an alternative gold-standard pain measure in neonates does not exist. Noxious-evoked behavioural activity, reflex withdrawal activity, physiological responses (such as heart rate change) and brain activity are necessarily surrogate measures of pain. However, since the conscious perception of pain is generated in the brain, noxious-evoked brain activity may provide an objective and sensitive surrogate measure of pain in infants. Brain-derived measures can be used to improve our understanding of the mechanisms of pain in the developing human brain, but they can also be translated into clinical tools to improve the assessment of analgesic interventions and to answer relevant clinical questions. The aim of the work in this thesis was to (i) develop generalisable tools to assess pain in infants; (ii) apply these tools to optimise analgesic clinical trials and (iii) to investigate the impact of concomitant conditions like inflammation on pain responses in hospitalised infants.

Brain activity was recorded from a sample of term infants using EEG and analysed in Chapter 3. Noxious-evoked brain activity following experimental noxious stimulation of the foot, hand and thigh was characterised by identifying the time window where the signal was significantly different from the background and the evoked potential identified with PCA. The latency of the noxious-evoked potentials was consistent with the location where the stimulus was applied in relation to the distance to the brain. Furthermore, the ERP morphology in

each independent site was correlated with the template of noxious-evoked brain activity developed by Hartley and collaborators [237]. The specific time window where the ERP is expected after a stimulus is applied to the thigh was used to characterise the response following immunisations and the generalizability of the template was demonstrated to quantify the noxious-evoked response to this clinical procedure. Thus, the template of noxious-evoked brain activity could be used to systematically characterise further acute clinical procedures commonly performed in the neonatal units and test analgesic interventions. Finally, the analgesic effect of paracetamol was investigated in a sample of 29 ex-preterm neonates during routine immunisations. The reduced noxious-evoked brain activity observed in the group that receive paracetamol before the immunisation needs to be further investigated in a randomised controlled trial. Having an objective outcome measure and standard tools for clinical trials would improve the quality of the evidence for pain management in neonates. Testing the reproducibility of this approach in multiple centres would provide insights regarding the generalisability across different clinical settings and patient populations. Additionally, it would allow for the conduction of systematic reviews and meta-analyses of neonatal pain interventions to summarize the evidence and guide evidence-based treatment decisions for this patient population.

Different factors can affect noxious-evoked responses in infants. These include age, sex, stress, prior pain experience and behavioural state [62, 197, 198, 235, 327, 355–357]. As a result, there is often large inter-individual variability in the measured noxious-evoked responses which translates to large sample sizes required to statistically demonstrate the expected effect in analgesic studies [279, 281, 284, 358–360]. Given the practical and ethical challenges of conducting neonatal research, it is important to optimise analgesic trial designs and minimise the number of participants required in the studies. In Chapter 4 I investigated whether EEG can be used to assess infant's individual baseline sensitivity by quantifying the noxious-evoked brain activity in response to a low-intensity experimental noxious

stimulus. This measure of baseline sensitivity was used to develop a paradigm to increase the power and reduce the sample size needed to assess the efficacy of analgesic interventions. This was achieved by adding the baseline sensitivity as a covariate to account for the inter-individual variability observed in the responses. The paradigm was tested with a pain-relieving intervention consisting of brushing the limb before the clinical procedure and it was demonstrated that accounting for inter-individual variability reduced the sample size needed to detect a true effect. Interestingly, the results showed that the neonates with larger magnitudes of noxious-evoked baseline sensitivity had the greatest reduction in response following the intervention. In contrast, neonates with low baseline sensitivity were less likely to demonstrate a benefit of the intervention, as the reduction in their responses was minimal. This suggests that assessing baseline sensitivity could be also used for identifying the infants that would benefit most from analgesic interventions, but further studies are needed to confirm this trend. Nonetheless, this could open the possibility for exploring precision medicine approaches. Being able to predict which patients are likely to experience more pain can be invaluable to prioritise resources and to improve management and subsequent outcome in newborns.

In the final experimental chapter of this thesis, electrophysiology measures were analysed to answer a clinically-relevant question regarding the effect of early life inflammation in spinal cord excitability and nociceptive sensitivity in term neonates. Immune function and sensitivity to pain are closely related, and adverse events during the neonatal period shape pain sensitivity later in life [163, 406–408, 411]. In this study, I showed that neonatal inflammation causes increased spinal excitability and hyperalgesia following clinically-necessary painful procedures in newborns. The degree of inflammation, quantified by measuring C-reactive protein level in the blood, directly relates to the magnitude of spinal hypersensitivity and evoked brain activity following nociceptive stimulation. One of the strengths of this study is the novel experimental design which meant that all neonates independent of the

group allocation presented with similar signs and risk factors of suspected early-onset neonatal infection and received the same regimen of intravenous antibiotics. This is important because potential confounding factors such as the type and dose of antibiotics were balanced between the groups. For instance, antibiotics could potentially influence the pain responses concomitant with the antibacterial effects as there is some evidence of the role of gut microbiota composition on the regulation of pain processing and other behaviours in animal models [410, 443, 444]. In the exploratory study, I evaluated a dataset of neonates who were treated with 5 days of intravenous antibiotics to describe the relationships and patterns in the observed noxious-evoked responses and generate a hypothesis that can be tested in a subsequent prospective confirmatory study. The higher mean magnitude of brain activity is a descriptive result and an interesting observation that warrants follow-up. If confirmed, it would be consistent with evidence from pre-clinical models [422, 441]. However, the effect observed in the exploratory study might be no larger than the difference that may be present due to random chance. Thus, an alternative interpretation of this observation is that early life infection that has been treated with antibiotics results in no long-term consequences in noxious evoked responses.

Overall, the research presented in this thesis is clinically relevant. The work presented here has the potential to improve our understanding of the variability in the response to nociceptive input and improve the assessment of analgesic interventions in neonates. It is also very timely, since the Divisions of Pediatrics and Maternal Health of the FDA are currently focused on the use of new tools to improve analgesic clinical trial designs, extrapolation, and endpoints in patients from birth to less than two years [445]. Some limitations of these studies and opportunities for future research directions are discussed in the next section.

6.2 Limitations and future work

The major limitations specific to each experimental chapter were considered in their respective discussion sections (Sections 3.4, 4.4 and 5.4). The points that will be discussed below concern topics related to the use and interpretation of EEG measures or noxious-evoked brain activity and the relationship with other surrogate measures of pain. These limitations are common to all the studies performed in this thesis and represent an opportunity to discuss potential solutions and further work.

Pain is a complex sensory and emotional experience. The array of responses triggered by a noxious input involves processing at different levels of the nervous system generating spinal cord reflexes, facial grimacing, physiological changes and the activation of brain regions (thought to be involved in both sensory and affective processing [128, 129]). Thus, a multimodal pain assessment approach might enable a more comprehensive estimate of the infant pain experience than the investigation of each of these measures alone [193, 351]. Most of the available clinical pain assessment tools incorporate both behavioural (facial features, body movements, cry) and physiological (changes in heart rate, oxygen saturation, respiratory rate, blood pressure) metrics. While some behavioural measures, particularly facial grimacing, are more likely to indicate pain than physiological measures [446], the scoring relies on subjective visual assessment. On the other hand, although the calculation of autonomic changes in heart rate and oxygen saturation is objective, these lack specificity to noxious stimulation [372]. As behavioural and physiological measures are not specific to pain it is challenging for clinicians and researchers to rely on these measures that can be truly indicative of pain but also of other states such as distress, discomfort, agitation or hunger [447]. The PIPP-R tool and other behavioural scales are adequate in the sense that infant behaviours including facial expressions and body movements are accepted as sensitive and valid indicators

of pain [372]. However, they can be impractical to apply in the clinical setting leading to poor inter-rater reliability and underestimation or overestimation of pain [448]. The subjectivity and lack of specificity are also important limitations and objective methods to assess changes in facial expression are needed in the research and clinical settings. For example, facial EMG can be used to detect muscle activity associated with pain-related features like brow bulge, eye squeeze and nasolabial furrow [449]. Automatic facial image analysis by computer vision is also a promising methodology that can aid in the coding of facial expressions in infants and other non-verbal populations [450].

The methods used to assess the magnitude of reflex withdrawal are also objective and are reliable indicators of spinal cord sensory processing; however, reflex withdrawal can occur without pain perception. The inclusion of an objective measure of noxious-evoked brain activity to this multimodal approach is valuable as it provides a more specific indication of the processing of nociceptive input at the cortical level.

How these measures are related to each other is not fully understood, especially when contextual and emotional factors known to influence pain perception are present. There are various examples in the infant literature demonstrating how these surrogate measures of pain are consistent between them. The magnitude of noxious-evoked brain activity measured by both EEG and NIRS is correlated with changes in facial features [237, 355, 356, 451]. Additionally, the magnitude of noxious-evoked brain activity recorded by EEG is associated with the magnitude of reflex withdrawal [186]. There is also evidence of concordance between the magnitude of EEG and NIRS noxious-evoked brain activity [199]. However, there are also many studies where discrepancies between different surrogate measures have been observed [199, 201, 247, 357, 452, 453]. During neonatal circumcision, changes in NIRS signal were observed during sharp and pressure induced pain but these were not reflected in the behavioural pain assessment [452]. Similarly, noxious-evoked

brain activity has been detected in the absence of facial expression responses [85, 186, 237]. The dissociations between these measures can be explained by multiple factors. Contextual elements, interventions and illness affect the independent modalities and can disrupt their associations. This was demonstrated in this thesis in Chapters 3 and 4 where the gentle touch and paracetamol interventions reduced the magnitude of noxious-evoked brain activity but not the reflex withdrawal and the behavioural scores respectively. Similarly, in Chapter 5 inflammation had an effect on brain activity and reflex responses but not on the behavioural pain scores. Other factors like the subjective versus objective nature of the assessments and the signal-to-noise ratios when measuring responses to single clinical procedures could also play a role. The use of automated tools to measure changes in facial activity like the use of electrodes to measure reflexes may help to overcome some of the current limitations [454]. Overall, this highlights the importance of adopting a comprehensive multimodal approach, considering that different indicators can provide additional information but could also be confounded by intrinsic and extrinsic elements.

To confirm the efficacy of both gentle touch and paracetamol, the conduction of randomised controlled trials in larger samples would be of great value. This is also the case for many commonly used analgesics where there is currently limited research, for example related to the use of topical anaesthetics, non-steroidal anti-inflammatory drugs and opioids. While there is a general agreement that an effective analgesic should ideally reduce the transmission of noxious input to the brain and the observed behavioural distress and physiological instability, there is no consensus on how to interpret the findings when multiple measures appear to show some disagreement [303]. For instance, two clinical trials assessing the efficacy of sucrose 15 years apart observed similar trends in the surrogate measures of pain (i.e. sucrose had an effect on pain-related behaviours but not on brain activity), but nevertheless reached opposing conclusions [247, 453]. Ultimately, the evaluation of the best approaches to measure pain should not be based on the popularity of the tools or

the feasibility for use in research studies but whether the measurements can produce reliable outcomes that can guide the treatment decisions and prevent long-term adverse consequences on neurodevelopment.

The ability to respond to painful stimuli varies greatly among early and late preterm neonates and full-term neonates. Additional considerations are needed for the assessment of pain responses in preterm infants as they may be exposed to longer periods of hospitalisation during a period of rapid neurodevelopment and high vulnerability to long-term consequences. Neonates with lower GA express less behavioural responses than more mature neonates [73, 183, 233, 355, 455]. By contrast, limb reflexes are exaggerated and disorganised in young infants and the magnitude, duration and latency reduce as GA increases [99]. The patterns of brain activity evoked by noxious stimulation also change greatly in latency and morphology across the developmental period with delta brushes commonly observed during early prematurity, followed by a transitory negative deflection in infants between 30-33 weeks PMA [233] which is replaced around 34 weeks PMA by the positive deflection characterised with the template used in this thesis [237]. The cumulative number of invasive procedures also influences the pain responses and in neonates with neurological conditions or receiving sedatives, pain may be weakly expressed, or not at all [244, 456]. Therefore, is important to assess PNA as well as PMA in studies with preterm infants to consider the differential influences of experience versus maturation.

Having discussed the value of multimodal pain assessment, the use of brain activity measured at one electrode site and quantified based on a pattern of activity detected in a small time frame is a reductionist approach. Nevertheless, while the template of noxious-evoked brain activity does not measure all nociceptive activity present in the infant brain, the activity at Cz has been shown to have the greatest and most reproducible amplitude compared to other electrodes sites across the brain [199, 237]. Moreover, EEG-derived patterns of noxious-evoked brain activity have

been observed in multiple national and international centres [235, 347, 357, 457, 458]. Researchers from independent groups have used the template developed by Hartley and collaborators and have shown consistent results [347, 459]. The generalisability and validity of this template are yet to be investigated with multicentre prospective studies. The replication of the findings obtained with this tool will corroborate the potential for its use as an objective outcome in analgesic clinical trials and will increase the confidence in the observations from previous studies. A validated brain-derived surrogate measure of pain would be of clinical significance especially for the younger and critically ill infants who display non-specific facial expressions.

Nonetheless, the interpretation of this measure of noxious-evoked brain activity in relation to a specific nociceptive process is not possible. Similarly, the measure of noxious-evoked baseline sensitivity characterised in Chapter 4 does not necessarily reflect specific nociceptive processing. In that context, other modalities, such as visual, auditory and tactile stimuli could be used to obtain a measure of baseline sensitivity, and this would account for some variance in the data. This is further supported by recent evidence that background resting-state brain activity is also predictive of an individual's responses to noxious stimuli [211]. However, the objective of the investigations presented in Chapter 4 was to develop a paradigm to optimise studies of analgesic efficacy. Therefore, the most appropriate stimulus to assess baseline sensitivity (which will likely account for the maximum variance in the data) is one with sensory modality matched to the subsequent clinically-required procedure. By recording baseline noxious-evoked activity before the painful procedure it is possible to (i) apply the experimental noxious stimuli to the same body site, (ii) record activity that is maximally evoked at the same electrode site and (iii) account for individual differences in somatosensory arousal. On the other hand, if the aim of the study was to isolate the nociceptive components of the observed activity, the use of appropriate control stimulus that can be matched in saliency and arousal to the noxious stimuli would have been necessary [124, 460].

