Improving the measurement and treatment of infant pain



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Abstract

Infants undergo numerous medically necessary painful procedures while in hospital, and this early life pain can lead to adverse effects. A range of pain relief interventions are available for use in neonatal units, including pharmacological analgesics, and non-pharmacological comfort measures. However, the literature regarding the efficacy of many of these pain relief interventions is inconclusive and inconsistent, and infant pain is consequently undertreated.

To assess infant pain, behavioural and physiological surrogate measures are utilised in the absence of verbal report. However, as these measures are not direct measures of pain, and can be influenced by other factors such as procedure-related distress, their use can lead to inconsistent conclusions regarding the efficacy of pain relief interventions. The perception of pain manifests in the brain, therefore it is plausible that measures of brain activity can provide specific and objective indications of the infant pain experience, and that these measures can be used to assess the efficacy of pain relief interventions. In this thesis, noxious-evoked brain activity is measured and used to investigate the effect of a pharmacological analgesic, paracetamol, and a non-pharmacological pain relief intervention, gentle touch targeted towards C-tactile (CT) fibres.

Noxious-evoked brain activity has been well characterised following medically required heel lances and experimental noxious stimuli, but brain activity evoked by alternative clinically relevant stimuli must also be characterised to assess the efficacy of a wider range of pain relief interventions for these procedures. In this thesis, brain activity evoked by immunisation is characterised, enabling the analgesic efficacy of paracetamol to be tested during routine infant immunisations.

Between-subject comparisons are often necessary when investigating the efficacy of pain relief interventions for medical procedures. However, there is great individual variability in nociceptive sensitivity, and controlling for this variability could enable better between-group comparisons. In the last section of this thesis, infant nociceptive sensitivity is measured and accounted for, in order to explore how this approach can be used to assess intervention efficacy, using the example of gentle touch targeted towards CT fibres.

Overall, the aims of the research described in this thesis are to advance knowledge regarding how infants respond to noxious clinical procedures, how pain relief interventions can modulate these responses, and how individual variability can be accounted for to enable better assessment of intervention efficacy. Research in the area of infant pain management is critical to inform clinical practise and improve the treatment of infant pain.

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Abbreviations

BIIP	Behavioural Indicators of Infant Pain
BOLD	Blood Oxygen Level-Dependent imaging
BNFc	British National Formulary for Children
CNS	Central Nervous System
COX	Cyclooxygenase
CT	C-tactile fibres
DC	Direct Current
ECG	Electrocardiography
EDIN	Echelle Douleur Inconfort Nouveau-Ne
EEG	Electroencephalography
EMG	Electromyography
EMLA	Eutectic Mixture of Local Anaesthetics
ERP	Event-Related Potential
fMRI	functional Magnetic Resonance Imaging
GABA	gamma-Aminobutyric acid
HDU	High Dependency Unit
HIE	Hypoxic ischaemic encephalopathy
HFT	High flow oxygen therapy
IASP	International Association for the Study of Pain
IQ	Intelligence Quotient

ITU	Intensive Care Unit
IV	Intravenous
IVH	Intraventricular haemorrhage
LDU	Low Dependency Unit
LED	Light Emitting Diode
LFT	Low flow oxygen therapy
MRI	Magnetic Resonance Imaging
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIPS	Neonatal Infant Pain Score
NIRS	Near Infra-Red Spectroscopy
PC	Principal Component
PCA	Principal Component Analysis
PIL	Patient Information Leaflet
PIPP-R	Premature Infant Pain Profile-Revised
RMS	Root Mean Squared
ROP	Retinopathy of Prematurity
SSC	Skin-to-skin care
SVIA	Self-ventilating in air

Chapter 1: Introduction

1.1 Thesis overview

The International Association for the Study of Pain (IASP) defines pain as 'an unpleasant sensory or emotional experience'[1], demonstrating that pain is a subjective perception. The task force specifically notes that 'the inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment'. This statement is of prime relevance in non-verbal infants and summarises the basis on which this thesis is grounded: infants cannot describe their pain experience, and therefore their pain levels, as well as the efficacy and safety of potential pain relief mechanisms, must be interpreted from surrogate measures of pain.

The issue of pain management in infants is critical, given the adverse short- and long-term side effects of early life pain, including physiological instability, altered pain processing and sensory thresholds, altered brain structure, and compromised neurocognitive development later in life [2] [3]. Historically, it was not recognised that infants feel pain, based on observations of immature nociceptive responses and the continued development of the nervous system in the postnatal period [4]. However, it is now accepted that infant pain needs to be effectively managed.

A range of interventions are available for use on neonatal units, including pharmacological interventions such as paracetamol and opioids, recommended based on their established efficacy in adults, and non-pharmacological tactile-

based interventions such as skin-to-skin contact (SSC) and swaddling, favoured for their lack of adverse effects [5]. However, there is a paucity of evidence regarding the efficacy and safety of many of these pain relief interventions, and infant pain is still under-detected and under-treated [6], potentially due to the lack of established guidelines and conclusions regarding intervention efficacy [7].

A possible reason for the inconsistencies in the findings regarding analgesic efficacy is the behavioural and physiological measures used to assess infant pain, as these can be influenced by distress [8 9]. As the brain is where the experience of pain is ultimately created [10], brain activity may provide a specific and objective indication of infant pain processing and the infant pain experience. Infant brain activity evoked by noxious stimulation has recently been characterised [11] and formed into a template of noxious-evoked brain activity [12]. This template has been demonstrated to be specific, sensitive, and correlated with pain-evoked facial expressions, and is validated for use in independent data sets, where it can be fitted to novel infant responses to quantify the magnitude of noxious-evoked brain activity present [12].

The template has also been used to assess analgesic efficacy: the effect of topical local anaesthetic has been evaluated [12], and the template has been validated for use in a clinical trial investigating the effect of morphine on infant brain activity [13 14]. In Chapter 3 of this thesis, this template of noxious-evoked brain activity is utilised to investigate the efficacy of the non-pharmacological tactile intervention, gentle touch targeted towards C-tactile (CT) fibres.

A limitation of the template is that it has only been validated for use on the previously characterised stimuli clinically relevant heel lances and experimental noxious stimuli. However, infants undergo many different clinically necessary procedures in the neonatal unit, and it is important to characterise further procedures in order to test alternative analgesics for these stimuli. In Chapter 4 of this thesis, the brain activity evoked by immunisation is characterised, and its suitability to be fit with the template is assessed. This would enable the use of this validated method to assess analgesic efficacy specifically for the process of immunisation. As infants receive paracetamol as an antipyretic to help manage the fever that may result for immunisation, in line with NHS guidelines [15], after charactering immunisation-evoked brain activity, the effect of paracetamol in reducing this evoked activity was investigated.

Many factors affect infant nociceptive sensitivity, including gestational age, prior pain, behavioural state, and illness [16], and the effects of gestational age on nociceptive responses have been well characterised [17] [18]. In the investigations composing this thesis, efforts are made to conduct within-subject comparisons to control for individual variability, or as in Chapter 3 of this thesis, to age-match infants in order to control for the effect of gestational age on nociceptive responses. However, in Chapter 5 of this thesis, a better alternative for more comprehensively accounting for individual pain variability is explored, by investigating whether taking infant nociceptive sensitivity into account can aid between-subject comparisons of pain intervention efficacy, using the example of CT-targeted touch.

While the primary surrogate measure of pain investigated in this thesis is the measurement of brain activity using EEG, signs of infant pain manifest at different levels of the nervous system. Therefore, reflex withdrawal and behavioural and physiological indicators of pain are also assessed to provide a comprehensive, multimodal estimate of the infant pain experience and how it is modulated by pharmacological and non-pharmacological pain relief interventions, and in response to novel clinically relevant stimuli.

Prior to presenting my results, the background information intrinsic to this thesis will be provided. In the Introduction, pain and nociception will first be defined, and the journey of noxious input from the periphery to the spinal cord and brain will be detailed. Next, the development of infant nociceptive circuitry and brain responses evoked by pain will be discussed, highlighting the many developmental changes that occur over the preterm period which must be considered when investigating infant pain. The immaturity of the nervous system at birth added to the now out-dated notion that infants do not feel pain, and these beliefs are reviewed, before discussing the current epidemiology of pain and its persisting prevalence. The consequences of early life pain are then reviewed, demonstrating the critical importance of managing infant pain. A range of different surrogate measures can be used to measure infant pain, including behavioural and physiological measures, as well as reflex withdrawal and several methods of brain imaging. The insights into the infant pain experience that each of these measures has provided, as well as the contextual factors that modulate these measures, are discussed. Lastly, the pharmacological and non-pharmacological pain relief interventions available for use on neonatal units are detailed, and examples in

which the measurement of infant brain activity has been used to assess analgesic efficacy are discussed, highlighting the previous work that forms the foundation on which this thesis is built.

1.2 The distinction between nociception and pain

Nociception is defined as 'the neural process of encoding noxious stimuli', and pain as 'an unpleasant sensory or emotional experience' [1] indicating that nociception refers to the transmission of noxious-related (nociceptive) information from the periphery along the spinal cord to brain regions, while pain is a subjective perception created in the cortex. The distinction between these terms is illustrated by the example that it is possible to feel pain in the absence of a noxious input or nociception, for example in neuropathic or chronic pain, while nociception does not necessarily result in the experience of pain, for example in soldiers wounded during battle [19], or in spinal cord transection animal models where nociceptive information can reach the spinal cord but pain behaviour is not displayed [20].

In the context of acute mechanical noxious stimuli, which will be investigated in this thesis, the journey from nociception to the experience of pain begins with the detection of noxious stimuli by primary sensory neurons in the skin [21] [22]. Nociceptive information is conveyed by A-delta or C-fibres; A-delta fibres are of larger diameter and are myelinated, meaning they can transmit information quickly and are believed to convey the immediate, alerting, localised 'sharp' sense of pain. A-delta fibre activation triggers reflex withdrawal, which is intended to protect the body from harm. The more numerous C-fibres are of smaller diameter and are

unmyelinated, meaning they transmit information more slowly, and convey the slower, less well localised, and 'dull' aspect of the pain experience [23] [24] [25] [26]. These A-delta and C-fibres carrying nociceptive information are distinct from A-beta, and C-tactile fibres, which convey information about touch stimulation of lower intensity [27].

A-delta and C-fibres synapse onto second order neurons in the dorsal horn of the spinal cord. This is the first point at which nociceptive information can be modulated in the central nervous system (CNS), as the dorsal horn integrates sensory information from a range of primary receptors, including those receiving touch stimulation [28], as well as receiving descending modulation from neurons originating in the brain [29] [26].

Second order neurons then decussate the spinal horn and ascend via the spinothalamic tract to the brainstem and thalamus [22] [26] [30], before reaching the cortex. Areas in the brainstem such as the periaqueductal gray and locus coereleus are involved in autonomic functions in response to threat such as increasing heart rate and respiration [31] [32] [33], and areas including the periaqueductal gray and rostroventral medulla are involved in the descending modulation of nociceptive signals [29] [34].

In contrast to the region-specific cortical processing of visual, touch, and auditory stimuli, there is no specific 'pain cortex'. Instead, nociceptive signals activate a range of areas that are associated with both sensory and emotional processing, including the somatosensory, insular, anterior cingulate, and frontal cortex [35] [36] [10].

The cortex is where the experience of pain arises [10], and the majority of modulation occurs here, with factors such as attention, mood, and prior expectations altering cortical activity and pain perception [37 38 39]. Cortical regions project back down via the periaqueductal gray and rostroventral medulla to the spinal cord to provide this descending pain modulation. Both inhibitory and facilitatory modulation are possible and have evolutionary value depending on the circumstances; it can be important to realise injury in order to avoid further damage and protect the affected area, but it can also be important to be able to focus on the goal of survival while ignoring the injury [29 40].

Past research has attempted to identify a 'pain matrix' of brain regions that reflect the experience of pain [41 42], and signatures have been formed that distinguish the perception of painful stimuli from non-painful warmth stimuli [43 44], pain expectations [45], and pain recall, and are reduced by analgesics [46]. However, the brain regions activated by noxious stimuli vary depending on contextual factors and individual differences [47 48], and other research has demonstrated that similar patterns of brain activity are active following non-painful auditory, tactile, and visual sensory stimulation [49]. Additionally, patients with a congenital insensitivity to pain also demonstrate activity in the supposed 'pain matrix' [50], indicating that the activation is not pain-specific [49] and may instead reflect the salience of a stimulus [51]. It is instead believed that nociceptive information is transmitted to the brain and pain is perceived by a fluid system comprised of multiple brain regions that are not solely pain-specific [52 10 53].

EEG can also be used to record evoked brain activity in the time domain following noxious stimulation. While the range of stimuli and time periods investigated have not yet led to a thorough understanding of adult noxious-evoked brain activity, event-related potentials (ERPs) can be observed following mechanical noxious stimulation that are composed of several components. An early deflection is comparable to activity evoked following non-noxious touch stimulation [54 55 56], and may be related to generalised tactile sensory processing, as the latency of this response is compatible with A-beta (tactile) as well as A-delta (nociceptive) fibre conduction velocities, and this response is consistently present regardless of the perception of pain [57]. The later component of the response may be reflective of pain perception, as the magnitude of this deflection is correlated with pain reports[57 58], and both the magnitude of the evoked response as well as the perception of pain are increased by application of the irritant substance capsaicin prior to noxious stimulation [58]. However, as mentioned above, this component could also be reflecting perceived stimulus intensity rather than reflecting pain specifically.

Given that pain is a subjective perception, it follows that verbal reports are the gold standard of pain reporting. Verbal pain reports generally correlate with brain activity characterised by fMRI, EEG-recorded evoked potentials, and EEG-recorded frequency changes following mechanical, laser and thermal noxious stimulation [59 60 58 61 57 62], and this brain activity is altered by factors expected to influence the experience of pain, including pain relief interventions [63] such as morphine [64], pregabalin and duloxetine [65], oxytocin [66], and emotional modulation [67]. In non-verbal infants who cannot vocalise their pain experience, brain imaging can

provide a good indication of nociceptive processing, even if it is not known exactly how this relates to the pain experience.

1.3 The early development of the nervous system

The many changes that occur over the developmental period influence the manner in which noxious stimuli are processed. In the first part of this section, the development of nociceptive processing is detailed, focussing on the peripheral cutaneous afferents that synapse in the dorsal horn and evoke reflex withdrawal, before considering the spinothalamic tract and its role in descending modulation, and finally reaching the development of the brain. In the second part of this section, research utilising brain imaging techniques to elucidate infant brain development is detailed, as the use of these methods can provide valuable information regarding developmental changes in brain structure, brain activity at rest, and brain activity in response to sensory stimuli. The use of EEG-recorded ERPs to characterise the development of sensory processing is of great relevance when later considering noxious-evoked potentials in infants.

1.3.1 The neurodevelopment of nociceptive circuitry

Peripheral nervous system neurons extend down the limbs to innervate the skin during embryonic development, and cutaneous nociceptors and mechanoreceptors are present in the skin and are functional from birth [68] [69] [70]. However, A- and C-fibre characteristics mature over the developmental period in rat pups, becoming less sluggish, decreasing in latency, increasing in peak firing rate, decreasing in

receptor field size, and demonstrating more convergence of multiple fibres onto dorsal horn cells with age [71].

Correspondingly, the reflexes that mechanical and noxious stimuli trigger in both rat pups and human infants become more refined, decrease in amplitude, duration and latency, and require higher intensities for activation with age [72 17 73 74 75]. Additionally, the sensitisation observed following repeated tactile stimulation in preterm infants (< 34 gestational weeks) and newborn rat pups changes to habituation during development [73 74]. The purpose of reflex withdrawal is to protect the body from harm, and this is exemplified by the gradual decrease in erroneous reflex movements (towards rather than away from the noxious stimulus) in rat pups during development [76].

It is postulated that the emergence of descending inhibition from the brain to the spinal cord underlies the development of reflex withdrawal. Although spinothalamic tract neurons are also present prior to birth in rat pups [77], these projections do not convey descending modulation in the early postnatal period. While stimulation of brain areas involved in descending modulation such as the periaqueductal gray and rostroventral medulla region in adult animals inhibits noxious-evoked spinal cord responses and reflexes [78 79], in rat pups stimulation of the periaqueductal gray and the dorsolateral funiculus (which receives input from brain regions involved in descending modulation) do not induce adult-like spinal cord cell or reflex withdrawal inhibition until after approximately three weeks of life, and higher intensities are needed to produce inhibition at younger postnatal ages [80 81].

The reason for this lack of descending modulation could be due to the excitatory, rather than inhibitory, effect of neurotransmitters in early stages of development. Hathway et al. [82] [83] have demonstrated that the neurotransmitter GABA, which is typically inhibitory in adults, has an excitatory influence on the rat pup spinal cord until approximately the third postnatal week. This effect is believed to be mediated by the rostroventral medulla, as administering a GABA antagonist to the rostroventral medulla decreases rat pup spinal nociceptive reflexes, and the switch is proposed to be opioid-dependent, as μ-opioid antagonist administration during the critical period (postnatal day 21 – 28) prevents the normal developmental switch to inhibitory control. The developmental switch of GABA to inhibitory control could be dependent on developmentally regulated ion transporters, which alter the intracellular potential such that GABA could cause depolarisation [84]. The postnatal emergence of inhibitory influences is also observed with respect to the neurotransmitter glycine, which does not exert inhibition until the second postnatal week [85] [86], and the excitatory-inhibitory switch of these neurotransmitters could contribute to the development of descending inhibition and the refinement of spinal cord responses and reflex withdrawal.

Thalamocortical and cortico-cortical connections are formed via the aid of the transient subplate structure; from the 24^{th} – 26^{th} gestational week neurons 'waiting' in the subplate penetrate the cortical plate, and synapse with cortical neurons until ~ 32 gestational weeks [87] [88] [89] [90] [91]. Interhemispheric connections are functional from 24 weeks post conception [92], and after ~ 30 weeks gestation the cortex is divided into six layers as seen in adults [93] [88]. The dissolution of the subplate begins

in the last weeks of gestation (33 – 35 weeks), but the existence of this structure is still evident postnatally in some cortical areas [93 88 94].

Likewise, cortical synaptic density peaks postnatally, and neurogenesis, neural migration, synaptic pruning, and myelination continue in the first postnatal year [92]. Although cerebral myelination begins in the second trimester of pregnancy, it peaks at 1 postnatal year, and along with synaptic pruning, has been demonstrated to continue into adolescence [95 96], indicating that the brain continues to develop well after the preterm period. While in-utero brain imaging is possible, and ultrasound and MRI have been used to image the foetal brain and detail age-related changes in grey matter, myelination, cortical thickness and brain region location [97 98 99 100 101], the majority of brain imaging research has focussed on postnatal infants, as detailed below.

1.3.2 Imaging the developing brain

Animal models and cytoarchitecture studies have been fundamental in investigating development, but the advent of non-invasive brain imaging has enabled remarkable insight into human development [92]. Multiple brain imaging techniques have been used in infants, including NIRS, fMRI, and EEG, and these techniques are discussed in more detail below (Section 1.6.3). MRI has been used to investigate the development of the brain, detailing changes in cortical gyrification and white mater tracts [102 103 95 104]. MRI images of the brain in infants at 28 weeks gestational age, 34 weeks gestational age, and 40 weeks gestational age have been constructed into 3D models of the brain (Figure 1.1) and

demonstrate the trajectory over which the brain develops in terms of size and cortical folding. MRI has also been used to investigate resting state brain activity [105] and demonstrate sensory-evoked functional responses in infants [106] [107], and likewise, NIRS has been able to demonstrate the cortical processing of sensory input, including visual [108] and olfactory stimuli [109], and ultrasound has been used to identify brain injury and predict developmental outcome in postnatal infants [110] [111].

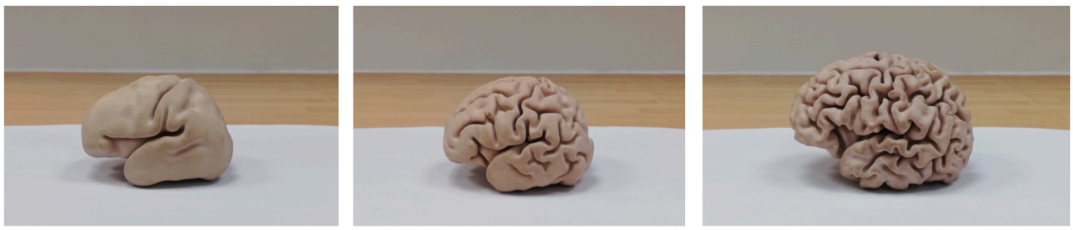


Figure 1.1. Cortical development. 3D models of the brain based on MRI scans at 28, 34, and 40 weeks' gestational age created by the Paediatric Neuroimaging Group, University of Oxford.

The early development of human brain activity has been well characterised using EEG [112] [113] [114], demonstrating that spontaneous brain activity, which is important for the development of cortical connectivity and brain-spinal cord connection [115] [116] [117] [118], changes over the postnatal preterm period. Inter-burst intervals (periods of electrical silence) and discontinuous patterns decrease with age, as do the occurrence of immature activity patterns such as delta brushes (delta waves with superimposed high frequency activity), and burst duration and continuous patterns increase with age [113] [119]. An example of EEG activity in a term infant, and in an ex-preterm infant who is displaying immature brain activity, are displayed in Figure 1.2. By characterising the maturation of the background brain activity and

brain structure, imaging techniques also enable clinical diagnoses and prognosis estimation for conditions such as epilepsy [120], hypoxic-ischaemic encephalography (HIE) [121] [122], intraventricular haemorrhage (IVH) [123], white matter injury [124], and neurodevelopmental delay [125] [126] [127].

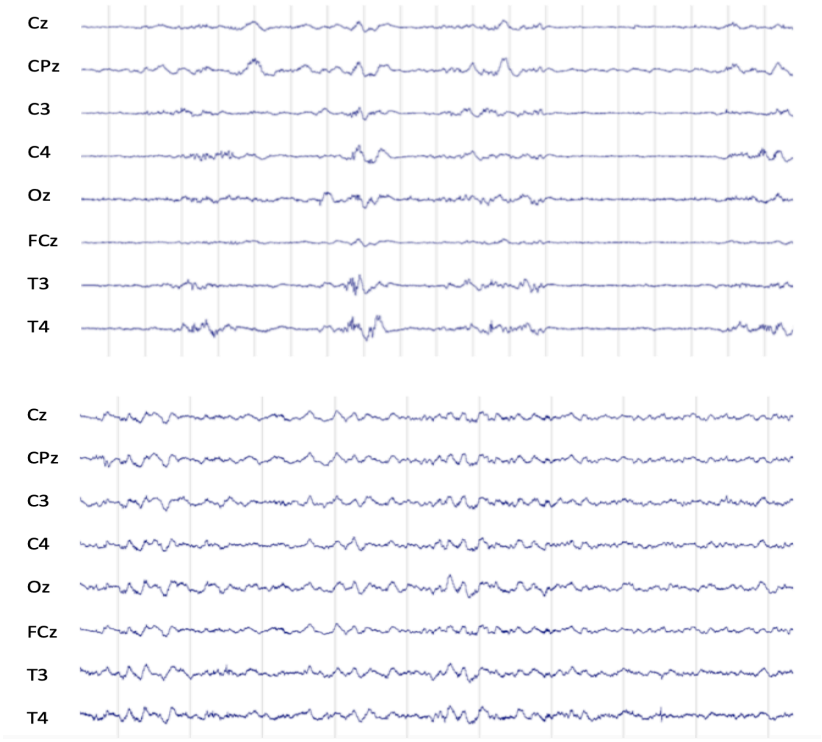


Figure 1.2 EEG activity of an ex-preterm and term infant. Top: ex-preterm infant born at 26+2 gestational weeks, currently at 36+6 gestational weeks, 74 postnatal days, displaying immature resting brain activity. Bottom: term infant at 42+5 gestational weeks, 4 postnatal days, displaying mature term brain activity. The contrast between these traces of electrical activity visually illustrates the developmental changes that occur over the preterm period.

ERPs can be observed following sensory stimulation, and can also be used to gain great insight into the development of sensory processing in typical and pathological states. Sensory event-related potentials mature with gestational age in morphology and latency, becoming sharper and larger in amplitude [128]; visual-

evoked responses have been observed in preterm infants starting at ~ 24 weeks gestational age, and become more defined and decrease in latency over development [129] [130] [131], auditory-evoked potentials are reported to become more consistent in the relative amplitudes of the evoked components after ~ 34 weeks [132] [133] [134], and while shorter-latency somatosensory-evoked potentials have characteristic topography from ~ 31 weeks post-menstrual age [135], longer-latency somatosensory-evoked potentials mature over the late preterm period and shift from contralateral to bilateral with age, possibly suggesting a reflection of higher-order somatosensory processing [136].

In young infants (< 30 weeks gestational age), the slow deflections evoked by different modalities of stimulation are comparable in morphology, but these responses become dissimilar with gestational age, and the development of defined primary components over the preterm period may follow different time-courses in the different modalities: somatosensory-evoked responses were well defined after 29 weeks gestation, whereas visual-evoked responses began to appear between 32 and 35 weeks [137]. Likewise, modality-specific development may follow difficult time-courses across the life span [138]: the visual response has been observed to mature over the first 6 months of life after which it is reported to be comparable to the adult response [139] [130], while auditory-evoked potentials continue to mature through childhood until adulthood: evoked responses are associated with significantly shorter latencies and differing component amplitudes in adults than newborns, toddlers, and 6-year old children, and newborns and toddlers demonstrate a diffuse response distribution across the scalp, while responses in children and adults are more focal [140].

ERP recording could be utilised as a bedside method of sensory system testing [141]; ERPs can be used to gauge the maturity of sensory processing relative to age, and ERPs are altered in infants with in-utero exposure to methadone [142]. Additionally, alterations in ERPs can predict neuromotor impairments in very preterm infants and full-term infants with birth asphyxia [143] [144] and in infants following therapeutic hypothermia for HIE [145]. While measurement of sensory ERPs is useful as it can provide insight into sensory development and can be used to aid diagnosis, measurement of noxious-evoked potentials can also provide information that can be used to answer important questions regarding infant pain processing, and can be used to help assess analgesic efficacy and manage infant pain. Noxious-evoked brain activity will be detailed in Section 1.6.3.3 below, after the history and importance of managing infant pain are highlighted.

1.4 History and prevalence of neonatal pain

Up until the 1980s it was widely believed that neonates did not feel pain, as they did not demonstrate signs of distress, or have memory of the occasion later in life [4]. Additionally, as the infant nervous system is not fully developed at birth and myelination is not complete [95], it was believed that pain processing did not occur. Several investigations into infant responses to noxious stimuli such as pin pricks concluded that the sensorimotor responses tested were 'imperfect at birth' but 'showed an increase in adequacy with the advance in age' [146], and that some infants exhibited 'no overt response to cutaneous irritation such as pin prick' [147]. Additionally, it was common practice to operate and perform clinical procedures

without pain relief [148] [149], as 'the facility of controlling a child of this age, together with the fact that it has neither the anticipation nor remembrance of suffering, however severe, seems to render this stage of narcotism unnecessary' [150].

In the mid 1980s, evidence demonstrating that anaesthesia improves post-surgical outcomes was published [151] [152], interest in neonatal pain increased [153], and the notion that infants are capable of perceiving pain was widely acknowledged [2]. Now, guidelines on neonatal pain management have been published, and surgery is no longer performed without anaesthesia [154] [155] [156] [157].

However, infants are still undergoing many painful procedures without adequate analgesia: records from European hospitals in 2008 and 2014 indicated that infants received on average 11 - 16 painful procedures per day (such as heel lances, venepuncture, cannulation, chest tube drainage, gastric tube insertion, and immunisations), summing to a total median of ~ 115 painful procedures during their hospitalisation. The majority (two thirds – three quarters) of these procedures were performed without analgesia [158] [159].

Additionally, analyses of a range of European hospitals have demonstrated variation in pain management administration both within and between countries, and have concluded that post-operative pain management, and interventions such as non-nutritive sucking, swaddling, and oral sweet solution are underused and highly variable [160] [161] [162]. It is likely that the large percentage of procedures performed without adequate pain management is due to the difficulties associated with interpreting infant pain and the uncertain efficacy of pain relief interventions

in infants, as well as a lack of pain education in clinical care teams [163]. However, the significant adverse short- and long-term consequences of neonatal pain, detailed in the following section, demonstrate the critical need to treat infant pain.

1.5 Consequences of neonatal pain

Short-term effects elicited by pain include increased heart rate [164] and decreased respiration and blood oxygen saturation [165] [166] [167] [2], which can be perilous as they can compromise the infant's physiological stability.

Numerous studies have investigated the long-term effects of early life pain, exploring sequelae including restricted growth, and altered sensory development, neurocognition, and brain structure and function from infancy to early adulthood. In infancy, greater exposure to painful procedures in early postnatal life is associated with lower weight at term age [168], and term infants who have experienced a higher number of prior painful procedures demonstrate altered brain activity, behaviour, and physiological responses to painful stimuli - brain activity and physiological changes are reported to be increased following prior pain [169] [170], but the effect of prior pain on behaviour has not been clearly characterised, with some studies reporting blunted facial expressions [170] [171], but others reporting exaggerated facial responses and crying [172], as is further detailed in the section regarding the effect of prior pain on surrogate measures of infant pain below (Section 1.7.2).

Prior pain has also been demonstrated to alter responses to light touch in the neonatal period [173], and differences in sensory processing have been demonstrated to persist into childhood and adolescence. These have not been clearly characterised; prior pain is associated with higher pain intensity reports [174], greater perceptual sensitisation, less habituation to tonic heat [175] [176], more pain catastrophising behaviour [176], and exaggerated responses to heat stimuli in school-age children [177], yet heat pain thresholds have also been reported to be elevated, prior pain did not alter mechanical pain thresholds or perceptual sensitisation [175], and number of early life surgeries is negatively correlated with pain intensity [174]. It may be that the wide range of procedures and time periods of prior pain exposure investigated are precluding a clear characterisation of the effect of early life pain, but this research nevertheless demonstrates that early life pain has the capacity to alter the development of sensory processing.

The development of brain structure, brain activity, and behaviour are also affected by early life pain [178]; neonatal pain has been associated with the delayed development of the corticospinal tract in infants [179], and exposure to painful procedures is associated with poor cognition and motor function at toddler age [180]. At school age, altered white matter structure has been demonstrated and is associated with reduced IQ [181], and cortical thickness was reduced in several regions including the frontal and parietal lobes [182]. A greater number of neonatal invasive procedures was associated with reduced volumes in the amygdala and thalamus, and this was related to poorer cognitive, visual-motor, and behavioural outcomes [183]. Additionally, background cortical rhythmicity was altered, which was associated with poorer visual-perception abilities [184]. Continued research is

now beginning to report differences in pain sensitivity, thresholds, and brain structure persisting into adulthood [185]. Given the detrimental long-term impacts of early life pain, the importance of better understanding infant responses to painful procedures and identifying effective pain relief strategies is clear. Accurate measurement of infant pain is crucial, and the range of measures utilised to assess neonatal pain are detailed below.

1.6 Measurement of neonatal pain

Assessment of neonatal pain necessarily relies on surrogate measures of the pain experience. Clinically, behavioural and physiological measures are used as indicators of infant pain, while methods of brain imaging are used for research. The different surrogate measures of pain used in infants are depicted in Figure 1.3.

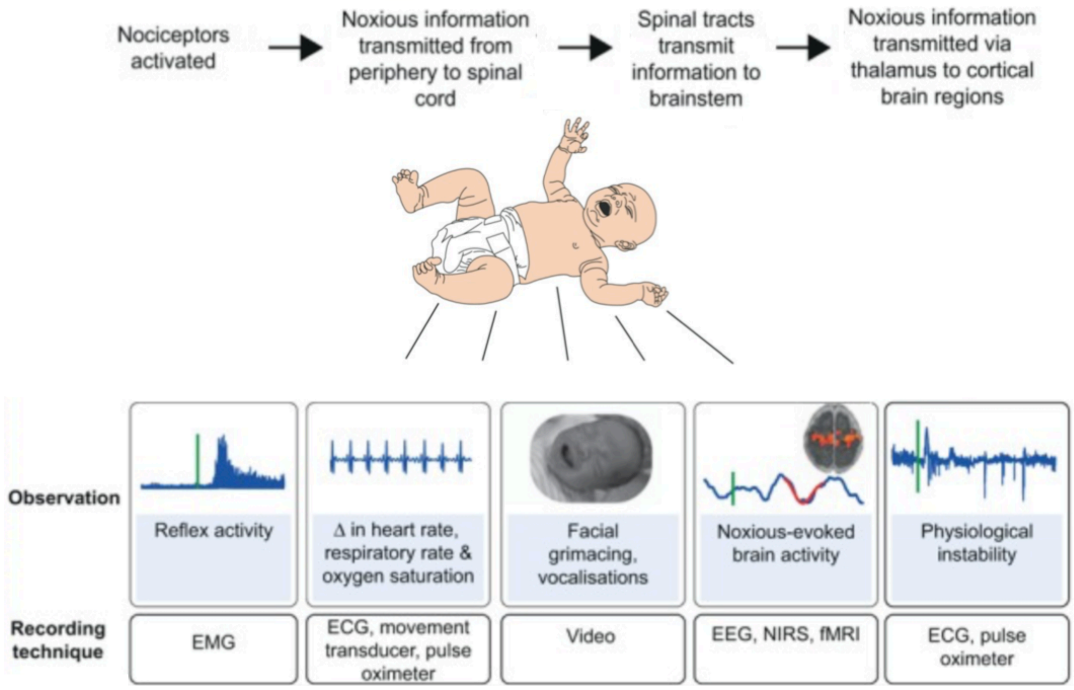


Figure 1.3 Schematic of the surrogate measures used to estimate pain in non-verbal infants. A range of indicators can be recorded, which measure different aspects of the response to pain across the body. Figure modified with permission from Moultrie et al., (2017)[7].

1.6.1 Behavioural and physiological measures

Behaviours used as surrogate measures of acute pain include facial expressions (such as brow bulge, nasolabial furrow, eye squeeze, facial muscle tension, and grimace), as well as limb and torso movements and crying. Physiological surrogate measures of pain refer to heart rate and oxygen saturations. Facial indicators and crying have likely evolved to alert the caregiver that attention is needed, while heart rate may increase to prepare the body for 'fight or flight'. These measures can be readily observed in the clinical setting, or in retrospective analysis [2 165]. There are many composite measures of pain, including the Premature Infant Pain

Profile-Revised (PIPP-R) [186] [187], Neonatal Infant Pain Score (NIPS) [188], Behavioural Indicators of Infant Pain (BIIP) [189], Echelle Douleur Inconfort Nouveau-Ne (EDIN) [190] and the COMFORT scale [191], which take into account a range of behavioural and physiological variables to provide a more comprehensive estimate of infant pain.

The PIPP-R profile, which measures facial expressions (brow bulge, eye squeeze, and nasolabial furrow) and physiology (heart rate and oxygen saturations) and also accounts for infant gestational age and behavioural state, is well validated for acute and procedural pain (heel lances, venipuncture, immunisations) in term and preterm (26 – 37+ weeks) infants [186] [187] [192] [193] [194] [195] [196]. The PIPP-R has been utilised in multiple investigations and randomised controlled trials [197] [198] [14] [199] [200] and is used in this thesis.

However, there are several limitations associated with the use of behavioural and physiological surrogate measures. There is low correlation between these two indicators of pain [201], with behavioural measures demonstrating more specificity and sensitivity than physiological indicators – facial expressions were more commonly observed solely after heel lancing, whereas heart rate was also increased by tactile contact [165], and interventions such as sucrose administration and rocking have been demonstrated to decrease facial expressions but not heart rate [202].

Behavioural and physiological measures are also influenced by distress: infants demonstrate pain scale score responses indicative of pain following a (non-painful)

nappy change [8] and cleaning of the skin prior to venipuncture [203], and pain scale scores do not discriminate between distressed infants (without any noxious input) and these same infants after immunisation [9]. Additionally, neonatal staff report difficulty with assessing pain using these indicators [204], and many contextual factors, such as age, behavioural state, and prior pain also influence these behavioural and physiological measures, as is detailed in the 'Factors affecting individual sensitivity to noxious stimulation' section below (Section 1.7), meaning that pain responses may be masked in some contexts.

1.6.2 Reflex withdrawal

Muscle movement in response to noxious stimulation can be evaluated by measuring reflex withdrawal using electromyography (EMG), which involves the placement of surface electrodes on the skin overlying muscle to quantify muscle activity. Reflexes occur in both adults and infants in response to noxious stimulation in order to protect the body by minimising contact with potential harm, and in adult studies, reflexes are measured to provide an objective quantification of spinal cord activity under the control of brain modulation [205]. Several studies have demonstrated correlation between reflex withdrawal threshold and pain perception [206 207], although other research suggests that the relationship between the two is not distinct [208] and that interventions do not always modulate the two measures in a consistent manner [205].

In infants, nociceptive reflexes exhibit intensity encoding, correlating with the intensity of the noxious stimulus eliciting the reflex [209], and the magnitude of the

reflex withdrawal is also correlated with the magnitude of EEG-recorded noxious-evoked brain activity [210]. However, like behavioural and physiological measures, reflexes are not specific to noxious input: reflexes also occur in response to tactile stimulation, albeit at lower amplitude, in younger gestational age infants and also in term infants [72] .

As detailed earlier, infant reflexes are refined over the developmental period, becoming more organised, and decreasing in amplitude, duration, and latency with age [72 17 73 74 75], and this occurs concomitantly with the maturation of brain regions involved in descending modulation of the spinal cord [17 80 81 82 83]. These developmental changes highlight the importance of accounting for gestational age and stage of developmental maturity when assessing nociceptive reflex responses.

1.6.3 Brain imaging

Behavioural and physiological measures of pain and reflex withdrawal do not solely reflect pain [201 9 203 8 72]. As the brain is where the experience of pain is ultimately created [10], it follows that in the absence of a verbal report, brain activity may be able to provide the most specific indication of pain processing and perception possible. Below are detailed the three methods of non-invasive brain imaging that have been used in neonates to measure noxious-evoked responses, and how they have advanced knowledge in the field of neonatal pain processing.