Little is known about the brain regions from where the EEG-recorded noxious potential originates in infants and the pain-related functions it reflects. The pattern of noxious-evoked activity identified with the use of the EEG template can be tentatively interpreted based on evidence from ERPs previously characterised in adults. The noxious-evoked potential fit by the template is a late, positive potential, which is centrally distributed and maximal at Cz [237]. Relating the brain activity evoked by the pinprick stimulus known to activate A-delta fibres [218, 241] to adult A-delta laser evoked potentials [322], could be useful to draw some superficial similarities between the infant template potential and the adult noxious-evoked P2 potential, which is also a late positive potential centrally distributed and maximal at Cz [320–322]. This adult potential originates mainly from the mid-cingulate cortex [320, 322, 323], a brain region with pain-related nocifensive behavioural functions such as avoidance behaviour and body orientation to stimulus [323]. Source localisation studies and complex experimental designs are lacking in infant pain research but these would be of great value to reduce the gaps in our knowledge.

Finally, an important limitation of the work in this thesis is the focus on the assessment of acute nociceptive pain. Electrophysiological noxious evoked potentials characterised in this thesis are valuable in the context of some of the most commonly performed procedures such as heel lances, cannulations and injections. The brief acute nature of these stimuli is ideal to have a precise time-locking of the recordings and to accurately measure the immediate millisecond post-stimulus period. However, further study using neuroimaging techniques is needed to characterise changes in brain activity concerning other types of pain such as post-operative pain and pain following more complex procedures like retinopathy of prematurity screening, lumbar puncture, tracheal intubation and chest drain insertions. The analysis of electrophysiological data collected from longer periods with decomposition in the frequency domain and machine learning tools could provide insights for the assessment of prolonged procedural and non-procedural pain in infants. Furthermore, there is an increasing concern regarding the transition from acute to chronic pain

that may be primed by early life experiences. The roles of non-neural glial cells in the nervous system extend beyond homeostasis and there is mounting evidence of neuroimmune regulation involved in the maintenance of chronic pain conditions [394]. As demonstrated in Chapter 5 the exposure to an immune challenge can alter pain sensitivity in newborn infants. Further work in longitudinal datasets would provide unique information regarding the potential long-term effects and the interaction between the immune and nervous systems during development.

6.3 Concluding remarks

Currently, there is a paucity of evidence regarding the efficacy of pain-relieving interventions used in neonatal practice. Despite the limitations discussed in the previous section, noxious-evoked brain activity measured with EEG can be used as an objective surrogate measure of pain in neonates. This thesis describes the characterisation of brain activity responses during commonly performed acute procedures for the evaluation of pharmacological and non-pharmacological interventions. By measuring infant's baseline sensitivity, the sample sizes to assess analgesic efficacy can be reduced and analgesic clinical trial designs optimised in neonates. This thesis also demonstrated that inflammation increases the sensitivity to pain and this effect can be detected from the first days of life. The identification of robust and developmentally sensitive surrogate measures of pain should continue to be a priority so that better safe and effective analgesic interventions are available for this population.

Appendices

A

Information leaflet and forms

A.1 Patient Information Leaflet

Your child is, or may be, eligible to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it involves. Please read the following information carefully and ask us if anything is unclear or if you would like more information.

1. **Study title:** Investigating pain in the developing human brain

2. What is the purpose of the study?
Infants in hospital often need to have many procedures as part of their routine medical treatment, which may cause discomfort. As they cannot tell us how much these procedures hurt, it is difficult to know how much pain they are feeling and to make sure that they receive the right medicines. We know that infants can process discomfort and pain in their brains and we have developed a method of assessing this pain-related brain activity. We also know that infants show that they are in pain using different behaviours. These may be indicated by changes in heart rate and breathing rate in response to pain.

The aim of this research is to understand more about how infants experience pain, so that better ways of treating pain can be developed. We are also interested in how infants respond to different stimuli from their environment, such as light and sound, and how this might change across development.

3. Does my child have to take part?
No, it is your decision whether or not your child takes part. If you decide to allow your child to take part, you will be asked to sign a consent form. If you decide you do not want your child to take part, this will not affect your child's care.

If you decide you would like your child to take part, you can change your mind at any time and withdraw your child from the study by telling the research team. You do not have to give a reason. You will be asked if we can use the data/images that have already been collected for analysis (all data) and for publication of results (anonymised data only).

4. What is involved in the study?
In this study we would like to understand how infants respond to clinically-required procedures, such as blood tests. **No clinical procedures will be carried out solely for research purposes.** We will only study your child during a procedure that is needed for clinical purposes.

As we are interested in how infants respond to different stimuli in their environment, we may also ask to study your baby during a control procedure and in response to sharp touch. These do not pierce the skin, but they stimulate the receptors that we are interested in generally without waking or upsetting the infant.

We will assess your child's response by measuring their activity. We may also video your child's face, and measure other responses such as muscle activity, heart rate and oxygen saturations. We will monitor your child before, during and after the clinical procedure. On rare occasions we may ask to monitor your child for up to 24 hours before and/or after the clinical procedure.

Clinical procedures will be completed in the routine way. The study will not interfere with your child's clinical care, nor will there be any delay if an emergency procedure is required. We may also explore the impact of pain relief and comfort measures on your child and may ask you to complete a questionnaire following the study.

As we are interested in how your child's response to pain changes as they grow, we may ask if we can study your child more than once during their stay in hospital. We will also ask you if we can contact you in the future, to ask if you would be happy for your child to take part in other research studies. If you agree that we can contact you in the future about other research studies, we will also record your contact details. Your contact details will not be passed onto anyone outside of the research team. You can opt-out of this at any point by contacting Dr Rebeccah Slater (details below). Your agreement for us to contact you does not form any obligation to participate in future research.

We may use the following recording measures for your child:

Measuring brain activity

Electroencephalography (EEG)**:** EEG is a portable imaging system to measure brain activity. It involves gently placing electrodes (small metal discs) on the head using a paste that can be washed off with soap and water. EEG is routinely used on the neonatal unit, children's wards and clinics.

Near Infrared Spectroscopy (NIRS): NIRS is a non-invasive technique to measure brain activity. It involves placing lights and detectors on the head to record changes in blood and tissue oxygen levels.

Ultrasound: an ultrasound machine uses sound waves to create images of the brain. Ultrasound is routinely used to monitor babies' development during pregnancy and to assess brain development on the neonatal unit. In our research we also use a special type of ultrasound called functional ultrasound. This can measure which areas of the brain are active. An ultrasound scan involves placing an ultrasound probe on your child's head. To make contact, some gel will be applied between the head and the probe.

Measuring other responses

Electromyography (EMG): EMG is a safe non-invasive technique to record muscle activity. Small electrodes will be placed on the skin over the muscle to see if your child pulls away during the stimulation (and clinical procedure if relevant).

Vital sign monitoring: Small adhesive electrodes may be placed on your child's chest to measure changes in heart rate (this is called an ECG) and breathing rate. A small probe may also be wrapped around your child's foot to measure changes in blood oxygen levels.

Videoing your child: We may also video your child during the study. This is so that we can assess changes in facial expression and body movements, and to record the exact timing of the stimulation or clinical procedure.

We may also approach you to ask if you are happy for us to use these images for teaching, publicity and/or scientific journals. If you agree, we will take separate consent for this as your child's face would be visible in the video footage. This is not a mandatory part of the study. If you choose not to allow us to use the images in this way, this will not affect your child's care or prevent your child from participating in this research.

5. Are there any additional risks or benefits for my child?

Obtaining video footage of your child is non-invasive and does not present any risk to your child. EEG, EMG and ECG have been used clinically for over 20 years without any adverse effects. Ultrasound is a tool that is routinely used in clinical practice. All studies have a dedicated team of healthcare professionals and researchers that will ensure the safety of your child at all times. We are not aware of any risks for your child taking part in this study.

The data collected are for research, so will not be reviewed by a doctor routinely. If any clinically significant findings are identified at the time of the study then the research team will report these to the clinical care team to handle as appropriate.

There are no direct benefits of participating in this research. This study is designed to gather information, to help guide improvements in care for infants in the future. If your child becomes distressed, the research study will be paused or stopped. Any clinically required procedures will still go ahead if the treating clinician feels that this is appropriate.

6. What information will be collected about my child?
We will collect clinical information about your child, for example their gestational age at birth and any medications they have received. This information helps us to determine which factors may influence the way an infant copes with pain. We will also collect information about your child's brain and it's activity, and may collect information about your child's muscle activity, vital signs (such as heart rate and breathing rate), and recordings of their facial expressions and body movements.

All information and videos that are collected during this research study will be kept strictly confidential. Each infant will be allocated a study number which will be used to label all data. This study has been registered with the data protection registration office and forms part of an educational programme.

7. What will happen to the results?
Results will be analysed and published in a journal. All publications will be made available on our website **https://neuroimaging.paediatrics.ox.ac.uk**. The findings may also be used for teaching or academic research presentations. No identifying information will be presented about you or your child, unless you have provided specific consent for us to use videos/images of your child in this way.

8. What will happen to my child's data?
We will be using information collected from your child and their medical records in order to conduct this study. Research is a task that we perform in the public interest. The University of Oxford, as Sponsor, is the data controller. This means that we, as University of Oxford researchers, are responsible for looking after the information collected and using it properly. We will use the minimum personally-identifiable information possible. We will keep identifiable information about your child for up to 5 years after the study has finished. This excludes any research documents with personal information, such as consent forms, which will be held securely at the University of Oxford for 25 years after the end of the study.

Data protection regulation provides you with control over your personal data and how it is used. When you agree to your information being used in research, however, some of those rights may be limited in order for the research to be reliable and accurate. Further information about your rights with respect to your personal data is available at
http://www.admin.ox.ac.uk/councilsec/compliance/gdpr/individualrights/

You can find out more about how we use your information from the contacts in section 12.

Research data may be shared with other researchers doing similar work, both here and abroad. Responsible members of the University of Oxford or the Oxford University Hospitals NHS Trust may be given access to data for monitoring and/or audit of the study to ensure we are complying with regulations.

9. Who is organising and funding this research?
This study is sponsored by University of Oxford and has been funded by The Wellcome Trust. Your doctor will not be paid for including you in this study.

10. Who has reviewed the study?

All research that involves NHS patients has to be approved by a Research Ethics Committee. Approval means that the Committee is satisfied that yours and your child's rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision about whether to take part. The South Central Oxford C Research Ethics Committee has reviewed and approved this study.

11. Comments or concerns during the study

The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor. NHS indemnity operates in respect of the clinical treatment with which your child is provided. If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Prof Rebeccah Slater (details below) or the University of Oxford Clinical Trials and Research Governance (CTRG) office (tel: 01865 (6)16480, email: ctrg@admin.ox.ac.uk).

12. Contact for further information

Prof Rebeccah Slater (Study Lead) *Professor of Paediatric Neuroscience* *University of Oxford*	01865 234537 rebeccah.slater@paediatrics.ox.ac.uk
Dr Eleri Adams (Clinical Lead) *Consultant Neonatologist* *Oxford University Hospitals NHS Trust*	01865 221356 eleri.adams@ouh.nhs.uk

Picture shows example of an EEG study.

Thank you for reading this information leaflet.

A.2 Parent Consent Form

Consent Form

Study ID: | | | | | | | | |

Infant's name:

Study Title: Investigating pain in the developing human brain

Chief Investigator: Prof Rebeccah Slater (01865 234537, rebeccah.slater@paediatrics.ox.ac.uk)

Please initial each box

Please complete in black ballpoint pen.

1	I confirm that I have read and understood the information sheet (clinical procedures) (**v9.0, dated 31/05/2019**), for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily.	☐
2	I understand that my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	☐
3	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by individuals from the University of Oxford or Oxford University Hospitals NHS Trust, where it is relevant to my child's taking part in this research. I give permission for these individuals to access to my child's records.	☐
4	I agree to my child being videoed during the study. I understand that recorded images will not be used for public use, only analysis. No identifiable information, including video recordings or imaging, will be used in any publications/presentations. Only anonymised data will be published or presented at meetings.	☐
5	I agree for the collected data to be used for teaching or academic research presentations.	☐
6	I agree to my child taking part in the above study.	☐
	OPTIONAL	
7	I agree to my child being studied on more than one occasion, up to a maximum of 5 occasions.	☐
8	I consent to being approached in the future about other research studies that my child may be eligible for.	☐
9	I agree to the images/videos of my child recorded during this study being used for publications and presentations.	☐

Name of parent:

Relationship to baby:

Signature:

Date:

Name of investigator taking consent:

Signature:

Date:

1 to be kept as part of the study documentation (original)
1 form for parent
1 to be kept with hospital notes

A.3 PIPP-R form

PIPP-R SCORING FORM

Section A: Video Number

☐☐☐☐

Section B: Participant Details

(To complete once video is un-blinded using Excel Spreadsheet)

B1: Study number:

☐☐☐☐☐☐☐☐

B2: Stimulus type:

Control Heel Lance ☐
Heel Lance ☐
Lumbar Puncture ☐
ROP Screen ☐
Cannulation ☐
Pin Pricks ☐
Immunisations ☐
Other *(please specify)* _____

Section C: Additional Info

C1: Latency to facial expression:

☐☐ secs

C2: Is infant sucking dummy/finger?

No ☐
Yes ☐

Section D: PIPP-R Scoring Table

Indicator		0	1	2	3
Gestational Age	CGA at study:	>= 36 weeks'	32 weeks' to 35+6 weeks'	28 weeks to 31+6 weeks	< 28 weeks
Behavioural State	Facial Features* present in baseline? No / Yes	Active awake: eyes open, facial movements	Quiet awake: eyes open, no facial movements	Active sleep: eyes closed, facial movements	Quiet sleep: eyes closed, no facial movements
Maximum Heart Rate	Baseline: Maximum:	0 to 4 beats per minute increase	5 to 14 beats per minute increase	15 to 24 beats per minute increase	>=25 beats per minute increase
Minimum Oxygen Saturations	Baseline: Minimum:	0% to 2% decrease	3% to 5% decrease	6% to 8% decrease	>8% decrease
Brow Bulge	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Eye Squeeze	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds
Nasolabial Furrow	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: >20 seconds

* Brow bulge, eye squeeze or nasolabial furrow

Section E: Total PIPP-R Score

Section F: Scorer Name

REC Ref: 12/SC/0447
v1.2 25-11-2020

References

1. Andersson, G *et al.* Do No Harm - The Balance of "Beneficence" and "Non-Maleficence". *Spine* **35,** S2–S8 (2010).
2. Unruh, A. M. Voices from the Past: Ancient Views of Pain in Childhood. *The Clinical Journal of Pain* **8,** 247–254 (1992).
3. Chadwick, J & Mann, W. *The medical works of Hippocrates: a new translation from the original Greek made especially for English readers.* 150–160 (Blackwell Scientific Publications, Oxford, 1950).
4. Ruhrah, J. *Pediatrics of the past* (ed Hoeber, P. B.) 196–220 (New York, 1925).
5. Philip, A. G. The evolution of neonatology. *Pediatric Research* **58,** 799–815 (2005).
6. Rutter, N. & Doyal, L. Neonatal care and management of pain: Historical and ethical issues. *Seminars in Neonatology* **3,** 297–302 (1998).
7. Schechter, N. L. The undertreatment of pain in children: An overview. *Pediatric Clinics of North America* **36** (1989).
8. Brown, T. & Fisk, G. *Anaesthesia for Children* 1st edn. (Blackwell Scientific Publications, Oxford, 1979).
9. Unruh, A. M. & J., M. P. in *Oxford Textbook of Pediatric Pain* (eds Stevens, B. J., Hathway, G. & Zempsky, W. T.) 2 ed, 1–20 (Oxford University Press, 2021).
10. Steward, D. J. Preterm infants are more prone to complications following minor surgery than are term infants. *Anesthesiology* **56,** 304–306 (1982).
11. Owens, M. E. & Todt, E. H. Pain in infancy: Neonatal reaction to a heel lance. *Pain* **20,** 77–86 (1984).
12. McGraw, M. Neural Maturation as Exemplified in the Changing Reactions of the Infant to Pin Prick Author (s): Myrtle B . McGraw Reviewed work (s): Published by : Wiley on behalf of the Society for Research in Child Development Stable URL : http://www.jstor.org/st. **12,** 31–42 (1941).
13. Dubois, J. *et al.* The early development of brain white matter: A review of imaging studies in fetuses, newborns and infants. *Neuroscience* **276,** 48–71 (2014).
14. Purcell-Jones, G., Dormon, F. & Sumner, E. Paediatric anaesthetists' perceptions of neonatal and infant pain. *Pain* **33,** 181–187 (1988).

15. Robinson, S. & Gregory, G. A. Fentanyl-air-oxygen anesthesia for ligation of patent ductus arteriosus in preterm infants. *Anesthesia and Analgesia* **60,** 331–334 (1981).

16. Collins, C. *et al.* Fentanyl pharmacokinetics and hemodynamic effects in preterm infants during ligation of patent ductus arteriosus. *Anesthesia and Analgesia* **64,** 1078–1080 (1985).

17. Friesen, R. H. & Henry, D. B. Cardiovascular changes in preterm neonates receiving isoflurane, halothane, fentanyl and ketamine. **64,** 238–242 (1986).

18. Anand, K. J. S. Randomised trial of fentanyl anaesthesia in preterm babies undergoing surgery: effects on the stress response. *The Lancet* **1,** 62–66 (1987).

19. Anand, K. J. S. & Hickey, P. Pain and its effects in the human neonate and fetus. *The New England Journal of Medicine* **317,** 1321–1329 (1987).

20. Anand, K. J. S. *et al.* Can the human neonate mount an endocrine and metabolic response to surgery? *Journal of Paediatric Surgery* **20,** 41–48 (1985).

21. Lawson, J. T. Letters. *Birth* **13,** 124–125 (1986).

22. McGrath, P. J. Science is not enough: The modern history of pediatric pain. *Pain* **152,** 2457–2459 (2011).

23. Poland, R. L., Roberts, R. J., Gutierrez-Mazorra, J. F. & Fonkalsrud, E. W. Neonatal anesthesia. *Pediatrics* **80,** 446 (1987).

24. Blencowe, H. *et al.* National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: A systematic analysis and implications. *The Lancet* **379,** 2162–2172 (2012).

25. Pierrat, V. *et al.* Neurodevelopmental outcomes at age 5 among children born preterm: EPIPAGE-2 cohort study. *The BMJ* **373** (2021).

26. Barker, D. & Rutter, N. Exposure to invasive procedures in neonatal intensive care unit admissions. *Archives of Disease in Childhood* **72,** 47–48 (1995).

27. Johnston, C. C., Collinge, J. M., Henderson, S. J. & Anand, K. J. A cross-sectional survey of pain and pharmacological analgesia in canadian neonatal intensive care units. *Clinical Journal of Pain* **13,** 308–312 (1997).

28. Simons, S. H. P. *et al.* Do We Still Hurt Newborn Babies? *Archives of Pediatrics & Adolescent Medicine* **157,** 1058 (2003).

29. Carbajal, R. *et al.* Epidemiology and Treatment of Painful Procedures in Neonates in Intensive Care Units. *JAMA* **300,** 60–70 (2008).

30. Johnston, C., Barrington, K. J., Taddio, A., Carbajal, R. & Filion, F. Pain in Canadian NICUs: Have we improved over the past 12 years? *Clinical Journal of Pain* **27,** 225–232 (2011).

31. Kyololo, O. M., Stevens, B., Gastaldo, D. & Gisore, P. Procedural pain in neonatal units in Kenya. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **99,** F464–F467 (2014).

32. Britto, C. D. *et al.* Pain-perception and assessment of painful procedures in the NICU. *Journal of Tropical Pediatrics* **60,** 422–427 (2014).

33. Chen, M. *et al.* A prospective study of pain experience in a neonatal intensive care unit of China. *Clinical Journal of Pain* **28,** 700–704 (2012).

34. Roofthooft, D. W., Simons, S. H., Anand, K. J., Tibboel, D. & Van Dijk, M. Eight years later, are we still hurting newborn infants? *Neonatology* **105,** 218–226 (2014).

35. Cruz, M. D., Fernandes, A. M. & Oliveira, C. R. Epidemiology of painful procedures performed in neonates: A systematic review of observational studies. *European Journal of Pain (United Kingdom)* **20,** 489–498 (2016).

36. Stevens, B. *et al.* Influence of risk of neurological impairment and procedure invasiveness on health professionals' management of procedural pain in neonates. *European Journal of Pain* **14,** 735–741 (2010).

37. Taylor, B. J. *et al.* Assessing postoperative pain in neonates: A multicenter observational study. *Pediatrics* **118** (2006).

38. Anand, K. J. S. Consensus Statement for the Prevention and Management of Pain in the Newborn. *Archives of Pediatrics & Adolescent Medicine* **155,** 173–180 (2001).

39. McNair, C., Yeo, M. C., Johnston, C. & Taddio, A. Nonpharmacologic Management of Pain During Common Needle Puncture Procedures in Infants: Current Research Evidence and Practical Considerations: An Update. *Clinics in Perinatology* **46,** 709–730 (2019).

40. Craig, K. D., Whitfield, M. F., Grunau, R. V., Linton, J. & Hadjistavropoulos, H. D. Pain in the preterm neonate: behavioural and physiological indices. *Pain* **52,** 287–299 (1993).

41. Morison, S. J., Grunau, R. E., Oberlander, T. F. & Whitfield, M. F. Relations between behavioral and cardiac autonomic reactivity to acute pain in preterm neonates. *Clinical Journal of Pain* **17,** 350–358 (2001).

42. Mitchell, A. J., Green, A., Jeffs, D. A. & Robertson, P. K. Physiologic Effects of Retinopathy of Prematurity Screening Examinations. *Adv Neonatal Care* **11,** 291–297 (2011).

43. Walker, S. M. Long-term effects of neonatal pain. *Seminars in Fetal and Neonatal Medicine* **24,** 101005 (2019).

44. Schwaller, F. & Fitzgerald, M. The consequences of pain in early life: Injury-induced plasticity in developing pain pathways. *European Journal of Neuroscience* **39,** 344–352 (2014).

45. Williams, M. D. & Lascelles, B. D. X. Early Neonatal Pain—A Review of Clinical and Experimental Implications on Painful Conditions Later in Life. *Frontiers in Pediatrics* **8** (2020).

46. Grunau, R. E. *et al.* Neonatal pain, parenting stress and interaction, in relation to cognitive and motor development at 8 and 18 months in preterm infants. *Pain* **143,** 138–146 (2009).

47. Chau, C. M. *et al.* Hippocampus, amygdala, and thalamus volumes in very preterm children at 8 years: neonatal pain and genetic variation. *Frontiers in Behavioral Neuroscience* **13** (2019).

48. Zwicker, J. G. *et al.* Score for neonatal acute physiology-II and neonatal pain predict Corticospinal tract development in premature newborns. *Pediatric Neurology* **48,** 123–129.e1 (2013).

49. Ranger, M. *et al.* Neonatal Pain-Related Stress Predicts Cortical Thickness at Age 7 Years in Children Born Very Preterm. *PLoS ONE* **8** (2013).

50. Vinall, J. *et al.* Invasive procedures in preterm children: brain and cognitive development at school age. *Pediatrics* **133,** 412–421 (2014).

51. Doesburg, S. M. *et al.* Neonatal pain-related stress, functional cortical activity and visual-perceptual abilities in school-age children born at extremely low gestational age. *Pain* **154,** 1946–1952 (2013).

52. Abernethy, L. J., Cooke, R. W. & Foulder-Hughes, L. Caudate and Hippocampal Volumes, Intelligence, and Motor Impairment in 7-Year-Old Children Who Were Born Preterm. *Pediatric Research* **55,** 884–893 (2004).

53. Ranger, M. *et al.* Neonatal Pain and Infection Relate to Smaller Cerebellum in Very Preterm Children at School Age. *Journal of Pediatrics* **167,** 292–298.e1 (2015).

54. Olsen, A. *et al.* Preterm birth leads to hyper-reactive cognitive control processing and poor white matter organization in adulthood. *NeuroImage* **167,** 419–428 (2018).

55. Walker, S. M. *et al.* Somatosensory function and pain in extremely preterm young adults from the UK EPICure cohort: sex-dependent differences and impact of neonatal surgery. *British Journal of Anaesthesia* **121,** 623–635 (2018).

56. Ranger, M. *et al.* Adverse behavioral changes in adult mice following neonatal repeated exposure to pain and sucrose. *Frontiers in Psychology* **9,** 1–14 (2019).

57. Henderson, Y. O., Victoria, N. C., Inoue, K., Murphy, A. Z. & Parent, M. B. Early life inflammatory pain induces long-lasting deficits in hippocampal-dependent spatial memory in male and female rats. *Neurobiology of Learning and Memory* **118,** 30–41 (2015).

58. Grunau, R. E., Holsti, L. & Peters, J. W. Long-term consequences of pain in human neonates. *Seminars in Fetal and Neonatal Medicine* **11,** 268–275 (2006).

59. Ranger, M. & Grunau, R. E. Early repetitive pain in preterm infants in relation to the developing brain. *Pain Manag.* **4,** 57–67 (2014).

60. Duerden, E. G. *et al.* Early procedural pain is associated with regionally-specific alterations in thalamic development in preterm neonates. *Journal of Neuroscience* **38,** 878–886 (2018).

61. Hohmeister, J. *et al.* Cerebral processing of pain in school-aged children with neonatal nociceptive input: An exploratory fMRI study. *Pain* **150,** 257–267 (2010).

62. Slater, R. *et al.* Premature infants display increased noxious-evoked neuronal activity in the brain compared to healthy age-matched term-born infants. *NeuroImage* **52,** 583–589 (2010).

63. Schmelzle-Lubiecki, B. M., Campbell, K. A., Howard, R. H., Franck, L. & Fitzgerald, M. Long-term consequences of early infant injury and trauma upon somatosensory processing. *European Journal of Pain* **11,** 799–809 (2007).

64. Hermann, C., Hohmeister, J., Demirakça, S., Zohsel, K. & Flor, H. Long-term alteration of pain sensitivity in school-aged children with early pain experiences. *Pain* **125,** 278–285 (2006).

65. Valeri, B. O. *et al.* Neonatal Invasive Procedures Predict Pain Intensity at School Age in Children Born Very Preterm. *Clinical Journal of Pain* **32,** 1086–1093 (2016).

66. Buskila, D. *et al.* Pain Sensitivity in Prematurely Born Adolescents. *Archives of Pediatrics and Adolescent Medicine* **157,** 1079–1082 (2003).

67. Hohmeister, J., Demirakça, S., Zohsel, K., Flor, H. & Hermann, C. Responses to pain in school-aged children with experience in a neonatal intensive care unit: Cognitive aspects and maternal influences. *European Journal of Pain* **13,** 94–101 (2009).

68. Anand, K. J., Coskun, V., Thrivikraman, K. V., Nemeroff, C. B. & Plotsky, P. M. Long-term behavioral effects of repetitive pain in neonatal rat pups. *Physiology and Behavior* **66,** 627–637 (1999).

69. Butkevich, I. P., Mikhailenko, V. A., Vershinina, E. A. & Aloisi, A. M. Effects of neonatal pain, stress and their interrelation on pain sensitivity in later life in male Rats. *Chinese Journal of Physiology* **59,** 225–231 (2016).

70. Van Den Hoogen, N. J. *et al.* Repeated touch and needle-prick stimulation in the neonatal period increases the baseline mechanical sensitivity and postinjury hypersensitivity of adult spinal sensory neurons. *Pain* **159,** 1166–1175 (2018).

71. Walker, S. M., Beggs, S. & Baccei, M. L. Persistent changes in peripheral and spinal nociceptive processing after early tissue injury. *Experimental Neurology* **275,** 253–260 (2016).

72. Low, L. A. & Fitzgerald, M. Acute pain and a motivational pathway in adult rats: Influence of early life pain experience. *PLoS ONE* **7** (2012).

73. Grunau, R. E., Oberlander, T. F., Whitfield, M. F., Fitzgerald, C. & Lee, S. K. Demographic and Therapeutic Determinants of Pain Reactivity in Very Low Birth Weight Neonates at 32 Weeks' Postconceptional Age. *Pediatrics* **107,** 105–112 (2001).

74. Donia, A. E.-S. & Tolba, O. A. Effect of early procedural pain experience on subsequent pain responses among premature infants. *Egyptian Pediatric Association Gazette* **64,** 74–80 (2016).

75. Taddio, A., Katz, J., Ilersich, A. L. & Koren, G. Effect of neonatal circumcision on pain response during subsequent routine vaccination. *Lancet* **349,** 599–603 (1997).

76. Taddio, A., Shah, V., Gilbert-MacLeod, C. & Katz, J. Conditioning and hyperalgesia in newborns exposed to repeated heel lances. *Journal of the American Medical Association* **288,** 857–861 (2002).

77. Cervero, F. & Wood, J. N. A history of pain research. *The Oxford Handbook of the Neurobiology of Pain,* 1–27 (2020).

78. IASP. IASP Terminology. *https://www.iasp-pain.org/resources/terminology/#pain,* Accessed: 15/04/2021 (2020).

79. Moayedi, M. & Davis, K. D. Theories of pain: From specificity to gate control. *Journal of Neurophysiology* **109,** 5–12 (2013).