1.6.3.1 NIRS (Near-infrared spectroscopy)

NIRS measures changes in cerebral oxygenation to infer functional brain activity, relying on the assumption that increased tissue oxygenation reflects an increase in blood flow due to increased cortical activity [211] [212] [213] [214], and is used in adults as well as infants [215]. Due to its portability and ease of application, NIRS was the first measure of brain activity used to investigate whether infants process nociceptive input at the level of the cortex. Increased cerebral oxygenation was observed following clinically necessary noxious stimuli (heel lance blood tests and venipuncture) [216] [217] [218] [219], but not non-noxious control stimuli [216] [217], demonstrating that nociceptive information is transmitted to the cortex, and dispelling any remaining notion that infants are not capable of processing painful stimuli.

Further research has demonstrated that pain-related NIRS signals are present in sedated infants following chest drain removal, despite dampened brain behaviours [220]. This demonstrates the ability of a brain-based imaging technique to provide information about the pain experience that cannot be detected by observation alone. However, NIRS has several limitations, including a low temporal resolution, and the reliance that the observed signals and their interpretation is based on the assumption that increased brain activity results in increased blood flow, though this neuro-vascular coupling relationship may be influenced by many factors [221].

1.6.3.2 fMRI (functional Magnetic Resonance Imaging)

Like NIRS, fMRI also provides an indirect measure of brain activity, as it also measures changes in blood oxygenation to infer changes in brain activity via a technique called blood oxygen level dependent (BOLD) imaging. While fMRI has a much higher spatial resolution than NIRS, it is not portable. Using fMRI, the brain regions active in adults in different behavioural states and following the application of different stimuli can be identified, as has been done following noxious stimulation and pain perception reporting, where regions including the primary somatosensory cortex, prefrontal cortex, anterior cingulate cortex, thalamus, insula, and amygdala were identified to be active [35] [36] [10]. In an early study characterising the infant response to noxious stimulation, the pattern of brain activity observed closely resembled that observed during the adult experience of pain, including brain regions involved in both sensory and affective processing [222] (Figure 1.4). Although this may suggest that the infant experience of pain is similar to that of an adults', it is not possible to draw this conclusion with any certainty, as infants cannot communicate the nature of their experience.

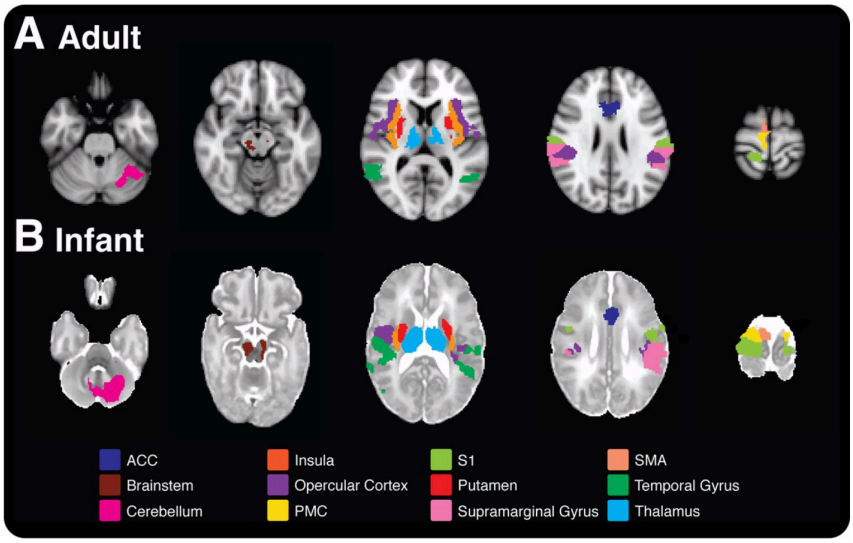


Figure 1.4 Similarity between brain region activation following noxious stimulation in adults and infants. Figure displayed with permission from Goksan et al. (2015)[222], demonstrating that similar brain regions are activated following experimental noxious stimulation in adults and infants.

Further investigation of the brain regions active in neonates after noxious stimulation has demonstrated that greater connectivity between the regions involved in descending modulation of pain signals is correlated with lower noxious-evoked brain activity [223], suggesting that from a young age, modulation in the brain may influence the magnitude of the response to noxious stimulation. This may appear to be in contrast with the animal studies detailed earlier demonstrating that in early development the descending modulation of the spinal cord from the brain is immature [80 81 82 83]; it may be that at birth, modulation of nociceptive input in the brain is more mature than modulation of the spinal cord, or these differences may be due to species differences. The relationship between surrogate measures of

pain at different developmental stages is important to consider when assessing infant pain.

1.6.3.3 EEG (Electroencephalography)

EEG can provide a more direct indication of brain activity than fMRI and NIRS, as it records neural electrical activity with an array of small electrodes placed on the scalp. Electrical activity is generated by the movement of positive and negative ions in and out of neurons firing action potentials, and the sum of activity in these neurons generates electric field potentials. EEG records this summed activity from neural populations; a single EEG electrode is estimated to detect synchronised activity from brain tissue containing 100 million – 1 billion neurons [224]. EEG therefore has low spatial resolution, and the extent of this spatial resolution is dependent on the number of electrodes applied, which in infants must be balanced with attempts to limit application time and handling. Conversely, EEG has a far higher temporal resolution than NIRS and fMRI, lending itself to the measurement of evoked potentials in the time domain. Lastly, EEG is easily portable and simple to apply cot-side, and a study investigating the effect of EEG application on infant cortisol levels as an indicator of stress found that EEG did not increase infant stress levels [225].

In 2010, EEG-recorded brain activity evoked by mechanical noxious stimulation was characterised by Slater et al., for the first time in neonates. This activity occurred at ~ 500 ms following heel lancing, but not non-noxious control stimulation, was maximal over the vertex of the scalp, and was evident in single

trials [11]. Subsequent research demonstrated that this potential is also evoked by lower intensity experimental noxious stimuli [210], which activate nociceptive A-delta as well as C-fibres [226 227]. The magnitude of the noxious-evoked brain activity elicited by these stimuli was correlated with their intensity [210], supporting the suggestion that this activity is related to the processing of noxious input. Similar patterns of evoked brain activity have been recorded in older infants aged 1 month – 1 year following immunisations and cannulation [228 229], demonstrating the commonality of this response following a range of stimuli and age groups. Infant noxious-evoked brain activity has been compared with adult noxious-evoked brain activity, demonstrating that while the first component of evoked activity is comparable in both age groups, the infant noxious-evoked response is composed of a longer latency deflection not present in adults [230]. This is consistent with the notion that stimulus-evoked responses mature with age, as discussed earlier when considering the development of sensory-evoked potentials [138 139 130 140].

In term infants, the brain activity evoked by heel lancing and experimental noxious stimulation has been formed into a template of noxious-evoked brain activity [12] (Figure 1.5). This template can be fitted to independent data, and is essentially scaled to quantify the noxious-evoked response, providing a 'weight' indicating the magnitude of noxious-evoked activity present. This template has been validated for use in several studies using independent data sets [12]: these studies have demonstrated that the template is specific, as it detects noxious-evoked brain activity following noxious, but not tactile, auditory, or visual stimuli of comparable intensity, and that the magnitude of noxious-evoked brain activity identified by the template correlates with facial expressions evoked by heel lancing, suggesting that

it is correlated with the infant pain experience. The template can also identify noxious-evoked activity in preterm infants, and is sensitive to analgesic modulation; topical local anaesthetic reduced noxious-evoked brain activity as quantified by the template, demonstrating its utility in assessing analgesic efficacy as will be discussed in greater detail in Section 1.8.3 below.

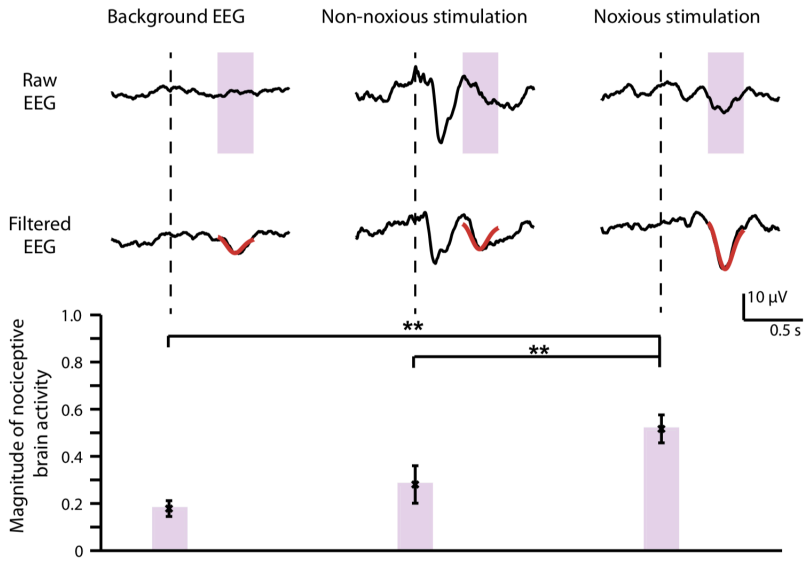


Figure 1.5 The template of noxious-evoked brain activity. Figure displayed with permission from Hartley et al. (2017)[12], demonstrating that fitting the template can characterise noxious-evoked brain activity that is significantly greater following noxious stimulation than following non-noxious tactile stimulation or in background brain activity.

While the majority of work utilising the template has occurred at the University of Oxford, researchers from independent institutions have recently used the template to quantify noxious-evoked brain activity [231] [232], demonstrating the applicability of this method. The template provides a useful objective and automated method for quantifying noxious-evoked brain activity, and is used in this thesis to assess the effect of potential pain relief interventions on responses to heel lancing and

experimental noxious stimuli. However, one limitation of the template is that it can only be used on previously characterised procedures, which is restricting given the wide range of procedures infants undergo on neonatal units. In this thesis, brain activity evoked by immunisation will also be assessed and compared with the template (Chapter 4), to assess the suitability of using this previously validated method to measure noxious-evoked brain activity following immunisation.

1.6.4 A multimodal approach to pain assessment

It is likely that the different surrogate measures reflect the transmission of noxious information from mechanoreceptor to brain at different levels: physiological recordings reflect autonomic responses [233] [234], and muscle reflex withdrawal relies on spinal cord circuitry and descending modulation [70]. Behavioural indicators may also reflect a reflex response [235], or may reflect a higher-level experience that can be influenced by emotions such as distress. The noxious-evoked brain activity recorded by fMRI may also be influenced by contextual and emotional factors, as it is in adults [236], while the EEG measures studied to date are likely to reflect a more specific aspect of the processing of noxious stimuli in the brain. There are likely to also be other noxious-related changes in brain activity, which could be investigated by increasing the number of electrodes, characterising changes in the frequency domain, or exploring a prolonged post-stimulus time window.

The different measures of pain-related brain activity are often concordant. For example, in single trials the magnitude of group-level NIRS and EEG recordings of noxious-evoked brain activity are correlated [219], and the magnitude of noxious-

evoked brain activity recorded by EEG is correlated with the magnitude of reflex withdrawal [210]. Additionally, the occurrence of noxious-evoked brain activity as measured by both EEG and NIRS correlates with the occurrence of facial expression changes [237 12 238 239].

However, surrogate measures of pain are not always consistent [240]; in some individuals, EEG and NIRS responses do not co-occur [219], and noxious-evoked brain activity has been observed in the absence of facial expression responses on numerous occasions [210 12 241]. Noxious-evoked brain activity does not correlate with cry presence or amplitude [242], and as detailed above, behavioural and physiological measures are not always harmonious [243 244]. While both brain activity and behaviour increase with age, the two are not always correlated [228]; brain activity is likely to be more sensitive than the scoring of facial expressions, which is often categorised into discrete variables. Furthermore, many contextual factors and interventions can disrupt the relationship between ordinarily concordant measures, including stress [245], prior pain [238], and sucrose administration [246], and these will be discussed in more detail in the section below.

The dissociations between these surrogate measures can be explained by a range of factors, including low signal-to-noise ratios when necessarily considering responses to single clinical procedures. This will be discussed further in the General Discussion, as will the specificity and sensitivity of each of the measures in distinguishing between pain and distress. The contrasts in these measures highlight the importance of taking a comprehensive multimodal approach to pain

assessment, as each indicator of pain can provide additional information with which to estimate the infant pain experience.

1.7 Factors affecting individual sensitivity to noxious stimulation

Many factors influence infant nociceptive responses [16] [247], including gestational age, sex, prior pain exposure, stress levels, illness severity, and behavioural state, and these contextual factors may influence the different surrogate measures of pain in a consistent manner, or may disrupt the correlation between them. It is important to take note of factors that may influence surrogate measures of pain in order to gain as accurate an estimation of the infant pain experience as possible. Additionally, accounting for the factors affecting infant sensitivity may enable better between-subject comparisons, as will be investigated in Chapter 5 of this thesis. Below are detailed the main factors which may affect individual sensitivity to noxious stimulation, and how they influence the different surrogate measures of pain.

1.7.1 Gestational age and sex

The effects of gestational age on noxious-evoked brain activity, reflex withdrawal, and facial expressions are to date the best characterised. Noxious-evoked brain activity measured by both EEG and NIRS increases in magnitude and decreases in latency during development following heel lancing and immunisation [216] [17] [228] [248], and noxious stimulation is more likely to elicit EEG-recorded noxious-evoked

potentials in older infants, as opposed to the non-modality specific delta brushes displayed in infants below ~ 34 – 37 weeks gestation [18 237].

While noxious-evoked brain activity increases in magnitude with age, reflex withdrawal in the same infants decreases [17] (Figure 1.6). This is consistent with the studies in animal models and human infants demonstrating that reflexes are refined postnatally, decrease in magnitude, duration, and latency, increase in activation threshold, and occur more discriminately after noxious rather than also tactile stimulation [72 17 73 74 75]. The concomitant development of postnatal noxious-evoked brain activity and reflex withdrawal in infants, along with the observation that brain regions involved in descending modulation influence noxious-evoked brain activity in human infants from birth [223] and begin to influence spinal activity in rat pups during early development [80 81 82 83], suggests that brain regions involved in descending modulation are likely to be modulating spinal cord activity and shaping the observed refinement in reflex withdrawal.

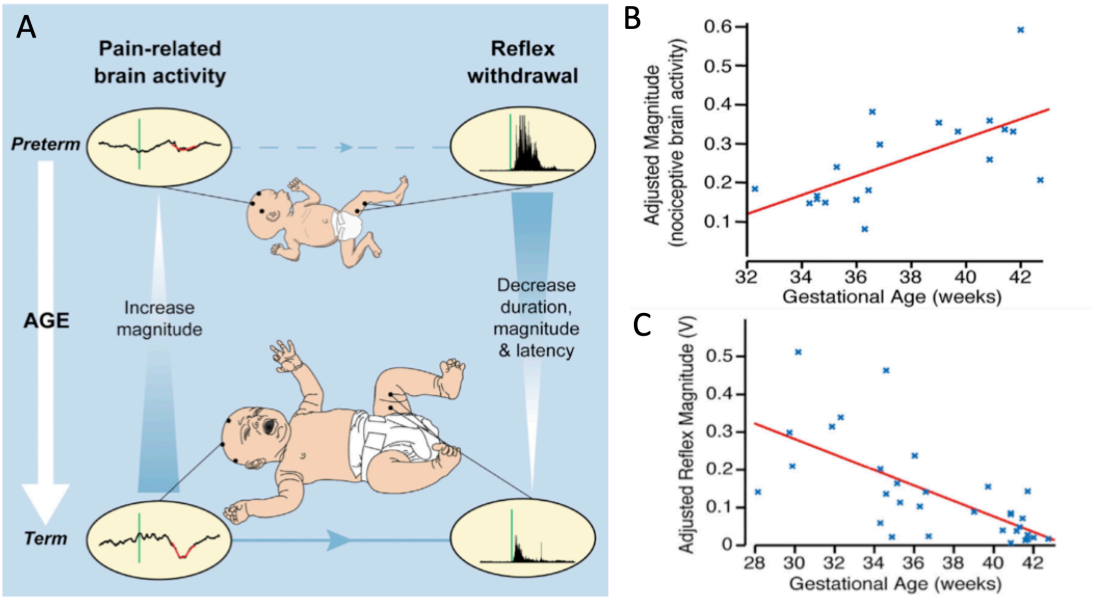


Figure 1.6 The effect of age on noxious-evoked responses. Figure modified with permission from Hartley et al. (2016)[17], demonstrating that the magnitude of noxious-evoked brain activity increases with age (A,B) and that the magnitude of reflex withdrawal decreases with age (A,C).

Older infants mount greater facial expression responses [247], and the latency to facial expression decreases with age over the postnatal period, similar to reflex withdrawal [249]. Facial expressions also occur more discriminately to noxious stimulation over touch stimulation in infants over ~ 33 weeks gestation, concomitant with the maturation of noxious-evoked brain activity [237]. Accounting for age when using several behavioural and physiological composite indicators of infant pain, such as the PIPP-R [187], EDIN6 scale [250], and Bernese Pain Scale for Neonates [251] has been explored, by assigning points based on gestational age to allow for the fact that younger infants are less likely to exhibit responses.

Sex-dependent differences in EEG-recorded noxious-evoked brain activity have been reported, with female infants more likely to exhibit a widespread rather than localised response to heel lancing [252]. In contrast with this, NIRS-measured brain activity evoked by venipuncture was more pronounced in male infants [217]. The behavioural literature is also divided, with several studies reporting that female infants exhibit more pain-related facial expressions [253] and higher pitch, more generally broadcast crying than male infants [254], but many other studies not demonstrating sex-dependent effects on infant brain activity [255] or behavioural or physiological pain responses [251 189 247 256]. The differences in these findings could be due to differences in the procedures investigated, the gestational age of the populations, or the sample sizes investigated, and further large-scale studies are needed to thoroughly investigate the potential effects of sex on pain responses.

1.7.2 Prior pain, stress and illness

Early life pain affects brain activity, behavioural, and physiological responses to pain in infancy. In ex-premature infants at term-corrected age, who have spent time in neonatal units undergoing painful procedures, EEG-recorded noxious-evoked brain activity to heel lancing was greater in magnitude than in age-matched term-born infants, who had not been exposed to prior pain [169], while NIRS-recorded brain activity to heel lancing is reported to be inversely related to the number of prior painful procedures [248]. Heart rate responses and oxygen saturation variability are reported to be increased with prior pain exposure [170], and the literature reporting the effect of prior pain on facial expressions is also divided; preterm infants exposed to previous pain are reported to display blunted facial expressions

to heel lancing [170] [171], while term infants exposed to prior heel lances exhibited more intense pain responses to venipuncture, but not immunisation [172] or the hip dysplasia screening test [257], and other research has also not observed an effect of prior pain on responses to immunisation in ex-premature infants [258].

Prior pain has also been demonstrated to disrupt the correlation between NIRS-measured prefrontal cortical activity and behavioural measures of pain [238], demonstrating the importance of a multimodal approach to pain assessment, and suggesting that prior pain should be taken into account when assessing the neonatal pain experience. Although the exact effects of prior pain exposure have not been characterised, and may be dependent on the extent of the prior painful exposure and the developmental period in which it occurs, these results indicate that prior pain can alter noxious processing.

Infants who are under stress also display altered responses to noxious stimuli and disrupted correlation between usually concordant measures. Using salivary cortisol as an indicator of stress, EEG-recorded noxious-evoked brain activity and behaviour were demonstrated to be dissociated in stressed infants, with an increase in noxious-evoked brain activity but not behaviour [245]. This is in line with the adult literature suggesting that stress enhances pain sensitivity [259], and could lead to an under-estimation of infant pain based on behavioural measures. Similarly, although the vasopressin (measured via the surrogate marker copeptin) produced during birth reduced noxious-evoked brain activity in infants born via vaginal delivery compared with caesarian section in the first few hours of life, birth-related fetal distress significantly increased the magnitude of noxious-evoked brain activity [232].

Lastly, illness also affects infant pain measurements, as sicker and weaker infants are less able to mount facial or behavioural responses [215]. Additionally, behavioural indicators of pain can be difficult to observe and assess when the presence of clinical equipment, such as intubation apparatus or feeding tubes, masks facial features or the ability to cry [260], or sedation blunts pain behaviours [220]. Further research is required to investigate noxious-evoked brain activity in these infants and provide further insight into their pain experience.

1.7.3 Behavioural state

Noxious-evoked brain activity measured by NIRS is modulated by sleep state, with sleeping infants exhibiting lower activity than awake infants [216]. Contrary to this, in a small sample of 10 infants, noxious-evoked potentials measured by EEG were not demonstrated to be modulated by sleep state, suggesting that they provide a consistent reflection of noxious-evoked neural activity in the cortex [11]. However, background cortical activity is modulated by behavioural state [261] and although the EEG-recorded noxious-evoked potential, which reflects the processing of noxious input, does not appear to be modulated by sleep state, the experience of pain could still be.

An earlier evoked potential identified using EEG, which is present following both noxious and sensory stimulation and may relate to attention processing, is sleep state dependent, with sleeping infants exhibiting larger magnitude earlier potentials [11]. The imaging methods used to measure the EEG-recorded and NIRS-recorded

markers of noxious-evoked activity could explain the differing effects of arousal state, as the haemodynamic activity recorded by NIRS is likely to be more widespread and affected by general brain state, encompassing both attention-related and noxious-related activity, while the EEG-recorded noxious-evoked potential is likely to reflect a more distinct and specific aspect of noxious processing.

Pain-related behaviours are also modulated by behavioural state, with less behavioural reactivity demonstrated in sleeping infants [247]. The PIPP-R [187] scale accounts for this by assigning points based on the arousal state of the infant in the baseline period.

In sum, there are many contextual factors that influence the different surrogate measures of pain, and the relationships between them. This further supports the use of a multimodal approach to infant pain assessment, as is used in this thesis, to gain the most comprehensive insight into the infant pain experience. In Chapter 3 of this thesis, infants have been matched based on gestational age in order to control for age-related effects on nociceptive responses when conducting between-group comparisons, as the influence of age on nociceptive responses has been well characterised as detailed in this section. However, in Chapter 5 of this thesis, a better approach to accounting for the many factors, in addition to age, which may influence nociceptive responses is explored, by considering infant nociceptive sensitivity itself.

1.8 Treatment of neonatal pain

Despite the importance of neonatal pain and its long-term effects, there is a paucity of conclusive evidence regarding the efficacy of many pain relief interventions in infants. This may stem from the lack of specificity associated with the commonly used behavioural and physiological indicators of infant pain, as well as the inconsistencies in procedures and dosing regimens investigated [7 262 263 264 265]. A range of pharmacological and non-pharmacological pain relief interventions are available for use on neonatal units, and these are supported by varying degrees of evidence, as detailed below. In the last part of this section, investigations into the efficacy of pain relief interventions using brain imaging are highlighted, demonstrating the increasing application of brain imaging techniques to investigate infant pain, and the insights they can provide.

1.8.1 Pharmacological interventions

Pharmacological interventions have typically been introduced based on their efficacy in adults, however, infant pharmacodynamics and pharmacokinetics differ from those of adults, and it cannot be assumed that the analgesics that are efficacious in adults are also effective in infants [266 267].

Investigation into the efficacy, and appropriate dosing regimens, of pharmacological analgesics is important; traditionally, dose calculation in infants has relied on the assumption that the development of the neonate into the adult occurs linearly and is predominantly based on body mass and/or surface area, and there are numerous methods of dose calculation which involve the scaling down of

adult doses based on weight and size [268]. However, it is clear that infants are not simply small adults and that the many physiological developmental changes that occur from the neonatal period to adulthood mean that drug profiles are different in infants [269] [270]. For example, the rate of enteral absorption is slower in neonates [271] and gastric pH is higher [272], affecting drug stability and absorption, and hepatic enzymes, which metabolise analgesic medications including opioids and paracetamol, are developmentally regulated [273] [274], influencing bioavailability. These differences could result in ineffective pain management or unexpected toxicity [270].

Investigations into the pharmacokinetics and pharmacodynamics of analgesics in infants are limited by the difficulties associated with conducting analgesic efficacy trials in infants, such as the caution central to altering drug doses in non-verbal infants, and the often necessarily invasive nature of data collection to assess drug pharmacokinetics and pharmacodynamics, as well as the difficulties associated with assessing neonatal pain. Behavioural measures of pain, which can be influenced by distress, may underestimate pharmacological interventions that reduce pain but do not alter procedure-related distress.

Based on the existing evidence, organisations including the British National Formulary for children (BNFc), National Health Service (NHS), and American Academy of Pediatrics have published guidelines on the recommended use and dosing regimens of pharmacological interventions in neonatal units, suggesting the use of opioids, paracetamol, ibuprofen, and topical local anaesthetic for varying levels of infant pain.

Opioids such as morphine and fentanyl are recommended for moderate to severe pain, for procedures such as central venous line placement, endotracheal intubation, chest tube insertion, and for ventilation [275] [276]. However, the literature reports inconclusive results regarding the analgesic efficacy of opioids in infants. Several investigations, utilising behavioural and physiological measures of infant pain, have reported that pain scores are reduced in ventilated infants [277] [278] [279], but other research has concluded that opioids do not provide analgesia in ventilated infants [280], for endotracheal suctioning [281] or for heel lancing [198], and a Cochrane review has concluded that there is insufficient evidence to recommend the routine use of opioids [263]. Additionally, the contraindications associated with opioids, including acute respiratory depression, cardiac arrhythmias, dependence, and tolerance [282] [283], as well as the potential longer-term effects of opioid administration in early life on neurocognitive development, demonstrated in animal models [284] and some [285] [286] though not other [287] [288] human studies, have resulted in a hesitation to administer these drugs. A trial utilising a multimodal approach to infant pain assessment, including measuring the effect of morphine on noxious-evoked brain activity as well as observing physiological effects [13] [14], is detailed in the final part of this section.

Paracetamol and ibuprofen are recommended for mild to moderate pain by the BNFc, for example for post-operative pain [289]. Liver damage and toxicity are the main adverse effects associated with paracetamol usage, and gastro-intestinal bleeding or ulceration as well as renal and cardiac impairment are concerns associated with ibuprofen administration (BNFc). Although paracetamol is one of

the most widely used drugs for all ages worldwide [290], the analgesic efficacy of paracetamol in neonates is disputed [265]. The existing literature utilising behavioural and physiological indicators of infant pain to assess the efficacy of paracetamol is detailed in Chapter 4, before measures of brain activity are utilised to investigate the analgesic efficacy of paracetamol in the research detailed in this chapter.

Topical local anaesthetics, such as lidocaine, tetracaine and EMLA (Eutectic Mixture of Local Anaesthetics, composed of lidocaine and prilocaine) are recommended for a range of acute, localised procedures, such as catheter insertion, central venous line placement, lumbar puncture, subcutaneous or intramuscular injection, and circumcision [289] [275]. Topical anesthetics are associated with less systemic side-effects than oral, rectal, or IV drugs; the main concerns associated with topical local anaesthetic application are skin reactions such as itching or swelling, and vasoconstriction. However, the literature regarding the analgesic efficacy of topical local anaesthetic for acute pain, which relies on behavioural and physiological measures of pain, is divided: several studies have concluded that local anaesthetic is effective for procedures such as venipuncture [291] [292] and lumbar puncture [293] but other studies have concluded that topical local anaesthetic is not effective for heel lancing [294], venipuncture [197], or immunisation [295] [296], and a Cochrane review in 2017 concluded that the existing evidence regarding the efficacy of topical local anaesthetic in infants was weak, its effects were of uncertain clinical significance, and that further high quality studies are required [264]. An investigation utilising noxious-evoked brain activity to assess the effect of local anaesthetic is detailed in the last part of this section [12]. It is imperative to assess the analgesic efficacy of pharmacological interventions, so the potential benefits

provided by analgesia can be carefully balanced with the potential for adverse side effects [7].

1.8.2 Non-pharmacological interventions

The use of non-pharmacological methods of pain relief has generally arisen from their use as infant comfort measures, and a range of techniques including sucrose administration, breastfeeding, and non-nutritive sucking, as well as touch-based techniques such as skin-to-skin care (SSC), facilitated tucking, and swaddling are recommended for pain relief in neonatal units.

Sucrose, and the administration of similar sweet solutions, has been observed to exert a calming effect of rapid onset, lasting several minutes, and has been widely demonstrated to reduce infant behavioural and physiological measures of pain [297]. Sucrose, or a pacifier with sucrose, is recommended for heel lancing, catheter insertion, central venous line placement, and injections [275] [298]. It is hypothesised that sucrose exerts its effect via the opioid system, as in rat models opioid antagonists negate the effect of sucrose [299], and infants with chronic antenatal exposure to opiates do not respond as strongly to sucrose [300]. However, not all research indicates that sucrose activates the opioid system [301], and the exact mechanisms by which sucrose operates are not certain. Recent research has suggested that repeated sucrose administration may be associated with adverse long term effects, including altered brain structure in animal models [302] and altered neurobehavioural development in some studies; sucrose appeared to be associated with poorer neurocognitive outcomes at term age when administered to very

preterm (< 31 weeks postconceptual age) [303], though not preterm (32 – 37 weeks gestational age) infants [304]. Therefore, the benefits of sucrose use need to be carefully balanced with possible negative side effects, and further research is required to determine the long-term effects of sucrose use in humans.

Breastfeeding has also been demonstrated to be effective in reducing infant pain [305], is purported to be of similar, or greater, efficacy than sweet solutions [306 307 308], and is recommended to alleviate procedural pain in neonates [309 310]. The efficacy of breastfeeding and sucrose, as assessed using behavioural and physiological indicators of pain, could be due to the distracting effect of sucking, as infants concentrating on sucking are less likely to demonstrate facial grimacing or crying, and non-nutritive sucking has also been demonstrated to reduce measures of infant pain [311].

The observed efficacy of breastfeeding could also stem from the comfort provided by maternal tactile contact. SSC, also known as kangaroo care after its resemblance with the method by which marsupials carry their young, is an established comfort measure and has been demonstrated to reduce behavioural and physiological indicators of infant pain to acute noxious procedures [312] including heel lancing [313 314 315], venepuncture [316], and intramuscular injection [317], and is recommended for use for acute pain [289]. Research has demonstrated that the difference between the pain relieving effect of SSC provided by the infants' mothers or a nonrelated female is small [318] suggesting that the touch aspects of SSC, independent of the familiar maternal presence, contribute to the effect of SSC for procedural pain.

Many other touch-based techniques, such as swaddling (the wrapping of an infant in blankets to limit limb movement) and containment or facilitated tucking (positioning the hands to manually hold the infant in a curled position, reminiscent of in-utero positioning) are also commonly used as comfort measures, and have also been demonstrated to reduce indicators of infant pain. Infants receiving these interventions were reported to feel less pain and calm down more quickly following acute noxious procedures including heel lancing [319] [320] and endotracheal suctioning [321] [322]. Swaddling, containment, and facilitated tucking are recommended for heel lancing, catheter insertion, central venous line placement, injections and endotracheal suctioning [275] [289]. Sensorial saturation, which typically involves sweet solutions and caregiver's voice as well as tactile contact, is also reported to be effective for heel lancing, eye examinations, and immunisations [323] [324].

Although the mechanisms underpinning the efficacy of touch-based techniques are not known, multiple theories exist, including effects exerted via descending modulation [325], as well as via the opiate [326] and oxytocin systems [327]. Touch-based interventions are advantageous as they are simple and easy to implement, and are not associated with the risk of adverse effects as in the case of many pharmacological analgesics. Massage of the heel is also recommended prior to heel lancing [289], and the literature investigating the efficacy of gentle touch as a pain relief intervention will be discussed in greater detail in Chapter 3, where the effect of gentle touch on infant nociceptive responses is investigated.

There may appear to be a divide between the results of investigations into pharmacological and non-pharmacological inventions, with inconclusive results regarding the efficacy of pharmacological analgesics, and the conclusion that non-pharmacological interventions are efficacious at reducing pain. As mentioned above, this may be due to the behavioural and physiological measures used, as these are influenced by distress, which is likely to be lessened by non-pharmacological interventions designed to comfort infants, but not necessarily by pharmacological pain relief interventions. It is important to reduce infant distress, as this has a positive impact on physiological stability and likely the experience of the procedure. However, as it is still possible to feel pain in the absence of distress, noxious input reaching the brain could still cause adverse long-term effects [328], and is therefore important to treat. In the next section, the use of brain imaging to shed light on additional aspects of infant pain processing and analgesic efficacy is discussed.

1.8.3 Brain activity as an assessment of pain relief intervention efficacy

Measurement of brain activity can provide an objective and specific indication of the effect of potential pain relief interventions on nociceptive processing. This section details how brain imaging has been used thus far to assess the efficacy of pharmacological and non-pharmacological methods of pain relief. To date, three potential methods of pain relief have been investigated using EEG-recorded noxious-evoked brain activity: topical local anaesthetic, morphine, and sucrose.

As detailed above, the literature using behavioural and physiological measures to assess the efficacy of topical local anaesthetic in infants is divided [264]. To date, there is one example of the use of brain activity to investigate the effect of local anaesthetic in infants. In this within-subject study, local anaesthetic was applied to one foot, and noxious-evoked brain activity following experimental noxious stimulation of both feet was compared. Local anaesthetic was reported to reduce the magnitude of the evoked brain activity [12], demonstrating that local anaesthetic is effective in blocking peripheral nociceptive input. This finding is inconsistent with many of the investigations relying on behavioural measures to assess acute pain detailed above, but this inconsistency, and the inconsistencies between the behavioural studies, could be due to a range of factors such as the choice of local anaesthetic and application time, the intensity of the noxious stimulation, and the specificity of the measures used to assess pain – infants may cry and exhibit facial expressions and elevated heart rate due to the general distress associated with clinical procedures (for example from being restrained for the clinical procedure), regardless of the level of pain they are perceiving [7 12]. Further work is needed to investigate whether local anaesthetic reduces noxious-evoked brain activity following clinical stimuli, which have a deeper penetration and higher intensity than the experimental noxious stimuli investigated here.

The literature regarding morphine is also divided [263]. In one randomised controlled trial investigating the effect of morphine on acute pain, noxious-evoked brain activity was validated for use as a co-primary outcome measure, along with clinical pain scores [13 14]. This trial took a multimodal approach to assess the effects of morphine, and after detecting profound adverse respiratory effects in these non-

ventilated infants, the trial was terminated early. This trial was therefore underpowered to investigate significant effects of morphine, however in the sample of infants collected, no trends in an effect of morphine on either noxious-evoked brain activity or clinical pain scores were observed. It is not possible to draw firm conclusions about the analgesic efficacy of morphine due to the early cessation of this trial, but the authors suggested that morphine should not be recommended for non-ventilated infants due to its risk of adverse effects, as well as the lack of suggestion of a trend towards pain relief. As the template was validated for use as a co-primary outcome measure in this clinical trial, this example demonstrates the validity of utilising the template of noxious-evoked brain activity as part of a multimodal approach to pain assessment.

The last pain intervention that has been investigated utilising this EEG-recorded noxious-evoked brain activity is sucrose. Noxious-evoked brain activity was not found to be altered by sucrose, although pain-related behaviour was reduced [246]. This effect on behaviour was consistent with the many reports of reduced behavioural and physiological indicators after sucrose and other sweet solution administration [297]. Several other studies have utilised alternative measures of brain activity to assess the impact of sucrose: consistent with the findings using this measure of EEG-recorded noxious-evoked brain activity, two studies utilising NIRS to look at changes in cerebral blood volume [329] [330] did not conclude that sucrose was effective in reducing brain activity. Contrary to this, several investigations into the effect of sucrose using both NIRS-recorded haemodynamic changes [331] [332] and EEG-recorded spectral frequency changes [333] did report reduced brain activity after sweet solution administration. The noxious-evoked brain

activity detailed and utilised in this thesis has been compared with a control stimulus to ensure that only the noxious-related component, rather than a reflection of general attention or salience is being analysed, while the other measures of brain activity described here may reflect more general stimulus processing, and this could explain the discrepancy in reported results. It seems possible that sucrose may reduce distress-related behaviour and physiology, and more generalised brain activity, but not alter noxious-related activity. The lack of impact on noxious-evoked brain activity could mean that sucrose does not protect against the long-term adverse consequences associated with early life pain and its effects on the brain [334]. The investigations detailed in this section illustrate the utility of an objective and specific measure of noxious-evoked brain activity, and highlight the importance of taking a multimodal approach to pain relief assessment in infants.

The EEG-based template [12] has not yet been applied to any tactile interventions, but other methods of brain imaging have been utilised to investigate the efficacy of comfort measures in providing pain relief. Using NIRS, pain-evoked brain activity was demonstrated to be lower following heel lancing in infants receiving maternal SSC than when the same infants were lying in their cot or incubator [335], and facilitated tucking reduced brain activity following punctate experimental stimuli [336]. These findings are consistent with behavioural results suggesting that touch-based interventions such as SSC, facilitated tucking, and infant massage reduce pain responses [312 337 338]. Infant massage has not yet been investigated using brain imaging, and in Chapter 3 of this thesis, the validated template of noxious-evoked brain activity is utilised to assess the efficacy of gentle touch in reducing infant pain, as part of a multimodal approach to pain relief intervention assessment.

1.9 Thesis aims

As detailed in this Introduction, the inability of non-verbal neonates to communicate their experiences of pain or pain relief, along with the continuing postnatal development of the infant nervous system and the former belief that infants did not perceive pain, has resulted in high rates of medically necessary painful procedures being performed without adequate analgesia. However, given the adverse short- and long-term consequences of early life pain, it is clear that pain management must be improved. A multimodal approach to neonatal pain assessment, including the measure of noxious-evoked brain activity, is likely to provide the best indication of the overall infant pain experience. This approach has been used to characterise responses to medically necessary procedures and assess analgesic efficacy, and in this thesis a multimodal approach is utilised to characterise further procedures, assess analgesic efficacy for additional pain relief interventions, and explore methods with which to improve the assessment of analgesic efficacy.

In the three results chapters of this thesis, I will:

1. Investigate the effect of slow touch targeted towards CT fibres on nociceptive responses,
2. Characterise nociceptive responses elicited by immunisation, and assess the effect of paracetamol on these responses,

3. Investigate whether individual sensitivity to noxious stimuli can be accounted for to aid between-subject comparisons of pain relief intervention efficacy.

Chapter 2: General Methods

This thesis is comprised of 3 results chapters in which data from 130 infants is presented. Many of the methods utilised to recruit infants, record their nociceptive responses following different stimuli, and analyse the resulting data are consistent across chapters. In this General Methods chapter, the common processes followed for infant recruitment, and the common techniques utilised to acquire and analyse nociceptive brain activity, reflex withdrawal, behaviour, and physiology data are outlined. Additionally, the methods with which the experimental noxious stimuli, clinically necessary heel lances, immunisations, and CT-optimal and non-optimal touch were applied are detailed.

2.1 Ethics

Ethical approval was obtained from the National Research Ethics Service. Studies were conducted as part of the ethics application titled 'Investigating pain in the developing human brain' (reference 12/SC/0447), and studies were carried out in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2 Recruitment

102 infants were recruited for the investigations in this thesis at the John Radcliffe Hospital, Oxford University Hospitals National Health Service Foundation Trust, UK from March 2015 to May 2019. In Chapter 3, 53 healthy ~ term infants were recruited, in Chapter 4, 27 ex-preterm infants who had reached ~ term age were recruited, and in Chapter 5, 22 healthy ~ term infants were recruited. In addition to

these infants who were recruited specifically for this thesis, 16 healthy term infants who had already been studied acted as age-matched controls in Chapter 3, pre-collected data from 11 infants were analysed in Chapter 5, and 15 control infants not receiving any intervention in Chapter 5 were co-recruited with another project investigating infant responses to heel lancing.

Infants were eligible for inclusion if they were clinically stable and did not have a history of neurological problems, intraventricular haemorrhage (IVH) of grade 3 or 4, or documented maternal substance abuse. None of the infants were receiving any analgesics or sedatives at the time of study, aside from the paracetamol administered prior to immunisations in the second part of Chapter 4. Other eligibility criteria required the infant's mothers to be over age 16 and to have custody of the infant, and for the mother, or father if the parents are married or the infant has been registered, to be able to provide informed consent.

After eligible infants were identified, a clinical member of staff was consulted to confirm that approaching parents regarding a research study would be appropriate. Parents were approached and asked if they would like to hear about an opportunity to take part in a research study. The study was explained, any questions were answered, and a Patient Information Leaflet (PIL, Appendix (i)) was given. If the study involved the use of the experimental noxious stimulus or the brush stimulus, these stimuli were shown to the parents. Parents in the Maternity Unit were given over an hour (ideally a few hours) to consider study participation. Recruitment from the Newborn Care Unit generally allowed a few days to be given to consider study participation, as infants are on the unit for longer and plans for

immunisations are made in advance. Parents were re-approached to ask if they had any further questions and if they would like to take part in the study. If parents chose to take part, they signed a consent form (Appendix (ii)) to evidence informed, written consent. It was stressed that research participation was entirely voluntary, would not affect clinical care, and that the parents could withdraw their infant at any time. A copy of the signed consent form was given to the parents, a copy was put in the infant's medical notes, and a copy was kept by the research team.

Figure 2.1 below displays an example of the number of eligible participants approached, consented, and studied who were due immunisations in the Neonatal Care Unit in Chapter 4 (left), and who were eligible to receive experimental noxious stimuli and CT-optimal touch prior to a clinically required heel lance on the Maternity Ward in Chapter 5 (right).

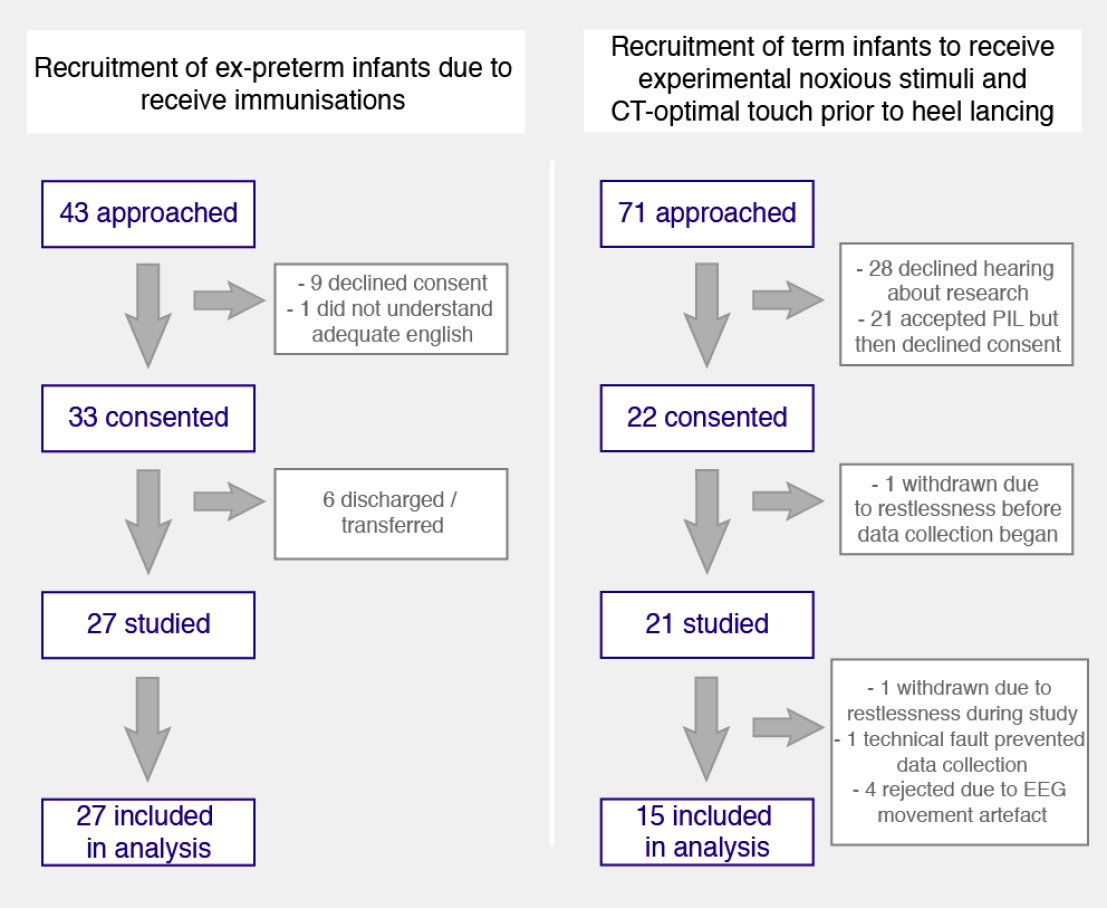


Figure 2.1: Recruitment timelines. The numbers of infants approached, consented, studied, and included in the analysis are displayed for ex-premature infants due immunisation in the Neonatal Care Unit in Chapter 4 (left) and term infants requiring medically essential heel lances in Chapter 5 on the Maternity Ward (right). This figure demonstrates the contrasts between recruiting on the neonatal unit and the maternity ward in terms of the number of parents who were approached and were interested in taking part in research; parents in the neonatal unit are more likely to take part in research than those on the Maternity Ward. The difference between the number of infants studied and included in the analysis in each study is due to the number of stimuli administered (3 immunisations versus 1 heel lance); recording responses to multiple stimuli means that the infant can still be included in the analysis even if the response to one stimulus has to be rejected. The strengths and limitations of the different types of recruitment, and the investigation of different types of stimuli, are discussed further in the General Discussion. (PIL = Patient Information Leaflet).

2.3 Recording techniques

2.3.1 Brain activity

EEG was used to measure noxious-evoked brain activity as it is non-invasive and can easily be applied cot-side without causing stress to infants [225]. As detailed in the General Introduction (Section 1.3.2 Imaging the developing brain; Section 1.6.3.3 EEG), EEG records summed neural electrical activity with an array of electrodes on the scalp [224], meaning that EEG can provide a more direct indication of brain activity than measures that record blood flow in the brain (e.g. NIRS, fMRI). EEG has low spatial resolution, which is dependent on the number of electrodes applied, and this in infants must be balanced with attempts to limit application duration and handling. EEG has precise temporal resolution and has been used to investigate the development of electrical brain activity in both typical [112 113 114] and atypical states (e.g. epilepsy [120], HIE [121 122], and neurodevelopmental delay [126 127]). The high temporal resolution of EEG means that it is ideally suited for investigating event-related activity [141], such as noxious-evoked potentials, with millisecond precision.

Electrophysiological activity from DC to 400 Hz was acquired with a SynAmps RT 64-channel headbox and amplifiers (Compumedics Neuroscan). Activity was recorded with a sampling rate of 2000 Hz using CURRYscan7 neuroimaging suite (Compumedics Neuroscan). All equipment conformed to the electrical safety standard for medical devices, IEC 60601-1.

EEG was recorded from eight locations on the scalp with Ambu Neuroline disposable Ag/AgCl cup electrodes. Electrodes were placed at Cz, CPz, C3, C4, Oz, FCz, T3, T4, with reference at Fz and ground at FPz (forehead) according to the modified international 10-20 system. (Figure 2.2A) The reference electrode was positioned at Fz because this site is minimally affected by muscle artefacts and located sufficiently distal to the central recording electrodes to ensure that noxious-evoked activity can be reliably recorded. Placement of the reference electrode at Fz (as opposed to on the nose or earlobes) was deemed acceptable by parents [11], and Fz has been used as the reference location in previous studies investigating noxious-evoked brain activity [11 17 210 237], including the validation of the template of noxious-evoked brain activity [12]. Preparation gel (Nuprep gel, D.O. Weaver and Co.) was gently rubbed on the scalp with a cotton bud to clean the area of skin, and conductive paste (Elefix EEG paste, Nihon Kohden) was applied to optimise electrode contact with the scalp.

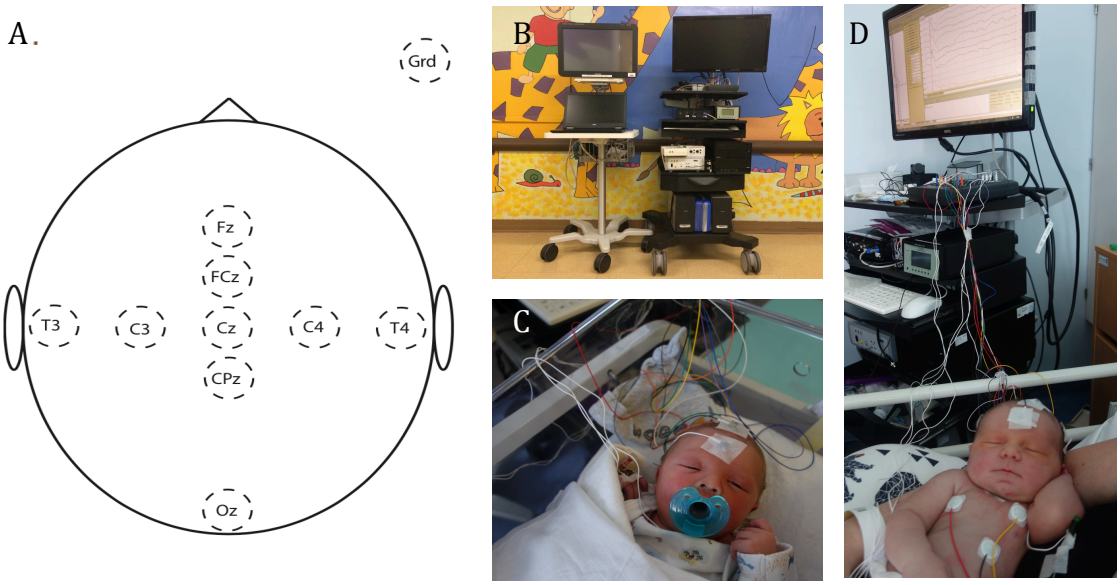


Figure 2.2: Acquisition set-up. (A) EEG montage, (B) Photograph of IntelliVue MX800 Philips monitor (left) and EEG recording computer (right). (C, D) Photographs of study set-up displayed with parental permission.

2.3.2 Reflex withdrawal

Bipolar EMG electrodes (Ambu Neuroline 700 solid gel surface electrodes) were placed on the skin overlying the biceps femoris of both legs. Reflexes were not recorded in the infants in Chapter 4, as the leg is restrained during immunisation administration, and electrode placement would have obscured access to the thigh for immunisation.

2.3.3 Behaviour and physiology

Facial expressions, heart rate, and oxygen saturations were recorded. This enabled utilisation of the PIPP-R [186187], a validated pain scale [192 193 194 195 196] which takes

into account changes in facial expression, heart rate, and oxygen saturation, as well as gestational age and behavioural state.

PIPP-R scores were calculated following heel lancing, immunisation, and experimental noxious stimuli in ex-preterm infants. PIPP-R scores were not calculated in response to experimental noxious stimuli in term infants, as previous studies have demonstrated that they do not elicit signs of behavioural distress or clinical concern [210].

Facial expressions were recorded using a hand-held video camera, and the point of the noxious stimulation was manually time-locked by the experimenter pressing a foot pedal to activate an LED light at the point of stimulation. 15 seconds of facial expressions were recorded prior to stimulation to assess the infant's baseline behavioural state, and 30 seconds were recorded post stimulation to score the occurrence of facial expressions, according to the PIPP-R scale.

Physiology was recorded using ixTrend software (ixellence GmbH) on a Philips neonatal monitor (IntelliVue MX800 patient monitor, Philips). SpO_2 was recorded using a pulse oximeter probe on the infant's foot, and heart rate was recorded using electrocardiogram (ECG) leads on the chest. The point of noxious stimulation was manually marked on the monitor.

2.4. Stimuli

A range of noxious and tactile stimuli were used in the studies that compose this

thesis, including experimental noxious stimuli, heel lances, immunisations, and CT-optimal and CT non-optimal touch. Heel lances and immunisations were only administered when clinically essential, and appropriate comfort measures such as swaddling or a dummy were provided to settle the infants.

2.4.1 Experimental noxious stimuli

The experimental noxious stimulus used was the PinPrick^{TM}, MRC Systems, Germany [339] (Figure 2.3), which was applied in blocks of approximately 10. The Pinprick has a flat tip of 0.25 mm diameter with controlled edge curvature, and was originally developed for quantitative sensoric testing of pain and perception thresholds in adults, as it administers a reproducible force that activates A- and C-fibre nociceptors in the skin [226 227 339]. The Pinprick is clinically safe and does not pierce the skin or cause tissue damage in adults or term infants: Pinpricks have been administered to term infants at a force of 128 mN in blocks of 10 in previous research studies, and have been well tolerated and do not cause behavioural distress [222 210 12]. Identifiable noxious-evoked brain activity responses have been observed in infants using lower intensity stimuli, but the signal-to-noise ratio is lower [210], and therefore a greater number of stimuli would need to be applied for the same power.



Figure 2.3 Experimental noxious stimulus. The PinPrick™, MRC Systems, Germany, force 128 mN used to provide experimental noxious stimulation.

The experimental noxious stimuli were applied to the medial or lateral plantar surface of the infant's heel, as this is where clinically relevant heel lancing is performed. There was a minimum inter-stimulus interval of 10 seconds, which was extended to allow the infant to settle if necessary.

Most of the experimental noxious stimuli were automatically time-locked to the EEG and EMG recordings using a contact trigger device (MRC Systems), but several early studies in Chapter 3 utilised a high-speed camera (220 frames per second; Firefly MV, Point Grey Research Inc.) that was directly linked to the recordings at the time of acquisition. Video recordings were reviewed post-acquisition and the time of stimulation was manually event-marked as the point where the barrel of the stimulator was first depressed [340 12].

2.4.2 Heel lance and control heel lance

The heel lances used were BD Microtainer Quikheel Infant Lancet (Becton, Dickinson and Company) with a penetration depth of 1.0 mm, which are used widely in neonatal care to obtain capillary blood samples. The heel lances

investigated were clinically required for the day 5 Guthrie test which all infants receive and which is done in hospital if either the infant or mother are still an inpatient, or to test for jaundice, sepsis, or blood gases in otherwise healthy term infants. The lancet was held against the infant's heel (medial or lateral plantar surface) by a clinical member of the research team (Figure 2.4), the button was pressed to release a retractable blade, and the blood was collected. The foot was not squeezed (as can be done to aid blood collection) for 30 seconds after the heel lance blade was released, to enable PIPP-R scoring specifically in response to the heel lance. If an insufficient blood sample was obtained with a single heel lance, a second heel lance was performed. This was also recorded if the parents were happy and the infant was settled.



Figure 2.4. Heel lance. Schematic of the lancet held in position against the infant's heel.

Control heel lances were performed as a non-noxious procedure with which to compare the heel lance. Control heel lances were performed on the same heel as the lance, by rotating the lancet by 90 degrees before releasing the blade so that the infant experienced the touch and sound sensation without the noxious input. Responses to the control heel lance and the heel lance could then be compared to ensure that the responses being investigated were noxious-evoked rather than a generalised response to a stimulus. Heel lances and control heel lances were

automatically time-locked to the EEG and EMG recordings using an event detection interface [341].

2.4.3 Immunisations

Intramuscular immunisations (25 mm, 23 gauge needle) were administered to the anterolateral thigh by a clinical member of staff. These immunisations are clinically required in infants at 8 and 12 weeks of age: at 8 weeks, infants receive 3 injections: the 6-in-1 vaccine (for diphtheria, hepatitis B, haemophilus influenza type b, polio, tetanus, and pertussis), the pneumococcal vaccine, and the meningitis B vaccine. The meningitis B immunisation is administered to one thigh, and the other two vaccinations to the other thigh, at least 2.5 cm apart[342]. At 12 weeks, infants receive the 2nd dose of the 6-in-1 vaccine [343].

Immunisation administration was recorded with a high-speed camera (220 frames per second; Firefly MV, Point Grey Research Inc.) time-locked to the EEG, for retrospective event marking of needle contact with skin [228]. There was an inter-stimulus interval of at least 30 seconds between immunisations to allow for PIPP-R scoring. The inter-stimulus interval was typically over a minute to allow the infants to settle after immunisations.

2.4.4 CT-optimal and CT non-optimal touch

CT-optimal touch (3 cm/s) and CT non-optimal touch (30 cm/s) were applied using the SENSELAb™ Brush-05, Somedic (Figure 2.5), prior to experimental noxious stimuli and heel lances. This brush is utilised for quantitative sensory testing in

adults, and is designed to provide a standardised force, which is applied by bringing the brush in contact with the skin until the filaments bend slightly before applying the stroking motion [344]. The velocities chosen were guided by the adult literature [345] [346], as is further detailed in Chapter 3.



Figure 2.5 Brush stimulus. SENSELab™ Brush-05, Somedic, Sweden, utilised to provide CT-optimal and CT non-optimal touch.

Brush stimulation was applied across approximately 10 cm of the infant's lower leg ipsilateral to the heel receiving noxious stimulation. This location was selected as it is the area of hairy skin (known to contain CT fibres [347] [348] [349] [350]) closest to the location of noxious stimulation. The researcher was cued to apply the brushing velocity and subsequent noxious stimulation by following a computer visualisation coded using PsychoPy. Brushing terminated ~ 1 second prior to the application of the noxious stimulus, and there was an inter-trial interval of at least 10 seconds, which was extended to allow the infant to settle if necessary. Brush stimulation was applied prior to, rather than during, noxious stimulation to minimise the possible effect of distraction, and the inter-stimulus interval of 1 second was selected in accordance with literature demonstrating that shorter intervals between CT-optimal touch and noxious stimulation are more effective in providing pain relief [346]. The brush stimulation itself was not time-locked to the EEG and EMG recordings; the noxious stimulation was time-locked as described below.

2.5 Analysis

2.5.1 Brain activity

MATLAB was used to analyse brain activity. EEG signals were baseline corrected to the pre-stimulus mean (the average baseline signal was deducted from the post-stimulus signal), and a notch filter at 50 Hz with harmonics was added to remove interference from mains electricity. Traces were filtered 0.5 – 70 Hz, except for in Study 1 of Chapter 3, where signals were filtered 0.5 – 30 Hz to remove interference from phototherapy lights in an infant receiving treatment for jaundice. Epochs of 5 seconds before and after the stimulus were analysed (Figure 2.6A). Individual epochs were rejected from the analysis if they contained gross movement artefact, or if there was movement in the baseline period. Artefacts and movement in the baseline period were identified by eye (Figure 2.6C).

A previously defined and validated template of noxious-evoked brain activity was used to calculate the magnitude of the noxious-evoked brain activity [12] following experimental noxious stimulation and clinically necessary heel lances. This template was formed by considering the topography and time course of the response to noxious stimuli (experimental noxious stimuli and clinical heel lances) compared to non-noxious stimuli (tactile and control heel lance). Noxious-evoked brain activity was maximal at the vertex electrode Cz, where it significantly differed from responses to non-noxious stimuli in the time window 446 – 611 ms post stimulation ($p=0.003$, cluster-corrected nonparametric test). Principal component analysis (PCA) was performed in this time window (400 – 700 ms) to

obtain a characteristic waveform of noxious-evoked brain activity, and a principal component (PC) was identified that was significantly greater in response to noxious stimuli than non-noxious stimuli or background brain activity ($p<0.01$).

The template can be projected onto individual infant's EEG activity in order to quantify the magnitude of noxious-evoked brain activity following noxious experimental stimuli and heel lances. Woody filtering (jittering of the data) can be used to maximise the correlation between the signal and the template in a pre-specified time window (e.g 400 – 700 ms post stimulation) in order to account for individual latency variation (Figure 2.6B). Jittering of the data using Woody filtering has been commonly performed when investigating noxious-evoked brain activity in infants, including in the investigations validating the template of noxious-evoked brain activity [12 17 210 237].

The template is scaled so that a magnitude of 1 represents the average response evoked by a heel lance in healthy, term infants, with responses to experimental noxious stimuli and in younger infants expected to be of smaller magnitude. In chapters 3 and 5, the magnitude of the noxious-evoked activity following heel lances and noxious experimental stimuli was determined by projecting the template onto the infants' EEG activity.

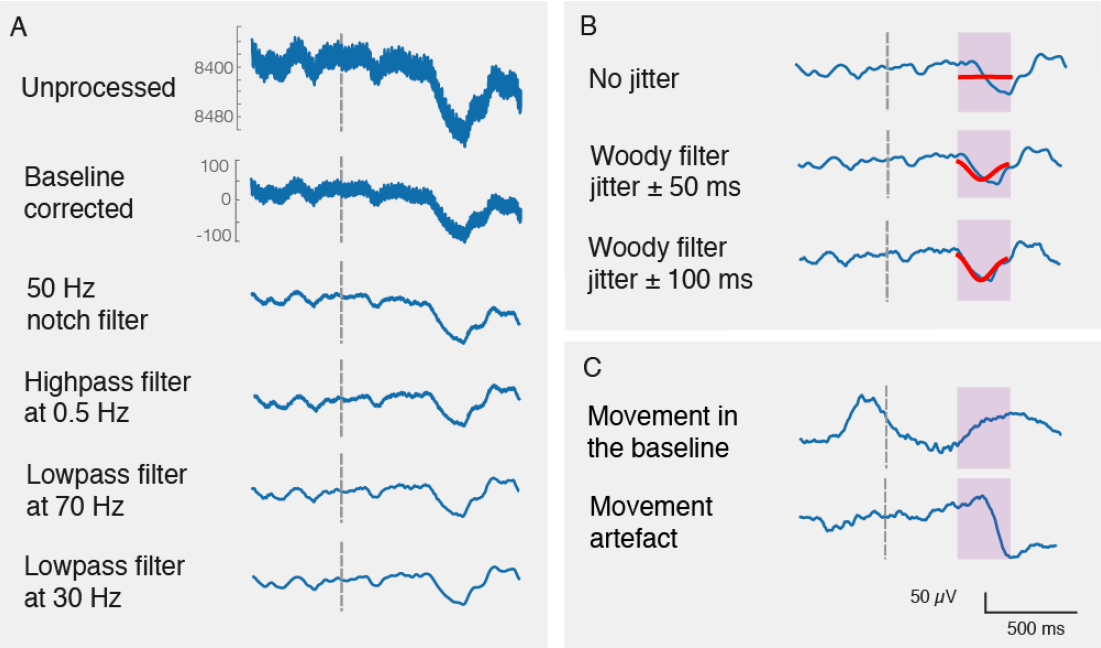


Figure 2.6. EEG analysis. (A) Processing of an example trace of evoked brain activity following heel lancing. Dashed line indicates the moment of heel lance stimulation. Unprocessed EEG activity is baseline corrected, and a notch filter at 50 Hz is applied to remove interference from mains electricity. A highpass filter at 0.5 Hz is applied to remove low frequency electrical activity, which is not physiological. A lowpass filter at 70 Hz is typically used to remove non-physiological high-frequency activity and smooth the trace; in Study 1 of Chapter 3 a 30 Hz lowpass filter was used to remove interference from an infant under jaundice treatment lights. (B) The trace is Woody filtered to allow a best fit between the template (red) and the evoked activity to be found; as can be seen, the template is projected in the window 400 – 700 ms post-stimulation (shaded box) when investigating responses evoked by noxious stimulation of the heel, and the data is jittered to find the best fit in the period allowed. (C) Examples of traces rejected due to movement in the baseline and window of interest.

2.5.2 Reflex withdrawal

Using MATLAB, EMG signals were filtered 10 – 500 Hz, with a notch filter at 50 Hz and harmonics. Epochs comprising of 5 seconds pre-stimulus and 15 seconds post-stimulus were rectified and analysed. Individual epochs were rejected due to

artefact, or if movement occurred in the baseline period (Figure 2.7). Two different methods of analysis were applied to quantify reflex withdrawal. The first method investigated was calculation of the root-mean-square (RMS), which can give an indication of the power of a signal in a set time period. The RMS was calculated in 250 ms windows, and the average RMS was calculated across the first post-stimulus second (i.e across 4 windows), a method which has been used previously [246] [210]. However, this method does not take into account the whole reflex response, which can vary in duration.

The second method investigated was an algorithm developed specifically to identify the start and end times of reflex withdrawal, and which has been used previously [17]. This algorithm filtered the signals with a low pass filter at 5 Hz, before smoothing the signal further by averaging across moving windows (each 250 ms long). The start and end points of the reflex withdrawal are then identified as the point when the magnitude of the differential goes above or falls below a certain threshold respectively. The algorithm end point was fixed at 15 seconds post stimulation if an end point was not found earlier. The differential (rate of change) was used to allow the muscle activity to settle at a constant level. The amplitude of the reflex is then calculated as the area under the smoothed curve (Figure 2.7).

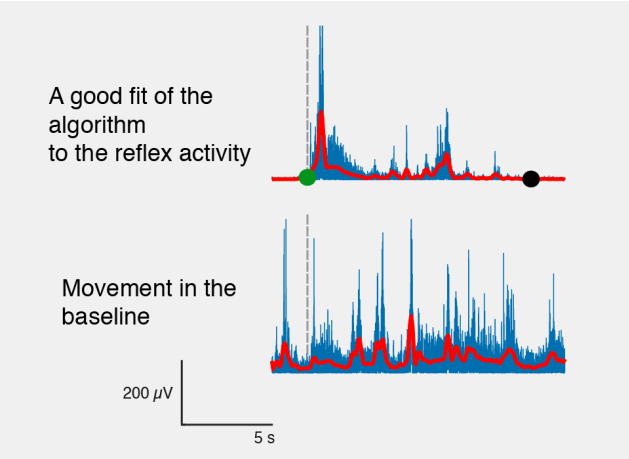
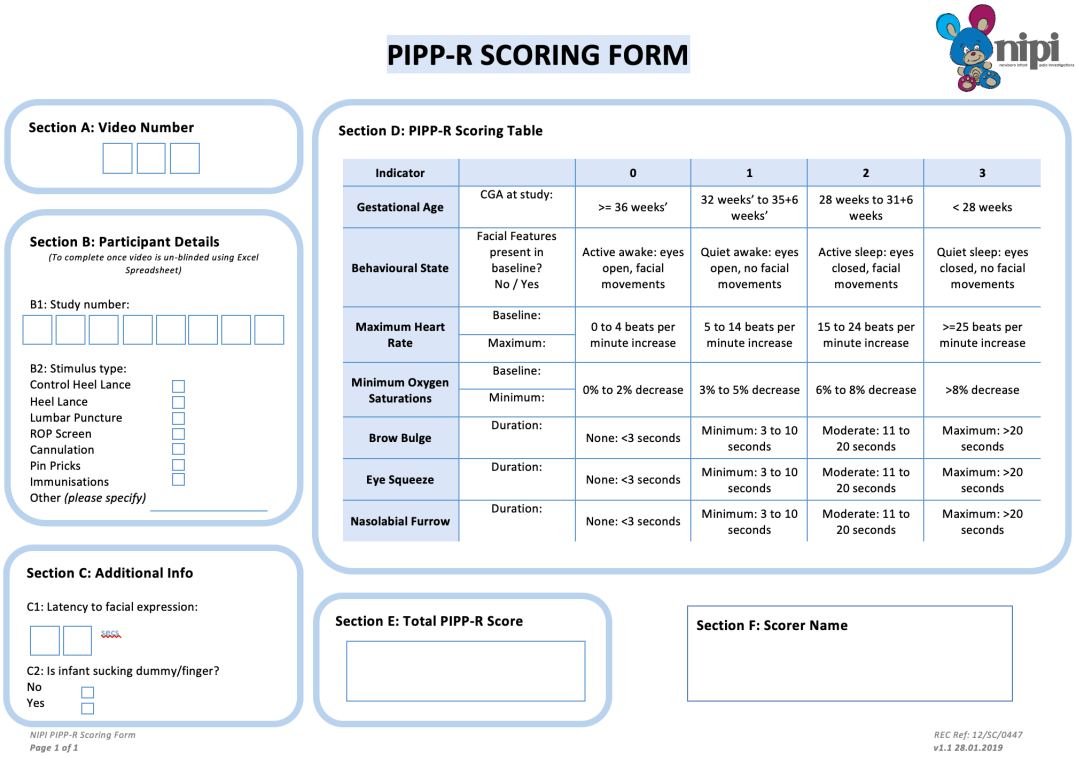


Figure 2.7: EMG analysis. Top: example of algorithm finding an appropriate start and end point of reflex withdrawal activity; green and black dots respectively. The red line is the differential below which the amplitude is calculated. Bottom: example of a rejected trace, in which there is movement in the baseline, and the algorithm is unable to find a start point.

2.5.3 Behaviour and physiology

Facial expressions and physiological data were scored in accordance with the PIPP-R [187]. The form used for PIPP-R scoring, which includes the criteria on which PIPP-R points are allocated is displayed below in Figure 2.8.



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:

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

Section E: Total PIPP-R Score

Section F: Scorer Name

NIPI PIPP-R Scoring Form
Page 1 of 1

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v1.1 28.01.2019

Figure 2.8: PIPP-R scoring form. Form designed by the Paediatric Neuroimaging Group for PIPP-R scoring, demonstrating the criteria used to assign points for infants' gestational age, behavioural state, facial changes, and physiological changes.

Facial videos were analysed retrospectively by myself and another trained observer; videos were randomised to ensure blinding to stimulus type. In the 15 seconds of baseline filming, the infants' behavioural state (active awake, quiet awake, active asleep, or quiet sleep) was assessed and assigned a score from 0 – 3. In the 30 seconds post-stimulation, the duration of nasolabial furrow, brow bulge and eye squeeze was assessed (Figure 2.9). A stopwatch was used to time the duration for which each facial expression could be observed. If the infant stopped exhibiting a certain facial expression, the stopwatch was paused, and the timer was resumed if the infant exhibited that expression again, giving a cumulative facial

expression duration. The duration of each facial expression was assigned a score 0 – 3, to give a total facial expression score 0 – 9.

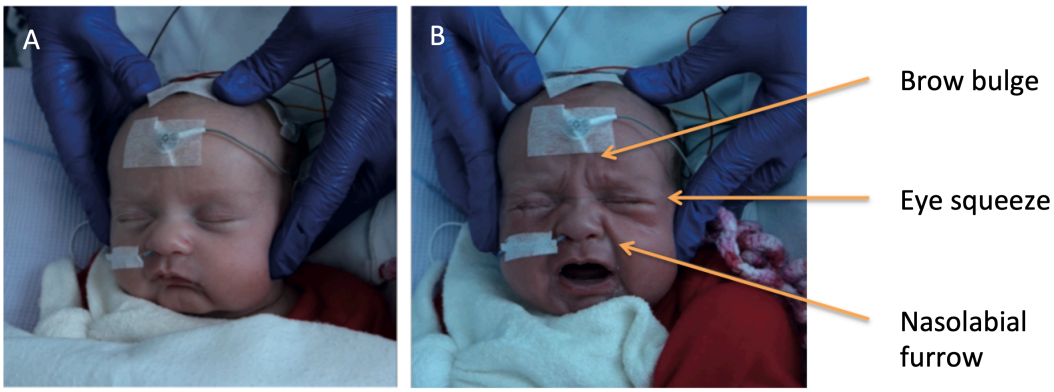


Figure 2.9 Behavioural state and facial feature scoring. Examples taken from a video recorded for PIPP-R scoring from (A) the baseline period in which behavioural state is assessed, and (B) the 30 seconds post stimulation in which the duration of brow bulge, eye squeeze, and nasolabial furrow is recorded. Photograph displayed with parental permission.

The physiological data was analysed using MATLAB. The baseline average heart rate and oxygen saturation were calculated, as well as the post-stimulus maximum heart rate and minimum oxygen saturation. The difference in these values was taken and assigned a score 0 – 3.

As indicated in the PIPP-R scoring form (Figure 2.8), points are also assigned depending on the gestational age of infants, with younger infants scoring more points (0 – 3). Finally, the gestational age, baseline behavioural state, facial expression change, and physiology scores are combined to provide a total score out of 21. Total scores of 6 or less indicate minimal or no pain, and scores greater than 12 indicate moderate to severe pain [186].

Intra-rater reliability of PIPP-R scoring was confirmed by scoring the videos of all infants receiving a heel lance in Chapter 3 on two occasions, and inter-rater reliability was confirmed in a subset of 18/30 infants from Chapter 3. Intra-rater reliability and inter-rater reliability were calculated using intra-class correlation and were 0.97 and 0.92 respectively.

As well as analysing total PIPP-R scores, the duration of facial expression response, the change in oxygen saturation, and the change in heart rate were compared without conversion to the PIPP-R scale. This allowed modality-specific comparisons. The duration of facial expression response was defined as the average duration of the 3 individual facial expressions. Additionally, in some studies further analysis of facial expression was performed: as not all infants mount facial expressions following heel lance stimulation [241] [12] [237], only the responses of 'responding' infants were considered. Infants were considered to be 'responders' if they exhibited any of the three facial behaviours for 3 seconds or more during the 30-second post-stimulation period.

Chapter 3: The effect of gentle touch on nociceptive responses in infants

3.1 Introduction

In infants, many touch-based techniques such as skin-to-skin contact (SSC), swaddling, and infant massage are established as comfort measures [351] [352] and are reported to reduce behavioural and physiological measures of infant pain [337] [312] [353] [338], as detailed in the General Introduction. While the efficacy of the tactile interventions SSC and facilitated tucking have been investigated using NIRS brain imaging, demonstrating a reduction in pain-evoked brain activity [335] [336], the validated template of noxious-evoked brain activity has not yet been utilised to investigate any tactile interventions.

Infant massage on body areas with hairy skin has been demonstrated to decrease facial expressions and heart rate at rest and following a range of acute clinical procedures including heel lancing and venipuncture in preterm and term infants [354] [355] [356] [357] [358], and massage of the heel is recommended by the Association of Paediatric Anaesthetists of Great Britain and Ireland for pain relief prior to heel lancing [289]. Despite the evidence for infant massage-induced comfort and pain relief, the mechanisms underpinning this effect are not known, and massage is not used widely on neonatal units [359].

CT fibre activation could be a possible mechanism underpinning the observed pain-relieving effects of infant massage. CT fibres are a subclass of slow-conducting unmyelinated sensory neurons from the C-fibre family, and are located in the hairy skin [347] [360] [350] [361]. CT fibres are activated by low-threshold stimuli such as gentle touch [348], and respond optimally to stroking at a velocity of 1 - 10 cm/s [345]. The firing rate of CT fibres is correlated with adult self-reports of pleasantness, with peak firing rates occurring at ~ 3 cm/s, concordant with the velocity that adults perceive as most pleasant [345] [362] [363].

CT fibres are believed to have evolved to serve a protective function by promoting affiliative behaviours and encouraging social touch [364] [365] [366] [367]; CT fibres respond optimally to touch at human skin temperature [368], and project to brain regions associated with reward-seeking and affective processing, such as the insular cortex, prefrontal cortex, superior temporal sulcus, and cingulate cortex [369] [370] [371] [372] [373]. Additionally, patients with a reduced density of CT fibres demonstrate less perceived pleasantness and empathy for CT-optimal touch [367], and CT fibre stimulation increases facial expressions of positive affect by inducing subtle increases in the muscles underlying smiling and reducing activity in the muscles responsible for frowning [374] [375].

Importantly, CT fibres may also play a role in modulating pain experience. In adults, CT-optimal touch has been demonstrated to reduce self-report of pain perception following acute noxious stimulation. Liljencrantz et al. [346] demonstrated that CT-optimal touch (3 cm/s), but not CT non-optimal touch (30 cm/s) or vibration, reduced pain ratings to acute heat pain, and Habig et al. [376] demonstrated

that CT fibres must be intact for slow touch to modulate pain intensity, as patients with small fibre neuropathy impairing CT fibres do not report a reduction in pain perception following CT-optimal touch. The influence of CT-optimal touch on pain perception has also been investigated using EEG in a study demonstrating that romantic partner delivery of CT-optimal, but not CT non-optimal touch, reduced event-related potentials following acute noxious laser stimulation, as well as reducing pain ratings [377].

CT fibres are also implicated in allodynia - the perception of pain following tactile stimulation that is not typically painful [378]. While the prevailing view suggested that A-beta tactile fibres were solely responsible for allodynia, research has demonstrated that nerve conduction block of myelinated A-beta fibres, but not unmyelinated C-fibres, allows the persistence of vibration-induced allodynia, suggesting that unmyelinated CT fibres are involved [379], and that during allodynia, there is reduced activity in the regions of the brain that typically process CT input [380]. Thus, it is suggested that CT fibres exert a normally inhibitory influence on the transmission of nociceptive signals, and that a reduction in CT fibre activity can cause allodynia [378] while an increase in CT fibre activity can reduce nociceptive signals.

CT fibres are believed to be functional from an early age: 9 month old infants are more attentive towards CT-optimal than CT non-optimal touch [381] [382], and CT-optimal touch activates brain regions known to be associated with affective touch processing in the first few weeks of life [383] [384]. Mothers instinctively stroke their infants at a CT-optimal rate [385] [386], however, it is unknown whether CT afferents

are responsive to tactile input from the first days of postnatal life, and whether they play a role in modulating pain perception at this early stage in development.