80. Eccleston, C. *et al.* Delivering transformative action in paediatric pain: a Lancet Child & Adolescent Health Commission. *The Lancet Child and Adolescent Health* **5,** 47–87 (2021).

81. Tracey, W. D. Nociception. *Current Biology* **27,** R129–R133 (2017).

82. Sandrini, G. *et al.* The lower limb flexion reflex in humans. *Progress in Neurobiology* **77,** 353–395 (2005).

83. Price, D. D. Central neural mechanisms that interrelate sensory and affective dimensions of pain. *Molecular interventions* **2,** 392–402 (2002).

84. Chambers, C. T. & Mogil, J. S. Ontogeny and phylogeny of facial expression of pain. *Pain* **156,** 798–799 (2015).

85. Tracey, I. & Mantyh, P. W. The Cerebral Signature for Pain Perception and Its Modulation. *Neuron* **55,** 377–391 (2007).

86. Apkarian, A. V., Bushnell, M. C., Treede, R. D. & Zubieta, J. K. Human brain mechanisms of pain perception and regulation in health and disease. *European Journal of Pain* **9,** 463 (2005).

87. Woolf, C. J. & Salter, M. W. Neuronal plasticity: Increasing the gain in pain. *Science* **288,** 1765–1768 (2000).

88. Raja, S. N., Meyer, R. A. & Campbell, J. N. Peripheral Mechanisms of Somatic Pain. *Anesthesiology* **68,** 571–590 (1988).

89. Julius, D. & Basbaum, A. I. Molecular mechanisms of nociception. *Nature* **413,** 203–210 (2001).

90. Ionescu, C. M., Copot, D. & Muresan, C. A multi-scale model of nociception pathways and pain mechanisms. *Applications in Engineering, Life and Social Sciences, Part A,* 55–68 (2019).

91. Basbaum, A. I., Bautista, D. M., Scherrer, G. & Julius, D. Cellular and Molecular Mechanisms of Pain. *Cell* **139,** 267–284 (2009).

92. Basbaum, A. I. & Woolf, C. J. Pain. *Current Biology* **9,** R429–31 (1999).

93. Reynolds, M. L., Fitzgerald, M. & Benowitz, L. I. GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb. *Neuroscience* **41,** 201–211 (1991).

94. Jackman, A. & Fitzgerald, M. Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. *Journal of Comparative Neurology* **418,** 281–298 (2000).

95. Fitzgerald, M. The development of nociceptive circuits. *Nature Reviews Neuroscience* **6,** 507–520 (2005).

96. Koltzenburg, M., Stucky, C. L. & Lewin, G. R. Receptive properties of mouse sensory neurons innervating hairy skin. *Journal of Neurophysiology* **78,** 1841–1850 (1997).

97. Andrews, K. & Fitzgerald, M. The cutaneous withdrawal reflex in human neonates: sensitization, receptive fields, and the effects of contralateral stimulation. *Pain* **56,** 95–101 (1994).

98. Fitzgerald, M. The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. *Journal of Physiology* **364,** 1–18 (1985).

99. Hartley, C. *et al.* Changing balance of spinal cord excitability and nociceptive brain activity in early human development. *Current Biology* **26,** 1998–2002 (2016).

100. Todd, A. J. Neuronal circuitry for pain processing in the dorsal horn. *Nature Reviews Neuroscience* **11,** 823–836 (2010).

101. Beggs, S., Torsney, C., Drew, L. J. & Fitzgerald, M. The postnatal reorganization of primary afferent input and dorsal horn cell receptive fields in the rat spinal cord is an activity-dependent process. *European Journal of Neuroscience* **16,** 1249–1258 (2002).

102. Woodbury, C. J. & Koerber, H. R. Widespread projections from myelinated nociceptors throughout the substantia gelatinosa provide novel insights into neonatal hypersensitivity. *Journal of Neuroscience* **23,** 601–610 (2003).

103. Fitzgerald, M. Spontaneous and evoked activity of fetal primary afferents in vivo. *Nature* **326,** 603–605 (1987).

104. Davidson, S., Truong, H. & Giesler, G. J. Quantitative analysis of spinothalamic tract neurons in adult and developing mouse. *Journal of Comparative Neurology* **518,** 3193–3204 (2010).

105. Craig, A. D. A new view of pain as a homeostatic emotion. *Trends in Neurosciences* **26,** 303–307 (2003).

106. Gauriau, C. & Bernard, J. F. A Comparative Reappraisal of Projections from the Superficial Laminae of the Dorsal Horn in the Rat: The Forebrain. *Journal of Comparative Neurology* **468,** 24–56 (2004).

107. Barr, G. A. Formalin-induced c-fos expression in the brain of infant rats. *Journal of Pain* **12,** 263–271 (2011).

108. Kostović, I. & Judaš, M. The development of the subplate and thalamocortical connections in the human foetal brain. *Acta Paediatrica, International Journal of Paediatrics* **99,** 1119–1127 (2010).

109. Ghosh, A. & Shatz, C. J. A role for subplate neurons in the patterning of connections from thalamus to neocortex. *Development* **117,** 1031–1047 (1993).

110. Krsnik, Ž., Majić, V., Vasung, L., Huang, H. & Kostović, I. Growth of thalamocortical fibers to the somatosensory cortex in the human fetal brain. *Frontiers in Neuroscience* **11** (2017).

111. Keunen, K., Counsell, S. J. & Benders, M. J. The emergence of functional architecture during early brain development. *NeuroImage* **160,** 2–14 (2017).

112. Vanhatalo, S. & Lauronen, L. Neonatal SEP - Back to bedside with basic science. *Seminars in Fetal and Neonatal Medicine* **11,** 464–470 (2006).

113. Vanhatalo, S. & Kaila, K. Development of neonatal EEG activity: From phenomenology to physiology. *Seminars in Fetal and Neonatal Medicine* **11,** 471–478 (2006).

114. Dubois, J. *et al.* Mapping the early cortical folding process in the preterm newborn brain. *Cerebral Cortex* **18,** 1444–1454 (2008).

115. Kostović, I. & Judaš, M. Transient patterns of cortical lamination during prenatal life: Do they have implications for treatment? *Neuroscience and Biobehavioral Reviews* **31,** 1157–1168 (2007).

116. Hanganu, I. L., Okabe, A., Lessmann, V. & Luhmann, H. J. Cellular mechanisms of subplate-driven and cholinergic input-dependent network activity in the neonatal rat somatosensory cortex. *Cerebral Cortex* **19,** 89–105 (2009).

117. Kostović, I. & Jovanov-Milošević, N. The development of cerebral connections during the first 20-45 weeks' gestation. *Seminars in Fetal and Neonatal Medicine* **11,** 415–422 (2006).

118. Stephenson, D. T. & Arneric, S. P. Neuroimaging of Pain: Advances and Future Prospects. *Journal of Pain* **9,** 567–579 (2008).

119. Vierck, C. J., Whitsel, B. L., Favorov, O. V., Brown, A. W. & Tommerdahl, M. Role of primary somatosensory cortex in the coding of pain. *Pain* **154,** 334–344 (2013).

120. Bushnell, M. C., Čeko, M. & Low, L. A. Cognitive and emotional control of pain and its disruption in chronic pain. *Nature Reviews Neuroscience* **14,** 502–511 (2013).

121. Gordon, E. M., Laumann, T. O., Adeyemo, B. & Petersen, S. E. Individual Variability of the System-Level Organization of the Human Brain. *Cerebral Cortex* **27,** 386–399 (2017).

122. Iannetti, G. D. & Mouraux, A. From the neuromatrix to the pain matrix (and back). *Experimental Brain Research* **205,** 1–12 (2010).

123. Mouraux, A., Diukova, A., Lee, M. C., Wise, R. G. & Iannetti, G. D. A multisensory investigation of the functional significance of the "pain matrix". *NeuroImage* **54,** 2237–2249 (2011).

124. Mouraux, A. & Iannetti, G. D. The search for pain biomarkers in the human brain. *Brain* (2018).

125. Ploner, M., Sorg, C. & Gross, J. Brain Rhythms of Pain. *Trends in Cognitive Sciences* **21,** 100–110 (2017).

126. Kucyi, A. & Davis, K. D. The dynamic pain connectome. *Trends in Neurosciences* **38,** 86–95 (2015).

127. Gursul, D., Hartley, C. & Slater, R. Nociception and the neonatal brain. *Seminars in Fetal and Neonatal Medicine* **24,** 101016 (2019).

128. Goksan, S. *et al.* fMRI reveals neural activity overlap between adult and infant pain. *eLife* **4,** e06356 (2015).

129. Goksan, S. *et al.* The influence of the descending pain modulatory system on infant pain-related brain activity. *eLife* **7,** 1–16 (2018).

130. Ossipov, M. H., Dussor, G. O. & Porreca, F. Review series Central modulation of pain. *The Journal of Clinical Investigation* **120,** 3779–3787 (2010).

131. Vanegas, H. & Schaible, H. G. Descending control of persistent pain: Inhibitory or facilitatory? *Brain Research Reviews* **46,** 295–309 (2004).

132. Beecher, H. K. Pain in men wounded in battle. *Annals of Surgery* **123,** 96–105 (1946).

133. Mayer, D. J. & Price, D. Central nervous system mechanisms of analgesia. *Pain* **2,** 379–404 (1976).

134. Reynolds, D. V. Surgery in the Rat during Electrical Analgesia Induced by Focal Brain Stimulation Sex Differences in Verbal and Performance IQ's of Children Undergoing Open-Heart Surgery. *Science* **164,** 444–445 (1969).

135. Tracey, I. Getting the pain you expect: Mechanisms of placebo, nocebo and reappraisal effects in humans. *Nature Medicine* **16,** 1277–1283 (2010).

136. Heinricher, M. M., Tavares, I., Leith, J. L. & Lumb, B. M. Descending control of nociception: Specificity, recruitment and plasticity. *Brain Research Reviews* **60,** 214–225 (2009).

137. Fields, H. L., Bry, J., Hentall, I. & Zorman, G. The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *The Journal of Neuroscience* **3,** 2545–2552 (1983).

138. Arvidsson, U. *et al.* Distribution and targeting of a μ-opioid receptor (MOR1) in brain and spinal cord. *The Journal of Neuroscience* **15,** 3328–3341 (1995).

139. Mansour, A., Fox, C. A., Akil, H. & Watson, S. J. Opioid-receptor mRNA expression in the rat CNS : anatomical and functional implications. *Trends in Neurosciences* **18,** 22–29 (1995).

140. Hathway, G. J., Koch, S., Low, L. & Fitzgerald, M. The changing balance of brainstem-spinal cord modulation of pain processing over the first weeks of rat postnatal life. *Journal of Physiology* **587,** 2927–2935 (2009).

141. Fitzgerald, M. & Koltzenburg, M. The functional development of descending inhibitory pathways in the dorsolateral funiculus of the newborn rat spinal cord. *Developmental Brain Research* **24,** 261–270 (1986).

142. Schwaller, F., Kwok, C. & Fitzgerald, M. Postnatal maturation of the spinal-bulbo-spinal loop: Brainstem control of spinal nociception is independent of sensory input in neonatal rats. *Pain* **157,** 677–686 (2016).

143. Hathway, G. *et al.* A postnatal switch in GABAergic control of spinal cutaneous reflexes. *European Journal of Neuroscience* **23,** 112–118 (2006).

144. Hathway, G. J., Vega-Avelaira, D. & Fitzgerald, M. A critical period in the supraspinal control of pain: Opioid-dependent changes in brainstem rostroventral medulla function in preadolescence. *Pain* **153,** 775–783 (2012).

145. Lu, Y., Sweitzer, S. M., Laurito, C. E. & Yeomans, D. C. Differential Opioid Inhibition of C- and Aδ- Fiber Mediated Thermonociception after Stimulation of the Nucleus Raphe Magnus. *Anesthesia and Analgesia* **98,** 414–419 (2004).

146. Koch, S. C. & Fitzgerald, M. The selectivity of rostroventral medulla descending control of spinal sensory inputs shifts postnatally from A fibre to C fibre evoked activity. *Journal of Physiology* **592,** 1535–1544 (2014).

147. Reardon, C., Murray, K. & Lomax, A. E. Neuroimmune communication in health and disease. *Physiological Reviews* **98,** 2287–2316 (2018).

148. Erickson, M. A., Dohi, K. & Banks, W. A. Neuroinflammation: A common pathway in CNS diseases as mediated at the blood-brain barrier. *NeuroImmunoModulation* **19,** 121–130 (2012).

149. Shatz, C. J. MHC Class I: An Unexpected Role in Neuronal Plasticity. *Neuron* **64,** 40–45 (2009).

150. Bhat, R. *et al.* Inhibitory role for GABA in autoimmune inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 2580–2585 (2010).

151. Borovikova, L. V. *et al.* Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* **405,** 458–462 (2000).

152. Stevens, B. *et al.* The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131,** 1164–1178 (2007).

153. Tenner, A. J., Stevens, B. & Woodruff, T. M. New tricks for an ancient system: Physiological and pathological roles of complement in the CNS. *Molecular Immunology* **102,** 3–13 (2018).

154. Schafer, D. P. *et al.* Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74,** 691–705 (2012).

155. Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330,** 841–845 (2010).

156. Salter, M. W. & Beggs, S. Sublime microglia: Expanding roles for the guardians of the CNS. *Cell* **158,** 15–24 (2014).

157. Salter, M. W. & Stevens, B. Microglia emerge as central players in brain disease. *Nature Medicine* **23,** 1018–1027 (2017).

158. Benson, S. *et al.* Neural circuitry mediating inflammation-induced central pain amplification in human experimental endotoxemia. *Brain, Behavior, and Immunity* **48,** 222–231 (2015).

159. Wegner, A. *et al.* Inflammation-induced hyperalgesia: Effects of timing, dosage, and negative affect on somatic pain sensitivity in human experimental endotoxemia. *Brain, Behavior, and Immunity* **41,** 46–54 (2014).

160. Chiu, I. M. *et al.* Bacteria activate sensory neurons that modulate pain and inflammation. *Nature* **501,** 52–57 (2013).

161. Ren, K. & Ronald Dubner. Interactions between the immune and nervous systems in pain. *Nature Medicine* **16,** 1267–1276 (2010).

162. Yoon, S. Y., Patel, D. & Dougherty, P. M. Minocycline blocks lipopolysaccharide induced hyperalgesia by suppression of microglia but not astrocytes. *Neuroscience* **221,** 214–224 (2012).

163. Zouikr, I. *et al.* Programming of formalin-induced nociception by neonatal LPS exposure: Maintenance by peripheral and central neuroimmune activity. *Brain, Behavior, and Immunity* **44,** 235–246 (2015).

164. Manworren, R. C. & Stinson, J. Pediatric Pain Measurement, Assessment, and Evaluation. *Seminars in Pediatric Neurology* **23,** 189–200 (2016).

165. FDA-NIH Biomarker Working Group. BEST (Biomarkers , EndpointS , and other Tools) Resource. *Silver Spring* **MD** (2021).

166. Grunau, R. V. & Craig, K. D. Pain expression in neonates: facial action and cry. *Pain* **28,** 395–410 (1987).

167. Grunau, R. V., Johnston, C. C. & Craig, K. D. Neonatal facial and cry responses to invasive and non-invasive procedures. *Pain* **42,** 295–305 (1990).

168. Anand, K. J. S., Roue, J., Rovnaghi, C. R., Marx, W. & Bornmann, L. Historical roots of pain management in infants: A bibliometric analysis using reference publication year spectroscopy. *Paediatric and Neonatal Pain* **2,** 22–32 (2020).

169. Grunau, R. E., Oberlander, T., Holsti, L. & Whitfield, M. F. Bedside application of the neonatal facial coding system in pain assessment of premature neonates. *Pain* **76,** 277–286 (1998).

170. Stevens, B. J. *et al.* The Premature Infant Pain Profile-Revised (PIPP-R): Initial Validation and Feasibility. *Clin J Pain* **30,** 238–243 (2014).

171. Cong, X., McGrath, J. M., Cusson, R. M. & Zhang, D. Pain assessment and measurement in neonates: An updated review. *Advances in Neonatal Care* **13,** 379–395 (2013).

172. Srouji, R., Ratnapalan, S. & Schneeweiss, S. Pain in Children: Assessment and Nonpharmacological Management. *International Journal of Pediatrics* **2010,** 1–11 (2010).

173. Foster, R. L., Yucha, C. B., Zuk, J. & Vojir, C. P. Physiologic Correlates of Comfort in Healthy Children. *Pain Management Nursing* **4,** 23–30 (2003).

174. Stevens, B., Johnston, C., Petryshen, P. & Taddio, A. Premature infant pain profile: Development and initial validation. *Clinical Journal of Pain* **12,** 13–22 (1996).

175. Stevens, B., Johnston, C., Taddio, A., Gibbins, S. & Yamada, J. The premature infant pain profile: Evaluation 13 years after development. *Clinical Journal of Pain* **26,** 813–830 (2010).

176. Carbajal, R. *et al.* Morphine does not provide adequate analgesia for acute procedural pain among preterm neonates. *Pediatrics* **115,** 1494–1500 (2005).

177. Nahum, Y., Tenenbaum, A., Isaiah, W. & Levy-Khademi, F. Effect of eutectic mixture of local anesthetics (EMLA) for pain relief during suprapubic aspiration in young infants: A randomized, controlled trial. *Clinical Journal of Pain* **23,** 756–759 (2007).

178. Lemyre, B. *et al.* How effective is tetracaine 4% gel, before a venipuncture, in reducing procedural pain in infants: A randomized double-blind placebo controlled trial. *BMC Pediatrics* **7,** 1–8 (2007).

179. Gibbins, S. *et al.* Validation of the Premature Infant Pain Profile-Revised (PIPP-R). *Early Human Development* **90,** 189–193 (2014).

180. Mörelius, E., Hellström-Westas, L., Carlén, C., Norman, E. & Nelson, N. Is a nappy change stressful to neonates? *Early Human Development* **82,** 669–676 (2006).

181. Shaw, S. C. *et al.* Assisted Physical Exercise and Stress in Preterm Neonates. *Indian Pediatrics* **55,** 679–682 (2018).

182. Kohut, S. A. & Pillai Riddell, R. Does the Neonatal Facial Coding System Differentiate Between Infants Experiencing Pain-Related and Non-Pain-Related Distress? *Journal of Pain* **10,** 214–220 (2009).

183. Gibbins, S. *et al.* Comparison of pain responses in infants of different gestational ages. *Neonatology* **93,** 10–18 (2008).

184. Slater, R. *et al.* Latency to facial expression change following noxious stimulation in infants is dependent on postmenstrual age. *Pain* **146,** 177–182 (2009).

185. Mogil, J. S. Animal models of pain: Progress and challenges. *Nature Reviews Neuroscience* **10,** 283–294 (2009).

186. Hartley, C. *et al.* The relationship between nociceptive brain activity, spinal reflex withdrawal and behaviour in newborn infants. *Scientific Reports* **5** (2015).

187. Andrews, K. & Fitzgerald, M. Cutaneous flexion reflex in human neonates: A quantitative study of threshold and stimulus-response characteristics after single and repeated stimuli. *Developmental Medicine and Child Neurology* **41,** 696–703 (1999).

188. Kühne, B., Stützer, H., Roth, B. & Welzing, L. The flexion withdrawal reflex reveals an increasing threshold during the first year of life and is influenced by the infant's state of consciousness. *Klinische Padiatrie* **224,** 291–295 (2012).

189. Cornelissen, L. *et al.* Postnatal temporal, spatial and modality tuning of nociceptive cutaneous flexion reflexes in human infants. *PLoS ONE* **8,** e76470 (2013).

190. Fitzgerald, M., Shaw, A. & MacIntosh, N. Postnatal development of the cutaneous flexor reflex: comparative study of preterm infants and newborn rat pups. *Developmental Medicine & Child Neurology* **30,** 520–526 (1988).

191. Waldenström, A., Thelin, J., Thimansson, E., Levinsson, A. & Schouenborg, J. Developmental learning in a pain-related system: Evidence for a cross-modality mechanism. *Journal of Neuroscience* **23,** 7719–7725 (2003).

192. Andrews, K. & Fitzgerald, M. Wound sensitivity as a measure of analgesic effects following surgery in human neonates and infants. *Pain* **99,** 185–195 (2002).

193. Van der Vaart, M. *et al.* Multimodal pain assessment improves discrimination between noxious and non-noxious stimuli in infants. *Paediatric and Neonatal Pain* **1,** 21–30 (2019).

194. Jöbsis, F. F. Noninvasive , Infrared Monitoring of Cerebral and Myocardial Oxygen Sufficiency and Circulatory Parameters. *Science* **198,** 1264–1267 (1977).

195. Hoshi, Y. Functional Near-Infrared Spectroscopy: Potential and Limitations in Neuroimaging Studies. *International Review of Neurobiology* **66,** 237–266 (2005).

196. Bunce, S. C., Izzetoglu, M., Izzetoglu, K., Onaral, B. & Pourrezaei, K. Functional near-infrared spectroscopy. *IEEE Engineering in Medicine and Biology Magazine* **25,** 54–62 (2006).

197. Slater, R. *et al.* Cortical pain responses in human infants. *The Journal of Neuroscience* **26,** 3662–3666 (2006).

198. Bartocci, M., Bergqvist, L. L., Lagercrantz, H. & Anand, K. J. Pain activates cortical areas in the preterm newborn brain. *Pain* **122,** 109–117 (2006).

199. Verriotis, M. *et al.* Mapping cortical responses to somatosensory stimuli in human infants with simultaneous near-infrared spectroscopy and event-related potential recording. *eNeuro* **3,** 663–673 (2016).

200. Slater, R., Cantarella, A., Franck, L., Meek, J. & Fitzgerald, M. How well do clinical pain assessment tools reflect pain in infants? *PLoS Medicine* **5,** 0928–0933 (2008).

201. Ranger, M. *et al.* A multidimensional approach to pain assessment in critically ill infants during a painful procedure. *Clin J Pain* **29,** 613–620 (2013).

202. Faris, F. *et al.* Non-invasive in vivo near-infrared optical measurement of the penetration depth in the neonatal head. *Clinical Physics and Physiological Measurement* **12,** 353–358 (1991).

203. Roche-Labarbe, N., Wallois, F., Ponchel, E., Kongolo, G. & Grebe, R. Coupled oxygenation oscillation measured by NIRS and intermittent cerebral activation on EEG in premature infants. *NeuroImage* **36,** 718–727 (2007).

204. Jezzard, P. & Toosy, A. T. in *MR Imaging in White Matter Diseases of the Brain and Spinal Cord* (eds Filippi, M, Stefano, N, Dousset, V & McGowan, J. C.) 93–100 (Springer-Verlag, Berlin, 2005).

205. Logothetis, N. K., Pauls, J., Augath, M., Trinath, T. & Oeltermann, A. Neurophysiological investigation of the basis of the fMRI signal. *Nature* **412,** 150–157 (2001).

206. Logothetis, N. K. What we can do and what we cannot do with fMRI. *Nature* **453,** 869–878 (2008).

207. Duerden, E. G. & Albanese, M. C. Localization of pain-related brain activation: A meta-analysis of neuroimaging data. *Human Brain Mapping* **34,** 109–149 (2013).

208. Jensen, K. B. *et al.* Brain activations during pain: A neuroimaging meta-analysis of patients with pain and healthy controls. *Pain* **157,** 1279–1286 (2016).

209. Garcia-Larrea, L. & Peyron, R. Pain matrices and neuropathic pain matrices: A review. *Pain* **154** (2013).

210. Williams, G. *et al.* Functional magnetic resonance imaging can be used to explore tactile and nociceptive processing in the infant brain. *Acta Paediatrica, International Journal of Paediatrics* **104,** 158–166 (2015).

211. Baxter, L. *et al.* Functional and diffusion MRI reveal the neurophysiological basis of neonates' noxious-stimulus evoked brain activity. *Nature Communications* **12,** 1–14 (2021).

212. Baxter, L. *et al.* Optimising neonatal fMRI data analysis: Design and validation of an extended dHCP preprocessing pipeline to characterise noxious-evoked brain activity in infants. *NeuroImage* **186,** 286–300 (2019).

213. Fitzgibbon, S. P. *et al.* The developing Human Connectome Project (dHCP) automated resting-state functional processing framework for newborn infants. *NeuroImage* **223,** 117303 (2020).

214. *Niedermeyer's Electroencephalography: Basic Principles, Clinical Applications, and Related Fields* 7 ed (eds Schomer, D. L. & Lopes da Silva, F. H.) (Oxford University Press, 2017).

215. Marcuse, L. V., Fields, M. C. & Yoo, J. J. in *Rowan's Primer of EEG* 1–37 (2016).

216. Key, A. P., Dove, G. O. & Maguire, M. J. Linking brainwaves to the brain: An ERP primer. *Developmental Neuropsychology* **27,** 183–215 (2005).

217. Worley, A., Fabrizi, L., Boyd, S. & Slater, R. Multi-modal pain measurements in infants. *Journal of Neuroscience Methods* **205,** 252–257 (2012).

218. Iannetti, G. D., Baumgartner, U., Tracey, I., Treede, R. D. & Magerl, W. Pinprick-evoked brain potentials: a novel tool to assess central sensitization of nociceptive pathways in humans. *Journal of Neurophysiology* **110,** 1107–1116 (2013).

219. Kakigi, R., Inui, K. & Tamura, Y. Electrophysiological studies on human pain perception. *Clinical Neurophysiology* **116,** 743–763 (2005).

220. Spreng, M. & Ichioka, M. Slow cortical potentials following pain stimulation in man. *Pflügers Archiv für die Gesamte Physiologie des Menschen und der Tiere* **279,** 121–132 (1964).

221. Bromm, B. & Treede, R. D. Human cerebral potentials evoked by CO2 laser stimuli causing pain. *Experimental Brain Research* **67,** 153–162 (1987).

222. Arendt-Nielsen, L. & Chen, A. C. Lasers and other thermal stimulators for activation of skin nociceptors in humans. *Neurophysiologie Clinique* **33,** 259–268 (2003).

223. Treede, R. D., Kief, S., Hölzer, T. & Bromm, B. Late somatosensory evoked cerebral potentials in response to cutaneous heat stimuli. *Electroencephalography and Clinical Neurophysiology* **70,** 429–441 (1988).

224. Kunde, V. & Treede, R. D. Topography of middle-latency somatosensory evoked potentials following painful laser stimuli and non-painful electrical stimuli. *Electroencephalography and Clinical Neurophysiology/ Evoked Potentials* **88,** 280–289 (1993).

225. Peyron, R. *et al.* Role of operculoinsular cortices in human pain processing: Converging evidence from PET, fMRI, dipole modeling, and intracerebral recordings of evoked potentials. *NeuroImage* **17,** 1336–1346 (2002).

226. Valeriani, M., Rambaud, L. & Mauguière, F. Scalp topography and dipolar source modelling of potentials evoked by CO2 laser stimulation of the hand. *Electroencephalography and Clinical Neurophysiology - Evoked Potentials* **100,** 343–353 (1996).

227. Tarkka, I. M. & Treede, R. D. *Equivalent electrical source analysis of pain-related somatosensory evoked potentials elicited by a CO2 laser* 1993.

228. Selton, D., Andre, M. & Hascoët, J. M. Normal EEG in very premature infants: Reference criteria. *Clinical Neurophysiology* **111,** 2116–2124 (2000).

229. Hayakawa, M. *et al.* Background electroencephalographic (EEG) activities of very preterm infants born at less than 27 weeks gestation: A study on the degree of continuity. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **84,** 163–167 (2001).

230. Kaminska, A., Eisermann, M. & Plouin, P. in *Handbook of Clinical Neurology* 1st ed., 125–142 (Elsevier B.V., 2019).

231. Pearl, P. L. *et al.* in *Niedermeyer's Electroencephalography: Basic Principles, Clinical Applications, and Related Fields* (eds Schomer, D. L. & Lopes da Silva, F. H.) 7 ed (Oxford University Press, 2017).

232. Hrbek, A., Karlberg, P. & Olsson, T. Development of visual and somatosensory evoked responses in pre-term newborn infants. *Electroencephalography and Clinical Neurophysiology* **34,** 225–232 (1973).

233. Van der Vaart, M. *et al.* Premature infants display discriminable behavioural, physiological and brain responses to noxious and non-noxious stimuli. *Cerebral Cortex,* 1–17 (2021).

234. Khazipov, R. *et al.* Early motor activity drives spindle bursts in the developing somatosensory cortex. *Nature* **432,** 758–761 (2004).

235. Fabrizi, L. *et al.* A shift in sensory processing that enables the developing human brain to discriminate touch from pain. *Current Biology* **21,** 1552–1558 (2011).

236. Slater, R. *et al.* Evoked potentials generated by noxious stimulation in the human infant brain. *European journal of pain* **14,** 321–326 (2010).