In this chapter, the effect of gentle touch, targeted towards CT fibres, on infant nociceptive responses is investigated. In the first of two studies composing this chapter, the effect of CT-optimal and CT non-optimal touch is investigated on noxious-evoked brain activity and reflex withdrawal following an experimental noxious stimulus. I hypothesised that CT-optimal touch, but not CT non-optimal touch, would reduce nociceptive responses, in line with the adult literature [346] [376] [387]. An order effect of repeated tactile and experimental noxious stimulation is also investigated: I hypothesised that the effect of CT-optimal touch would diminish with repeated stimulation, consistent with the effect of repeated touch stimulation on CT fibre firing rates [348] [388] and ratings of pleasantness [363].

In the second study composing this chapter, the effect of CT-optimal touch prior to a clinically relevant stimulus, heel lancing, was investigated. In this study noxious-evoked brain activity and reflex withdrawal were again recorded, along with behavioural and physiological measures of infant pain. In line with the literature demonstrating that infant massage reduces clinical measures of infant pain [354] [355] [356] [357] [358], I hypothesised that these indicators would be reduced.

The main findings presented in this Chapter have been published in Gursul et al. 2018, *Current Biology* [389].

3.2 Chapter overview

This chapter is composed of two studies:

1. A within-subject study, in which 30 healthy, term infants received blocks of CT-optimal (3 cm/s), CT non-optimal (30 cm/s), and no touch prior to experimental noxious stimulation. Noxious-evoked brain activity and reflex withdrawal was measured.
2. A between-subject study, in which CT-optimal touch was administered prior to a medically essential heel lance. 32 healthy term infants received heel lances; half received CT-optimal touch prior to lancing and half received no touch. Noxious-evoked brain activity, reflexes, facial expressions, and physiology were recorded.

3.3 Methods

3.3.1 Participating infants

Infants were recruited across 2 studies. Infant demographics are shown in Table 3.1. 33 infants were recruited to take part in Study 1, but on 3 occasions infants were excluded due to restlessness, illness, or technical issues. Data from 30 infants were therefore analysed in this exploratory investigation. A power calculation was then performed using the results from Study 1 to estimate the sample size required to observe an effect of CT-optimal touch prior to heel lancing in Study 2. In Study 1, the magnitude of noxious-evoked brain activity was reduced by ~ 60 % following the application of CT-optimal touch prior to the first experimental

noxious stimulus. Assuming a reduction of this size following heel lancing (magnitude of noxious-evoked brain activity following heel lancing in an independent sample of infants = 1 ± 0.9 [12] (mean ± standard deviation)), 32 infants would be needed in a paired analysis to see a significant effect of CT-optimal touch with 80% power at a significance level of 0.05.

20 infants were recruited to receive CT-optimal touch prior to heel lancing. In 4 instances the EEG response to the lance had to be rejected due to artefact, leaving 16 infants in the CT-optimal touch group. 16 age-matched infants who had not received any intervention prior to lancing were selected from a database of infants whose responses to lances had been recorded as part of previous research studies. Age-matching was based on gestational age at study and also aimed to minimise differences in postnatal age between the paired infants.

	Study 1	Study 2	
		CT-optimal touch	No touch
Number of infants	30	16	16
Gestational age at study (weeks)	40.0 (39.0, 41.0)	39.9 (38.0, 41.7)	39.8 (37.9, 41.3)
Postnatal age at study (days)	2 (1, 3)	5 (3.8, 5)	2.5 (1, 4)
Birthweight (g)	3557 (604)	3391 (577)	3685 (659)
Number of males	14 (46.7 %)	10 (62.5 %)	5 (31.3 %)
Number born by normal vaginal delivery	14 (46.7 %)	10 (62.5 %)	5 (31.3 %)
Average Apgar score at 1 minute	8.7 (1.5)	8.6 (2.6)	8.2 (1.8)

Table 3.1: Demographics of infants studied in Chapter 3.
Values provided are mean (standard deviation), median (lower quartile, upper quartile) or number (percentage).

3.3.2 Experimental design

In Study 1, experimental noxious stimuli were applied to the heel in 3 blocks. Each stimulus was preceded by 5 seconds of CT-optimal (3 cm/s), CT non-optimal (30 cm/s), or no touch in a randomised order (Figure 3.1A).

The velocities 3 cm/s and 30 cm/s were chosen based on the velocities investigated in the adult literature; touch at 1 – 10 cm/s elicits peak CT fibre firing rates, pleasantness ratings, and pain reduction, while touch at 30 cm/s does not [345] [346].

The 5-second duration of the brush stimulation was also guided by adult studies [374], and was selected to ensure that the total study duration would be well tolerated by infants. 10 stimuli were originally planned to be administered per block in line with previous studies [210 12], but an error in an early version of the code cueing the stimuli meant that only 9 stimuli were applied in some instances, and therefore only the first 9 stimuli from each condition are included in the analysis.

In Study 2, half of the infants did not receive any touch prior to lancing, and in the other half CT-optimal touch was applied for 10 seconds (Figure 3.1B). The duration of the brush stimulation was increased from 5 seconds to be consistent with recently published literature following the demonstration that longer periods of CT-optimal stimulation are more effective [346], and because the single-trial nature of medically required heel lances meant that the duration of the study was substantially shorter than in Study 1 and so a longer duration brush stimulation could be applied.

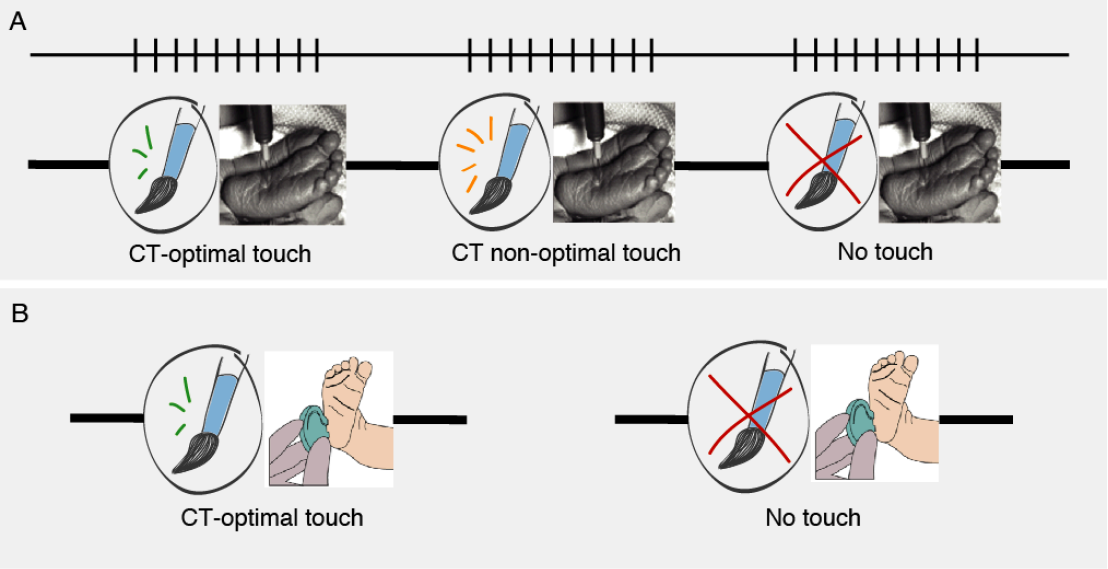


Figure 3.1: Experimental protocol followed in Chapter 3. (A) Study 1: 30 infants received blocks of CT-optimal touch, CT non-optimal touch, or no touch (randomised order) prior to noxious experimental stimuli. (B) Study 2: 16 infants received 10 seconds of CT-optimal touch prior to a clinically required heel lance, and the other 16 infants did not receive touch prior to lancing.

3.3.3 Recording techniques

Brain activity and reflex withdrawal were recorded in Study 1 and Study 2, and behavioural and physiological measures were also recorded in Study 2. These measures were not recorded in Study 1 as infants do not react behaviourally to experimental noxious stimuli [210]. The recording techniques used are detailed in General Methods (Chapter 2).

3.3.4 Analysis

3.3.4.1 Brain activity

Noxious-evoked brain activity was recorded following experimental noxious stimulation and clinically required heel lances. The template of noxious-evoked brain activity, validated for use on both of these stimuli [12], was fitted to quantify the magnitude of the noxious-evoked response, as detailed in General Methods, Chapter 2.

In Study 1, the mean number of stimuli considered in the analysis was 8.7 ± 1.3 (mean ± standard deviation) of the 9 stimuli applied in each block. The brain activity recorded in response to the first stimulus was rejected due to artefact in 8 infants in the CT-optimal touch condition, 10 infants in the CT non-optimal touch condition, and 10 infants in the no touch condition. In Study 2, 4 of the 20 recruited infants' EEG responses to the heel lance contained artefact and were rejected.

Before template fitting, the data was Woody filtered by a maximum shift of ± 50 ms in Study 1 and ± 100 ms in Study 2. These filter settings ensured that the magnitude of noxious-evoked brain activity characterised by the template following noxious stimulation (in the no touch condition) was significantly greater than in the background brain activity in Study 1, and significantly greater than both background brain activity and responses to the control heel lance in Study 2. In Study 1, the mean ± standard error of the magnitude of noxious-evoked brain activity was 0.14 ± 0.06 in background brain activity, and 0.42 ± 0.12 following the first experimental noxious stimulus: these values are significantly different ($p=0.022$, two-sided t-test).

In Study 2, the mean ± standard error magnitude of noxious-evoked brain activity was 0.42 ± 0.08 in the background brain activity, 0.78 ± 0.16 following the control heel lance, and 1.21 ± 0.17 following the heel lance: the noxious-evoked brain activity characterised following heel lancing is significantly greater than background brain activity ($p<0.001$ two-sided t-test), and also significantly greater than the noxious-evoked brain activity characterised following the control lance ($p=0.011$, paired two-sided t-test). Although a Woody filter of 50 has been used as standard in previous studies [12 210 17], the higher Woody filter jitter of 100 ms was used in Study 2 to provide a better fit to the template. In this study the 50 ms jitter did not allow adequate fitting of the template to the clearly visible evoked activity following heel lancing (see Figure 3.2), and the noxious-evoked activity identified was not significantly different than background EEG activity or the evoked activity following the control heel lance ($p = 0.11$ and $p=0.68$ respectively). Individual waveforms following the first experimental noxious stimulus in all infants in Study 1, and following heel lance stimulation in all infants in Study 2 are provided in Figure 3.2. Highlighted are examples demonstrating the better fit with the Woody filter of ± 100 ms in Study 2.

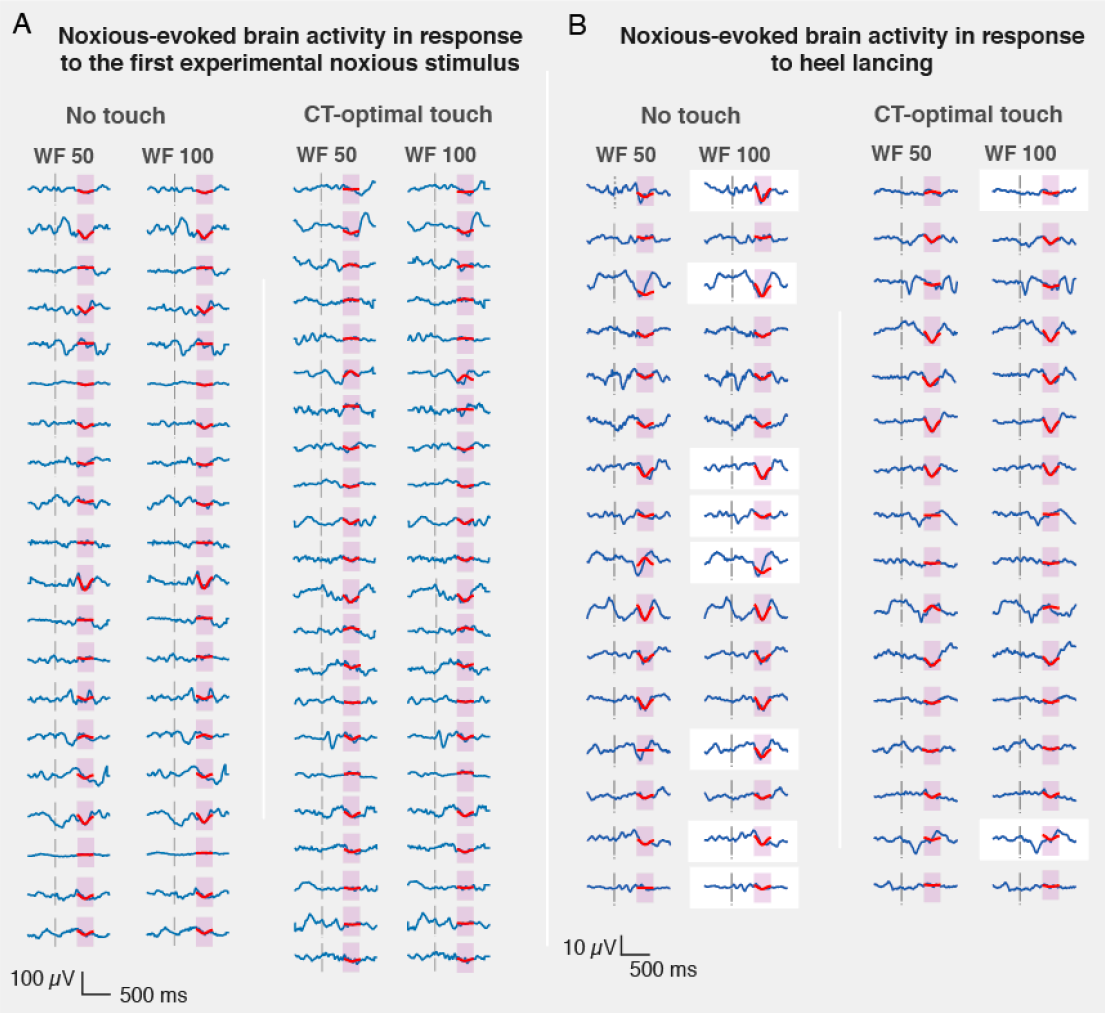


Figure 3.2: Altering the Woody filter jitter allows the template of noxious-evoked brain activity to be most appropriately fit to the evoked brain activity. Figure demonstrating the fitting of the noxious-evoked template (red) when permitting a Woody filter jitter of ± 50 ms and ± 100 ms to the brain activity evoked by the first experimental noxious stimulus in Study 1 (A) and to the heel lance in Study 2 (B). Examples of occasions where a jitter of ± 100 ms permits a better fit of the template to the evoked response in Study 2 are highlighted. Dashed line indicates the point of noxious stimulation; shaded box indicates the time window of interest (400 – 700 ms post stimulation when considering noxious stimulation of the heel).

3.3.4.2 Reflex withdrawal

General analysis of reflex withdrawal is detailed in General Methods, Chapter 2; both the RMS method and the algorithm method were applied to quantify the reflex withdrawal. In Study 1, reflex withdrawal responses to the first trial in each block were rejected from 9 infants in the CT-optimal touch condition, 9 infants in the CT non-optimal touch condition, and 6 infants in the no touch condition. In Study 2, responses from 3 infants from the CT-optimal touch group and 2 infants in the no touch group were rejected due to movement artefact.

3.3.4.3 Behaviour and physiology

The behavioural and physiological measures investigated in Study 2 were recorded as described in General Methods, Chapter 2. All 16 infants in the CT-optimal touch group had recorded facial expression videos, heart rate, and oxygen saturation. In the no touch group, 2 infants did not have facial videos that could be analysed due to technical difficulties, and 3 did not have oxygen saturation recordings due to a fault with the pulse oximeter. Therefore, full PIPP-R scores for 5/16 infants in the no touch group could not be calculated.

3.3.5 Statistical analysis

The magnitude of noxious-evoked brain activity following experimental noxious stimulation was compared to background brain activity in the no touch group, and the magnitude of noxious-evoked brain activity following heel lancing was compared to both background brain activity and the control heel lance in the no

touch group. These comparisons were performed to ensure that the noxious-evoked brain activity was well characterised using the relevant Woody filter settings. Two-sided t-tests were performed, and p-values of <0.05 in Study 1 and 0.025 in Study 2 (to account for multiple comparisons) were taken to indicate statistical significance.

In Study 1, the primary question was to investigate whether CT-optimal touch reduced noxious-evoked brain activity following a single experimental noxious stimulus relative to the no touch condition. A single experimental noxious stimulus was considered, as this is more clinically relevant. The magnitude of noxious-evoked brain activity following the first experimental noxious stimulus in the CT-optimal touch condition and the no condition were compared using a linear mixed-effects model (using the nlme R package) with modality set as a fixed effect and individual infants taken as random effects. A p-value of 0.05 was taken to indicate statistical significance.

Secondary questions involved investigating the effect of CT non-optimal touch on the first experimental noxious stimulus, whether the effect of CT-optimal touch on noxious-evoked brain activity was maintained with repetition (i.e whether CT-optimal touch reduced noxious-evoked brain activity when all trials where considered together), and the effect of CT-optimal and CT non-optimal touch on reflex withdrawal to the first experimental noxious stimuli. When reporting the magnitude of noxious-evoked brain activity in all trials, trials were first averaged within-subject before taking the group average. To adjust for multiple comparisons, a more stringent p-value of 0.01 was taken to indicate statistical significance for

these analyses. Linear mixed-effects models were used to analyse the EEG data, and Wilcoxon signed rank tests were used to analyse reflex withdrawal responses as the data were positively skewed. Standard error of the median was calculated using 1000 bootstrap samples of the data.

A post-hoc test was performed to compare the effect of CT-optimal touch on noxious-evoked brain activity to the first experimental noxious stimulus (trial 1) with the effect of CT-optimal touch on the following experimental noxious stimuli (trials 2-9), after observing that CT-optimal touch reduced noxious-evoked brain activity in the first trial but not in all trials. A linear mixed-effects model with trial as a fixed effect and individual infants as random effects was used. A more stringent p-value of 0.01 was taken to indicate significance.

In Study 2, the effect of CT-optimal touch on noxious-evoked brain activity was investigated by comparing the magnitude of noxious-evoked brain activity following heel lancing in the CT-optimal touch group and the no touch group using a two-tailed t-test. Reflex withdrawal magnitude, behavioural, and physiological responses were compared using Mann-Whitney U tests as the data were skewed. The proportion of infants who exhibited facial expression responses in the CT-optimal touch and no touch group were compared using a chi-squared test.

The primary outcome of Study 2 was to investigate the effect of gentle touch on responses to heel lancing in age-matched infants. A post-hoc test was conducted to

investigate the influence of gestational age on the magnitude of noxious-evoked brain activity in these age-matched infants; linear correlation was used.

To investigate the percentage of time a significant effect of gentle touch on the magnitude of noxious-evoked brain activity following heel lancing would have been observed if infants were not age-matched, a post-hoc test was conducted. Random samples (x 100) of 16 non age-matched infants were taken from the pool of pre-collected data containing 36 infants who had not received any intervention prior to heel lancing, and the average magnitude of noxious-evoked brain activity was compared with the gentle touch group using a t-test. The number of occasions on which $p < 0.05$ was recorded and expressed as a percentage.

3.4 Results

3.4.1 Study 1: The effect of gentle touch on nociceptive responses evoked by experimental noxious stimuli

3.4.1.1 Noxious-evoked brain activity is reduced by CT-optimal touch, but not CT non-optimal touch

Initially, only the first response to the experimental noxious stimulation and preceding touch was considered, as this was considered to be more clinically relevant. The mean ± standard error of noxious-evoked brain activity was 0.14 ± 0.06 in background brain activity, 0.42 ± 0.12 in the no touch condition, 0.18 ± 0.09 in the CT-optimal touch condition, and 0.35 ± 0.09 in the CT non-optimal

touch condition.

Noxious-evoked brain activity in the CT-optimal touch condition was significantly reduced compared with noxious-evoked brain activity in the no touch condition ($p=0.029$, linear mixed effects analysis). In contrast, CT non-optimal touch did not significantly reduce noxious-evoked brain activity ($p=0.57$, linear mixed effects analysis, Figure 3.3A). Compared to the noxious-evoked brain activity in the no touch condition, the evoked potential following the first CT-optimal touch was approximately 60% lower in magnitude.

3.4.1.2 Reflex withdrawal is not altered by CT-optimal or CT non-optimal touch

Using the RMS method to calculate the magnitude of the reflex withdrawal, the median ± standard error was 1.9 ± 0.31 μV in background muscle activity, 6.7 ± 3.0 μV in the no touch condition, 4.9 ± 4.8 μV in the CT-optimal touch condition, and 10.3 ± 9.5 μV in the CT non-optimal touch condition. When considering the amplitude of the reflex response, the median ± standard error was 0 ± 0 μV in the background period, 12,713 ± 14,265 μV in the no touch condition, 0 ± 14,663 μV in the CT-optimal touch condition, and 28,752 ± 24,688 μV in the fast touch condition.

Neither CT-optimal nor CT non-optimal touch significantly altered the reflex withdrawal response compared to the no touch condition when considering either

the RMS (p=0.20 and p=0.40 respectively, Figure 3.3B) or the amplitude (p=0.52 and p=0.38 respectively).

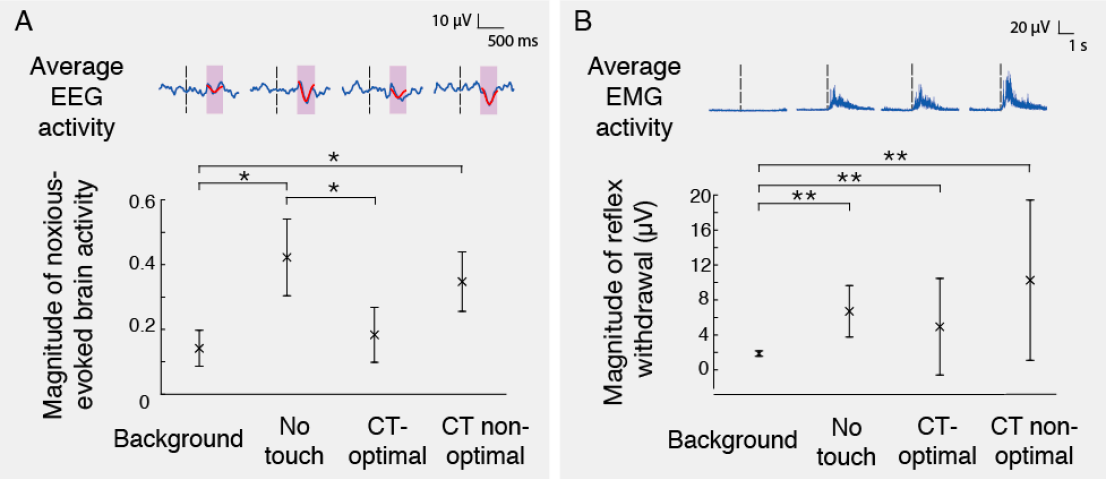


Figure 3.3: CT-optimal touch reduces noxious-evoked brain activity, but not reflex withdrawal, in response to an experimental noxious stimulus. (A) Top: average background brain activity, and responses following the first experimental noxious stimulus in the no touch, CT-optimal, and CT non-optimal conditions. Traces are Woody filtered and shown overlaid with the template of noxious-evoked brain activity. Black dashed lines indicate the point of noxious stimulation and pink shading highlights the time window of interest for noxious-evoked brain activity following heel stimulation (400 – 700 ms post stimulation). Bottom: Noxious-evoked brain activity in the no touch condition and CT non-optimal condition, but not the CT-optimal condition, are significantly greater than background brain activity. CT-optimal touch significantly reduces noxious-evoked brain activity compared to the no touch condition. Error bars indicate mean ± standard error. (B) Top: average background reflex withdrawal activity, and responses to the first experimental noxious stimulus in the no touch, CT-optimal, and CT non-optimal conditions. Bottom: reflex withdrawal magnitude, calculated using the RMS, is not altered by CT-optimal or CT non-optimal touch. Error bars indicate median ± standard error; * indicates $p < 0.05$; ** indicates $p < 0.001$.

3.4.1.3 Investigating an order effect of successive stimulation

When considering all trials, noxious-evoked activity following CT-optimal touch

was not significantly different to noxious-evoked activity with no prior touch ($p = 0.62$, linear mixed effects analysis, Figure 3.4A). In the no touch condition, the noxious-evoked brain activity was of comparable magnitude following each stimulus, consistent with previous results [210]. However, in the CT-optimal touch condition the magnitude of noxious-evoked brain activity was not consistent across stimuli: the magnitude of noxious-evoked brain activity following the first noxious stimulus was significantly lower than the magnitude of the noxious-evoked brain activity in subsequent trials ($p = 0.019$, Figure 3.4B, linear mixed effects analysis). There was no difference in reflex withdrawal magnitude with repeated stimuli in either condition (Figure 3.4B).

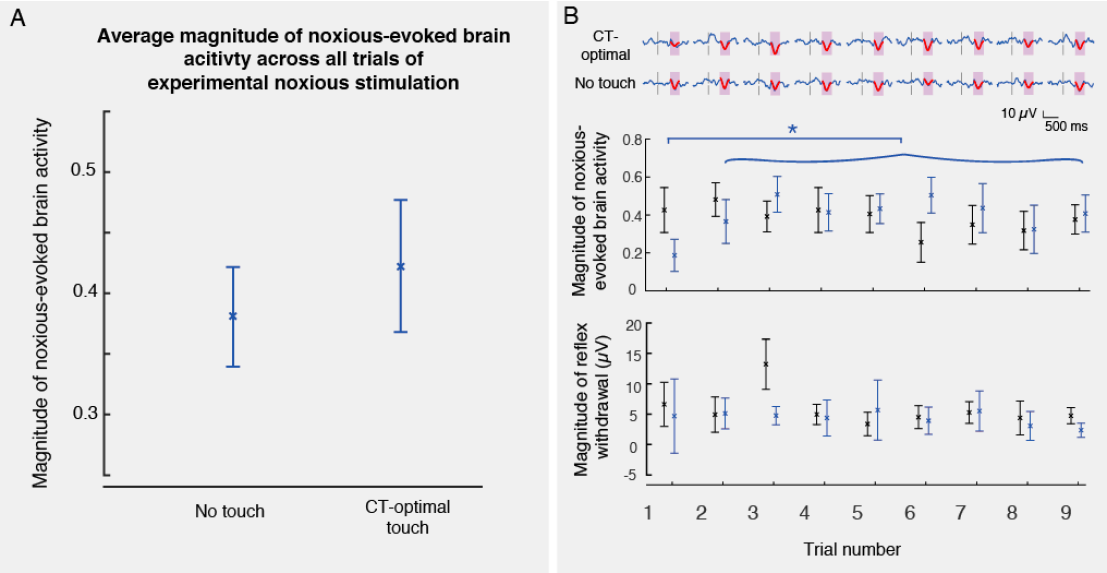


Figure 3.4: Order effect of CT-optimal touch. (A) When considering all trials, CT-optimal touch does not reduce noxious-evoked brain activity compared with the no touch condition. (B) Top: average noxious-evoked brain activity in each trial in the CT-optimal and no touch conditions. Middle: Noxious-evoked brain activity with trial number in the no touch condition (black) and CT-optimal touch condition (blue). Noxious-evoked activity following the first experimental noxious stimulus in the CT-optimal touch condition is significantly lower than noxious-evoked brain activity following the subsequent stimuli. Bottom: reflex withdrawal magnitude, calculated using the RMS method, does not change with trial number. Error bars indicate mean ± standard error for brain activity and median ± standard error for reflex withdrawal; * indicates $p < 0.05$.

3.4.2 Study 2: The effect of gentle touch on nociceptive responses evoked by clinically necessary heel lancing

3.4.2.1 Noxious-evoked brain activity following a clinical procedure is reduced by CT-optimal touch

The mean ± standard error of noxious-evoked brain activity was 1.12 ± 0.17 in the no touch condition, and 0.72 ± 0.17 in the CT-optimal touch group. Consistent

with Study 1, noxious-evoked brain activity was significantly reduced by CT-optimal touch (p=0.045, two-sided t-test, Figure 3.5A). An approximately 40% reduction in the magnitude of noxious-evoked brain activity was observed.

One infant required two heel lances for sufficient blood collection and these were performed ~ 4 minutes apart. CT-optimal touch was applied prior to each lance, and contrary to the attenuation of activity with trial number observed in Study 1 (where stimuli were on average ~ 15 seconds apart), the magnitude of noxious-evoked brain activity was similar on each occasion, though conclusions cannot be drawn from only one infant (Figure 3.5C).

When investigating the effect of gestational age on responses to heel lancing in the age-matched control infants, the two were found to be significantly correlated (p=0.024, R^2 = 0.31, Figure 3.6). When the magnitude of noxious-evoked brain activity following heel lancing in the infants receiving gentle touch was compared with the magnitude of noxious-evoked brain activity in random samples of non age-matched infants who had not received any intervention prior to lancing, a significant difference was found only 3 % of the time.

3.4.2.2 Reflex withdrawal is not altered by CT-optimal touch

The median ± standard error of the RMS was 35.2 ± 7.8 μV in the no touch condition, and 32.8 ± 5.7 μV in the CT-optimal touch condition. The amplitude of reflex withdrawal was 100,160 ± 23,786 μV in the no touch group, and 110,400 ± 73,848 μV in the CT-optimal touch group; CT-optimal touch did not significantly

alter reflex magnitude when considering either the RMS (p=1, Mann-Whitney U-test, Figure 3.5B) or the amplitude (p=0.70), in accordance with the results from Study 1.

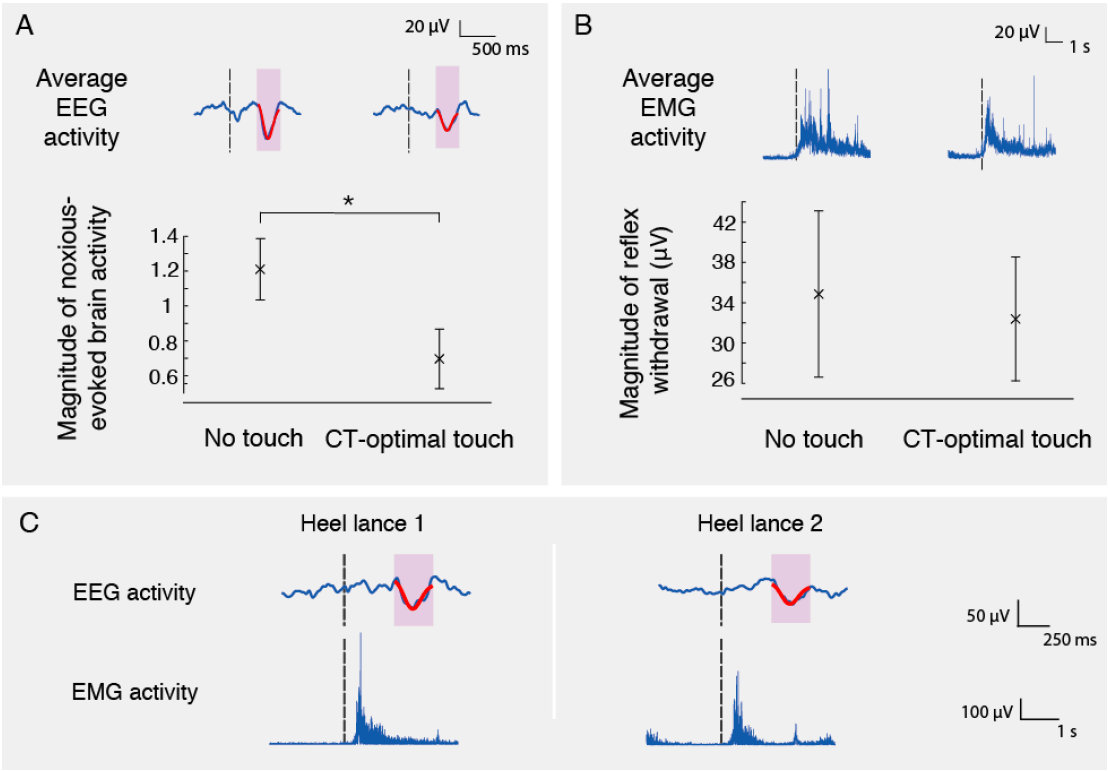


Figure 3.5: CT-optimal touch reduces noxious-evoked brain activity, but not reflex withdrawal, prior to a clinically necessary heel lance. (A) Top: average brain activity following heel lancing in the no touch condition and CT-optimal touch condition. Bottom: Noxious-evoked brain activity in the CT-optimal touch group is significantly lower than noxious-evoked brain activity in the no touch group. (B) Top: average reflex withdrawal activity in the no touch and CT-optimal touch condition. Bottom: CT-optimal touch does not alter reflex withdrawal. (C) In one infant requiring two heel lances approximately 4 minutes apart, the magnitude of the noxious-evoked brain activity (and reflex withdrawal) are comparable. Error bars indicate mean ± standard error for brain activity and median ± standard error for reflex withdrawal; * indicates $p < 0.05$.

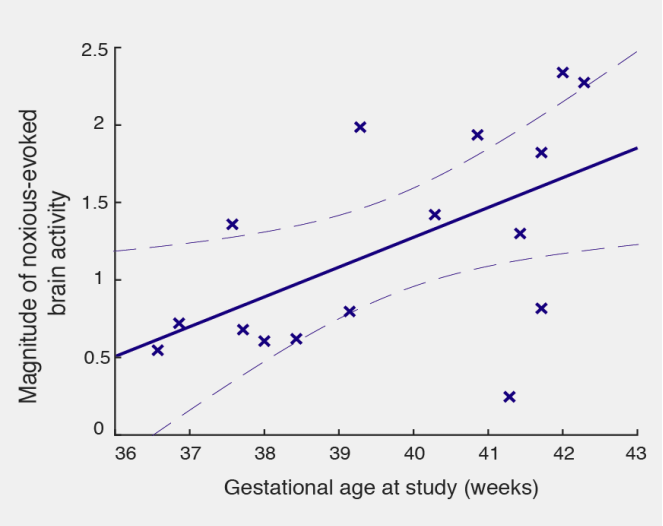


Figure 3.6 Gestational age is positively correlated with noxious-evoked brain activity in control infants. Gestational age correlates with noxious-evoked brain activity in the 16 age-matched control infants who did not receive any intervention prior to lancing. Regression line (solid) and 95% confidence intervals (dashed) are displayed.

3.4.2.3 Effects of CT-optimal touch on facial expressions and physiology

The PIPP-R score for infants in the CT-optimal touch group was 8 (5, 10) (median (lower quartile, upper quartile)), and 7 (6, 11) in the no touch group. These values are not significantly different (p=1, Mann-Whitney U-test). However, PIPP-R scores for only 11 out of 16 infants could be calculated in the no touch group due to missing data, and so the smaller sample size must be noted.

Separate facial expression, heart rate, and oxygen saturation analyses were conducted to allow modality-specific analyses and the analysis of larger sample sizes. Regarding facial expressions, the average expression duration was 4 (2, 12) seconds in the CT-optimal group, and 6 (0, 14) seconds in the no touch group. These durations are not significantly different (p=0.93, Mann-Whitney U-test). Some infants do not respond facially to a noxious stimulus [12 241 237], and similar proportions of infants exhibited facial expression responses in the CT-optimal

touch group and the no touch group (12/16 in the CT-optimal touch group, and 9/14 in the no touch group; p=0.52, chi-squared test, not significantly different). When considering only the infants who responded facially, the duration of the facial expression response was 7 (4, 13) seconds in the CT-optimal touch group, and 13 (9, 15) in the no touch group. These responses are not significantly different (p=0.30, Mann-Whitney U-test) although the median response duration was almost 50% shorter in the CT-optimal touch group compared with the no touch group (Figure 3.7).

Regarding physiological changes, the heart rate increase of 25 (14, 33) BPM in the CT-optimal touch group was not significantly different from the increase of 26 (15, 32) BPM in the no touch group (p=0.91, Mann-Whitney U-test), and the oxygen saturation decrease of 1 (0, 3) % in the CT-optimal touch group was not significantly different from the change of 0 (0, 1) % observed in the infants in the no touch group (p=0.29, Mann-Whitney U-test) (Figure 3.7).

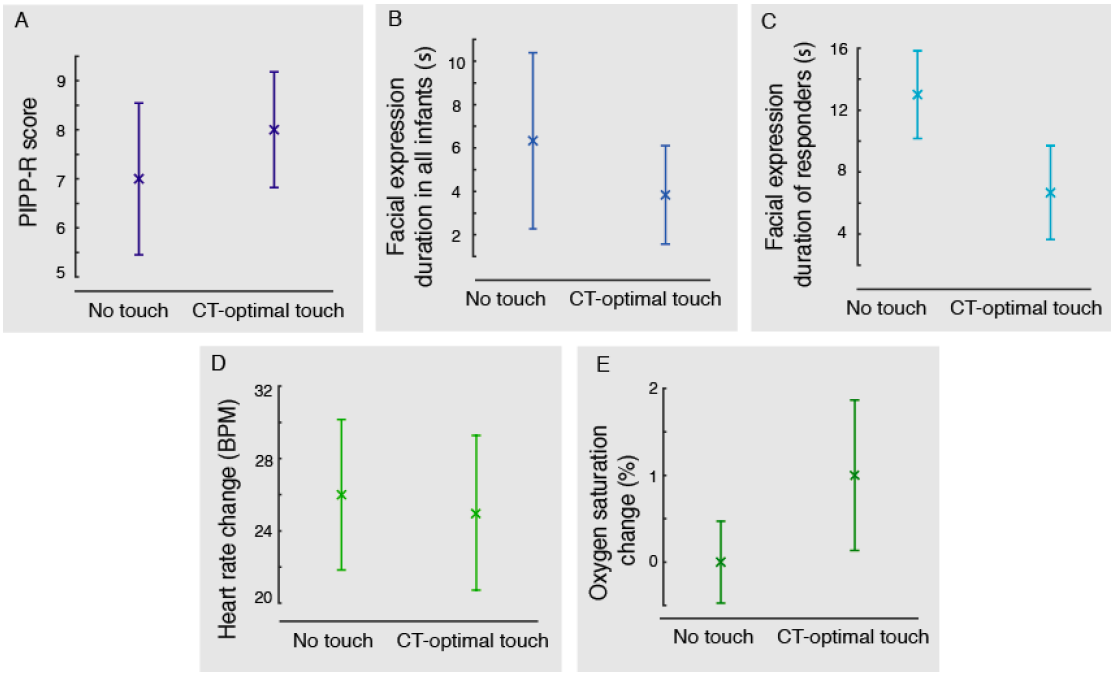


Figure 3.7 CT-optimal touch prior to heel lancing does not significantly alter behaviour or physiology. PIPP-R scores (A), facial expression response duration in all infants (B), facial expression response duration in only the infants who exhibited a facial expression response (C), heart rate change (D), and oxygen saturation change (E) in the no touch condition and CT-optimal touch condition. Error bars indicate median ± standard error.

3.5 Discussion

3.5.1 Overview

Study 1 demonstrates that gentle touch prior to an experimental noxious stimulus, at a velocity that activates CT fibres in adults, can attenuate noxious-evoked brain activity in infants. Touch that is not targeted towards CT fibres did not significantly modulate noxious-evoked brain activity. The effect of CT-optimal touch was clinically relevant, as stroking at a CT-optimal velocity prior to heel lancing reduced noxious-evoked brain activity. These findings are consistent with the adult literature demonstrating that CT fibre activation can modulate the

perception of pain [346 376 387]. CT-fibres are believed to be involved in encouraging social bonding [364 367], and it is plausible that the role of CT fibres in mediating pain reduction is related to their role in conveying the affective and rewarding properties of human touch.

3.5.2 Order effect of CT-optimal touch

When considering the magnitude of noxious-evoked brain activity with trial number in the no touch condition, no habituation or sensitisation was observed, as previously reported [210]. However, the reduction in noxious-evoked brain activity caused by the pre-stimulus CT-optimal touch appears to attenuate with repeated stimulation, suggesting that CT fibre activity may habituate. This is in line with evidence from previous studies using microneurography to demonstrate that CT fibres fatigue and become less responsive to following stimuli [348 388], and behavioural studies reporting that ratings of liking and wanting to be touched at a CT-optimal rate decrease with repetitions [363].

In Study 2, an infant requiring two heel lances (~ 4 minutes apart) demonstrated noxious-evoked brain activity of comparable size after each instance of CT-optimal touch and noxious stimulation. Although these results come from only one infant, they suggest that CT fibre activity may recover in the space of a few minutes. Most research investigating CT fibre fatigue in human adults has utilised intertrial intervals in the seconds range [348 388], but one study recording from cat C-mechanoreceptors has demonstrated that while responses are reduced following rapid successive stimulation, at intervals of over 4 minutes, there was no

interaction between successive stimuli [390]. Human CT fibres are believed to be less dependent on previous history than the C-mechanoreceptors in other mammals [348], and the timings of CT fibre fatigue should be characterised in infants if CT-optimal touch is to be considered as a therapeutic intervention. The time-course of CT recovery is especially relevant when considering practices such as clustered care in hospitalised infants, in which procedures and routine care are delivered at the same time rather than being spaced over time, in order to allow longer periods for the infant to rest and minimise episodes of handling [391].

3.5.3 Dissociation of nociceptive responses

Despite observing a significant effect of CT-optimal touch on noxious-evoked brain activity, no effect was observed on reflex withdrawal, behaviour, or physiology. This was not anticipated, as reflex withdrawal is generally correlated with noxious-evoked brain activity [210], and infant massage has been commonly reported to reduce body movements, facial expressions, and physiology [354 355 356 357 358].

Reflex withdrawal develops concomitantly with noxious-evoked brain activity during the preterm period [17] and the two measures are correlated in term infants [210]. Furthermore, animal work demonstrates that CT fibres make numerous connections in the dorsal horn through which inhibitory influences can be exerted [392 393 394 378], reminiscent of the proposed gate control theory of pain [395], suggesting that CT-optimal touch would alter reflex withdrawal. Based on the lack of effect of gentle touch on reflex withdrawal observed here, and studies suggesting that the

regions involved in descending modulation in the brain are mature in infants [223] but that the corticospinal connections involved in spinal modulation are not yet mature [80] [81] [82] [83], it was hypothesised that CT-optimal touch influences the more mature brain systems but does not affect the still developing spinal circuitry in infants. However, this study may also be underpowered to detect an effect on reflex withdrawal, as will be discussed further in Chapter 5.

PIPP-R scores between groups were also unaltered by CT-optimal touch. However, this study was not powered to investigate behavioural and physiological effects – previous power calculations have indicated that sample sizes of ~ 66 infants in each group would be required to observe a clinically meaningful reduction in PIPP-R score of 2 points [13]. When only facial responders were considered, there was a trend towards a decrease in facial expression duration. This difference was not significant, but is consistent with observations that tactile stimulation, such as infant massage and kangaroo care significantly reduce behaviour and pain scores [338] [353] [354] [355] [356] [357] [358]: further investigation into the behavioural effects of CT-specific touch is warranted in a larger sample, as will be further discussed in Chapter 5.

Previous studies investigating the effect of infant massage on surrogate measures of pain have provided longer periods of massage than that applied in this study (2 minutes or more) [354] [355] [357] [358]. It is possible that a period of stimulation longer than the 5 or 10 seconds of CT-optimal touch applied here is necessary before effects on behaviour and physiology are observed. In future studies, the effects of a longer period of CT-optimal touch should be explored, and the period of CT-optimal

touch that could provide the greatest effect without habituation or fibre fatigue occurring should be investigated.

Lastly, it is not surprising that CT-optimal touch did not alter oxygen saturation changes, as in the no touch condition the heel lance caused negligible changes (0 (0, 1) % (median (lower quartile, upper quartile))), meaning that an improvement was not possible.

3.5.4 Potential mechanisms of action

While in adults microneurography can be used to identify CT fibres [345] this technique is not ethical or safe for use in infants, and it is therefore not possible to determine for certain which fibres are being activated during tactile interventions. Nonetheless, the lack of effect of CT non-optimal touch on noxious-evoked brain activity suggests that the observed effects may be CT-specific, and that CT fibre activation could be responsible for mediating the pain reduction observed following tactile interventions such as infant massage.

CT fibres are known to project via the dorsal horn in the spinal cord to cortical regions involved in the processing of both sensory and affective aspects of stimuli in adults, such as the insular cortex, prefrontal cortex, superior temporal sulcus, and cingulate cortex [369 370 371 372 373]. The specific neurophysiological mechanisms by which CT fibres modulate pain are not known, but the literature on the interactions between touch and pain suggest that tactile stimuli influence nociceptive processing at both the level of the spinal cord and the brain. Animal

work suggests that tactile (vibratory) stimuli depress nociceptive signalling at the level of the spinal cord [396], in accordance with the gate theory of pain [395], and similarly, adult studies administering non-painful electrical stimulation concomitant with painful laser stimulation have concluded that this non-nociceptive input dampens the nociceptive input at the level of the spinal cord [397], as it is only effective if the two inputs were ipsilateral [398]. Additionally, further work suggests that touch exerts its pain-modulatory influence at both the subcortical and cortical level, as the blink reflex (generated by the subcortical brainstem) is reduced by tactile stimulation, as is cortical activity recorded by both EEG [325] and magnetoencephalography (MEG) [399].

In infants, an effect on reflex withdrawal was not observed, and this could be due to immature spinal cord and brain-spinal cord circuitry [80 81 82 83], as seen in animal models. Two recent imaging studies, though not a third [400], have demonstrated that in the first few months of life, CT-optimal touch activates brain regions involved in affective processing as in adults, including the insular cortex and middle temporal gyrus. These studies suggest that infants process the affective and emotionally valent aspects of CT-optimal touch in an adult-like manner [384 383], and this could underlie the adult-like reduction in cortical pain processing following CT-optimal touch observed here.

In adults, two endogenous substrates have been proposed to be involved in mediating the pain-relieving effects of CT-optimal touch. The first is the hormone and neuromodulator oxytocin, which has been implicated in the effects of tactile stimulation due to its role in the social aspects of touch, and its observed pain-

modulatory ability. Oxytocin is released in response to skin stimulation [401] including massage [402] [403] [404], and is involved in social behaviour [405] [406] [407] and modulating the stress response [408] [409] [410]. Oxytocin administration has been demonstrated to reduce pain perception to both chronic and acute painful stimuli in several studies [411] [66] [412] [413]. As the actions of oxytocin resemble the effects observed following CT fibre activation, oxytocin has been hypothesised to mediate CT-optimal touch [327].

The opioid system, well known to be involved in pain modulation, has also been implicated in underlying the effects of tactile stimulation. In infants, massage (as well as music therapy) is reported to increase the concentration of beta-endorphins [414], and in adults massage delivered by a romantic partner has been shown to alter opioid receptor availability in brain areas involved in processing social touch [415]. Another study in adults investigated the relationship between CT fibres and the opioid system, and demonstrated that fibromyalgia patients with chronic pain and altered opioid receptor availability display a blunted dissociation between CT-optimal and CT non-optimal touch. This study also demonstrated that in healthy participants the opioid antagonist naloxone increases ratings of touch pleasantness and decreases the preference for CT-optimal touch, suggesting that opioids may be involved in the processing of CT-optimal touch, though this relationship is not yet well characterised [326]. However, other research reports that administration of μ-opioid receptor agonists and antagonists does not alter the perceived pleasantness of CT-optimal touch, indicating that μ-opioid signalling is unlikely to underpin the effects of CT-optimal touch [416]. The research in this thesis does not allow conclusions to be made about the neurophysiological mechanisms underpinning

CT-optimal touch, and further work, informed by animal models and adult studies, is needed to explore this.

Despite the existence of hypotheses regarding the neurophysiological underpinnings of CT-optimal touch, CT fibres may not be involved, or may not comprise a large contribution of the effect of gentle touch. Touch to the glabrous skin, which is not believed to contain CT fibres [347] [360] is also perceived as pleasant [370], massage of the glabrous heel is recommended in clinical guidelines for pain relief prior to heel lancing in infants [289], hand-holding is observed to reduce pain perception in adults [417], and other forms of non-affective touch and fibre activation have been demonstrated to reduce pain ratings and pain-evoked brain activity, as detailed above [397] [418] [398] [325] [399]. This suggests that there are mechanisms other than CT fibres that convey information about pleasant and social touch. One proposed theory is that CT fibres constitute an innate system for social touch, while touch to the glabrous skin is a learned response (for example the association between hand holding and the psychological feeling of support), as these two types of touch are processed in different brain regions; touch to the glabrous skin activates brain regions associated with sensory processing e.g. the somatosensory cortex, while touch to the hairy skin activates regions associated with emotional processing such as the insular cortex and orbitofrontal cortex [370]. Further research is needed to ascertain the relative contributions of different mechanisms to the perception of pleasantness and pain relief following touch. Nevertheless, the observation that gentle touch can reduce pain processing in infants is an important finding, due to the numerous benefits associated with tactile interventions detailed below.

3.5.5 Benefits of tactile therapies

As a non-pharmacological intervention, gentle touch is not associated with a risk of adverse effects, is easy to implement and cost-free. The reduction in noxious-evoked brain activity following CT-optimal touch is comparable to the effect size reported in a previous study of topical local anaesthetic [12], however the effect of topical anaesthetic is likely to have been conservative in this study due to the limited time allowed for absorption (30 minutes) prior to application of the noxious stimuli.

Infant massage is associated with numerous specific beneficial impacts [419] [420]. Infant massage has been demonstrated to cause increases in infant weight gain [421] [422] [423] [424] [425], decreased stress levels [426] [427] [428], reduced length of hospital stay [429], improved sleep patterns [430] [431], and a reduction in bilirubin levels, decreasing infant jaundice [432] [433] [434].

Additionally, social touch is important for parent-infant bonding: parents instinctively stroke infants at a CT-optimal velocity [385] [386], and report that infant massage improves the parent-infant bond [435] [436] [437] and reduces maternal symptoms of anxiety [438] [439] and depression [440] [441].

Research also indicates that early caring touch is important for infant development: supportive touch in premature infants affects responses to later procedural touch and pain [173], and maternal touch frequency predicts the development of functional connectivity between brain regions associated with social processing in 5 year olds

[442]. Additionally, institutionalised infants who have received reduced early life touch display altered sensory processing later in life [443] [444] [445]. Parents may not be able to touch or hold their hospitalised premature infants due to safety, infection control, or cultural reasons [446], but this research suggests that early caring touch is important from the first stage of life.

3.5.6 Strengths and limitations

As detailed above, a crucial limitation in this study is the inability to determine the contribution of CT fibres to the observed effects of gentle touch. It cannot be concluded CT fibre activation is underlying the reduction in noxious-evoked brain activity, but nevertheless the demonstration that gentle touch can modulate noxious-evoked brain activity is important due to its clinical relevance, safety, and ease of application. Further research to determine the contribution of CT fibres, and other possible mechanisms underlying the effects of gentle touch is important, in order to be able to optimise tactile interventions.

The use of experimental noxious stimuli in Study 1 of this chapter allowed a within-subject study design, in which the stimuli could be applied on multiple occasions. The within-subject design allowed the effects of individual variability to be accounted for, and the application of trains of multiple stimuli enabled an increased signal to noise ratio. In Study 2 of this chapter, the effect of gentle touch on the clinically relevant stimuli heel lances was investigated. The single trial nature of investigating clinical procedures resulted in more rejections due to artefact, prolonging the process of recruitment (data from 4 infants receiving gentle

touch were rejected due to artefact, meaning that 20 infants had to be recruited and studied to obtain a sample size of 16 infants).

The single trial nature of investigating medically required stimuli also meant that a between-subject comparison was required. Infants were matched based on gestational age, as nociceptive responses are known to be altered with gestational age [17 72 18 237] and age-matching can account for the variation associated with age. This allowed a paired analysis to be performed, which reduced the sample size required from 58 infants (29 per group) for a non-paired analysis to 32 infants (16 per group). The observation that in the age-matched control infants the magnitude of noxious-evoked brain activity following heel lancing was significantly correlated with gestational age, and the calculation that a significant effect of gentle touch would only have been observed 3 % of the time if infants were not age-matched, emphasises the importance of age-matching to account for factors affecting individual variation to nociceptive stimuli. However, age-matching does not account for individual variation associated with other factors, such as behavioural state, prior pain, and genetic variability and their effects on nociceptive sensitivity, and also lengthens the recruitment process, as eligible infants requiring blood tests must be of the correct age. Therefore, in Chapter 5 of this thesis, an alternative method of matching infants is investigated, by accounting for nociceptive sensitivity itself.

3.5.7 Future directions

Preterm infants undergo a higher frequency of medically necessary painful procedures than older infants [447], and a safe and simple method of pain relief would be invaluable in this population. In these infants skin is more fragile [448], sensory systems are still developing postnatally [17 72], and handling may have detrimental effects on physiological stability and pain responses [449 450 391]. However, more recent research suggests that comforting touch has positive stress-reducing effects in preterm infants [451], many of the benefits of infant massage on pain responses, weight gain, and stress levels detailed above have been demonstrated in preterm infants [354 355 423 426 429], and it would be interesting to investigate whether CT-optimal touch is also effective in modulating the response to pain in these infants.

Additionally, the efficacy of CT-optimal touch prior to other medically necessary stimuli, including those of higher intensity such as immunisation, must still be investigated. Whether CT-optimal touch delivered by a parent is more effective than CT-optimal touch delivered by a researcher is also of interest: although CT-optimal touch delivered to adults by a robot has been shown to result in a comparable perception of pleasantness to CT-optimal touch delivered manually [452], other research has demonstrated that 9 month old infants attend more to CT-optimal touch they believe to be delivered by their mother than an investigator [382].

Lastly, the neurophysiological underpinnings of CT-optimal touch-induced pain relief should be explored, including the cellular and molecular signalling mechanisms as well as the optimal period of application and the optimal location of administration. It is not yet known whether CT-optimal touch must be

administered to a nearby dermatome to the noxious stimulation to have a pain-relieving effect [397], or whether CT-optimal touch is also effective in reducing pain perception when applied to areas of the body such as the back, which have a high density of CT fibres and is the location at which adults predict CT-optimal touch will feel the most pleasant [453] [350]. This research would increase understanding of how CT fibres exert their influence, and could aid the development of targeted pain relief therapies.

3.6 Conclusion

These findings provide a potential explanation for the pain-relieving effects of infant massage and demonstrate that this effect is clinically applicable. A better understanding of the role of CT fibres in early life and the neurophysiological mechanisms that may be underpinning tactile pain interventions could lead to the more widespread use of optimised non-pharmacological pain management interventions in neonatal care.

Chapter 4: Characterisation of immunisation-evoked responses and investigation of the effect of paracetamol

4.1 Introduction

Characterisation of clinical procedures is necessary to enable the assessment of pain relief interventions for these procedures. While responses to experimental noxious stimuli and heel lances have been well characterised [11] [210] [17], and a template of noxious-evoked brain activity has been validated to assess the magnitude of the responses to these stimuli [12], this template cannot be applied to additional stimuli without prior validation. Investigation into whether the already existing template provides a suitable fit to brain activity following alternative acute medically-required procedures is advantageous, as it means that the brain activity evoked by these stimuli does not need to be formed into a new template, but that the already validated template can be utilised. In a previous study of 12 term infants, the template was shown to provide a suitable fit to brain activity evoked by cannulation [229], however, the applicability of the template to brain activity evoked by additional stimuli, for example immunisation, has not yet been assessed.

Intramuscular immunisations are administered to infants at postnatal weeks 8, 12, and 16, and at 1 year [343] to provide protection against a range of diseases. Prematurely born infants still in the neonatal unit receive these immunisations as inpatients. Evoked brain activity in response to immunisations has been recorded previously in (term-born) term infants in outpatient clinics [228], and visually resembles that seen in response to previously characterised noxious stimuli.

However, it remains to be seen whether this response is consistent in hospitalised ex-preterm infants who have reached immunisation age at a much younger post-menstrual age than term-born infants and whose brain activity is likely to be less mature (Figure 1.2, General Introduction), and whether the template can reliably fit this response. As well as characterising brain activity, immunisation-evoked facial expressions and physiology are also recorded in this study to enable a multimodal assessment of infant pain.

Paracetamol is administered as an antipyretic immediately after the week 8 immunisations in line with the NHS guidelines, to help manage the fever that may result from the Meningitis B vaccine that is administered at this time [15]. In adults, paracetamol is an established antipyretic and analgesic, and although its exact mechanisms of action have not been fully elucidated, paracetamol is believed to act both peripherally, inhibiting cyclooxygenase (COX) synthesis of prostaglandins, and centrally, where it also influences COX, as well as acting on the descending serotonergic system, nitric oxide pathway, and influences the formation of active metabolites in the cannabinoid system [454] [455].

In adults, paracetamol has been demonstrated to reduce pain ratings to both chronic and acute pain. Following surgeries including lumbar discectomy, breast surgery, hysterectomy, and dental surgery, paracetamol administration has consistently been demonstrated to reduce pain ratings over the 24 post-operative hours according to patient-rated visual analogue scales [456] [457] [458] [459] [460], and patients receiving paracetamol reported increased satisfaction with their pain management [458]. Several studies have also indicated that paracetamol administration decreases

the post-operative need for morphine [459] [461]. However, the results regarding the effect of paracetamol on morphine consumption are mixed, with some research reporting that the reduced need for morphine is subtle and not statistically significant [456]. In addition, investigations have concluded that although morphine was consumed at a lower rate in the paracetamol group, the total amount consumed was not different [457], and similarly, that although a lower proportion of patients received morphine with prior paracetamol administration, the total amount of morphine consumed did not differ [462]. Nevertheless, based on this evidence, a Cochrane review has concluded that there is high quality evidence to suggest that a single dose of paracetamol can provide effective analgesia for ~ 4 hours for roughly 36 % of patients with post-operative pain, and that there is a ~ 26 % reduction in opioid administration over the 24 hours post-surgery [463].

Regarding the effect of paracetamol on acute pain, there are also several studies demonstrating that paracetamol increases the threshold at which painful electrical [464], mechanical [465] [466], and laser [467] stimuli are detected, and decreases the severity of pain ratings following electrical stimulation [468] and mechanical stimulation of areas of hyperalgesia [469].

Research has also demonstrated that paracetamol reduces EEG-recorded pain-related brain activity [470]. Following thermal noxious stimulation, the magnitude of evoked potentials (calculated as the peak-to-peak amplitude) was reduced [471], and following electrical stimulation, the peak-to-peak amplitude of evoked potentials, as well as stimulus-evoked delta frequency changes, were reduced by ~ 20 %, concomitant with a 6 % decrease in pain ratings [468]. Following laser stimulation,

the evoked potential (quantified as the power of the signal) was reduced, along with a concomitant increase in pain thresholds [472], and following painful carbon dioxide stimulation of nasal mucosa, pain-related evoked potentials (calculated as the base-to-peak amplitude) were reduced by paracetamol, although an effect on pain ratings was not observed [473]. However, not all studies have found an effect of paracetamol on brain activity or pain thresholds following noxious stimulation [474].

fMRI has also been used to investigate the effect of paracetamol on the processing of acute noxious thermal stimuli in healthy adult participants; a reduction in activity in cortical regions typically involved in pain processing including the prefrontal cortices, insula, thalami, anterior cingulate cortex, and periaqueductal gray was reported, and paracetamol significantly reduced numerical pain ratings [475]. The effect of paracetamol on acute mechanical stimulation in osteoarthritis patients has also been investigated using fMRI, reporting a reduction in activation of the sensory cortex and supramarginal gyrus along with reduced pain ratings [476].

While the majority of the adult literature indicates that paracetamol is an effective analgesic, in infants the evidence regarding the analgesic efficacy of paracetamol is inconsistent. Paracetamol has been shown to be ineffective for circumcision-related pain, although it may provide some benefit in the later post-operative period (> 6 hours) [477]. In line with this, several, though not all [478], studies have demonstrated a reduced post-operative need for opioids after paracetamol administration [479] [480] [481]. Additionally, one study has reported reduced pain scores in infants in mild to moderate chronic pain (e.g from delivery-related trauma) [482],

and another study suggests that paracetamol modestly reduces pain scores, but not cry duration, during ROP eye examinations [483].

However, many other research studies suggest that paracetamol is not effective for procedural pain including heel lances [484 485], peripherally inserted central catheters [486] and eye screening [487] in infants. Consequently, a Cochrane review [265] concluded that there is insufficient evidence to determine the efficacy of paracetamol on infant procedural pain. It may be that the behavioural measures used to assess the efficacy of paracetamol for acute procedures, such as facial expressions or crying duration, do not solely reflect pain but are influenced by distress, as discussed previously [203 9 8], or it may be that paracetamol is not effective in infants due to developmentally regulated pharmacokinetics and pharmacodynamics.

There is a paucity of information regarding the pharmacokinetics and pharmacodynamics of paracetamol in ex-premature neonates, however there is evidence of its immaturity; glucuronidation, a step in the metabolism of paracetamol, and clearance of paracetamol, are reduced in neonates, and although their increase over development can be predicted by gestational age and weight, there is also great age-independent inter-individual variability in drug metabolism [488 489 490 491 492]. Research has suggested that doses of 15 – 20 mg / kg should be administered to infants ~ 36 weeks gestational age [493 494], and that paracetamol reaches peak plasma concentrations within 1 hour following IV administration [495 489 496] and just over ~ 1 hour following oral and rectal administration [497 498].

During the course of this DPhil, the Neonatal Consultants at the John Radcliffe Hospital, Oxford made a change to the clinical practice guidelines to move the administration of oral paracetamol from immediately after immunisation administration to 1 hour prior to immunisation administration. This earlier administration would allow paracetamol to exert its influence prior to the process of immunisation [497] [498], and could allow fever to be managed more effectively and potentially decrease the physiological instability that can occur post-immunisation [499] [500] [501]. Although some evidence has indicated that prophylactic paracetamol administration may decrease antibody concentrations in infants [502], this effect is not believed to be clinically significant as protective antibody levels were still reached when assessed at follow-up [503], and more recent studies, by the same authors and others, demonstrate that paracetamol administration before or at the time of immunisation does not affect the clinical efficacy of the immunisations on the immune system [504] [505] [506] [507].

According to the Oxford Newborn Care Services Guidelines [342], a paracetamol dose of 15 mg/kg was given, in line with the National Institute for Health and Care Excellence (NICE) and British National Formulary for Children (BNFc) guidelines for infants born at less than 4 kg: this recommended dose is the same for paracetamol being given for the purpose of pyrexia management and for the purpose of relief from mild-to-moderate pain, is in line with the research outlined above regarding the pharmacokinetics [493] and time of action of oral paracetamol [497] [498], and suggests that paracetamol would be efficacious ~ 1 hour post administration.

This clinical guideline change at the John Radcliffe Hospital, Oxford, provided the opportunity to first characterise immunisation-evoked responses, and then investigate the analgesic efficacy of paracetamol on immunisation-evoked responses in a between-subject comparison in the second study of this chapter. In the final study of this chapter, the effect of paracetamol on responses evoked by experimental noxious stimuli was investigated in a within-subject comparison, by applying the stimuli prior to paracetamol administration, and approximately 1 hour post paracetamol administration (prior to immunisation). These stimuli are well characterised and validated to be fit with the template of noxious-evoked brain activity, and a within-subject analysis means that variability in individual sensitivity to noxious input can be controlled for.

I hypothesised that immunisation-evoked brain activity in these ex-preterm infants would be similar to previously characterised noxious-evoked brain activity, and suitable for fitting with the template of noxious-evoked brain activity. I also hypothesised that paracetamol would reduce the magnitude of immunisation-evoked brain activity, as well as the magnitude of noxious-evoked brain activity following experimental noxious stimuli.

4.2 Chapter overview

This chapter is composed of 3 studies:

1. Characterisation of immunisation-evoked brain activity, behaviour, and physiological responses,

2. Investigation into the effect of paracetamol on immunisation-evoked responses in an independent sample of infants,
3. Investigation into the effect of paracetamol on nociceptive responses evoked by experimental noxious stimuli in a within-subject comparison.

4.3 Methods

4.3.1 Participating infants

As described in Chapter 2 (Section 2.2 Recruitment), eligible infants were clinically stable, and did not have a history of neurological problems, IVH grade 3 or above, or documented maternal substance abuse. Infants were not receiving any sedatives or analgesics at the time of study, aside from the infants receiving paracetamol for immunisation in this study.

Parents of 43 infants were approached. 9 did not want to take part in research, 1 did not appear to understand adequate English, and 6 were interested in taking part but were discharged or transferred before immunisations were administered (as illustrated in Figure 2.1, General Methods chapter). 27 infants were studied, and their demographic characteristics are described below (Table 4.1). Ventilation method and ward location are recorded to provide a more detailed characterisation of these ex-preterm infants, who are a more heterogeneous population than the term infants studied in Chapters 3 and 5 in terms of the range of their gestational ages and weights, and their prior complications and experiences during their time in the neonatal unit.

	Immunisation characterisation	Effect of paracetamol
Number of infants	17	10
Number of immunisations received	3 (1)	3 (0)
Gestational age at study (weeks)	38 (37, 39)	36 (36, 38)
Gestational age at birth (weeks)	28 (25, 29)	27 (26, 28)
Postnatal age at study (days)	64 (59, 94)	63 (59, 70)
Weight at study (g)	2770 (499)	2350 (575)
Weight at birth (g)	1040 (387)	910 (221)
Number of males	10 (59 %)	4 (40 %)
Ventilation	SVIA: 8 (47 %) LFT: 5 (29 %) HFT: 4 (24 %)	SVIA: 4 (40%) LFT: 2 (20 %) HFT: 4 (40 %)
Location	LDU: 11 (65 %) HDU: 5 (29 %) ITU: 1 (6 %)	LDU: 3 (30 %) HDU: 7 (70 %) ITU: 0

Table 4.1: Demographics of infants studied in Chapter 4. Values given are mean (standard deviation), median (lower quartile, upper quartile) or number (percentage). (Ventilation: SVIA = self-ventilating in air, LFT = low flow, HFT = high flow therapy; Location: LDU = low dependency unit, HDU = high dependency unit, ITU = intensive care unit).

Infants were recruited to Study 1 until the clinical practice guidelines were changed to move paracetamol administration prior to immunisation. After the guideline change, as many infants as possible were recruited for Studies 2 and 3 in the available time, but this number was limited by the infrequency with which ex-preterm infants require immunisation while still in hospital (~ 1-2 infants per month).

4.3.2 Experimental design

In Study 1 of this chapter, infants received 1-3 clinically necessary immunisations at week 8 or week 12. For the week 8 immunisations where paracetamol was due, this was administered immediately after the immunisations. In Studies 2 and 3, oral paracetamol was administered 1 hour prior to the week 8 immunisations.

Depending on parental preference, infants recruited after the guideline change either only took part in an observational investigation into the effect of paracetamol on immunisation, or also received 10 experimental noxious stimuli prior to and 1 hour post paracetamol administration (just prior to immunisation) (Figure 4.1). 10 experimental noxious stimuli were applied in line with previous investigations [210] [12] and the work in this thesis. The within-subject design of this analysis means that this comparison is better able to detect changes in brain activity due to paracetamol (as will be discussed further in Chapter 5). Experimental noxious stimuli were applied prior to immunisation administration, as immunisations can cause physiological instability and it would not have been appropriate to apply experimental stimuli post-immunisation in these ex-preterm hospitalised infants. Therefore it was not possible to use this paradigm in the infants in Study 1 who received paracetamol after immunisation. Methods relating to the administration of immunisations and experimental noxious stimuli are detailed in the General Methods chapter.

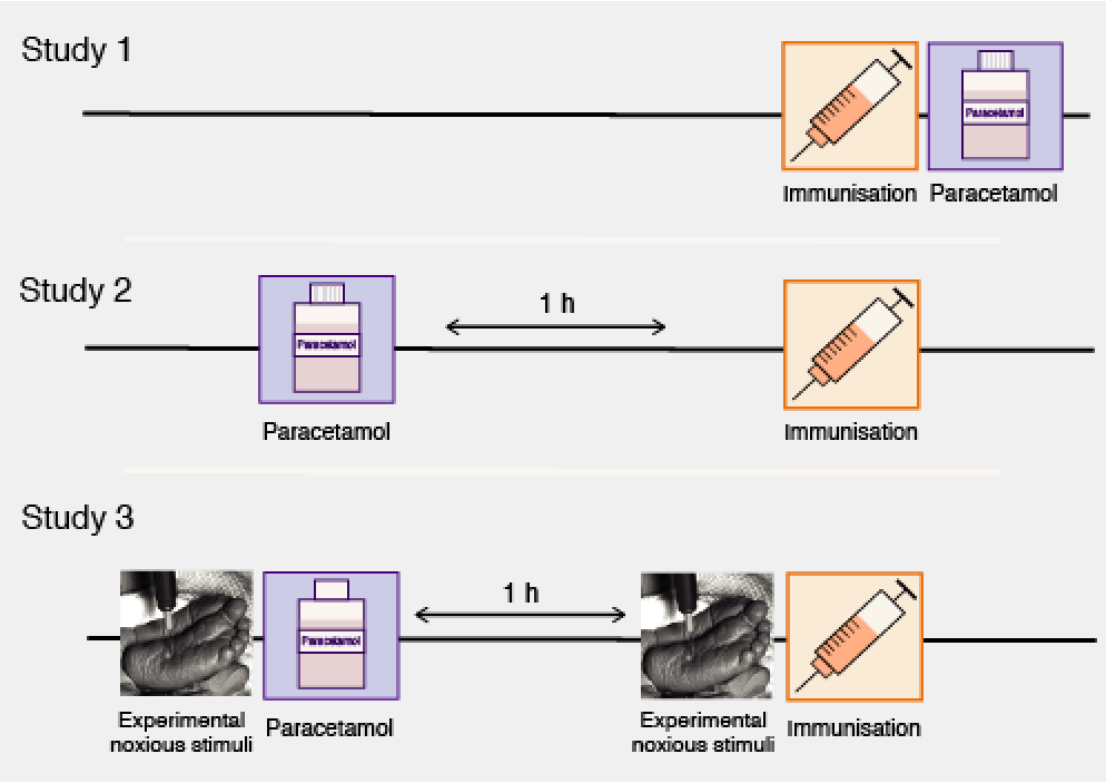


Figure 4.1: Experimental design followed in the studies detailed in Chapter 4. Infants in Study 1 received immunisations followed by paracetamol. Infants in Studies 2 and 3 received paracetamol 1 hour prior to immunisation, and infants in Study 3 received experimental noxious stimuli before and ~ 1 hour post paracetamol administration.

4.3.3 Recording techniques

Brain activity was recorded in response to both immunisation and experimental noxious stimuli. As immunisations cannot be automatically time-locked to the EEG recordings, a high-speed video camera (220 frames per second) that was directly linked to the EEG acquisition was used to film immunisation administration. The moment the immunisation needle first made contact with the skin was determined retrospectively (Figure 4.2). This point was chosen as stimuli including immunisations [228], experimental noxious stimuli [210], and cannulas [340 229]

have previously been time-locked to the EEG successfully using the high-speed video camera by finding the moment of first contact with skin, and this moment can be clearly identified from high-speed video camera recordings. Time-locking additional points such as moment of fluid administration was considered, but it was not possible to see this clearly enough from the high-speed video camera recordings.

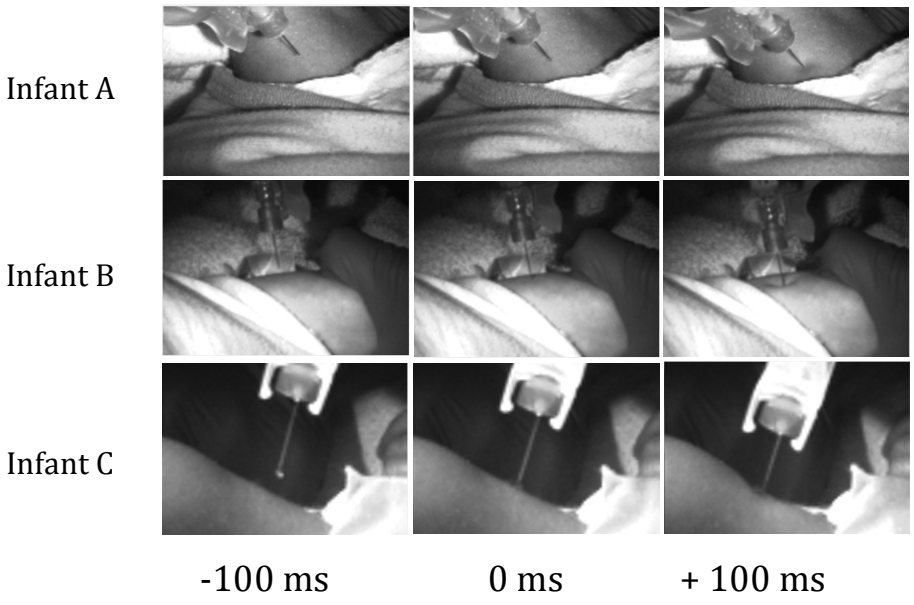


Figure 4.2: Identification of needle contact with skin. Examples from 3 infants demonstrating the use of the high-speed camera to identify the moment of stimulus contact with skin (t=0).

Intra-rater and inter-rater reliability of event-marking were checked in a sample of 10 videos: my event-markings were not significantly different from each other ($p=0.15$, Wilcoxon signed rank test, difference in ratings = 4.5 (2.5, 7) ms (median (lower quartile, upper quartile)) or from the markings of a second investigator ($p=0.89$, difference between ratings = 8.5 (2.3, 12) ms). Note that the frame rate of

the high-speed camera was 220 frames per second, so 4.5 ms is the inter-frame interval.

Reflexes were not recorded as the thigh was held still for immunisation administration. PIPP-R scores were recorded following immunisations and experimental noxious stimuli, as although experimental stimuli do not cause behavioural distress in term infants [210], the behavioural and physiological responses to these stimuli in ex-premature infants, two of whom were still preterm (below 37 weeks gestational age), have not been investigated. Further details of recording techniques are provided in the General Methods, Chapter 2.

4.3.4 Analysis

4.3.4.1 Brain activity

A total of 73 immunisations were administered to 27 infants; 43 immunisations in the 17 infants who had not received paracetamol prior to immunisation, and 30 immunisations in the 10 infants who did receive paracetamol prior to immunisation. In the infants who had not received paracetamol, 34 of the 43 immunisations across the 17 infants had adequate video footage from which the moment of immunisation could be successfully identified. In the paracetamol group, brain activity was not recorded in 2 infants (each receiving 3 immunisations) due to technical faults, and in a further infant only 1 of 3 immunisations was recorded. Of the 22 recorded immunisations, 18 immunisations across 8 infants had adequate video footage. The remaining immunisations could not be identified due to unclear

footage or because the process of immunisation obscured the camera view of the event. The use of the high-speed camera to obtain clear recordings improved with practice; in the first few infants, only one of three immunisations could be successfully event-marked, and this number, as well as the clarity of the recordings, increased over time with practice of obtaining the video footage and better communication with the clinical team administering the immunisations.

EEG recorded in response to immunisations and experimental noxious stimuli was exported and filtered as detailed in General Methods. The EEG epochs of 1 immunisation in the no paracetamol group (from an infant with only 1 immunisation identifiable from the video footage) and 1 immunisation in the paracetamol group (from an infant with 3 immunisations identifiable from the video footage) were rejected due to movement artefact.

Immunisation-evoked brain activity was characterised, and assessed to investigate the suitability of fitting the template of noxious-evoked brain activity. As infants received variable number of immunisations, responses were first Woody filtered and averaged within-subject to ensure that the group response was not biased towards individuals who had received a greater number of immunisations. A post-hoc test confirmed that there was no order effect of immunisation-evoked brain activity; the magnitude of the evoked response to the first immunisation was not different to the magnitude evoked by the last immunisation in infants receiving 3 immunisations ($p=0.62$). Woody filtering to the individual's average by ± 100 ms was permitted due to the possible imprecision associated with manually event-marking the moment of first contact of immunisation needle with skin – the largest

inter-rater difference in event mark timings was 67 ms, suggesting that allowing a Woody filter jitter of ± 100 ms to account for this is appropriate. Woody filtering of ± 100 ms to the average was also done when obtaining the group average response, to account for individual differences in the latency of the evoked response.

Cluster analysis was performed to identify periods where brain activity evoked by immunisation was significantly different from background brain activity. T-statistics were calculated between immunisation-evoked and background brain activity at each time point, and were considered as significant if they were above a predefined threshold of the 97.5^{th} percentile of the t-distribution. The start point and end point of the clusters were defined as the timepoint when the t-statistics fell above and below this threshold respectively.