237. Hartley, C. *et al.* Nociceptive brain activity as a measure of analgesic efficacy in infants. *Science Translational Medicine* **9** (2017).

238. Truini, A. *et al.* Laser-evoked potentials: Normative values. *Clinical Neurophysiology* **116,** 821–826 (2005).

239. Fabrizi, L. *et al.* Cortical activity evoked by an acute painful tissue-damaging stimulus in healthy adult volunteers. *Journal of Neurophysiology* **109,** 2393–2403 (2013).

240. Verriotis, M. *et al.* Cortical activity evoked by inoculation needle prick in infants up to one-year old. *Pain* **156,** 222–230 (2015).

241. Magerl, W., Fuchs, P. N., Meyer, R. A. & Treede, R.-D. Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. *Brain* **124,** 1754–1764 (2001).

242. Gursul, D. *et al.* Stroking modulates noxious-evoked brain activity in human infants. *Current Biology* **28,** R1380–R1381 (2018).

243. Duley, L., Uhm, S. & Oliver, S. Top 15 UK research priorities for preterm birth. *The Lancet* **383,** 2041–2042 (2014).

244. AAP Committee on Fetus and Newborn and Section on Anesthesiology and Pain Medicine. Prevention and Management of Procedural Pain in the Neonate: An Update. *Pediatrics* **137,** e20154271 (2016).

245. Wielenga, J. M., Tume, L. N., Latour, J. M. & Van Den Hoogen, A. European neonatal intensive care nursing research priorities: An e-delphi study. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **100,** F66–F71 (2015).

246. Stevens, B., Yamada, J., Ohlsson, A., Haliburton, S. & Shorkey, A. Sucrose for analgesia in newborn infants undergoing painful procedures. *Cochrane Database of Systematic Reviews* **7** (2016).

247. Slater, R. *et al.* Oral sucrose as an analgesic drug for procedural pain in newborn infants: A randomised controlled trial. *The Lancet* **376,** 1225–1232 (2010).

248. Anseloni, V., Ren, K., Dubner, R. & Ennis, M. Ontogeny of analgesia elicited by non-nutritive suckling in acute and persistent neonatal rat pain models. *Pain* **109,** 507–513 (2004).

249. Anseloni, V. C., Ren, K., Dubner, R. & Ennis, M. A brainstem substrate for analgesia elicited by intraoral sucrose. *Neuroscience* **133,** 231–243 (2005).

250. Schneider, J. *et al.* Procedural pain and oral glucose in preterm neonates: brain development and sex-specific effects. *Pain* **159,** 515–525 (2018).

251. Tremblay, S. *et al.* Repeated exposure to sucrose for procedural pain in mouse pups leads to long-term widespread brain alterations. *Pain* **158,** 1586–1598 (2017).

252. Meek, J. & Huertas, A. Cochrane review: Non-nutritive sucking, Kangaroo care and swaddling/facilitated tucking are observed to reduce procedural pain in infants and young children. *Evidence-Based Nursing* **15,** 84–85 (2012).

253. Benoit, B., Martin-Misener, R., Latimer, M. & Campbell-Yeo, M. Breast-Feeding Analgesia in Infants. *The Journal of Perinatal & Neonatal Nursing* **31,** 145–159 (2017).

254. de Sousa Freire, N. B., Santos Garcia, J. B. & Carvalho Lamy, Z. Evaluation of analgesic effect of skin-to-skin contact compared to oral glucose in preterm neonates. *Pain* **139,** 28–33 (2008).

255. Gray, L., Watt, L. & Blass, E. M. Skin-to-skin contact is analgesic in healthy newborns. *Pediatrics* **105** (2000).

256. Johnston, C. C. *et al.* Kangaroo Care Is Effective in Diminishing Pain Response in Preterm Neonates. *Archives of Pediatrics and Adolescent Medicine* **157,** 1084–1088 (2003).

257. Akcan, E., Yiğit, R. & Atici, A. The effect of kangaroo care on pain in premature infants during invasive procedures. *Turkish Journal of Pediatrics* **51,** 14–18 (2009).

258. Chermont, A. G., Falcão, L. F. M., Silva, E. H. L. D. S., Balda, R. D. C. X. & Guinsburg, R. Skin-to-skin contact and/or oral 25% dextrose for procedural pain relief for term newborn infants. *Pediatrics* **124** (2009).

259. Association of Paediatric Anaesthetists of Great Britain and Ireland. Good practice in postoperative and procedural pain management, 2nd edition. *Pediatric Anesthesia* **22** (2012).

260. Johnston, C *et al.* Skin-to-skin care for procedural pain in neonates (Review). *Cochrane Library,* 113 (2017).

261. Liu, Q. *et al.* Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. *Nature Neuroscience* **10,** 946–948 (2007).

262. Vallbo, Å., Olausson, H., Wessberg, J. & Norrsell, U. A system of unmyelinated afferents for innocuous mechanoreception in the human skin. *Brain Research* **628,** 301–304 (1993).

263. Vallbo, A. B., Olausson, H., Wessberg, J. & Kakuda, N. Receptive field characteristics of tactile units with myelinated afferents in hairy skin of human subjects. *The Journal of Physiology* **483,** 783–795 (1995).

264. Bennett, R. H., Bolling, D. Z., Anderson, L. C., Pelphrey, K. A. & Kaiser, M. D. fNIRS detects temporal lobe response to affective touch. *Social Cognitive and Affective Neuroscience* **9,** 470–476 (2014).

265. Gordon, I. *et al.* Brain mechanisms for processing affective touch. *Human Brain Mapping* **34,** 914–922 (2013).

266. McGlone, F. *et al.* Touching and feeling: Differences in pleasant touch processing between glabrous and hairy skin in humans. *European Journal of Neuroscience* **35,** 1782–1788 (2012).

267. Olausson, H. *et al.* Unmyelinated tactile afferents signal touch and project to insular cortex. *Nature Neuroscience* **5,** 900–904 (2002).

268. McGlone, F., Wessberg, J. & Olausson, H. Discriminative and Affective Touch: Sensing and Feeling. *Neuron* **82,** 737–755 (2014).

269. Olausson, H., Wessberg, J., Morrison, I., McGlone, F. & Vallbo, Å. The neurophysiology of unmyelinated tactile afferents. *Neuroscience and Biobehavioral Reviews* **34,** 185–191 (2010).

270. Von Mohr, M., Kirsch, L. P. & Fotopoulou, A. The soothing function of touch: Affective touch reduces feelings of social exclusion. *Scientific Reports* **7,** 1–9 (2017).

271. Essick, G. K. *et al.* Quantitative assessment of pleasant touch. *Neuroscience and Biobehavioral Reviews* **34,** 192–203 (2010).

272. Löken, L. S., Wessberg, J., Morrison, I., McGlone, F. & Olausson, H. Coding of pleasant touch by unmyelinated afferents in humans. *Nature Neuroscience* **12,** 547–548 (2009).

273. Triscoli, C., Ackerley, R. & Sailer, U. Touch satiety: differential effects of stroking velocity on liking and wanting touch over repetitions. *PLoS ONE* **9,** e113425 (2014).

274. Von Mohr, M., Krahé, C., Beck, B. & Fotopoulou, A. The social buffering of pain by affective touch: a laser-evoked potential study in romantic couples. *Social cognitive and affective neuroscience* **13,** 1121–1130 (2018).

275. Courtois, E. *et al.* Epidemiology and neonatal pain management of heelsticks in intensive care units: EPIPPAIN 2, a prospective observational study. *International Journal of Nursing Studies* **59,** 79–88 (2016).

276. Field, T., Hernandez-Reif, M., Feijo, L. & Freedman, J. Prenatal, perinatal and neonatal stimulation: A survey of neonatal nurseries. *Infant Behavior and Development* **29,** 24–31 (2006).

277. Van den Anker, J., Reed, M. D., Allegaert, K. & Kearns, G. L. Developmental Changes in Pharmacokinetics and Pharmacodynamics. *Journal of Clinical Pharmacology* **58,** S10–S25 (2018).

278. Smits, A. *et al.* Current knowledge, challenges and innovations in developmental pharmacology: A combined conect4children Expert Group and European Society for Developmental, Perinatal and Paediatric Pharmacology White Paper. *British Journal of Clinical Pharmacology,* 1–20 (2021).

279. Anand, K. J. *et al.* Effects of morphine analgesia in ventilated preterm neonates: primary outcomes from the NEOPAIN randomised trial. *Lancet* **363,** 1673–1682 (2004).

280. Aranda, J. V. *et al.* Analgesia and sedation during mechanical ventilation in neonates. *Clinical Therapeutics* **27,** 877–899 (2005).

281. Taddio, A. *et al.* Intravenous morphine and topical tetracaine for treatment of pain in preterm neonates undergoing central line placement. *Journal of the American Medical Association* **295,** 793–800 (2006).

282. Simons, S. *et al.* Routine Morphine Infusion in Preterm. *JAMA : the journal of the American Medical Association* **290,** 2419–2427 (2003).

283. Bellù, R. *et al.* Opioids for newborn infants receiving mechanical ventilation. *Cochrane Database of Systematic Reviews* (2021).

284. Hartley, C. *et al.* Analgesic efficacy and safety of morphine in the Procedural Pain in Premature Infants (Poppi) study: randomised placebo-controlled trial. *The Lancet* **392,** 2595–2605 (2018).

285. Pacifici, G. M. Clinical Pharmacology of Fentanyl in Preterm Infants. A Review. *Pediatrics and Neonatology* **56,** 143–148 (2015).

286. Wu, Y. *et al.* Pre- and Postnatal Maturation are Important for Fentanyl Exposure in Preterm and Term Newborns: A Pooled Population Pharmacokinetic Study. *Clinical Pharmacokinetics* **4** (2021).

287. Lago, P., Benini, F., Agosto, C. & Zacchello, F. Randomised controlled trial of low dose fentanyl infusion in preterm infants with hyaline membrane disease. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **79,** 194–197 (1998).

288. Orsini, A. J., Leef, K. H., Costarino, A., Dettorre, M. D. & Stefano, J. L. Routine use of fentanyl infusions for pain and stress reduction in infants with respiratory distress syndrome. *Journal of Pediatrics* **129,** 140–145 (1996).

289. Pacifici, G. M. & Allegaert, K. Clinical Pharmacology of Paracetamol in Neonates: A Review. *Current Therapeutic Research - Clinical and Experimental* **77,** 24–30 (2015).

290. Ceelie, I. *et al.* Effect of intravenous paracetamol on postoperative morphine requirements in neonates and infants undergoing major noncardiac surgery. *JAMA* **309,** 149–154 (2013).

291. Härmä, A., Aikio, O., Hallman, M. & Saarela, T. Intravenous paracetamol decreases requirements of morphine in very preterm infants. *Journal of Pediatrics* **168,** 36–40 (2016).

292. Ohlsson, A. & Shah, P. S. Paracetamol (acetaminophen) for prevention or treatment of pain in newborns. *Cochrane Database of Systematic Reviews* (2020).

293. Roofthooft, D. W. *et al.* Randomized controlled trial comparing different single doses of intravenous paracetamol for placement of peripherally inserted central catheters in preterm infants. *Neonatology* **112,** 150–158 (2017).

294. Seifi, F., Peirovifar, A. & Gharehbaghi, M. M. Comparing the efficacy of oral sucrose and acetaminophen in pain relief for ophthalmologic screening of retinopathy of prematurity. *American Journal of Medical Sciences and Medicine* **1,** 24–27 (2013).

295. Badiee, Z. & Torcan, N. Effects of high dose orally administered paracetamol for heel prick pain in premature infants. *Saudi Medical Journal* **30,** 1450–1453 (2009).

296. Bonetto, G. *et al.* Prevención del dolor en recién nacidos de término: estudio aleatorizado sobre tres métodos. *Arch Argent Pediatr* **106,** 392–396 (2008).

297. Shah, V., Taddio, A. & Ohlsson, A. Randomised controlled trial of paracetamol for heel prick pain in neonates. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **79,** F209–F211 (1998).

298. Baarslag, M. A. *et al.* Paracetamol and morphine for infant and neonatal pain; still a long way to go? *Expert Review of Clinical Pharmacology* **10,** 111–126 (2017).

299. Brummelte, S. *et al.* Procedural pain and brain development in premature newborns. *Annals of Neurology* **71,** 385–396 (2012).

300. Vinall, J. & Grunau, R. E. Impact of repeated procedural pain-related stress in infants born very preterm. *Pediatric Research* **75,** 584–587 (2014).

301. Dunne, J. *et al.* Extrapolation of adult data and other data in pediatric drug-development programs. *Pediatrics* **128** (2011).

302. Craig, S. S. *et al.* Lidocaine and phenylephrine versus saline placebo nasal spray for the pain and distress of nasogastric tube insertion in young children and infants: a randomised, double-blind, controlled trial. *The Lancet Child and Adolescent Health* **3,** 391–397 (2019).

303. Slater, R. The challenge of distinguishing pain from distress in young children. *The Lancet Child and Adolescent Health* **3,** 367–368 (2019).

304. Slater, R. *et al.* A blinded randomised placebo-controlled trial investigating the efficacy of morphine analgesia for procedural pain in infants: Trial protocol. *Wellcome Open Research* **1** (2017).

305. Oakes, L. M. Sample Size, Statistical Power, and False Conclusions in Infant Looking-Time Research. *Infancy* **22,** 436–469 (2017).

306. Public Health England. Routine childhood immunisations Autumn 2019. *https://www.gov.uk/government/publications/routine-childhood-immunisation-schedule,* Accessed: 19/04/2021.

307. Hartley, C. *et al.* Noxious stimulation in children receiving general anaesthesia evokes an increase in delta frequency brain activity. *Pain* **155,** 2368–2376 (2014).

308. MRC Systems. QST products - PinPrick stimulators. *https://www.mrc-systems.de/en/products/pinprick,* Accessed: 15/04/2021 (2021).

309. Ziegler, E. A., Magerl, W., Meyer, R. A. & Treede, R. D. Secondary hyperalgesia to punctate mechanical stimuli. Central sensitization to A-fibre nociceptor input. *Brain* **122,** 2245–2257 (1999).

310. Rolke, R. *et al.* Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): Standardized protocol and reference values. *Pain* **123,** 231–243 (2006).

311. MRC Systems. PinPrick stimulators with contact trigger. *https://www.mrc-systems.de/downloads/en/pinprick,* Accessed: 16/04/2021 (2020).

312. Public Health England & NHS England. *Introduction of meningococcal B immunisation for infants.* 2015.

313. Somedic SenseLab AB. SENSELab Brush-05. *Available at: http://somedic.com/en-doc/SENSELab-Brush-05-User-Manual-EN.pdf,* Accessed: 20/04/2021 (2017).