Cluster analysis involves measuring the difference between numerous timepoints, and these multiple comparisons must be corrected for, as clusters of significant activity could form by chance even if there was no real difference between the two groups being compared. Therefore, Monte Carlo Simulation was performed to model the probability of obtaining different cluster sizes by chance: background brain activity was randomly shuffled with immunisation-evoked brain activity, and t-statistics between the two groups were calculated at every time point as was done in the original cluster analysis. The size of the largest cluster that formed was noted. This was done 1000 times to build a distribution of cluster sizes formed by chance. Then, the likelihood of the original cluster having formed due to chance if there

was no true difference between the two groups could be assessed by comparison with this distribution. A p-value < 0.05 was considered significant.

Next, Principal Component Analysis (PCA) was performed to obtain the waveform components (principal components, PCs) that explain the variance in the data within the time window defined by the significant cluster. The presence of these PCs was compared in the immunisation-evoked and background brain activity to ensure that the PC was present in higher levels in the immunisation-evoked than background brain activity, and correlation between the PCs and the previously characterised template of noxious-evoked brain activity was investigated.

Experimental noxious stimuli were also applied pre- and post- paracetamol administration. These are well characterised and validated to be fit with the template. A Woody filtered jitter of ± 50 ms was allowed to find a best fit with the template, consistent with previous studies [12 210] and Chapter 3.

4.3.4.2 Behaviour and physiology

Facial expressions, heart rate changes, and oxygen saturation changes were analysed and PIPP-R scores were calculated as detailed in General Methods, Chapter 2.

The facial videos of 1 infant were not analysed as the infant was distressed and crying throughout the baseline period, and in 2 other infants facial view was obscured following administration of 1 (of 3) immunisations. In another infant,

facial video was not recorded due to parental request. Physiology was not recorded in a further 3 infants - due to technical issues in 2 instances, and because the immunisation was administered without sufficient warning and so the monitor was not accurately marked in 1 infant.

Full PIPP-R scores to at least 1 immunisation could be calculated in 13 of 17 infants in the no-paracetamol group, and 9 of 10 infants in the paracetamol group. When infants received more than one immunisation, a within-subject average was calculated before obtaining the group average, as the PIPP-R, facial duration, heart rate change, and oxygen change values in infants receiving 3 immunisations were not different following the first and last immunisation ($p=1$, $p=0.42$, $p=0.95$, $p=0.88$ respectively). PIPP-R scoring was also performed in the infants receiving experimental noxious stimuli.

4.3.5 Statistical analysis

A two-sided t-test was utilised to determine whether the first principal component (PC1) identified from principal component analysis (PCA) was present in higher levels in immunisation-evoked than background brain activity. Linear correlation was used to assess the correlation of PC1 and the previously characterised template of noxious-evoked brain activity; p-values < 0.05 were considered significant.

Following projection of the template onto immunisation-evoked brain activity, the magnitude of the immunisation-evoked response as characterised by the template was compared with the magnitude characterised in background brain activity to

determine whether the characterised activity was distinct from background; a paired t-test was used.

A post-hoc diagnostic test was also performed on the magnitude of immunisation-evoked brain activity as quantified by the template to ensure there was no difference between the response following the first and last immunisation (paired two-sided t-test). Linear regression was used to investigate the effect of gestational age on immunisation-evoked brain activity.

Diagnostic tests were run on the PIPP-R responses to ensure that an order effect resulting from receiving 3 immunisations was not observed on behavioural and physiological responses; non-parametric Mann-Whitney U tests were used to compare the response to the first and last immunisation

Statistical tests were not conducted on the immunisation-evoked responses with prior paracetamol due to the small sample size.

4.4 Results

4.4.1 Characterisation of immunisation-evoked responses

4.4.1.1 Brain activity

Immunisation-evoked brain activity from 16 infants was averaged within-subject prior to obtaining the group average (Figure 4.3A). The group average response to

immunisation was compared to background brain activity (both Woody filtered to the average in the same manner to allow fair comparison) in order to visualise the evoked response (Figure 4.3B).

Cluster analysis [508] identified a period at 369 – 524 ms post stimulation in which immunisation-evoked brain activity significantly differed from background brain activity (p=0.038, cluster-corrected non-parametric test) (Figure 4.3C). No other clusters demonstrated significant differences after accounting for multiple comparisons with the cluster-corrected test.

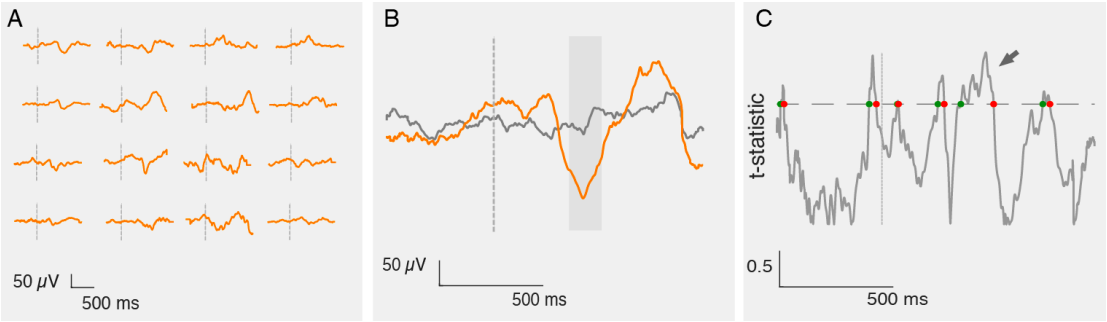


Figure 4.3: Immunisation-evoked brain activity. (A) Average subject traces, Woody filtered to the average by ± 100 ms, are shown. Dashed lines indicate the point when the immunisation needle first made contact with the skin. (B) Group average trace of immunisation-evoked brain activity (orange) overlaid on background brain activity (grey), both Woody filtered by ± 100 ms to the average. Grey box highlights the region in which immunisation-evoked brain activity and background brain activity are significantly different (369 – 524 ms post stimulation), derived from the cluster analysis depicted in C. (C) T-statistics indicate the difference in immunisation-evoked and background brain activity at consecutive timepoints; horizontal dashed line indicates a pre-defined threshold of the 97.5th percentile of the t-distribution, above which t-statistics were considered to indicate a significant difference. Green and red dots indicate the respective start and end points of clusters that are above the threshold. Arrow indicates the only cluster, also depicted by the grey box in B, which was significant following the cluster-corrected non-parametric test.

Principal component analysis (PCA) was then performed on the immunisation-evoked and background brain activity to find the waveform components that explain the variance in the data (Figure 4.4A). PCA was performed in the window 296 – 596 ms post noxious stimulation - this window was determined by taking the midpoint of the cluster and extending the beginning and end to obtain a 300 ms long window, consistent with the length of the original noxious-evoked template to enable comparison.

The first principal component (PC1) explained 45% of the variance in the data and was present in significantly higher levels in the immunisation-evoked data than the background data ($p=0.034$, t-test, Figure 4.4B). PC2 was not present in significantly different levels in immunisation-evoked compared with background brain activity ($p=0.12$), and the other PCs explained very small proportions of the variance (7 % and below).

The correlation between PC1 and the template of noxious-evoked brain activity was investigated, and the two were found to be highly correlated ($p<0.001$, $R^2=0.52$, Figure 4.4C), suggesting that immunisation-evoked brain activity is suitable to be fit with the previously validated template of noxious-evoked brain activity.

Immunisation-evoked responses were therefore fit with the template in the determined time window, which was rounded up from 296 – 596 ms to 300 – 600

ms post stimulation (Figure 4.4D). Subject traces were Woody filtered to the template in this time window. The mean ± standard error of the magnitude of immunisation-evoked brain activity was 0.70 ± 0.094. The magnitude of the response characterised by the template was significantly higher in response to immunisation compared with the background brain activity ($p<0.001$, Figure 4.4D).

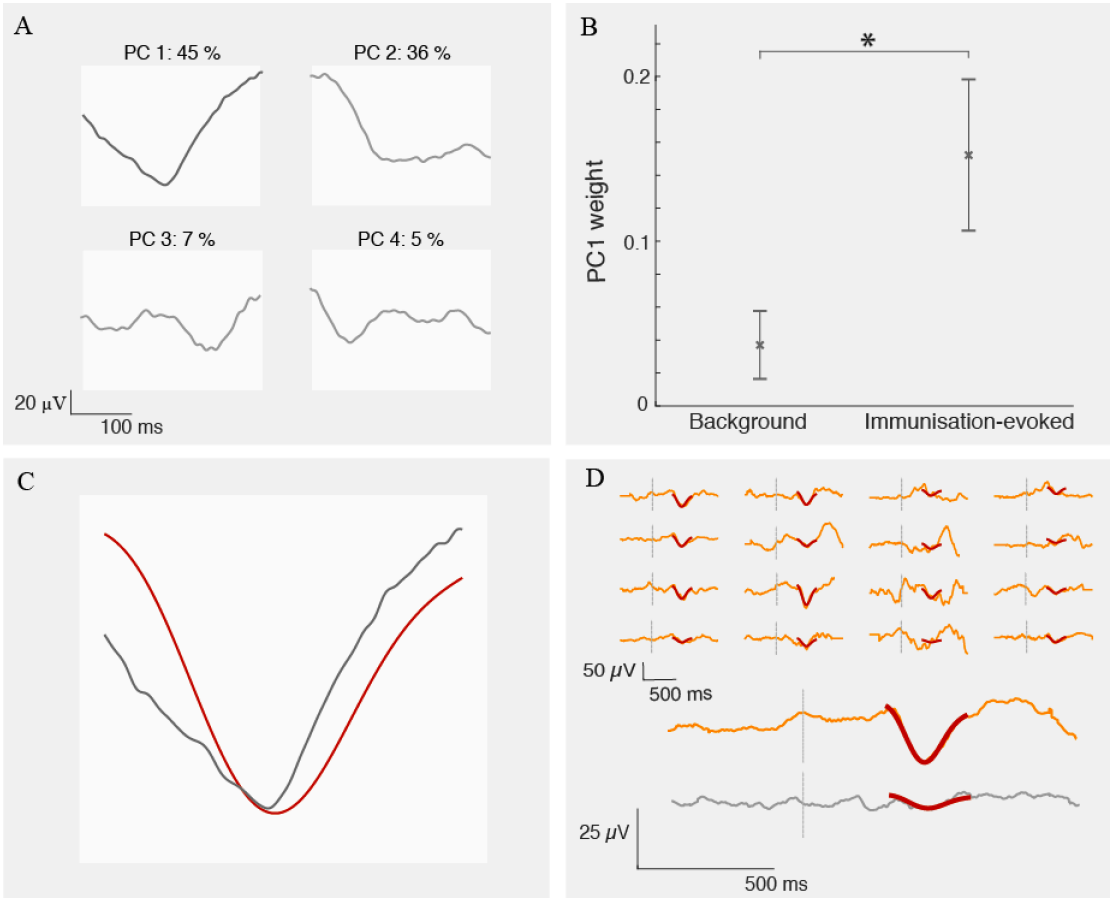


Figure 4.4: Immunisation-evoked brain activity can be fit with the template of noxious-evoked brain activity. (A) The 4 PCs that explained the most variance in the immunisation-evoked and background brain activity. The percentage of the variance each PC can explain is indicated. (B) PC1 is present at a significantly higher level in immunisation-evoked than background brain activity; * indicates $p < 0.05$; error bars indicate mean ± standard error. (C) PC1 (grey) is highly correlated with the template (red). (D) Subject average (top) and group average (bottom) immunisation-evoked brain activity, both Woody filtered to and fit with the template (orange). Group average background brain activity, also Woody filtered to the template, is shown for comparison (grey).

4.4.1.2 Behaviour and physiology

Infants had PIPP-R scores of 11 (9, 12) (median (lower quartile, upper quartile)) in response to immunisation. Infants exhibited facial expression durations of 17 (15, 24) seconds, heart changes of 16 (12, 25) BPM, and oxygen saturation changes of -1 (-2, 1) %.

4.4.2 The effect of paracetamol on immunisation-evoked responses

4.4.2.1 The effect of paracetamol on immunisation-evoked brain activity

In the infants who had received paracetamol prior to immunisation, the template of noxious-evoked brain activity was fitted in the time window determined from the cluster analysis of immunisation-evoked activity in the no-paracetamol group in Study 1, 300 – 600 ms post stimulation (Figure 4.5A). The mean ± standard error of the magnitude of the evoked activity was 0.41 ± 0.14, suggesting a trend towards a reduction when compared with the no paracetamol group (mean ± standard error of 0.70 ± 0.094, Figure 4.5B). While statistical analysis is not conducted to draw conclusions in this section due to the small sample size, a t-test between the magnitude of immunisation-evoked brain activity in the no paracetamol and paracetamol groups was performed to provide an indication of the strength of the trend; the p-value was 0.093, suggesting that following continued recruitment until full sample sizes are reached, paracetamol is likely to reduce immunisation-evoked brain activity, as is discussed further in the Discussion.

The magnitude of noxious-evoked brain activity characterised by the template was investigated in the background brain activity for comparison with immunisation-evoked brain activity following paracetamol; immunisation-evoked brain activity appears to be of larger magnitude than background brain activity (0.41 ± 0.14 versus 0.13 ± 0.032 respectively), suggesting that immunisations are still producing responses greater than background brain activity following paracetamol even in this small sample size. Additionally, the magnitude of background brain activity as characterised by the template in the paracetamol and no paracetamol group was compared and appear to be comparable (0.13 ± 0.032 versus 0.16 ± 0.44 respectively), suggesting that paracetamol does not affect the magnitude of background brain activity as characterised by the template and that an effect on background brain activity is not influencing the apparent trend in the immunisation-evoked response following paracetamol administration. However, larger sample sizes must be investigated before firm conclusions can be reached.

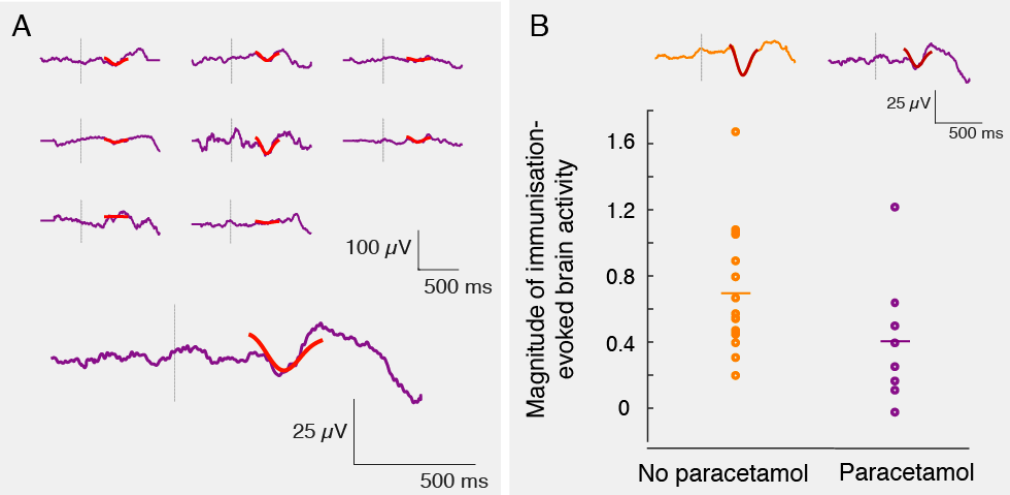


Figure 4.5. Using the template to investigate the effect of paracetamol on immunisation-evoked brain activity. (A) Subject average (top) and group average (bottom) traces, Woody filtered to and fit with the template. (B) Top: group average traces without paracetamol (orange) and with paracetamol (purple). Bottom: plot demonstrating a trend towards a reduction in the magnitude of immunisation-evoked brain activity in the paracetamol group. Horizontal lines indicate the group average magnitude of immunisation-evoked brain activity.

Infants were administered immunisations when clinically appropriate, and therefore age at time of study was not a factor that could be controlled. A slightly higher median (lower quartile, upper quartile) gestational age at study was noted in the infants receiving immunisation without paracetamol 38 (37, 39) compared with the paracetamol group (36 (36, 38), although both groups were of similar median postnatal age (64 (59, 94) in the no paracetamol group, compared with 63 (59, 70)). As age is a factor that can influence responses to noxious stimuli [17] [18], the correlation between gestational age at study and immunisation-evoked brain activity, as characterised by the template, was investigated to check whether age was influencing the magnitude of the evoked activity. Gestational age at study was

not found to be correlated with immunisation-evoked brain activity in either the no paracetamol (p=0.60, R^2=0.020) or paracetamol group (p=0.73, R^2=0.023), suggesting that differences in age range did not influence the magnitude of the responses in the two groups (Figure 4.6).

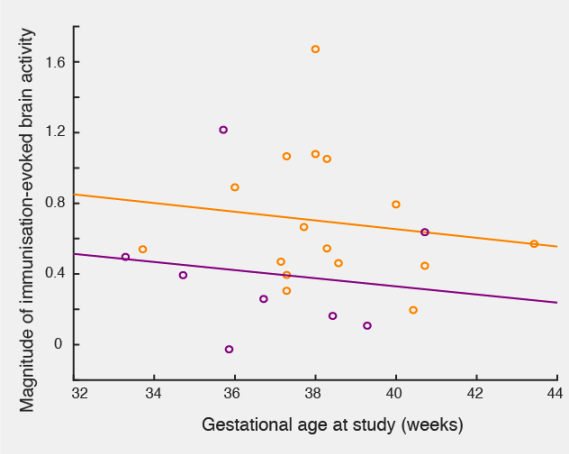


Figure 4.6 Immunisation-evoked brain activity is not correlated with age. Gestational age at study does not appear to influence the magnitude of immunisation-evoked brain activity in either the no paracetamol (orange) or paracetamol (purple) group. Regression lines are displayed.

4.4.2.2 The effect of paracetamol on behaviour and physiology

PIPP-R responses from the infants who had and had not received paracetamol prior to immunisation were compared, and are displayed below (Table 4.2). Additionally, the duration of facial expression, heart rate change, and oxygen saturation change are displayed.

	No paracetamol	Paracetamol
PIPP-R	11 (9, 12)	13 (12, 14)
Facial expression duration (s)	17 (15, 24)	22 (18, 24)
Heart rate change (BPM)	16 (12, 25)	18 (12, 29)
Oxygen saturation change (%)	-1 (-2, 1)	-3 (-5, 0)

Table 4.2: Behavioural and physiological responses to immunisations in the no paracetamol and paracetamol group.

4.4.3 The effect of paracetamol on noxious-evoked responses elicited by experimental noxious stimuli

4.4.3.1 Brain activity

During the recruitment of infants receiving paracetamol prior to immunisation, parents were given the choice to also allow the application of experimental noxious stimuli prior to and ~ 1 hour post paracetamol administration. Of 10 parents agreeing to take part in the immunisation study, 5 also agreed to the application of experimental noxious stimuli. Due to a technical fault, only 4 infants have recorded brain activity to experimental noxious stimuli. There appears to be a trend towards a reduction in the magnitude of noxious-evoked brain activity post paracetamol administration with 3 out of 4 infants average magnitude of the noxious-evoked response decreasing following paracetamol administration and the group average decreasing from 0.40 ± 0.13 pre-paracetamol to 0.23 ± 0.066 post-paracetamol

(Figure 4.7), however, the small sample size prohibits conclusions from being reached.

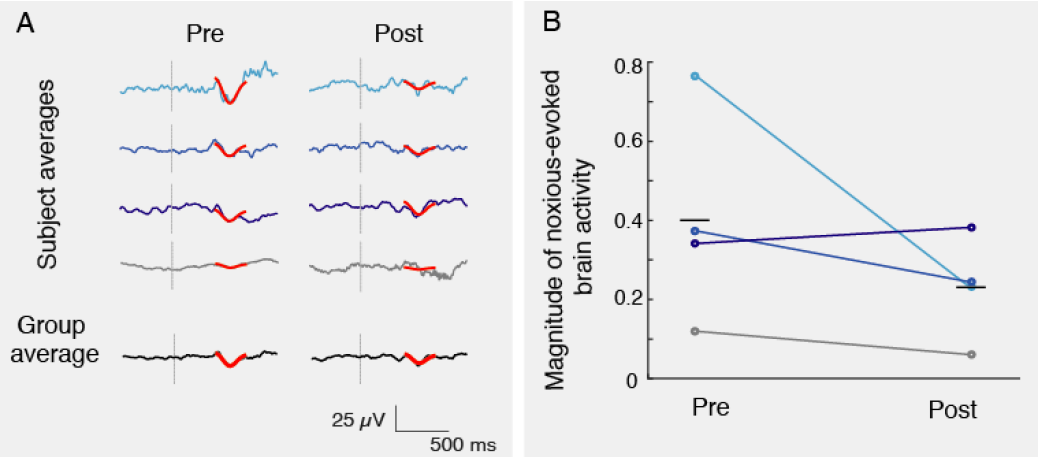


Figure 4.7 The effect of paracetamol on noxious-evoked brain activity following experimental noxious stimulation. (A) Subject average EEG activity in 4 infants (top) and group average EEG activity (bottom) to experimental noxious stimuli pre and ~1 hour post paracetamol administration. Traces are Woody filtered to, and fit with the template (red). (B) Plot demonstrating the effect of paracetamol on the magnitude of noxious-evoked brain activity in individual infants. Horizontal lines indicate the group average magnitude of noxious-evoked brain activity.

4.4.3.2 Behaviour and physiology

Behavioural and physiological measures were recorded in 4 ex-preterm infants receiving experimental noxious stimuli prior to and post paracetamol administration, and a further 2 ex-preterm infants receiving experimental noxious stimuli prior to their week 12 immunisation, for which paracetamol is not required. 2 of these infants were still preterm (< 37 weeks gestational age).

PIPP-R scores without paracetamol were 3 (1, 7), facial expression durations were 0 (0, 3) seconds, heart rate changes were 6 (-1, 12) BPM, and oxygen saturation changes were -1 (-2, 0) %. With paracetamol, PIPP-R scores were 4 (0, 4), facial expression durations were 0 (0, 0) seconds, heart rate changes were 5 (2, 8) BPM, and oxygen saturation changes were -1 (-1, 1) %.

4.5 Discussion

4.5.1 Overview

In this chapter, immunisation-evoked brain activity has been characterised and demonstrated to correlate with the template of noxious-evoked brain activity. This is of importance, as it means that the already validated template, which has been demonstrated to be specific and sensitive, is suitable for use in preterm infants, and correlates with behavioural responses [12], can be used to assess the magnitude of immunisation-evoked brain activity, rather than having to validate immunisation-evoked responses anew. Importantly, the template is also already validated to investigate analgesic efficacy [12 13 14], and therefore can also be used here to investigate the effect of paracetamol.

4.5.2 Immunisation-evoked responses

The sample size in which immunisation-evoked brain activity was characterised in this exploratory analysis was in line with previous studies characterising responses

to immunisations [228], as well as responses to heel lances [11], cannulation [229], and experimental noxious stimuli [210].

When applying the template to immunisation-evoked responses in these ex-preterm infants, the mean (± standard error) magnitude of the response was 0.70 ± 0.094. This lies in between the average response to a heel lancing in newborn term infants, 1[12], and the average response to 128 mN experimental noxious stimuli observed in Chapter 3 in newborn term infants (0.42 ± 0.12), and is similar to the response characterised following cannulation in 2 -3 month old term infants (~ 0.65) [229]. Despite the expectation that the more intense stimulus of immunisation may elicit a higher magnitude of evoked brain activity than heel lancing, the lower value could be because the brain activity measured is evoked by the first contact of the needle to the skin, not to the whole immunisation process. This could explain the similarity of the magnitude in response to immunisation with the response to cannulation, as these both involve identifying the moment of needle contact with skin. Other parts of the immunisation process, such as needle entry into muscle or fluid injection may be more noxious, but were not possible to identify from the high-speed video recording.

Regarding behavioural and physiological responses, the PIPP-R scores to immunisation in these ex-preterm infants (11 (9, 12), median (lower quartile, upper quartile)) are consistent with previous reports of increased pain-associated behavioural and physiological changes to immunisation in healthy infants in their first postnatal days and months [509 317 228 510 511], and also to PIPP-R scores reported following cannulation in term infants (11 ± 4, mean ± standard error) [229].

Recommendations from the PIPP-R authors [186], as well as NHS guidelines [512] advise that scores of 7-12 indicate slight to moderate pain and suggest institution of comfort measures, while scores of over 12 may indicate severe pain, and suggest the use of pharmacological intervention. These recommendations indicate that paracetamol administration prior to immunisations may be apt, especially as paracetamol is given for immunisations anyway due to its antipyretic properties.

The values observed here in response to immunisation are higher than the median (lower quartile, upper quartile) values of 7 (6, 11) reported following heel lancing in the no touch group in Chapter 3. Although this comparison of PIPP-R scores to immunisation and lancing is between prematurely-born and term-born infants, PIPP-R scores account for age, and the scores to heel lancing in preterm infants in the literature are comparable with the scores reported in Chapter 3 following lancing in term infants [193]. Notably, all infants here exhibited facial expressions in response to immunisation, while only 9 of 14 infants responded facially following lancing in Chapter 3. Immunisation-evoked facial expressions were also of longer duration; 17 (15, 24) seconds compared with 6 (1, 14) seconds in all infants in Chapter 3 and 13 (9, 15) seconds in only facially responding infants in response to lancing in Chapter 3.

The higher behavioural response to immunisations than heel lancing is in contrast with the lower magnitude of evoked brain activity, however this is likely to be because the behavioural and physiological responses reflect the entire immunisation process (which is likely to be a more intense stimulus than heel lancing), while the evoked brain activity measured is in response to only the first

contact of needle to skin (which may be a less intense stimulus than heel lancing). In contrast to this, when investigating responses evoked by heel lancing, the foot is not squeezed for the 30 second duration of PIPP-R scoring, and the magnitude of evoked brain activity is correlated with facial expressions [12] as both the brain activity and behavioural score are in response to the same acute stimulus.

4.5.3 Latency of evoked brain activity

The activity characterised here in response to immunisations on the thigh occurs earlier (369 – 524 ms post stimulation) than the activity previously characterised in response to experimental noxious stimuli and lances on the heel (~ 446 – 611 ms post stimulation) [11 18 12 232]. The latencies associated with experimental noxious stimulation at different body sites in infants have previously been investigated, and noxious-evoked activity of comparable magnitude was observed at 289 – 438 ms following experimental noxious stimulation of the hand in 14 infants [232], while noxious-evoked activity was observed at 338 – 534 ms following thigh stimulation in 10 infants [513]. This time window is consistent with the cluster observed following immunisation administration to the thigh here.

The shift in latency following stimulation of different body sites is also concordant with work in adults demonstrating evoked activity in different time windows following experimental noxious stimulation at different sites [514 515 516 517], and work in infants demonstrating evoked activity following tactile stimulation at different body locations [341 518]. The latency shift can be explained by the distances

of the sites of stimulation from the brain; it takes more time for a signal to travel from the foot to the brain then from the thigh, hand, or face.

4.5.4 The effect of paracetamol

Given the evidence of reduced noxious-evoked brain activity and pain perception in adults detailed above [465 468 470 475], I hypothesised that paracetamol would also reduce infant brain activity evoked by noxious stimuli. While the collected data suggest that paracetamol may reduce the brain activity evoked by immunisation, consistent with the adult literature, these analyses are underpowered due to the limited number of infants receiving paracetamol prior to immunisation in the available time, and so firm conclusions cannot be drawn.

The previously validated template of noxious-evoked brain activity was used to assess the efficacy of paracetamol. This methodology has the potential to cause bias towards detecting a reduction in brain activity in the infants receiving paracetamol prior to immunisation, because the time window in which the template of noxious-evoked brain activity was fit was identified as well as applied in the infants receiving immunisations without paracetamol in Study 1 of this study. In a situation with no time constraints, a more robust approach may have been to characterise the response to immunisations and determine the relevant time window in an independent sample of infants, before investigating the effect of paracetamol on responses to immunisation in separate samples of infants. However, the template is already well validated in an independent sample of infants, has been demonstrated to fit responses to multiple types of noxious stimulation (cannulation

as well as heel lancing and experimental noxious stimuli) [12] [229], and the time window of evoked activity found here is consistent with the time window determined in an independent sample of infants to experimental noxious stimuli applied to the thigh [513]. This evidence of consistency in independent samples suggests that in the exploratory pilot study conducted here, it is a reasonable approach to fit the existing template to this data in the selected time window.

Following observation of a slight difference in median gestational ages in the two groups, the effect of gestational age on the magnitude of immunisation-evoked activity was investigated, to ensure that the slightly lower median age in the paracetamol group was not explaining the observed reduction in the magnitude of evoked activity. With further recruitment, it is likely that the gestational ages in the two groups would become more similar, however in the sample size analysed here, gestational age was not correlated with immunisation-evoked activity, suggesting that the age difference does not explain the lower magnitude of evoked activity. While age has been demonstrated to correlate with noxious-evoked brain activity previously [17], and is demonstrated to correlate with responses to heel lancing in term infants in this thesis (Chapter 3, Figure 3.6), it is likely that in these ex-preterm infants who have many weeks of experience of interventions on the neonatal unit, age explains less of the observed individual variability in responses to noxious stimulation.

Based on the pilot data presented here, a power calculation was performed to estimate the number of infants that would be needed to detect an effect of paracetamol on immunisation-evoked brain activity. Assuming that paracetamol

can reduce brain activity by ~ 40 % as observed here, 30 infants would be needed in each group to see a significant effect of paracetamol with 80% power at a significance level of 0.05. A power calculation was also performed to estimate the number of infants needed to see an effect of paracetamol in a within-subject analysis utilising experimental noxious stimuli; for this investigation 17 infants would be needed to conduct a paired analysis, with each infant receiving experimental noxious stimuli prior to and post paracetamol administration. Recruitment of infants without prior paracetamol is no longer possible in this study due to the change in local clinical practice, however this information can be used to guide recruitment into future studies investigating the analgesic efficacy of paracetamol.

Regarding the effect of paracetamol on behaviour and physiology, no reduction is observed when considering facial expression, heart rate, or oxygen saturation changes, consistent with studies demonstrating that paracetamol does not alter behavioural or physiological responses to heel lancing [484] [485] [265]. This is also concordant with some of the adult literature suggesting that the effect of paracetamol on brain activity is more apparent than on behaviour [468] [473].

PIPP-R scores appear to be slightly increased, however the lack of apparent difference between the facial expression durations, heart rate changes, and oxygen saturation scores suggests that the slight increase in PIPP-R scores following paracetamol administration could potentially be due to the points allocated for gestational age and behavioural state in the two groups, as more infants in the paracetamol group received points for gestational age and behavioural state; a

median (lower quartile, upper quartile) of 3 (1, 3) points was given for behavioural state compared to 2 (0, 3) in the no paracetamol group, and 5 infants in the paracetamol group were given points for gestational age (< 36 weeks), compared to 1 in the no paracetamol group, although several more infants in the no paracetamol group were close to the boundary for receiving points based on age. Additionally, the sample sizes here are small, and it is likely that with larger sample sizes and a more even distribution of ages and other factors affecting nociceptive responses such as behavioural state, the PIPP-R scores would not be elevated following paracetamol administration.

When considering the lack of effect of paracetamol on behavioural and physiological measures observed here, and the lack of effect reported in the literature, it may appear that there is discord between the suggestion that paracetamol affects immunisation-evoked brain activity but not behaviour or physiology. However, as the brain activity characterised is in response to the first contact of the needle to the skin, while the behavioural and physiological changes are in response to the full process of immunisation – including the needle piercing the skin, entering the muscle, and fluid injection. Therefore, paracetamol could affect responses to the less intense first contact of the needle with skin, but not the more intense process of full immunisation [519]. Additionally, infant distress, resulting from being handled and restrained, is likely to influence and elevate behavioural and physiological measures, but not evoked brain activity. The influence of distress on behavioural scores, but not brain activity, was also apparent in an investigation into the effect of local anaesthetic [12], where application of local anaesthetic induced a reduction in noxious-evoked brain activity but not

behaviour following experimental noxious stimulation, as detailed in the Introduction.

4.5.5 Responses to experimental noxious stimuli in ex-preterm infants

In the small number of ex-premature infants studied in this study, the average brain activity response to experimental noxious stimuli without paracetamol (0.40 ± 0.13, mean ± standard error) was of a similar magnitude to that observed in term infants in Chapter 3 (0.42 ± 0.12, mean ± standard error), suggesting that their evoked responses are not immature. Concordant with the effect of paracetamol on immunisation-evoked brain activity, the responses with paracetamol administration demonstrated a trend towards a reduction (0.23 ± 0.066), but the very small sample size does not permit conclusions to be reached, and recruitment must be continued.

The PIPP-R response without paracetamol was 3 (1, 7) (median (lower quartile, upper quartile)). The authors of the PIPP-R suggest that scores of 6 or less generally indicate minimal or no pain [186], and the values observed here in ex-preterm infants are consistent with the low PIPP-R scores reported following experimental noxious stimulation in term infants [210]. In this paper, PIPP-R scores to experimental noxious stimuli (4 ± 3) were compared with PIPP-R scores during rest (3 ± 2), and given that the two were not significantly different, it was concluded that term infants are not distressed by this stimulation. Similarly here, the results suggest that, like term infants, ex-preterm infants do not show signs of behavioural distress or pain following experimental noxious stimulation.

The PIPP-R values with and without paracetamol were comparable (3 (1, 7) and 4 (0, 4) respectively), concordant with the effect of paracetamol on immunisation-evoked responses. Although this is a very small sample size, it is not surprising that paracetamol did not have an effect on these scores, as the facial expression, heart rate, and oxygen saturation changes were negligible, with a noteworthy component of the PIPP-R score often originating from the points attributed for behavioural state (0 – 3) and gestation age (1) for preterm infants.

4.5.6 Strengths and limitations

In this chapter responses to immunisations were first characterised before testing the effect of paracetamol on these responses, and experimental noxious stimuli were also utilised to assess the efficacy of paracetamol. While the characterisation of immunisation-evoked brain activity is important to be able to test paracetamol specifically for the clinically relevant procedure of immunisation, a strength of this study was the aim to include experimental noxious stimuli in the experimental design, as these stimuli are already well-validated, can be automatically time-locked to the EEG recordings, can be applied by practiced members of the research team, and can be applied on multiple occasions to increase the signal to noise ratio [12] [210]. Additionally, the opportunity to apply these stimuli on multiple occasions enables a within-subject comparison which can account for individual variation, in contrast with the immunisation study in which two independent groups of infants were studied at the time point they were due immunisations, and had differing gestational ages, prior pain exposure, and other factors affecting nociceptive

responses. This limitation of between-subject comparisons will be the subject of Chapter 5.

To record immunisation-evoked responses, a clinical member of staff was required to give the immunisations, while members of the research team used the high-speed camera to time-lock the moment of administration to the brain activity recordings, and behavioural and physiological recordings were manually marked. This is in contrast to data collection following experimental noxious stimuli and heel lance stimulation, as these stimuli can be automatically time-locked to the brain activity recordings and are performed by a member of the research team who is accustomed to the experimental design. In this study, the clinical member of staff administering the immunisations was often unfamiliar with our research practices, was often rushed as they also had other duties on the neonatal unit, or perhaps did not fully comprehend the amount of warning that was required prior to immunisation administration to be able to obtain clear high-speed video camera recordings and mark the physiology and behavioural recordings. Therefore, some immunisations could not be accurately event-marked, resulting in a loss of data.

Additionally, as the clinical staff member administering the immunisations varied between studies, immunisation administration styles varied in speed and force, meaning that the force of the first contact of the needle to skin was not consistent between infants. This could affect between-subject comparisons, and was one of the reasons why the effect of paracetamol on responses evoked by the experimental noxious stimulus, which provides a constant force, was also investigated.

Ensuring that paracetamol was administered as near to 1 hour as possible prior to immunisations was also difficult in some instances, as the prescription and administration of paracetamol and the preparation, checking, and administration of immunisations required multiple individuals to be available at the relevant times. The average time between paracetamol administration and experimental noxious stimuli application was 63 ± 7 minutes (mean ± standard deviation), and between paracetamol administration and immunisation was 79 ± 10 minutes, with 2 infants receiving immunisations ~ 1 hour 30 minutes – 1 hour 40 minutes post paracetamol administration. This again demonstrates the strengths of utilising two types of noxious stimuli to investigate the efficacy of paracetamol, the clinically relevant immunisations as well as the experimental noxious stimuli whose application timing can be more closely regulated. However, the delays in immunisation administration are not likely to significantly influence the efficacy of paracetamol, as peak plasma paracetamol concentrations are reached over 1 hour post oral administration in neonates [497 498].

Regarding the analysis of the immunisation-evoked data, a limitation is that there is a higher uncertainty associated with manually event-marking the moment of needle contact with the skin than with the automated time-locking of experimental stimuli and heel lances, especially in instances where the high-speed videos were not of high quality. This led to the use of a larger Woody filter jitter of ± 100 ms, which could introduce the inclusion of more noise. However, background data was also Woody filtered by ± 100 ms, confirming the specificity of the evoked response, and the confirmed intra- and inter-rater reliability suggests that event-marking was reliable.

There are also limitations associated with the interpretation of this characterisation of immunisation-evoked brain activity; when investigating the brain activity evoked by a heel lance in order to form the template [12], and in Chapters 3 and 5, the control heel lance condition was used to ensure that the brain activity being characterised after the real lance was nociceptive. Here, this study did not involve a control condition, as there is no stimulus that resembles the touch of a sharp needle to skin but is not nociceptive. Therefore, it cannot be concluded with certainty that the characterised brain activity is nociceptive; it may relate to arousal or attention. However, as the time window in which the evoked activity is identified is consistent with the time window in which noxious-evoked brain activity following experimental noxious stimulation of the thigh is observed [513], and other noxious medical stimuli such as cannulas [229] evoke similar patterns of brain activity, it is likely that this immunisation-evoked activity does relate to nociceptive processing. Additionally, the suggestion that paracetamol may dampen this activity is consistent with it being noxious-related.

The last limitation is that while the immunisation-evoked response characterised can be fit with the previously validated and objective template, the brain activity identified here is likely to be in response to the first contact of the needle to the skin, not to the whole process of immunisation. The first contact of needle to skin is not likely to be the most painful aspect of this procedure, and therefore alternative methods of analysis that can detect changes elicited by the entire process of immunisation, such as frequency analysis over a period of seconds, may be more suited to detecting changes associated with the entire immunisation

procedure. It would be interesting to characterise the frequency changes evoked by immunisation and investigate whether these changes could be used as a future measure of analgesic efficacy, in order to investigate the effect of paracetamol on the whole immunisation process.

4.5.7 Future directions

In Study 1 of this chapter, the template of noxious-evoked brain activity is demonstrated to provide a suitable fit to immunisation-evoked brain activity, indicating that as well as responses to experimental noxious stimuli, heel lances, and cannulas, the template can be fit to responses to additional clinically relevant acute stimuli. In the future it would be interesting to see if other clinically relevant acute stimuli, such as lumbar punctures, also evoke responses that can be fit with this template, in order to compare evoked responses and assess pain relief interventions for a wider range of procedures.

Given that paracetamol is administered prophylactically for immunisation-related pyrexia following week 8 immunisations, and that earlier administration does not affect immunisation efficacy [503] [504] [505] [506], it seems logical that paracetamol can be given prior to immunisation to allow for any potential analgesic effects. In outpatient clinics, paracetamol administration is advised immediately after, rather than prior to immunisation as this can be more easily enforced by the clinical team and does not risk parental administration of paracetamol prior to immunisations masking any infant illness which may mean that immunisations should be delayed [520]. However, in the hospital environment of the neonatal unit, this is not an issue,

and if paracetamol were found to be efficacious, then neonatal guidelines could be changed at a wider level to allow paracetamol administration prior to immunisation. As discussed in the General Discussion, a randomised controlled trial, including the assessment of noxious-evoked brain activity, is necessary to thoroughly investigate the analgesic efficacy of paracetamol.

As well as investigating the analgesic efficacy of paracetamol, other methods of pain relief, such as local anaesthetic, gentle touch, and SSC, can also be assessed to see if they reduce immunisation-evoked brain activity as measured by the template. Investigating the efficacy of a wider range of pain relief interventions could be useful in providing pain relief for immunisations for which paracetamol is not administered (e.g those at 12 weeks of age, or the vitamin K injection given on the first postnatal day[521]), or in cases in which paracetamol administration is not appropriate (e.g to infants with liver dysfunction[342]), as other interventions, especially non-pharmacological interventions, may be associated with less risk of adverse effects.

4.6 Conclusion

The suggestion that noxious-evoked brain activity following application of different noxious stimuli is common and can be fit with the template proffers the possibility of assessing a range of procedures and analgesics with an already validated and objective measure. Although recruitment must continue before any firm conclusions about the efficacy of paracetamol can be reached, the work in this chapter demonstrates how pain relief interventions can be assessed by

utilising an already validated template of noxious-evoked brain activity, and suggests that paracetamol may reduce noxious-evoked brain activity.

Chapter 5: Can accounting for individual sensitivity to noxious stimuli aid between-subject comparisons?

5.1 Introduction

Many factors can influence the response to noxious stimulation in infants as detailed in the General Introduction, including gestational age [17 18 237], sex [252 217], prior pain [169 238], stress [245], illness [215], and behavioural state [216]. In within-subject comparisons, as are possible when using experimental noxious stimuli that can be applied on multiple occasions, individual variability in nociceptive sensitivity is not an issue. However, when performing clinically relevant procedures such as heel lancing or cannulation that are only required on one occasion, between-subject comparisons are necessary. In these instances, differing individual sensitivity between groups could obscure intervention effects.

In Chapter 3, gentle touch was demonstrated to reduce noxious-evoked brain activity following both experimental noxious stimuli and heel lances. In the within-subject analysis using experimental noxious stimuli to investigate the effect of gentle touch, the reduction in noxious-evoked brain activity was clear to observe, likely due to the combination of the increased sample size and the within-subject nature of the comparison. When investigating the effect of gentle touch prior to heel lancing in the between-subject comparison, infants were matched based on gestational age in order to conduct a paired analysis. This decreased the sample size required for a non-paired analysis from 58 infants (29 per group) to 32 infants (16 per group) for a paired analysis.

The 16 age-matched infants in Chapter 3 were chosen from a pool of 36 infants who had not received any intervention prior to heel lancing. Consistent with previous results [17], it was found that in the 16 age-matched control infants who had not received touch prior to lancing, age correlated significantly with the noxious-evoked response (Figure 3.6, Chapter 3), highlighting the influence of age on nociceptive responses. Additionally, when the infants who had received gentle touch prior to lancing were compared with random samples of non-age-matched infants from the pre-collected data, a significant difference was found only 3% of the time, demonstrating the importance of age-matching to observe an intervention effect.

Another example of age affecting nociceptive responses is evident in a previously conducted investigation into the effect of topical local anaesthetic on noxious-evoked brain activity [12 229]. In this work, local anaesthetic reduced the brain activity evoked following experimental noxious stimulation in a within-subject analysis (Figure 5.1A,B), however the same effect was not observed when investigating the effect of local anaesthetic on responses evoked by cannulation in a between-subject analysis (Figure 5.1C). While the lack of effect of local anaesthetic on cannulation-evoked brain activity could be due to the increased intensity of this stimulus, or an inadequate characterisation of cannulation-evoked brain activity, the brain activity evoked by cannulation also demonstrated positive correlation with gestational age (Figure 5.1D). The infants receiving local anaesthetic were on average slightly older, suggesting that this age difference could explain the lack of effect observed [229].

These examples demonstrate the benefits of age-matching in ensuring that the effects of interventions are not obscured. However, as seen in Chapter 4 when investigating the responses of infants receiving immunisations, it is not always possible to age-match infants when the time of study is dependent on medical procedures which are only performed when the infant is clinically stable. Additionally, while age is one factor that can influence the magnitude of noxious-evoked brain activity, there are also many factors, both known, as detailed above, and as yet unknown, that can influence noxious-evoked brain activity. This is illustrated by the variation in the correlation between age and noxious-evoked brain activity as can be seen below in Figure 5.1D and in Figure 3.6, Chapter 3.

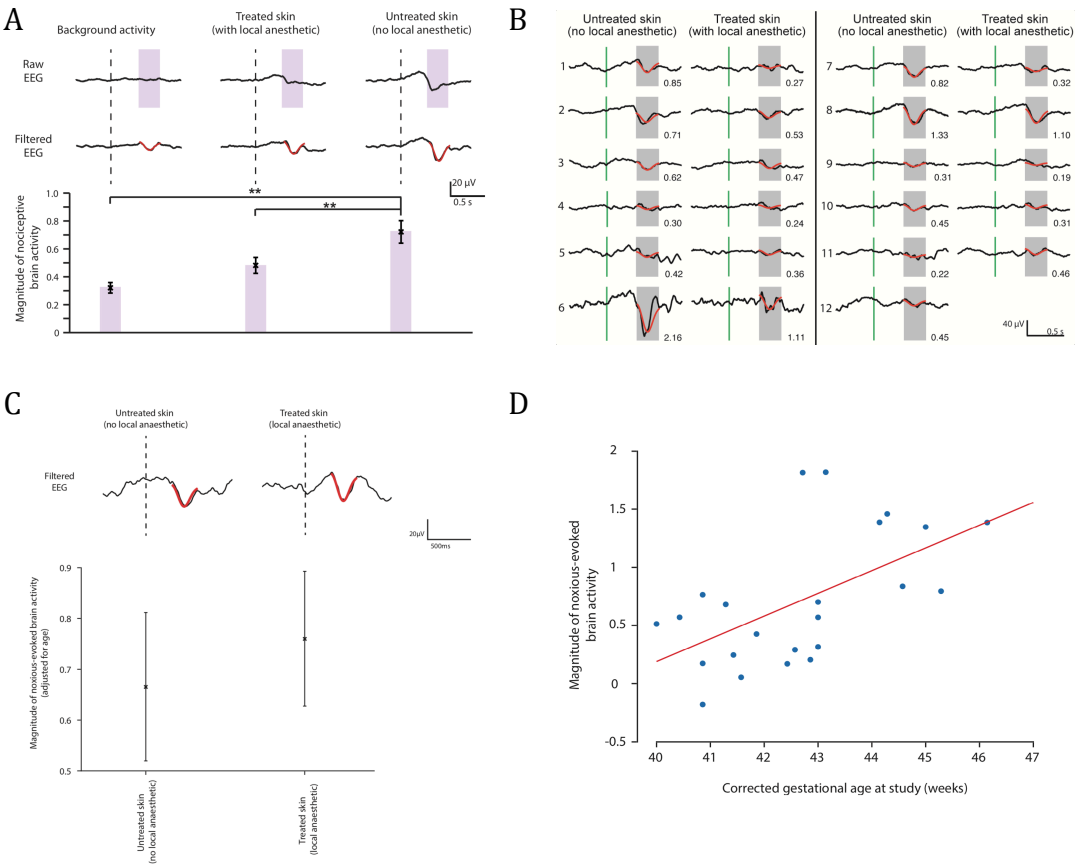


Figure 5.1 Individual variability in noxious-evoked responses may obscure observation of the effect of local anaesthetic in between-subject comparisons. Figures (A) and (B) are adapted with permission from Hartley et al. 2017 [12]; figures (C) and (D) are reproduced with permission from Green 2018 [229]. (A) Local anaesthetic reduces noxious-evoked brain activity following experimental noxious stimulation in a within-subject comparison. (B) Responses to experimental noxious stimuli in individual infants span a wide range of magnitudes, (weights of 0.22 – 2.16 without local anaesthetic, and weights of 0.19 – 1.11 with local anaesthetic). (C) Local anaesthetic does not have a significant effect on the magnitude of noxious-evoked brain activity following cannulation in a between-subject comparison. (D) Gestational age is correlated with the magnitude of noxious-evoked brain activity following cannulation.

In the analysis of local anaesthetic on responses evoked by experimental noxious stimuli, the magnitude of noxious-evoked brain activity without local anaesthetic is

very varied between individuals (Figure 5.1B). This between-subject variation is not an issue here, due to the within-subject design, however this example demonstrates how the considerable variability in individual responsiveness to noxious stimuli could obscure intervention effects in between-subject analyses where the groups are not well matched. I performed a post-hoc analysis of this data and demonstrated that the magnitude of noxious-evoked brain activity of each infant when local anaesthetic had been applied is correlated with the response of each infant without local anaesthetic ($p<0.001$, $R^2 = 0.72$, Figure 5.2), demonstrating the important point that responses to noxious stimuli are highly correlated within-subject.

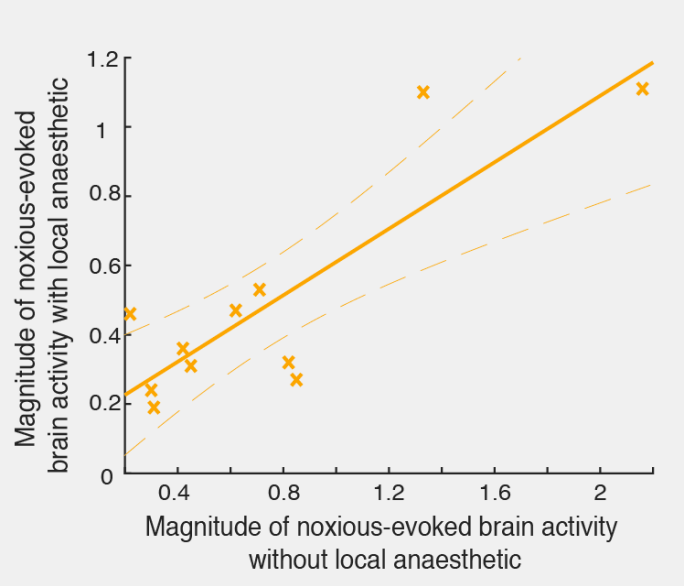


Figure 5.2 Individual responses to experimental noxious stimulation are correlated with post-treatment responses. I performed a post-hoc analysis of the data from infants receiving experimental noxious stimuli with and without topical local anaesthetic in Hartley et al., 2017 [12]; the magnitude of noxious-evoked brain activity with local anaesthetic application is correlated with the response without local anaesthetic.

In adults, there are also many factors that have been demonstrated to influence pain perception [37], including mood [38], expectation, attention [39], ethnicity [522], gender [523] and genetic variability [524] [525], and these are likely to contribute to the inter-individual variability in pain-evoked brain activity [526]. To overcome differences in individual nociceptive processing and pain perception, adult studies involving EEG and fMRI imaging adjust the intensity of the noxious stimulation between participants to achieve a consistent perception of pain [527] [528] [529]; for example, prior to imaging, individuals would be asked to verbally rate a range of noxious stimuli to determine intensities at which a certain level of pain (e.g. 6 out of 10) is perceived. Then, in the imaging session, the intensity of noxious stimuli would be adjusted so that the perception perceived, rather than the stimulus applied, is consistent between participants. This is illustrated in a schematic in Figure 5.3: the red and pink lines indicate differing relationships between the intensity of the stimulus and pain perception in two individuals. When administering a stimulus of consistent intensity (intensity-locking), different levels of pain perception are evoked in each individual (panel A), and intervention effects can be difficult to observe (for example, the pre-intervention response in the second individual (pink) is smaller than the post-intervention response in the first individual (red), although an intervention-induced reduction is still seen in this individual) (panel B). In contrast, when administering a stimulus that may vary in intensity but elicits a consistent perception of pain between participants (perception-locking) (panel C), intervention effects are more clearly observed (panel D).

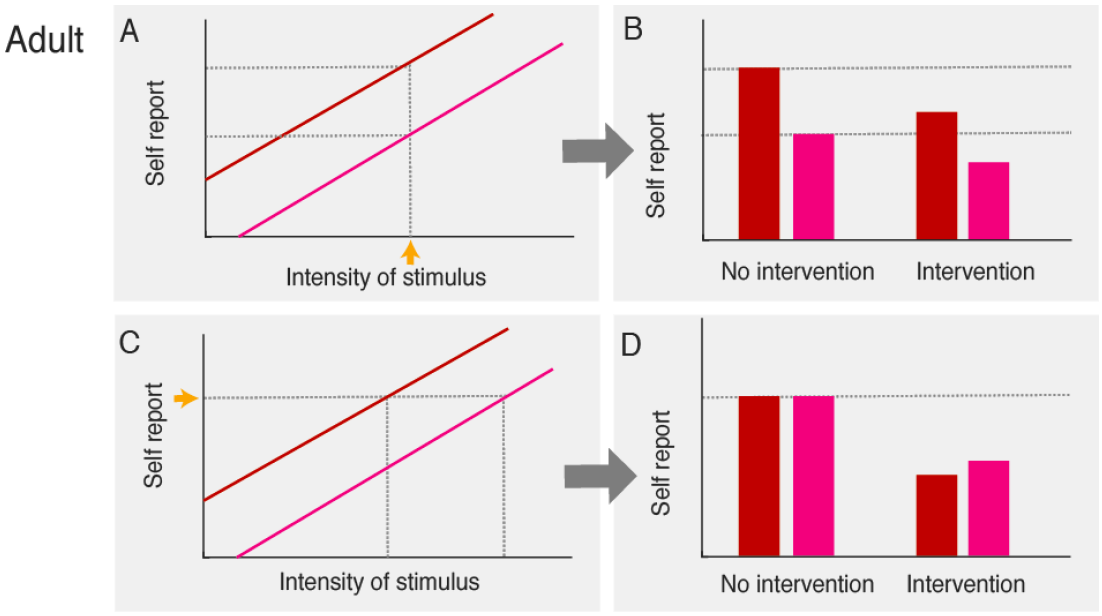


Figure 5.3 Schematic demonstrating the method by which individual sensitivity to pain is accounted for in adult studies. (A) Individual variability means that the application of a stimulus of consistent intensity (intensity-locking) can elicit differing magnitudes of pain perception in different individuals. Red and pink lines represent the differing relationships between stimulus intensity and pain perception in two individuals. (B) When utilising an intensity-locking paradigm to investigate the efficacy of an intervention, effects can be difficult to see due to this individual variability. (C) In many studies, the intensity of the stimulus is scaled between individuals so that a consistent perception of pain is elicited (perception-locking). (D) When utilising a perception-locking paradigm to investigate the efficacy of pain relief interventions, effects are clearer to observe.

In infants, indication of pain perception and the ensuing adjustment of stimulus intensity is not possible. Instead, in this chapter I develop a novel paradigm to account for individual variability in nociceptive sensitivity and test its applicability for assessing pain relief interventions in infants. The basis of this paradigm is that the magnitude of individual responses to experimental noxious stimuli will be highly correlated with responses to more intense clinically-required noxious

stimuli, such as heel lancing. The within-subject relationship between the two stimuli can then be used to account for individual variability in nociceptive sensitivity, by a) considering the ratio of the responses following the two stimuli, and b) by using linear regression to explore the contribution of responses to experimental noxious stimuli and gentle touch on heel lance responses. The utility of this approach is that nociceptive sensitivity can be tested in infants prior to the application of an analgesic intervention and can then be accounted for when considering between-subject comparisons of analgesic efficacy.

I first test the hypothesis that responses to experimental noxious stimuli and heel lancing are correlated. While it is known that the magnitude of noxious-evoked brain activity reflects the intensity of noxious stimulation at the group level in infants [210], this has not been demonstrated within-subject in infants. Then, using the example of the pain relief intervention of gentle touch, I investigate whether accounting for individual nociceptive sensitivity aids the between-subject investigation into analgesic efficacy.

I hypothesised that noxious-evoked brain activity following experimental noxious stimuli and clinically necessary heel lances would be correlated at an individual level (as illustrated in the schematic in Figure 5.4A), a necessity for the employment of this paradigm. I also hypothesised that in relatively small sample sizes, differences in individual sensitivity to noxious stimuli between infants may lead to high levels of variance in the reported results, which could mask the effects of the intervention (Figure 5.4B). This is likely to be especially problematic when dealing with small sample sizes, as it is plausible that (by chance) infants in the

intervention group may have a higher (or lower) average nociceptive sensitivity and therefore skew the results. I predicted that upon taking into account individual nociceptive sensitivity, the effect of the intervention may be observed. I hypothesised that the pain relief intervention of gentle touch would elicit a decrease in the magnitude of responses to heel lancing that would either be consistent in effect size across all infants (Figure 5.4C), or would be larger in infants with higher nociceptive sensitivity (Figure 5.4D). In both instances, it is advantageous to take into account nociceptive sensitivity in order to be able to observe an effect of gentle touch on responses to heel lancing.

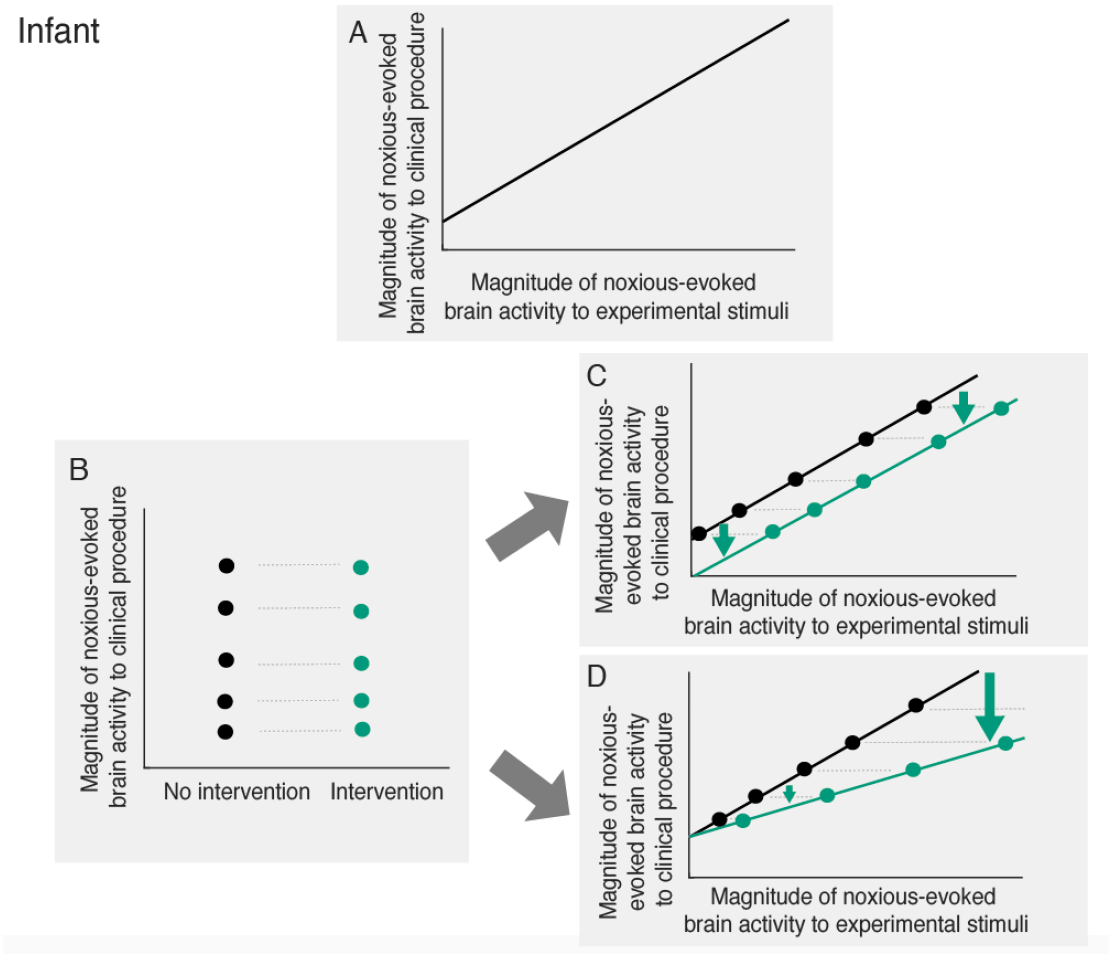


Figure 5.4 Schematic demonstrating the paradigm being investigated in this chapter for accounting for individual nociceptive sensitivity in infants. (A) I hypothesised that noxious-evoked brain activity responses to experimental noxious stimuli and clinical procedures will be correlated; infants with responses in the bottom left region would be 'low responders' with low nociceptive sensitivity, while infants in the top region would be 'high responders' with high nociceptive sensitivity. (B) I predicted that although intervention effects may be obscured in small sample sizes without taking into account individual nociceptive sensitivity, considering responses to experimental noxious stimuli would allow observation of intervention effects. I hypothesised that the size of the intervention effect would either be consistent across infants with differing nociceptive sensitivity (C), or that the effect of the intervention would be dependent on nociceptive sensitivity, with larger intervention effects in infants with higher nociceptive sensitivity (D). Each point in panels (B–D) indicates an infant with different response to heel lance and nociceptive sensitivity; in both cases (C) and (D), taking into account individual nociceptive sensitivity by considering the magnitude of responses to experimental noxious stimuli allows observation of intervention effects.

Given that the outcome measure here is noxious-evoked brain activity, I hypothesised that accounting for individual nociceptive sensitivity by gauging infant noxious-evoked responses to experimental noxious stimuli will be an efficacious and comprehensive method of accounting for individual nociceptive variability, and that a touch-induced reduction in noxious-evoked brain activity following heel lancing would be observed when taking into account individual responsiveness, but not when simply comparing the responses to lancing in the two groups.

5.2 Chapter overview

This chapter is composed of two studies:

1. Investigating the within-subject relationship between noxious-evoked responses to experimental noxious stimuli and heel lances,
2. Exploring whether accounting for individual nociceptive sensitivity aids the between-subject investigation into the efficacy of gentle touch for heel lancing.

5.3 Methods

5.3.1 Participating infants

In Study 1, data from previously collected studies was analysed. Infant demographics are displayed in Table 5.1. Based on simulations of this data,

assuming a 50 % wider range of responses to increased intensity experimental noxious stimuli and a reduction of 40 % following gentle touch (as was seen following heel lancing in Chapter 3), a sample size of 15 infants per group would be needed to detect a significant reduction in heel lance responses following gentle touch by taking into account individual nociceptive sensitivity or conducting a t-test 85% of the time.

Number of infants	11
Gestational age at study (weeks)	40.9 (39.9, 41.6)
Postnatal age at study (days)	4 (2,4)
Birthweight (g)	3930 (520)
Number of males	5 (45.5 %)
Number born by normal vaginal delivery	2 (18.2 %)
Average Apgar score at 1 minute	8.5 (2.1)

Table 5.1: Demographics of infants in Study 1 of Chapter 5. Values given are mean (standard deviation), median (lower quartile, upper quartile) or number (percentage).

In Study 2, 15 infants were recruited to receive experimental noxious stimuli and gentle touch prior to lancing. 15 additional infants receiving experimental noxious stimuli and no intervention prior to lancing were co-recruited with another research project investigating responses to lances. To recruit the infants receiving gentle touch prior to lancing, 71 parents were approached; 28 did not want to hear about research or receive an information leaflet, 21 did not want to take part in the study, and 22 consented for study. Following this, 1 infant was withdrawn before data collection and 1 was withdrawn during data collection due to restlessness, 1

technical fault prevented data collection, and data from 4 infants had to be rejected due to EEG movement artefact (as illustrated in Figure 2.1, General Methods, Chapter 2). One of the reasons explaining why a high number of parents did not want to hear about research opportunities was because they were likely to be discharged from hospital imminently.

	No touch	**Gentle touch**
Number of infants	15	15
Gestational age at study (weeks)	40.6 (37.4, 40.9)	39.9 (37.6, 41.1)
Postnatal age at study (days)	2 (1, 5)	4 (3,5)
Birthweight (g)	3520 (615)	3460 (663)
Number of males	9 (60.0 %)	6 (40.0 %)
Number born by normal vaginal delivery	5 (33.3 %)	5 (33.3 %)
Average Apgar score at 1 minute	8.6 (1.7)	8.1 (2.5)

Table 5.2: Demographics of infants in Study 2 of Chapter 5. Values given are mean (standard deviation), median (lower quartile, upper quartile) or number (percentage).

5.3.2 Experimental design

In Study 1, infants received 32 mN and 64 mN experimental noxious stimuli (approximately 20 of each) and clinically necessary heel lances. Responses to these noxious stimuli were analysed to investigate the relationship between the noxious stimuli of different intensities.

In Study 2, infants received approximately 10 - 15 experimental noxious stimuli of 128 mN intensity and a clinically required heel lance. Half of the infants received

gentle touch prior to heel lancing, and half did not receive touch prior to lancing. None of the infants received touch prior to the experimental noxious stimuli; these were administered to gauge individual nociceptive sensitivity without any intervention effects. The decision was made to increase the intensity of the noxious experimental stimuli from 32 or 64 mN to 128 mN as infants do not demonstrate behavioural distress to this intensity, and a higher intensity means greater signal to noise ratio and that less repetitions are likely to be needed for the same power (Figure 5.5).

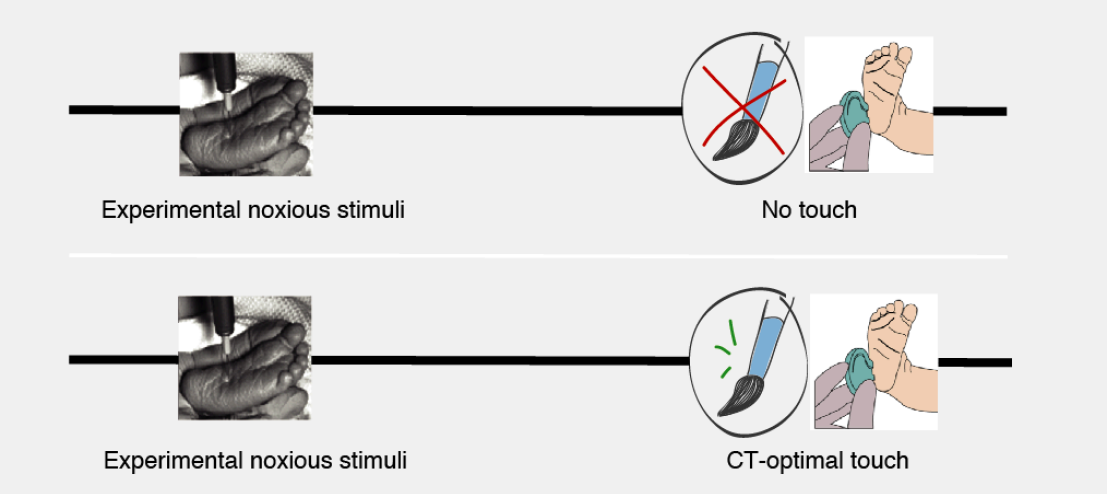


Figure 5.5 Experimental design followed in Study 2 of Chapter 5. All infants received ~10 - 15 experimental noxious stimuli to gauge nociceptive sensitivity. Half of the infants then received gentle touch prior to heel lancing, the other half did not receive touch prior to heel lancing.

5.3.3 Recording techniques

In Study 1, brain activity was recorded to the experimental noxious stimuli and the heel lance. In Study 2, brain activity and reflex withdrawal were recorded to both the experimental noxious stimuli and the clinically necessary heel lance, and facial

expressions and physiology were also recorded to the heel lance. These techniques are detailed in General Methods (Chapter 2) of this thesis.

5.3.4 Analysis

5.3.4.1 Brain activity

Brain activity analysis is detailed in the General Methods chapter and was conducted as in Chapter 3. In both studies, the template of noxious-evoked brain activity was fitted to responses evoked by experimental noxious stimuli and clinically required heel lances.

In Study 1, the response to 1 heel lance was rejected due to movement artefact. In the remaining infants, each infant received ~ 20 experimental noxious stimuli at 32 mN intensity, and ~ 20 at 64 mN intensity. Due to movement artefact, an average of 4 ± 2 (mean ± standard deviation) responses to 32 mN stimuli were rejected per infant, and an average of 3 ± 3 responses to 64 mN stimuli were rejected. In Study 2, responses to 4 heel lances were rejected in the gentle touch group, and 3 responses were rejected in the no touch group. 10 – 15 experimental noxious stimuli at force 128 mN were applied; in the gentle touch group responses to an average of 2 ± 2 stimuli were rejected and in the control group an average of 3 ± 3 stimuli were rejected. Responses were rejected due to movement artefact or if they skewed the average response (if the mean was not comparable to the median), in order to attain the best possible characterisation of each individual infant's average response to experimental noxious stimulation.

A Woody filter jitter of ± 50 was allowed to find the best fit of the template to responses evoked by experimental noxious stimuli, and a jitter of ± 100 ms was allowed to find a best fit to the responses evoked by heel lances, consistent with Chapter 3. This ensured that in Study 1, the noxious-evoked brain activity characterised following the 32 mN stimuli, 64 mN stimuli, and heel lances was significantly different from background brain activity Woody filtered by the same jitter ($p<0.001$, $p=0.0011$, and $p<0.001$ respectively). In Study 2, the magnitude of noxious-evoked brain activity following the 128 mN stimuli in all infants and heel lances in control infants (with no prior touch) was also different from background brain activity Woody filtered by the same amount (response to experimental noxious stimuli compared with background brain activity $p=0.012$; response to heel lance compared with background brain activity $p<0.001$; response to heel lance compared with response to control lance $p=0.025$).

5.3.4.2 Reflex withdrawal

Reflex withdrawal analysis is detailed in the General Methods chapter and was conducted on the data in Study 2 using both the RMS and the algorithm method. No responses to heel lancing or experimental noxious stimuli were rejected in the no touch group; in the gentle touch group 1 infant did not have EMG applied due to infant restlessness during the study, and 1 response to lancing was rejected due to movement artefact in the baseline period. Responses to 2 experimental noxious stimuli were rejected in the gentle touch group.

5.3.4.3 Behaviour and physiology

PIPP-R scores and facial expression responses were calculated to the heel lance in both the gentle touch and the no touch groups for comparison with the results from Chapter 3, as a trend towards a reduction in facial expression duration in responders was observed in Chapter 3. In the no touch group, 1 infant did not have recorded heart rate and oxygen saturations due to technical issues, and therefore 1 PIPP-R score could not be calculated. In the gentle touch group, 1 infant did not have a facial video due to technical issues, and in 1 infant the face was obscured, preventing facial expression scoring. Additionally, in 2 infants physiological recordings could not be analysed due to a technical issue at the time of recording, meaning that 4 PIPP-R scores could not be calculated.

5.4.5 Statistical analysis

A post-hoc test was also conducted on the data from an independent investigation into the efficacy of topical local anaesthetic [12]. Linear correlation was used to investigate the relationship between the magnitude of noxious-evoked brain activity with and without local anaesthetic.

In the data from both studies, the magnitude of noxious-evoked brain activity in response to the experimental noxious stimuli was compared to background brain activity, and the responses to heel lances with no prior touch were compared to background brain activity and control heel lances with consistent jitter settings to

ensure that the Woody filter jitter utilised adequately characterised the noxious-evoked brain activity.

The correlation between the noxious-evoked brain activity in response to experimental noxious stimuli and heel lancing in both studies was investigated using Pearson's parametric linear correlation. Correlation between reflex withdrawal responses in Study 2 was investigated using Spearman's non-parametric linear correlation. Noxious-evoked responses to experimental noxious stimuli in the two studies were compared using a t-test.

When comparing the magnitude of the noxious-evoked brain activity following heel lancing in the no touch and gentle touch groups without taking into account nociceptive sensitivity, a t-test was used when investigating brain activity, and the Mann-Whitney U-test was used for reflex withdrawal. Two methods of accounting for individual nociceptive sensitivity were investigated initially; calculation of the ratio of the response following experimental noxious stimuli to the response following heel lancing (magnitude of noxious-evoked response to heel lance / magnitude of noxious-evoked response to experimental noxious stimuli), and linear regression investigating the contribution of the interaction between gentle touch and the response to experimental noxious stimuli to the response to heel lancing. Consideration of the ratio of responses to the two noxious stimuli was useful in allowing outliers in this relationship to be identified; outliers were defined as being under the lower quartile - (1.5 x the interquartile range), or above the upper quartile + (1.5 x the interquartile range) of the ratio of the two responses. Mann-Whitney U-tests were used to compare ratios as the data were skewed.

Upon observation that the effect of gentle touch appears to be greater in infants with higher nociceptive sensitivity, a post-hoc analysis was performed in which infants were only included in the analysis if their response to the experimental noxious stimulus was above 80% of their background activity. Thresholded analyses have also been conducted previously in order to ensure only infants with substantial nociceptive brain activity are included in the analysis [17].

5.4 Results

5.4.1 Noxious-evoked responses to experimental noxious stimuli and clinically essential heel lances are correlated

In Study 1, infants received experimental noxious stimulation at 32 mN intensity, 64 mN intensity, and clinically necessary heel lances (Figure 5.6A). The magnitude of the noxious-evoked brain activity following the 64 mN stimuli and heel lances was significantly correlated ($p=0.0072$, $R^2=0.58$, Figure 5.6B), and a trend was observed in the correlation between responses to the 32 mN stimuli and the heel lance ($p=0.23$, $R^2=0.15$, Figure 5.6C) and between the 32 mN and 64 mN experimental stimuli ($p=0.099$, $R^2=0.27$, Figure 5.6D). This suggests that this relationship could be used to account for individual variability.

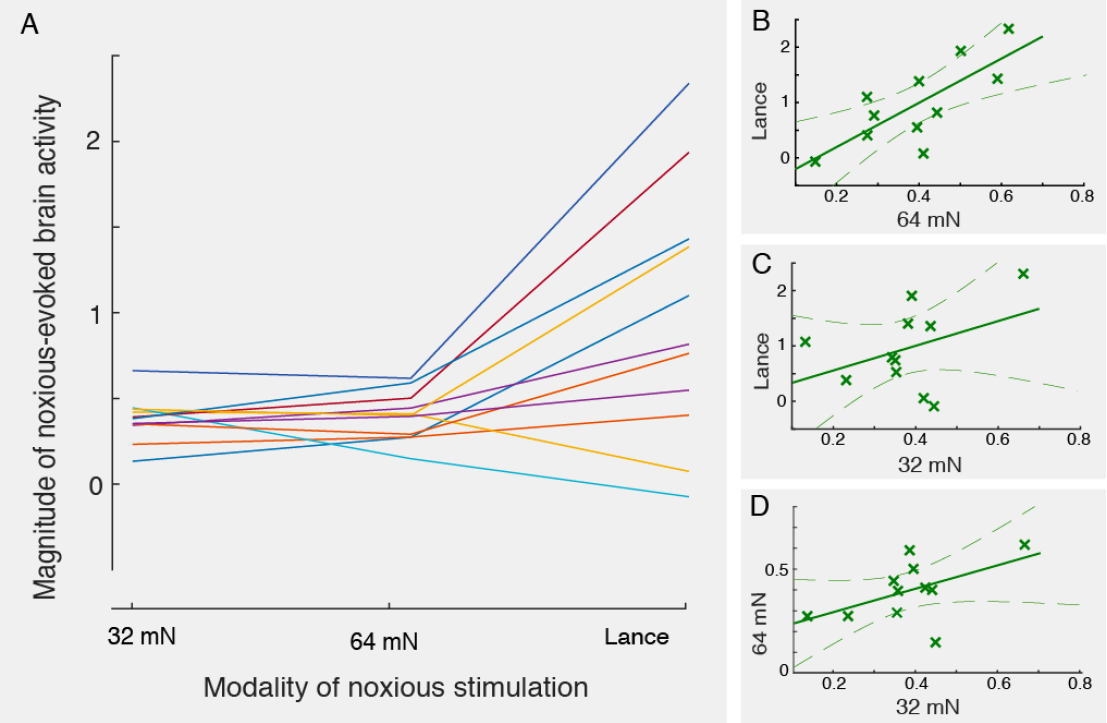


Figure 5.6. Relationship between responses to experimental noxious stimuli and heel lances in Study 1. (A) The magnitude of the noxious-evoked response following 32 mN experimental noxious stimuli, 64 mN experimental noxious stimuli and heel lances. Each colour represents an individual infant's responses. (B) Significant correlation between the magnitude of the noxious-evoked response following the 64 mN experimental stimulus and the heel lance. (C, D) Trends towards correlation between responses to the 32 mN experimental noxious stimuli and the lance, and the 32 mN and 64 mN experimental noxious stimuli.

In Study 2, infants were then recruited to receive the 128 mN experimental noxious stimulus prior to heel lancing, as responses to the slightly more intense experimental stimulus are expected to provide a higher signal to noise ratio. Noxious-evoked responses following heel lancing are displayed in Figure 5.7. In these infants (who did not receive any touch prior to the heel lancing), the magnitude of the noxious-evoked brain activity to this higher intensity experimental noxious stimulus and the clinically required heel lance were

significantly correlated ($p=0.033$, $R^2=0.30$, Figure 5.8A), replicating the results of Study 1.