314. Peirce, J. *et al.* PsychoPy2: Experiments in behavior made easy. *Behavior Research Methods* **51,** 195–203 (2019).

315. Malhotra, R. K. & Avidan, A. Y. in *Atlas of Sleep Medicine* (eds Chokroverty, S. & Thomas, R. J.) Second Edi, 77–99 (Elsevier Inc., 2014).

316. Nitschke, J. B., Miller, G. A. & Cook, E. W. Digital filtering in EEG/ERP analysis: Some technical and empirical comparisons. *Behavior Research Methods, Instruments, and Computers* **30,** 54–67 (1998).

317. Koutroumanidis, M., Sakellariou, D. & Tsirka, V. in *Oxford Textbook of Clinical Neurophysiology* 119–130 (2016).

318. Widmann, A., Schröger, E. & Maess, B. Digital filter design for electrophysiological data - a practical approach. *Journal of Neuroscience Methods* **250,** 34–46 (2015).

319. Niemarkt, H. J. *et al.* Maturational changes in automated EEG spectral power analysis in preterm infants. *Pediatric Research* **70,** 529–534 (2011).

320. Garcia-Larrea, L., Frot, M. & Valeriani, M. Brain generators of laser-evoked potentials: From dipoles to functional significance. *Neurophysiologie Clinique* **33,** 279–292 (2003).

321. Valentini, E. *et al.* The primary somatosensory cortex largely contributes to the early part of the cortical response elicited by nociceptive stimuli. *NeuroImage* **59,** 1571–1581 (2012).

322. Valeriani, M., Pazzaglia, C., Cruccu, G. & Truini, A. Clinical usefulness of laser evoked potentials. *Neurophysiologie Clinique* **42,** 345–353 (2012).

323. Vogt, B. A. & Sikes, R. W. in *Cingulate neurobiology and disease* (ed Vogt, B. A.) 312–339 (Oxford University Press, New York, 2009).

324. Stevens, B. Premature Infant Pain Profile-revised (PIPP-R) e-Learning Module. *The Hospital for Sick Children ("SickKids") - Academy Online - https://lab.research.sickkids.ca/stevens/pipp-r-module/,* Accessed: 10–11–2021 (2021).

325. Hall, R. W. & Anand, K. J. Pain management in newborns. *Clinical Perinatology* **41,** 895–924 (2014).

326. Lim, Y. & Godambe, S. Prevention and management of procedural pain in the neonate: An update, American Academy of Pediatrics, 2016. *Archives of Disease in Childhood: Education and Practice Edition* **102,** 254–256 (2017).

327. Verriotis, M. *et al.* The distribution of pain activity across the human neonatal brain is sex dependent. *NeuroImage* **178,** 69–77 (2018).

328. Friston, K. J. *et al.* Statistical parametric maps in functional imaging: A general linear approach. *Human Brain Mapping* **2,** 189–210 (1994).

329. Arichi, T. *et al.* Development of BOLD signal hemodynamic responses in the human brain. *NeuroImage* **63,** 663–673 (2012).

330. Schmidt Mellado, G. *et al.* The impact of premature extrauterine exposure on infants' stimulus-evoked brain activity across multiple sensory systems. *NeuroImage: Clinical* **33,** 102914 (2021).

331. Kakigi, R., Watanabe, S. & Yamasaki, H. Pain-Related Somatosensory Evoked Potentials. *Journal of Clinical Neurophysiology* **17,** 259–308 (2000).

332. Spiegel, J & Hansen, C. Laser-evoked potentials after painful hand and foot stimulation in humans" evidence for generation of the middle-latency component in the secondary somatosensory cortex. *Neuroscience Letters* **216,** 179–182 (1996).

333. Donadio, A. *et al.* A novel sensor design for accurate measurement of facial somatosensation in pre-term infants. *PLOS ONE* **13** (ed Tu, W.-J.) e0207145 (2018).

334. National Institute for Health and Care Excellence (NICE). *Paracetamol. BNFc* 2020.

335. Jozwiak-Bebenista, M. & Nowak, J. Z. Paracetamol: Mechanism of action, applications and safety concern. *Acta Poloniae Pharmaceutica - Drug Research* **71,** 11–23 (2014).

336. Hyllested, M., Jones, S., Pedersen, J. L. & Kehlet, H. Comparative effect of paracetamol, NSAIDs or their combination in postoperative pain management: A qualitative review. *British Journal of Anaesthesia* **88,** 199–214 (2002).

337. Krebs, E. E. *et al.* Effect of opioid vs nonopioid medications on pain-related function in patients with chronic back pain or hip or knee osteoarthritis pain the SPACE randomized clinical trial. *JAMA - Journal of the American Medical Association* **319,** 872–882 (2018).

338. Malver, L. P. *et al.* Electroencephalography and analgesics. *British Journal of Clinical Pharmacology* **77,** 72–95 (2014).

339. Arendt-Nielsen, L., Nielsen, J. C. & Bjerring, P. Double-blind, placebo controlled comparison of paracetamol and paracetamol plus codeine - a quantitative evaluation by laser induced pain. *European Journal of Clinical Pharmacology* **40,** 241–247 (1991).

340. Bromm, B, Forth, W, Richter, E & Scharein, E. Effects of acetaminophen and antipyrine on non-inflammatory pain and EEG activity. *Pain* **50,** 213–221 (1992).

341. Pickering, G. *et al.* Paracetamol and opioid pathways: A pilot randomized clinical trial. *Fundamental and Clinical Pharmacology* **27,** 339–345 (2013).

342. Pickering, G. *et al.* The brain signature of paracetamol in healthy volunteers: A double-blind randomized trial. *Drug Design, Development and Therapy* **9,** 3853–3862 (2015).

343. Höck, M. *et al.* Prophylactic low-dose paracetamol administration associated with lowered rate of patent ductus arteriosus in preterm infants – Impact on outcome and pain perception. *Pediatrics and Neonatology* **61,** 84–91 (2020).

344. Maris, E. & Oostenveld, R. Nonparametric statistical testing of EEG- and MEG-data. *Journal of Neuroscience Methods* **164,** 177–190. arXiv: `0404501 [arXiv:cond-mat]` (2007).

345. Bromm, B & Scharein, E. Principal Component Analysis of pain related cerebral potentials to mechanical and electrical stimulation in man. *Electroencephalography and Clinical Neurophysiology* **53,** 94–103 (1982).

346. Cobo, M. M. *et al.* Quantifying individual noxious-evoked baseline sensitivity to optimise analgesic trials in neonates. *eLife* **10,** 1–24 (2021).

347. Kasser, S. *et al.* Birth experience in newborn infants is associated with changes in nociceptive sensitivity. *Scientific Reports* **9,** 4117 (2019).

348. Mouraux, A. *et al.* Nociceptive Steady-State Evoked Potentials Elicited by Rapid Periodic Thermal Stimulation of Cutaneous Nociceptors. *Journal of Neuroscience* **31,** 6079–6087 (2011).

349. Jiang, X., Bian, G. B. & Tian, Z. Removal of artifacts from EEG signals: A review. *Sensors (Switzerland)* **19,** 1–18 (2019).

350. Laudiano-Dray, M. P. *et al.* Quantification of neonatal procedural pain severity: a platform for estimating total pain burden in individual infants. *Pain* **161,** 1270–1277 (2020).

351. Moultrie, F., Slater, R. & Hartley, C. Improving the treatment of infant pain. *Current Opinion in Supportive and Palliative Care* **11,** 112–117 (2017).

352. Bishop, D. V. The psychology of experimental psychologists: Overcoming cognitive constraints to improve research: The 47th Sir Frederic Bartlett Lecture. *Quarterly Journal of Experimental Psychology* **73,** 1–19 (2020).

353. Wanigasekera, V. *et al.* Disambiguating pharmacological mechanisms from placebo in neuropathic pain using functional neuroimaging. *British Journal of Anaesthesia* **120,** 299–307 (2018).

354. European Medicines Agency. *CPMP/ICH/2711/99 - ICH topic E 11: clinical investigation of medicinal products in the paediatric population* 2001.

355. Green, G. *et al.* Behavioural discrimination of noxious stimuli in infants is dependent on brain maturation. *Pain* **160,** 493–500 (2019).

356. Ozawa, M., Kanda, K., Hirata, M., Kusakawa, I. & Suzuki, C. Influence of repeated painful procedures on prefrontal cortical pain responses in newborns. *Acta Paediatrica, International Journal of Paediatrics* **100,** 198–203 (2011).

357. Jones, L. *et al.* Nociceptive cortical activity is dissociated from nociceptive behavior in newborn human infants under stress. *Current Biology* **27,** 3846–3851 (2017).

358. Ancora, G. *et al.* Efficacy and safety of continuous infusion of fentanyl for pain control in preterm newborns on mechanical ventilation. *Journal of Pediatrics* **163,** 645–651 (2013).

359. Kabataş, E. U. *et al.* Efficacy of single dose oral paracetamol in reducing pain during examination for retinopathy of prematurity: a blinded randomized controlled trial. *Indian Journal of Pediatrics* **83,** 22–26 (2016).

360. Sindhur, M. *et al.* Intranasal fentanyl for pain management during screening for retinopathy of prematurity in preterm infants: a randomized controlled trial. *Journal of Perinatology* **40,** 881–887 (2020).

361. Cooper, S. A. *et al.* Research design considerations for single-dose analgesic clinical trials in acute pain: IMMPACT recommendations. *Pain* **157,** 288–301 (2016).

362. McEntegart, D. J. The pursuit of balance using stratified and dynamic randomization techniques: an overview. *Therapeutic Innovation & Regulatory Science* **37,** 293–308 (2003).

363. Demant, D. T. *et al.* The effect of oxcarbazepine in peripheral neuropathic pain depends on pain phenotype: a randomised, double-blind, placebo-controlled phenotype-stratified study. *Pain* **155,** 2263–2273 (2014).

364. Smith, S. M. *et al.* The potential role of sensory testing, skin biopsy, and functional brain imaging as biomarkers in chronic pain clinical trials: IMMPACT considerations. *Journal of Pain* **18,** 757–777 (2017).

365. Vollert, J. *et al.* Stratifying patients with peripheral neuropathic pain based on sensory profiles: algorithm and sample size recommendations. *Pain* **158,** 1446–1455 (2017).

366. Lane, N. E. *et al.* Tanezumab for the treatment of pain from osteoarthritis of the knee. *New England Journal of Medicine* **363,** 1521–1531 (2010).

367. Sanga, P. *et al.* Efficacy, safety, and tolerability of fulranumab, an anti-nerve growth factor antibody, in the treatment of patients with moderate to severe osteoarthritis pain. *Pain* **154,** 1910–1919 (2013).

368. Davis, K. D. *et al.* Discovery and validation of biomarkers to aid the development of safe and effective pain therapeutics: challenges and opportunities. *Nature Reviews Neurology* **16,** 381–400 (2020).

369. Coghill, R. C., Mchaffie, J. G. & Yen, Y. Neural correlates of interindividual differences in the subjective experience of pain. *Proceedings of the National Academy of Sciences of the United States of America* **114,** 8538–8542 (2017).

370. Lorenz, J., Beck, H. & Bromm, B. Cognitive performance, mood and experimental pain before and during morphine-induced analgesia in patients with chronic non-malignant pain. *Pain* **73,** 369–375 (1997).

371. Winkler, A. M., Ridgway, G. R., Webster, M. A., Smith, S. M. & Nichols, T. E. Permutation inference for the general linear model. *NeuroImage* **92,** 381–397 (2014).

372. Hatfield, L. A. & Ely, E. A. Measurement of acute pain in infants: a review of behavioral and physiological variables. *Biological Research for Nursing* **17,** 100–111 (2015).

373. Johnston, C. C. & Stevens, B. J. Experience in a neonatal intensive care unit affects pain response. *Pediatrics* **98,** 925–930 (1996).

374. Van den Broeke, E. N. *et al.* Characterizing pinprick-evoked brain potentials before and after experimentally induced secondary hyperalgesia. *Journal of Neurophysiology* **114,** 2672–2681 (2015).

375. Croy, I. *et al.* Interpersonal stroking touch is targeted to C tactile afferent activation. *Behavioural Brain Research* **297,** 37–40 (2016).

376. Abraira, V. E. *et al.* The Cellular and Synaptic Architecture of the Mechanosensory Dorsal Horn. *Cell* **168,** 295–310 (2017).

377. Marshall, A. G., Sharma, M. L., Marley, K., Olausson, H. & McGlone, F. P. Spinal signalling of c-fiber mediated pleasant touch in humans. *eLife* **8,** 1–15 (2019).

378. Marshall, A. G. & McGlone, F. P. Affective Touch: The Enigmatic Spinal Pathway of the C-Tactile Afferent. *Neuroscience insights* **15,** 1–5 (2020).

379. Sailer, U. *et al.* Temporal dynamics of brain activation during 40 minutes of pleasant touch. *NeuroImage* **139,** 360–367 (2016).

380. Morrison, I. ALE meta-analysis reveals dissociable networks for affective and discriminative aspects of touch. *Human Brain Mapping* **37,** 1308–1320 (2016).

381. Salter, M. W. & Henry, J. L. Differential responses of nociceptive vs. non-nociceptive spinal dorsal horn neurones to cutaneously applied vibration in the cat. *Pain* **40,** 311–322 (1990).

382. Lu, Y. & Perl, E. R. A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. *Journal of Neuroscience* **23,** 8752–8758 (2003).

383. Liljencrantz, J. *et al.* Slow brushing reduces heat pain in humans. *European Journal of Pain (United Kingdom)* **21,** 1173–1185 (2017).

384. Habig, K. *et al.* Low threshold unmyelinated mechanoafferents can modulate pain. *BMC Neurology* **17,** 1–11 (2017).

385. Meijer, L. L., Ruis, C., van der Smagt, M. J., Scherder, E. J. & Dijkerman, H. C. Neural basis of affective touch and pain: A novel model suggests possible targets for pain amelioration. *Journal of Neuropsychology* **16,** 38–53 (2022).

386. Scher, M. S., Johnson, M. W. & Holditch-Davis, D. Cyclicity of neonatal sleep behaviors at 25 to 30 weeks' postconceptional age. *Pediatric Research* **57,** 879–882 (2005).

387. Blumberg, M. S. & Seelke, A. M. H. in *Oxford Handbook of Developmental Behavioral Neuroscience* (eds Blumberg, M. S., Freeman, J. H. & Robinson, S. R.) 390–423 (Oxford University Press, 2014).

388. Axelin, A., Kirjavainen, J., Salanterä, S. & Lehtonen, L. Effects of pain management on sleep in preterm infants. *European Journal of Pain* **14,** 752–758 (2010).

389. De Asis-Cruz, J. *et al.* Functional brain connectivity in ex utero premature infants compared to in utero fetuses. *NeuroImage* **219** (2020).

390. Clifton, L. & Clifton, D. A. The correlation between baseline score and post-intervention score, and its implications for statistical analysis. *Trials* **20,** 4–9 (2019).

391. Vickers, A. J. The use of percentage change from baseline as an outcome in a controlled trial is statistically inefficient: A simulation study. *BMC Medical Research Methodology* **1,** 1–4 (2001).

392. Vickers, A. J. & Altman, D. G. Analysing controlled trials with baseline and follow up measurements. *British Medical Journal* **323,** 1123–1124 (2001).

393. Niculescu, A. B. *et al.* Towards precision medicine for pain: diagnostic biomarkers and repurposed drugs. *Molecular Psychiatry* **24,** 501–522 (2019).

394. Hore, Z. & Denk, F. Neuroimmune interactions in chronic pain – An interdisciplinary perspective. *Brain, Behavior, and Immunity* **79,** 56–62 (2019).

395. Dowling, D. J. & Levy, O. Ontogeny of early life immunity. *Trends in Immunology* **35,** 299–310 (2014).

396. Olin, A. *et al.* Stereotypic Immune System Development in Newborn Children. *Cell* **174,** 1277–1292 (2018).

397. Laforest-Lapointe, I. & Arrieta, M. C. Patterns of early-life gut microbial colonization during human immune development: An ecological perspective. *Frontiers in Immunology* **8,** Article 788 (2017).

398. Zouikr, I., Bartholomeusz, M. D. & Hodgson, D. M. Early life programming of pain: Focus on neuroimmune to endocrine communication. *Journal of Translational Medicine* **14,** 1–15 (2016).

399. Binshtok, A. M. *et al.* Nociceptors are interleukin-1β sensors. *Journal of Neuroscience* **28,** 14062–14073 (2008).

400. Lidow, M. S., Song, Z. M. & Ren, K. Long-term effects of short-lasting early local inflammatory insult. *NeuroReport* **12,** 399–403 (2001).

401. Wang, G., Ji, Y., Lidow, M. S. & Traub, R. J. Neonatal hind paw injury alters processing of visceral and somatic nociceptive stimuli in the adult rat. *Journal of Pain* **5,** 440–449 (2004).

402. Ruda, M. A., Ling, Q. D., Hohmann, A. G., Peng, Y. B. & Tachibana, T. Altered nociceptive neuronal circuits after neonatal peripheral inflammation. *Science* **289,** 628–630 (2000).

403. Cailes, B. *et al.* Epidemiology of UK neonatal infections: The neonIN infection surveillance network. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **103,** 547–553 (2018).

404. Mukhopadhyay, S., Eichenwald, E. C. & Puopolo, K. M. Neonatal early-onset sepsis evaluations among well-appearing infants: Projected impact of changes in CDC GBS guidelines. *Journal of Perinatology* **33,** 198–205 (2013).

405. Puri, D. *et al.* Prevalence of clinical signs of possible serious bacterial infection and mortality associated with them from population-based surveillance of young infants from birth to 2 months of age. *PLoS ONE* **16,** 1–14 (2021).

406. Barr, G. A. & Hunter, D. A. Interactions between glia, the immune system and pain processes during early development. *Developmental Psychobiology* **56,** 1698–1710 (2014).

407. Campbell, E. J., Watters, S. M., Zouikr, I., Hodgson, D. M. & Dayas, C. V. Recruitment of hypothalamic orexin neurons after formalin injections in adult male rats exposed to a neonatal immune challenge. *Frontiers in Neuroscience* **9,** 1–10 (2015).

408. Tadros, M. A., Zouikr, I., Hodgson, D. M. & Callister, R. J. Excitability of rat superficial dorsal horn neurons following a neonatal immune challenge. *Frontiers in Neurology* **9** (2018).

409. Wang, K. C. *et al.* Interleukin-1 receptor antagonist ameliorates neonatal lipopolysaccharide-induced long-lasting hyperalgesia in the adult rats. *Toxicology* **279,** 123–129 (2011).

410. Yan, S. & Kentner, A. C. Mechanical allodynia corresponds to Oprm1 downregulation within the descending pain network of male and female rats exposed to neonatal immune challenge. *Brain, Behavior, and Immunity* **63,** 148–159 (2017).

411. Karshikoff, B., Tadros, M. A., Mackey, S. & Zouikr, I. Neuroimmune modulation of pain across the developmental spectrum. *Current Opinion in Behavioral Sciences* **28,** 85–92 (2019).

412. Hunter, D., Chai, C. & Barr, G. A. Effects of COX inhibition and LPS on formalin induced pain in the infant rat. *Developmental Neurobiology* **75,** 1068–1079 (2015).

413. Zouikr, I. *et al.* Altered formalin-induced pain and fos induction in the periaqueductal grey of preadolescent rats following neonatal LPS exposure. *PLoS ONE* **9** (2014).

414. Gomes, C. I. & Barr, G. A. Local injury and systemic infection in infants alter later nociception and pain affect during early life and adulthood. *Brain, Behavior, & Immunity - Health* **9,** 100175 (2020).

415. Mestas, J. & Hughes, C. C. W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology* **172,** 2731–2738 (2004).

416. National Institute for Health and Care Excellence (NICE). Antibiotics for early-onset neonatal infection : antibiotics for the prevention and treatment of early-onset neonatal infection. NICE Guideline No 149 (2012).

417. Jones, G. T., Power, C. & Macfarlane, G. J. Adverse events in childhood and chronic widespread pain in adult life: Results from the 1958 British Birth Cohort Study. *Pain* **143,** 92–96 (2009).

418. Faul, F., Erdfelder, E., Buchner, A. & Lang, A. G. Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods* **41,** 1149–1160 (2009).

419. Hutchinson, M. R. *et al.* Low-dose endotoxin potentiates capsaicin-induced pain in man: Evidence for a pain neuroimmune connection. *Brain, Behavior, and Immunity* **30,** 3–11 (2013).

420. Holm, S. A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian Journal of Statistics* **6,** 65–70. arXiv: `arXiv:1011.1669v3` (1979).

421. Cobo, M. M. *et al.* Early life inflammation is associated with spinal cord excitability and nociceptive sensitivity in human infants. *Nature Communications,* 1–8 (2022).

422. Zouikr, I. & Karshikoff, B. Lifetime modulation of the pain system via neuroimmune and neuroendocrine interactions. *Frontiers in Immunology* **8** (2017).

423. Zouikr, I. *et al.* Altered nociceptive, endocrine, and dorsal horn neuron responses in rats following a neonatal immune challenge. *Psychoneuroendocrinology* **41,** 1–12 (2014).

424. Baba, H., Kohno, T., Moore, K. A. & Woolf, C. J. Direct activation of rat spinal dorsal horn neurons by prostaglandin E2. *Journal of Neuroscience* **21,** 1750–1756 (2001).

425. Trang, T., Beggs, S. & Salter, M. W. Brain-derived neurotrophic factor from microglia: A molecular substrate for neuropathic pain. *Neuron Glia Biology* **7,** 99–108 (2011).

426. Watkins, L. R., Martin, D., Ulrich, P., Tracey, K. J. & Maier, S. F. Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. *Pain* **71,** 225–235 (1997).

427. Benson, S., Engler, H., Wegner, A., Schedlowski, M. & Elsenbruch, S. Elucidating vulnerability to inflammation-induced hyperalgesia: Predictors of increased musculoskeletal pain sensitivity during experimental endotoxemia. *Brain, Behavior, and Immunity* **88,** 302–307 (2020).

428. Karshikoff, B. *et al.* Why sickness hurts: A central mechanism for pain induced by peripheral inflammation. *Brain, Behavior, and Immunity* **57,** 38–46 (2016).

429. Karshikoff, B. *et al.* Modality and sex differences in pain sensitivity during human endotoxemia. *Brain, Behavior, and Immunity* **46,** 35–43 (2015).

430. Martucci, K. T. & Mackey, S. Neuroimaging of Pain. *Anesthesiology,* 1241–1254 (2018).

431. Relland, L. M., Gehred, A. & Maitre, N. L. Behavioral and Physiological Signs for Pain Assessment in Preterm and Term Neonates During a Nociception-Specific Response: A Systematic Review. *Pediatric Neurology* **90,** 13–23 (2019).

432. Buyuktiryaki, M. *et al.* Evaluation of prolonged pain in preterm infants with pneumothorax using heart rate variability analysis and edin (Échelle douleur inconfort nouveau-né, neonatal pain and discomfort scale) scores. *Korean Journal of Pediatrics* **61,** 322–326 (2018).

433. Cremillieux, C., Makhlouf, A., Pichot, V., Trombert, B. & Patural, H. Objective assessment of induced acute pain in neonatology with the Newborn Infant Parasympathetic Evaluation index. *European Journal of Pain (United Kingdom)* **22,** 1071–1079 (2018).

434. Konsman, J. P., Parnet, P. & Dantzer, R. Cytokine-induced sickness behaviour: Mechanisms and implications. *Trends in Neurosciences* **25,** 154–159 (2002).

435. Grossberg, A. J. *et al.* Inflammation-induced lethargy is mediated by suppression of orexin neuron activity. *Journal of Neuroscience* **31,** 11376–11386 (2011).

436. Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. & Kelley, K. W. From inflammation to sickness and depression: When the immune system subjugates the brain. *Nature Reviews Neuroscience* **9,** 46–56 (2008).

437. Bueno, M., Eriksson, M. & Stevens, B. J. in *Oxford Textbook of Pediatric Pain* (eds Stevens, B. J., Hathway, G. & Zempsky, T) 2 edn, 1–45 (Oxford University Press, 2021).

438. The Young Infants Clinical Signs Study Group. Clinical signs that predict severe illness in children under age 2 months: a multicentre study. *The Lancet* **371,** 135–142 (2008).

439. Shimada, A. & Hasegawa-Ishii, S. Histological architecture underlying brain-immune cell-cell interactions and the cerebral response to systemic inflammation. *Frontiers in Immunology* **8** (2017).

440. Boissé, L., Spencer, S. J., Mouihate, A., Vergnolle, N. & Pittman, Q. J. Neonatal immune challenge alters nociception in the adult rat. *Pain* **119,** 133–141 (2005).

441. Denk, F., McMahon, S. B. & Tracey, I. Pain vulnerability: a neurobiological perspective. *Nature Neuroscience* **17,** 192–200 (2014).

442. Sproston, N. R. & Ashworth, J. J. Role of C-reactive protein at sites of inflammation and infection. *Frontiers in Immunology* **9,** 1–11 (2018).

443. Cryan, J. F. & Dinan, T. G. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience* **13,** 701–712 (2012).

444. Mayer, E. A., Knight, R., Mazmanian, S. K., Cryan, J. F. & Tillisch, K. Gut microbes and the brain: Paradigm shift in neuroscience. *Journal of Neuroscience* **34,** 15490–15496 (2014).

445. FDA. FDA-M CERSI: Analgesic Clinical Trial Designs, Extrapolation, and Endpoints in Patients from Birth to Less Than Two Years of Age Public Workshop. *https://www.fda.gov/drugs/news-events-human-drugs/fda-m-cersi-analgesic-clinical-trial-designs-extrapolation-and-endpoints-patients-birth-less-two,* Accessed: 18/01/2022 (2021).

446. Välitalo, P. A. *et al.* Pain and distress caused by endotracheal suctioning in neonates is better quantified by behavioural than physiological items: A comparison based on item response theory modelling. *Pain* **157,** 1611–1617 (2016).

447. Ranger, M., Johnston, C. C. & Anand, K. J. Current Controversies Regarding Pain Assessment in Neonates. *Seminars in Perinatology* **31,** 283–288 (2007).

448. Maxwell, L. G., Fraga, M. V. & Malavolta, C. P. *Assessment of Pain in the Newborn: An Update* **4,** 693–707 (Elsevier Inc., 2019).

449. Roué, J. M., Morag, I., Haddad, W. M., Gholami, B. & Anand, K. J. Using sensor-fusion and machine-learning algorithms to assess acute pain in non-verbal infants: A study protocol. *BMJ Open* **11,** 1–7 (2021).

450. Cohn, J. F. & Ekman, P. in *The New Handbook of Methods in Nonverbal Behavior Research* (eds Harrigan, J., Rosenthal, R. & Scheder, K.) 9–64 (Oxford University Press, 2008).

451. Roué, J. M. *et al.* Multi-modal pain assessment: Are near-infrared spectroscopy, skin conductance, salivary cortisol, physiologic parameters, and Neonatal Facial Coding System interrelated during venepuncture in healthy, term neonates? *Journal of Pain Research* **11,** 2257–2267 (2018).

452. Yuan, I. *et al.* Functional near-infrared spectroscopy to assess pain in neonatal circumcisions. *Paediatric Anaesthesia,* 1–9 (2021).

453. Bucher, H. U. *et al.* Sucrose reduces pain reaction to heel lancing in preterm infants: A placebo-controlled, randomized and masked study. *Pediatric Research* **38,** 332–335 (1995).

454. Kaiser, J., Crespo-Llado, M. M., Turati, C. & Geangu, E. The development of spontaneous facial responses to others' emotions in infancy: An EMG study. *Scientific Reports* **7,** 1–10 (2017).

455. Johnston, C. C., Stevens, B., Craig, K. D. & Grunau, R. V. Developmental changes in pain expression in premature, full-term, two- and four-month-old infants. *Pain* **52,** 201–208 (1993).

456. Hummel, P. & van Dijk, M. Pain assessment: Current status and challenges. *Seminars in Fetal and Neonatal Medicine* **11,** 237–245 (2006).

457. Jones, L. *et al.* EEG, behavioural and physiological recordings following a painful procedure in human neonates. *Scientific Data* **5,** 1–10 (2018).

458. Maitre, N. L. *et al.* Cry presence and amplitude do not reflect cortical processing of painful stimuli in newborns with distinct responses to touch or cold. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **102,** F428–F433 (2017).

459. Klarer, N., Rickenbacher, H., Kasser, S., Depoorter, A. & Wellmann, S. Electrophysiological measurement of noxious-evoked brain activity in neonates using a flat-tip probe coupled to electroencephalography. *Journal of Visualized Experiments* **2017,** 1–5 (2017).

460. Hu, L. & Iannetti, G. D. Painful Issues in Pain Prediction. *Trends in Neurosciences* **39,** 212–220 (2016).