In Study 2, the RMS and the amplitude of reflex withdrawal were also considered. The magnitude of the reflex withdrawal in response to the experimental noxious stimuli and the heel lance was significantly correlated when using the RMS method ($p=0.029$, $R^2=0.32$), and showed a trend towards correlation when considering the amplitude ($p=0.083$; $R^2=0.21$, Figure 5.8B). These results demonstrate that noxious-evoked brain activity and reflex withdrawal responses following this higher intensity noxious stimulation, in an independent data set, are also correlated with responses to heel lancing.

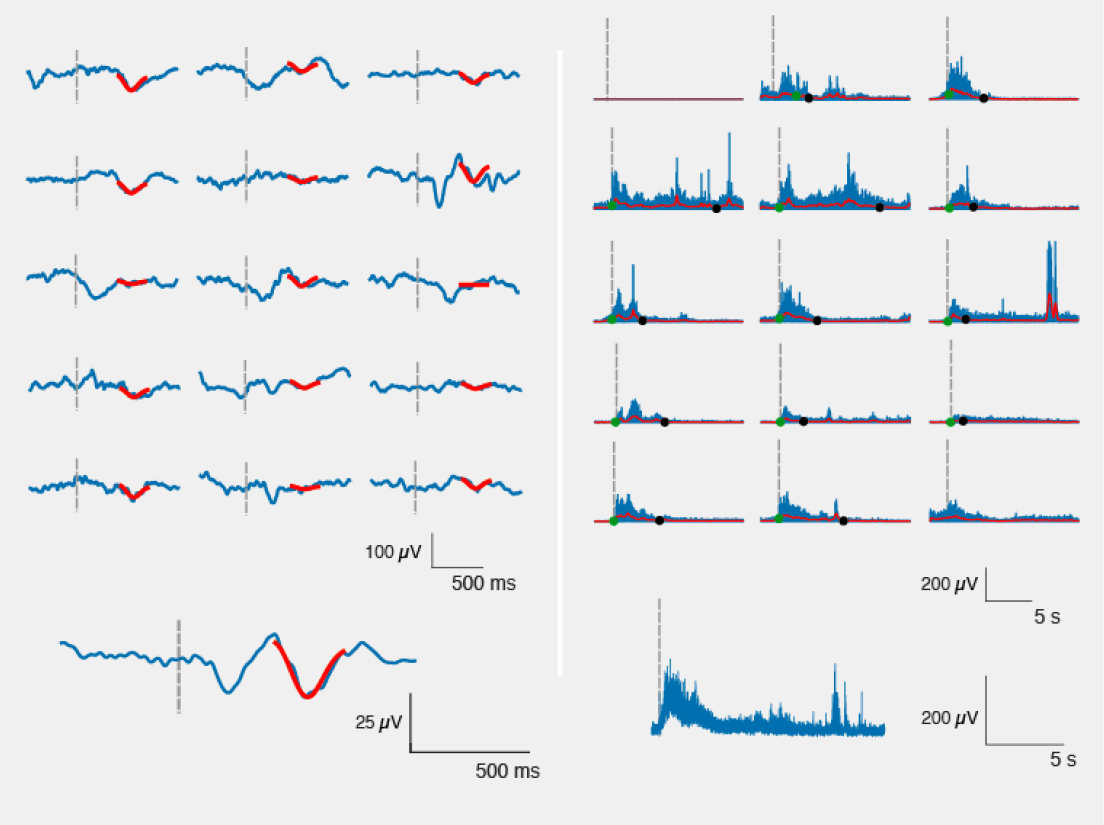


Figure 5.7. Noxious-evoked brain activity and reflex withdrawal responses following heel lancing in control infants in Study 2. EEG traces following heel lancing (left) are jittered by ± 100 ms to, and fitted with, the template of noxious-evoked brain activity (red). Reflex withdrawal traces (right) are displayed in blue; the green and black dots indicate the start and end points found by the algorithm respectively, and the red line depicts the smoothed curve from which the amplitude is calculated. Dashed line indicates the moment of heel lance stimulation. Group averages are displayed at the bottom.

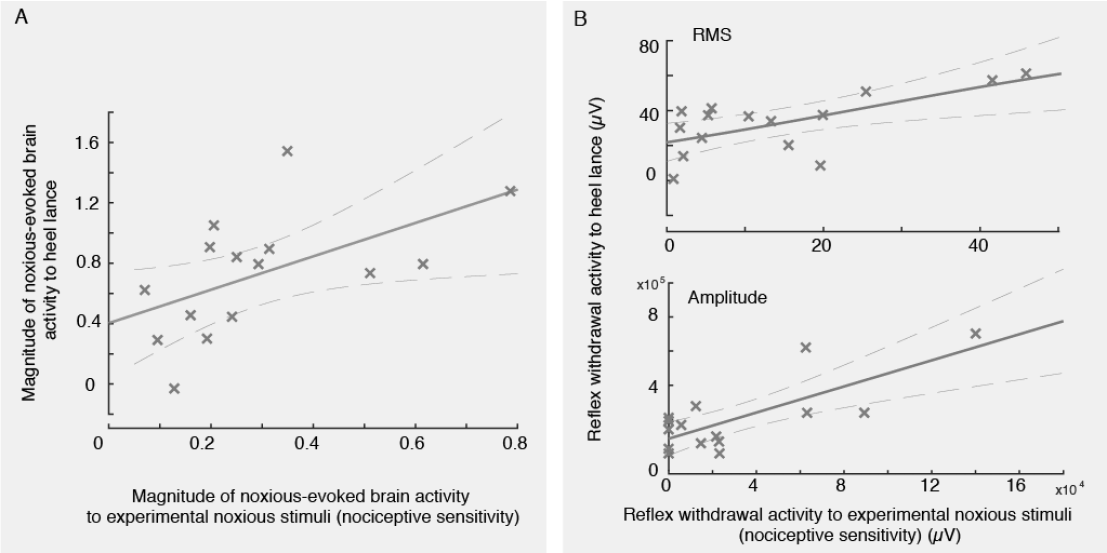


Figure 5.8. Noxious-evoked brain activity and reflex withdrawal responses to experimental stimuli and heel lances demonstrate within-subject correlation. (A) Significant correlation is observed between the magnitude of noxious-evoked brain activity to 128 mN experimental noxious stimuli and heel lances in infants receiving no intervention prior to lancing. (B) The relationship between the magnitude of the reflex withdrawal response to experimental noxious stimuli and heel lancing demonstrates significant correlation when quantified using the RMS method (top), and a trend towards correlation when considering the amplitude of the response (bottom).

5.4.2 Investigating the effect of gentle touch by accounting for individual nociceptive sensitivity

First, the magnitude of noxious-evoked brain activity in response to heel lancing in the no touch and gentle touch groups was compared without taking into account individual nociceptive sensitivity; responses to heel lancing in the gentle touch group are displayed below in Figure 5.9. As expected given the relatively small sample size, no significant difference was observed without taking into account

individual nociceptive sensitivity (mean ± standard error magnitude of noxious-evoked brain activity in no touch group = 0.73 ± 0.10, magnitude in gentle touch group = 0.67 ± 0.12, p=0.70, Figure 5.10A).

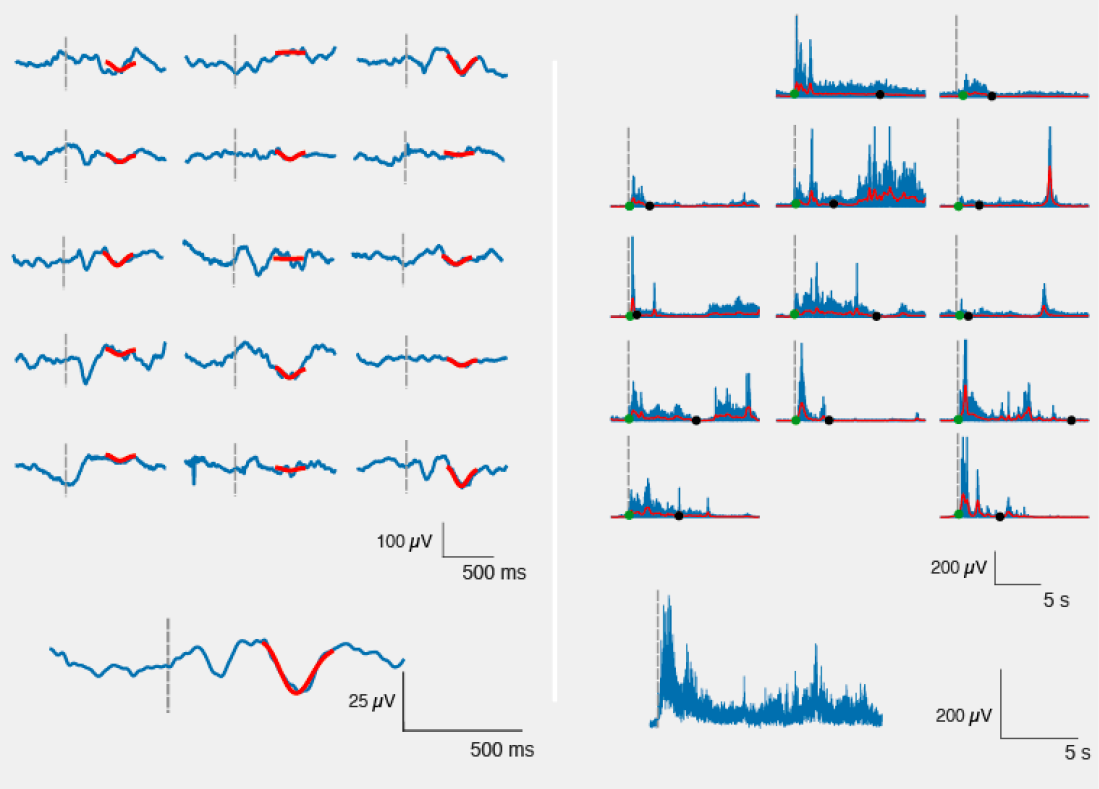


Figure 5.9. Noxious-evoked brain activity and reflex withdrawal responses following heel lancing in the gentle touch group in Study 2. The reflex withdrawal of the first infant in the gentle touch group was rejected due to artefact, and in the penultimate infant EMG was not recorded due to infant restlessness during the study.

To investigate the effect of individual sensitivity and to identify any outliers with regards to the relationship between the two noxious stimuli, the ratio of the response following experimental noxious stimuli to the response following heel lancing was investigated. Two outliers were noted in the gentle touch group and one outlier was noted in the control group (Figure 5.10B). Despite noxious-evoked

brain activity that looked typical and was not influenced by artefact (Figures 5.7, 5.9), the relationship between the responses to experimental noxious stimuli and heel lancing in these infants did not follow the typical correlation, and these points were defined as outliers (as detailed in the Statistical Analysis section) and removed from the subsequent analysis.

Following removal of the outliers, when accounting for individual nociceptive sensitivity by considering the ratio of the response following noxious experimental stimuli to the response following heel lancing, gentle touch significantly reduced the response to heel lancing ($p=0.049$, Figure 5.10C). Linear regression did not demonstrate a significant effect of an interaction between gentle touch and individual sensitivity ($p=0.17$, Figure 5.10D), however, Figure 5.10D is reminiscent of the hypothesised schematic in Figure 5.4D; a trend towards an effect of gentle touch when considering individual nociceptive sensitivity is apparent. It is likely that the noise in the data (illustrated by the wide distribution of points around the regression line) and the relatively low sample size are preventing a significant effect from being observed, suggesting that increasing the sample size may help.

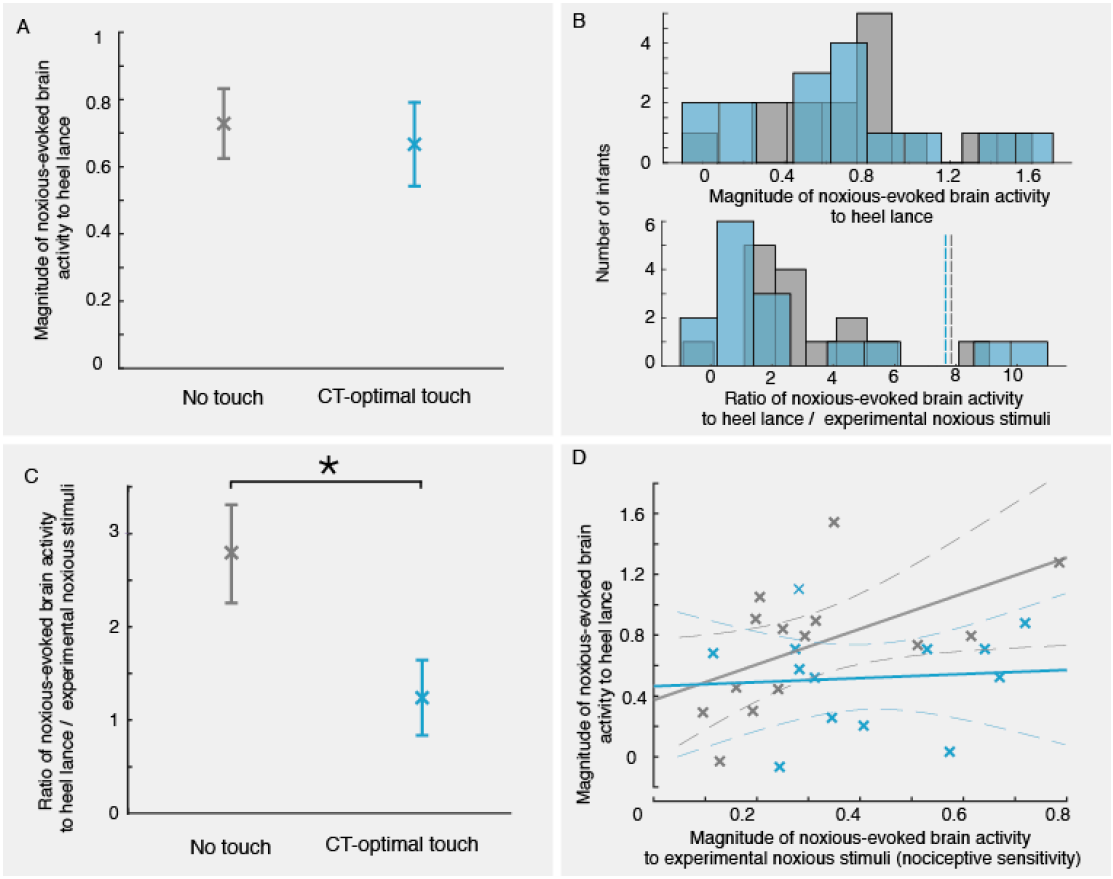


Figure 5.10 Investigating taking into account nociceptive sensitivity (A) When comparing responses to heel lancing in the no touch and gentle touch group without taking into account nociceptive sensitivity, there is no difference between the two groups. Error bars indicate mean ± standard error. (B) Histograms depicting the distribution of heel lance responses (top), and the distribution of the ratio of the response to the heel lance divided by the response to experimental noxious stimuli (bottom) in all infants in the no touch (grey) and gentle touch (blue) groups. Dashed lines indicate the limit over which the ratio of the responses to the two stimuli were defined as outliers and removed from the subsequent analysis; 3 outliers are evident. (C) Taking into account individual nociceptive sensitivity by considering the ratio of the response to heel lancing divided by the response to experimental noxious stimuli indicates a significant effect of gentle touch. Error bars indicate median ± standard error. (D) Taking into account individual nociceptive sensitivity by considering the relationship between responses to experimental noxious stimuli and heel lancing (linear regression) suggests a trend towards an effect of gentle touch on heel lance responses; no touch group in grey, gentle touch group in blue.

As the data in Study 1 and the control data in Study 2 both demonstrated within-subject correlation between responses to experimental noxious stimuli and heel lancing (Figure 5.11), and there was no significant difference between the responses to experimental noxious stimuli in the two datasets (p=0.21, t-test), the two datasets were pooled to investigate whether increasing the sample size and spread of the control data would increase the power of this investigation. Without taking into account individual nociceptive sensitivity, the comparison of the responses to heel lancing without gentle touch in the pooled data (n=26) and with gentle touch in the Study 2 data (n=15) did not indicate a significant effect of gentle touch (p=0.35, Figure 5.12A), as above.

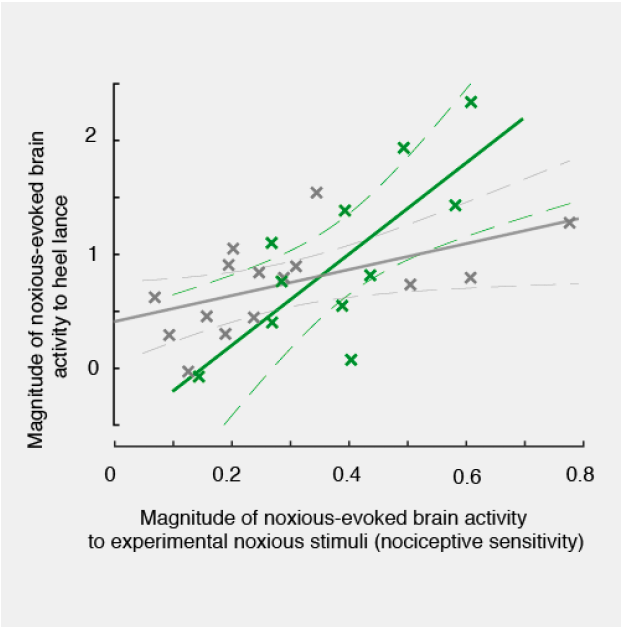


Figure 5.11 Responses to experimental noxious stimuli and heel lancing are correlated in both Study 1 and Study 2. There was no significant difference between the responses to experimental noxious stimuli in the two datasets, and they were therefore pooled. Study 1 in green; Study 2 in grey. There is a larger range of heel lance responses in Study 1 than Study 2.

Concordant with the above analysis of just the Study 2 data, the same 3 infants were outliers when considering the ratio of their responses to heel lancing and experimental noxious stimuli. Following removal of these infants from the analysis, accounting for individual sensitivity both by considering the ratio of responses to

the two stimuli and by using linear regression (Figure 5.12B) indicated that gentle touch influences responses to heel lancing (p=0.056 and p=0.029 respectively).

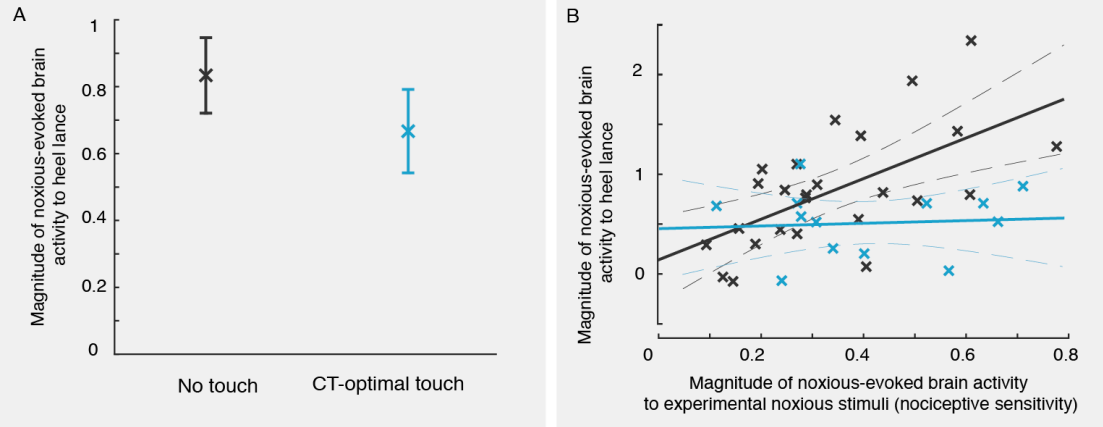


Figure 5.12 Investigating taking into account nociceptive sensitivity in pooled data (A) Gentle touch does not significantly alter the magnitude of noxious-evoked brain activity following heel lancing without taking into account nociceptive sensitivity. (B) Taking into account individual nociceptive sensitivity by considering the relationship between responses to experimental stimuli and heel lances (linear regression) in the pooled data demonstrates a significant effect of gentle touch.

As may be expected, it appears that the impact of gentle touch has a greater effect in infants where the magnitude of noxious-evoked brain activity is greater, consistent with the schematic in Figure 5.4D; as well as a reduction in the magnitude of lance responses (a translation down the y-axis) following removal of outliers (Figures 5.10D; 5.12B) the gradient of the line of best fit is less steep in the gentle touch group than control group. In the data from Study 2, when considering the relationship between responses to experimental noxious stimuli and heel lances, many infants are low responders: there is a cluster of responses to noxious stimulation with magnitudes ~ 0.2 – 0.3, which is comparable to the mean ± standard error magnitude of background brain activity, 0.23 ± 0.098. In these

lower responding infants, gentle touch would be expected to have a smaller effect than in larger responding infants, due to a floor effect.

To investigate whether gentle touch was effective in only infants demonstrating substantial noxious-evoked brain activity responses and who are sensitive to noxious stimulation (defined as having a response to experimental noxious stimuli which was above 80% of their average background brain activity), the average magnitude of noxious-evoked brain activity elicited by the experimental noxious stimuli was compared with the average activity characterised by the template in the background brain activity in each infant. If the response evoked by experimental noxious stimulation was not above 80% of their background brain activity, the infant was excluded from this section of the analysis to investigate the effect of gentle touch after removing non-responding infants. Thresholding at 80% of the background is a technique that has been used previously to define whether noxious-evoked brain activity is present [17]. 2 infants in the gentle touch group, and 3 infants in the no touch group were removed from the analysis (Figure 5.13A).

Following removal of these infants that are less sensitive to nociceptive stimuli, the comparison of the magnitude of noxious-evoked brain activity to heel lancing (without any other method of taking into account individual nociceptive sensitivity) in the gentle touch and no touch group is significant ($p=0.033$, Figure 5.13B). This demonstrates that in infants who are more sensitive to noxious stimuli, gentle touch significantly reduces noxious-evoked brain activity.

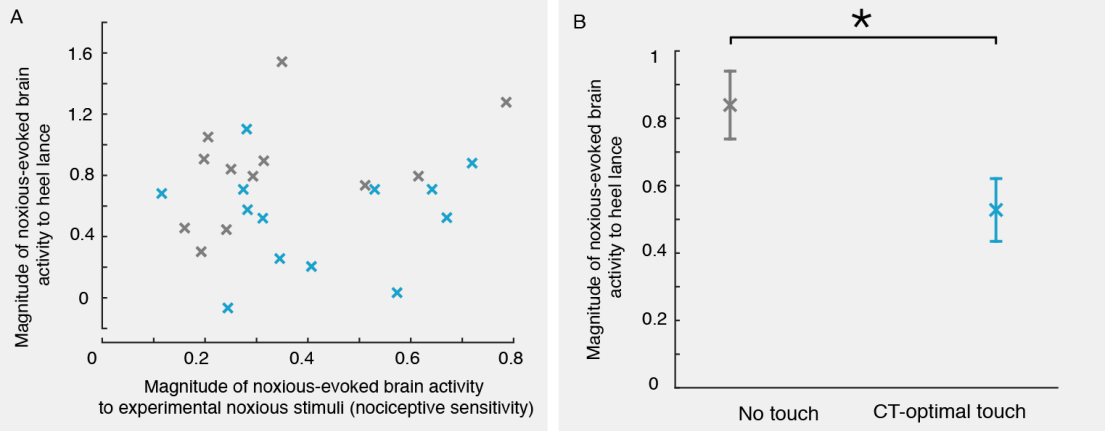


Figure 5.13 Gentle touch is effective in infants who have higher nociceptive sensitivity. (A) The relationship between experimental noxious stimuli and heel lance responses (in Study 2) following thresholding to remove infants with low nociceptive sensitivity whose responses to experimental noxious stimuli were not greater than their background brain activity. (B) Following thresholding and removal of infants with low nociceptive sensitivity, gentle touch significantly reduces noxious-evoked brain activity following heel lancing.

Reflex withdrawal was also investigated, by considering both the RMS and the amplitude. When considering the RMS of the reflex withdrawal response, gentle touch did not have a significant effect when comparing heel lance responses without accounting for individual nociceptive sensitivity ($p=0.15$, Figure 5.14A).

When accounting for individual nociceptive sensitivity by considering the ratio of the reflex withdrawal response following experimental noxious stimuli to that following heel lancing, in 3 infants in the gentle touch group, and 2 in the control group, the values of the ratio of the RMS of the reflex response following experimental stimuli to the response following heel lancing were outliers, and these 5 infants were therefore rejected from the subsequent analysis (Figure 5.14B). Following rejection of these outliers, when taking into account individual

nociceptive sensitivity by considering the ratio of the reflex response following experimental stimuli to that following heel lancing, gentle touch elicited an effect that is approaching significance (p=0.051, Figure 5.14C), although linear regression did not (p=0.13, Figure 5.14D). Consistent with the analysis of brain activity, this may be due to the noise in the data and relatively low sample size, but there was no Study 1 reflex withdrawal data with which to pool this data.

When considering the amplitude of the reflex response, gentle touch did not have an effect when comparing the response to heel lancing without taking into account individual sensitivity in the two groups (p=0.38, Figure 5.14E). Consideration of the ratio of responses cannot be performed here, because it is not possible to divide by a response to experimental noxious stimuli of 0, but accounting for individual variability using linear regression indicates a significant effect of gentle touch (p=0.0015, Figure 5.14F). The relationship between reflex responses to experimental noxious stimuli and heel lancing in Figure 5.14(D) and (F) is also reminiscent of the hypothesised schematic in Figure 5.4D, indicating that gentle touch elicits a larger reduction in reflex withdrawal in infants who are larger responders.

These results demonstrate that taking into account individual nociceptive sensitivity by considering the ratio of responses or using linear regression does indicate an effect of gentle touch on the magnitude of reflex withdrawal activity.

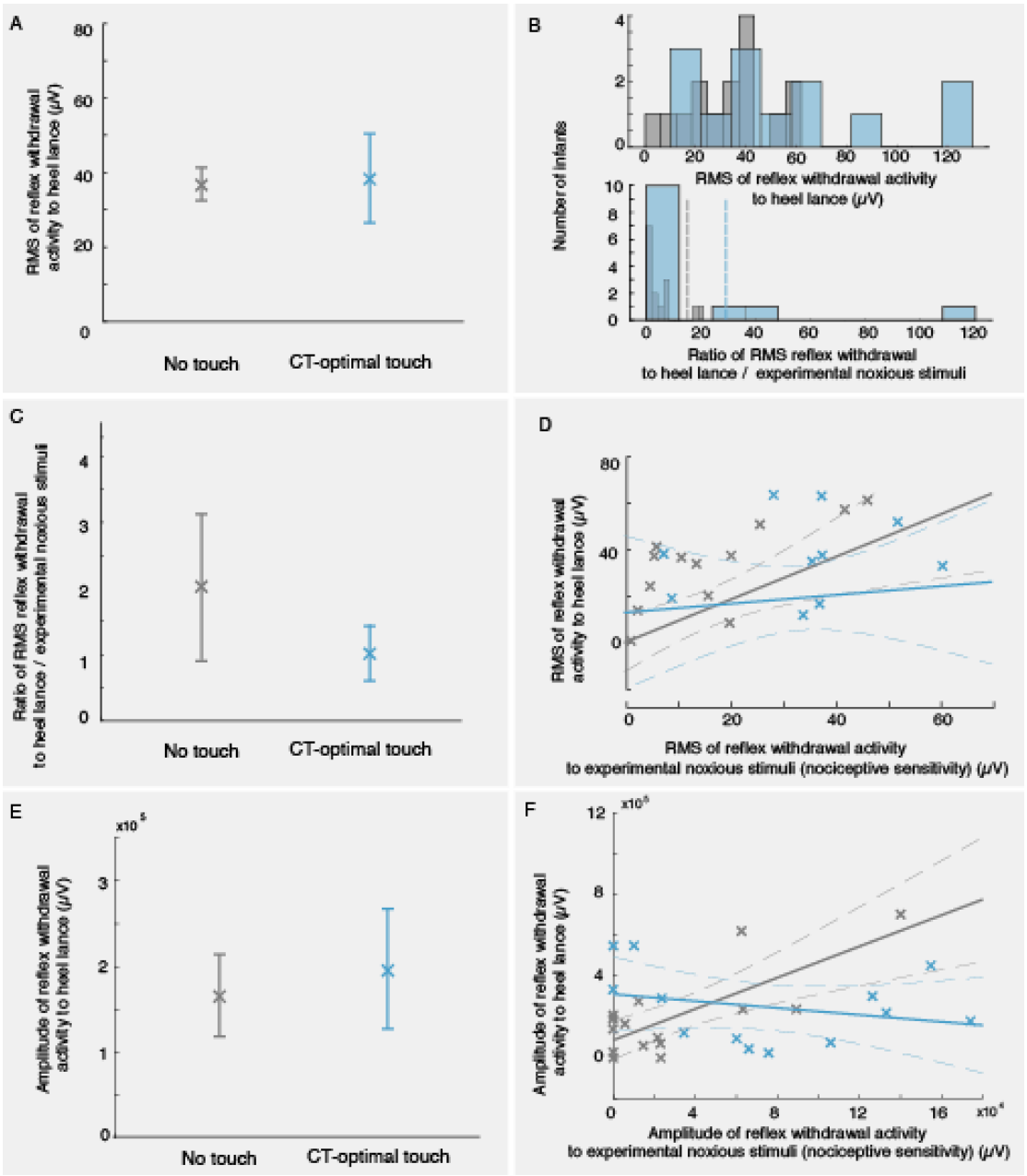


Figure 5.14 Investigating the effect of taking into account individual nociceptive sensitivity on reflex withdrawal responses. (A) When using the RMS to quantify reflex withdrawal activity, gentle touch does not significantly affect the magnitude of the reflex withdrawal response without taking into account individual nociceptive sensitivity. Error bars indicate median ± standard error. (B) Histograms displaying the distribution of heel lance reflex responses (top), and the ratio of the response to the two noxious stimuli (bottom), quantified using the RMS. 3 outliers in the gentle touch group, and 2 in the control group were rejected from the subsequent analysis. (C) Taking into account individual nociceptive sensitivity when considering the ratio of the responses following experimental stimulation to that following heel lancing indicates a trend towards an effect

of gentle touch on reflex withdrawal magnitude quantified using the RMS. (D) Taking into account individual sensitivity by considering the relationship between responses to experimental noxious stimuli and heel lancing quantified using the RMS indicates a trend towards an effect. (E) When considering the amplitude of the reflex withdrawal without taking into account individual nociceptive sensitivity, the effect of gentle touch is on the brink of significance. (F) Taking into account individual nociceptive sensitivity by considering the relationship between responses to experimental noxious stimuli and heel lancing (linear regression) indicates a significant effect of gentle touch on reflex withdrawal responses to heel lancing.

5.4.3 The effect of gentle touch on behavioural and physiological responses

As infants do not display behavioural responses to experimental noxious stimuli, it was not possible to investigate the relationship between behavioural responses to the two noxious stimuli. The effect of gentle touch on PIPP-R scores and facial expressions in all infants and in just responding infants was nonetheless recorded for comparison with Chapter 3, to further investigate the observed trend towards a touch-induced reduction in facial expressions in responding infants observed in Chapter 3. A similar proportion of infants mounted facial expression responses in both groups; 13 of 15 (87%) in the no touch group, and 11 of 13 (85%) in the gentle touch group. PIPP-R scores and facial expression durations are displayed in Table 5.3 and were not significantly different when comparing the no touch and the gentle touch group.

	No touch	Gentle touch	p-value
PIPP-R	6 (5, 9)	9 (7, 12)	0.14
Facial duration in all infants (s)	6 (4, 12)	8 (3, 12)	0.68
Facial duration in responding infants (s)	7 (4, 13)	14 (4, 9)	0.54

Table 5.3 The effect of gentle touch on PIPP-R scores and facial expressions following heel lancing.

5.5 Discussion

5.5.1 Overview

The results detailed in this chapter support the hypothesis that taking into account individual nociceptive sensitivity, by considering the correlation between responses to experimental noxious stimuli and clinical procedures, is useful in observing intervention effects. Concordant with the hypothesised schematic in Figure 5.4D, the effect of gentle touch is greater in infants with higher nociceptive sensitivity. The need for smaller sample sizes to observe intervention effects when accounting for individual variation in nociceptive sensitivity is useful given the challenges of neonatal recruitment, as discussed further in the General Discussion.

5.5.2 Within-subject correlation of responses to noxious stimuli

While intensity encoding has been demonstrated previously in infants between 32 mN, 64 mN, 128 mN experimental noxious stimuli and heel lances, this has not been demonstrated within-subject as the same infants did not receive both experimental stimuli as well as a clinically necessary heel lance [210]. In this analysis of Study 1 data, within-subject correlation of noxious-evoked brain activity is demonstrated.

Compared to the responses to the 64 mN stimulus, the responses to the 32 mN stimulus were less strongly correlated with the response to the heel lance. This is likely to be due to the higher signal to noise ratio following the 64 mN stimuli. This led to the decision to recruit infants to receive the 128 mN stimulus prior to heel lancing in Study 2 to improve the signal to noise ratio still further. Additionally, the 128 mN stimulus, but not the 64 or 32 mN stimuli can be automatically time-locked to the brain activity recordings, resulting in less uncertainty regarding the moment of stimulation. Consistent with Study 1, noxious-evoked brain activity responses to the 128 mN experimental noxious stimuli and heel lance in the Study 2 data were correlated. The response to the 128 mN stimulus in the Study 2 data was not significantly different from the response to the 64 mN stimulus in the Study 1 data, which allowed pooling of the data for increased power.

Intensity coding of the magnitude of reflex withdrawal has also been demonstrated between noxious stimuli of differing intensities previously [210] [209], and in the Study 2 data is demonstrated within-subject between experimental noxious stimuli and

heel lances. Both the RMS and the amplitude of the reflex withdrawal activity were considered; the RMS indicated significant correlation, while the amplitude indicated a trend towards correlation, possibly due to the many responses to experimental noxious stimuli which were classified as '0'.

5.5.3 Accounting for individual sensitivity aids the between-subject investigation into the effect of gentle touch

Consideration of the ratio of the response following experimental noxious stimulation to the response following heel lancing was used to investigate the relationship between the responses to the two stimuli and identify outliers regarding this relationship. Following rejection of outliers, considering the ratio of the responses to the two stimuli demonstrated that gentle touch was effective, but linear regression did not indicate a statistically significant effect. This is likely due to a combination of being underpowered after the removal of the outliers from the analysis, and the noise in the data as illustrated in Figure 5.10D. However, the trends in the data were reminiscent of the hypothesised schematic in Figure 5.4D.

Therefore, the data from Study 2 was pooled with the data from Study 1, in which infants demonstrated a larger spread of heel lance responses, to investigate whether increasing the sample size and spread of the data would aid the investigation into accounting for individual nociceptive sensitivity when considering the effect of gentle touch. Following pooling of the data, taking into account individual nociceptive sensitivity using linear regression, as well as considering the ratio of the two responses following outlier rejections, both indicated an effect of gentle

touch. This demonstrates the importance of adequate power and spread of data, as well as identifying outliers, in the process of analysis; in an ideal situation more infants would be recruited who received the same intensity stimulus, as will be discussed further below.

Lastly, thresholding of the data was performed to include only the infants who responded to experimental noxious stimuli at levels above their background brain activity, i.e the infants who are more sensitive to the noxious stimulus. These are the infants in which interventions are expected to have an effect, as concordant with the hypothesised schematic in Figure 5.4D, and as can be seen in Figure 5.10D and 5.12B, the effect size of interventions appears to be larger when infants are larger responders. This is expected, as low responders do not have scope for large reductions and can only have their responses reduced by small amounts. From this data, it is not possible to determine whether this is due to a floor effect (whereby responses cannot be decreased further as they are already at a minimum), or whether the effect size of the intervention is always proportional to the original magnitude of the response.

When comparing only the high responders, a comparison of the magnitude of noxious-evoked brain activity following heel lancing (without any other method of taking into account individual sensitivity) demonstrated a significant effect of gentle touch, indicating that gentle touch is effective in these higher responding infants.

Regarding the identification of outliers by considering the ratio of their responses, 2 infants in the gentle touch group, and 1 infant in the control group were identified. No factors were identified which could explain these outliers (no difference in gestational age, postnatal age, sepsis levels, sickness levels, or prior pain). However, a possible explanation could be a change in behavioural state. There is a paucity of information regarding how behavioural state influences noxious-evoked brain activity; only one study to date has investigated the effect of sleep state on EEG-recorded infant noxious-evoked brain activity in a relatively small sample size of 12 infants and did not find an effect [11], however, sleep state alters background EEG activity [261], and is reported to influence NIRS-recorded pain-evoked brain activity [216] and behaviour [247], as detailed in the General Introduction. Although the experimental noxious stimuli and heel lances were applied in the same session and care was taken to settle the infant prior to the application of all stimuli, infants can change behavioural and sleep state rapidly, meaning that the infant could be in different behavioural states during the application of the two types of stimuli, which could disrupt the within-subject relationship. Another possible explanation is local sensitisation, as when applying the experimental noxious stimuli, care was taken to avoid areas of the foot that appeared inflamed due to previous procedures (most infants had previous experience of heel lancing). However, this was not always possible when performing the clinically necessary heel lance, and localised sensitisation of the skin could have affected responses to heel lances and influenced the relationship between responses to the two stimuli.

In this chapter, three methods of accounting for individual nociceptive sensitivity (calculation of the ratio of responses to noxious stimuli, linear regression, and thresholding), have demonstrated that gentle touch reduces noxious-evoked brain activity following heel lancing. This is consistent with the conclusion reached in Chapter 3, using the technique of age-matching to account for individual variability, that gentle touch is effective in reducing noxious-evoked brain activity. However, the method used here of accounting for individual variability in sensitivity is likely to be a much more comprehensive method of accounting for nociceptive sensitivity than age-matching or attempting to account for the many other contextual factors that may influence nociceptive responses, as the feature of interest itself, nociceptive sensitivity, is being accounted for here. Future research is needed to see if this method is also applicable over a wider age range or a population of infants with a more diverse background (e.g including ex-premature infants with experience on the neonatal unit).

Interestingly, accounting for individual sensitivity in reflex withdrawal also demonstrated that gentle touch reduces reflex withdrawal prior to heel lancing. Consistent with the analysis of noxious-evoked brain activity, when using the RMS method to quantify reflex withdrawal, several infants in both the gentle touch and the control group were outliers regarding the ratio of their responses following experimental noxious stimuli to their responses following heel lancing. Following removal of these outliers, both consideration of the ratio of responses and linear regression indicated an effect of gentle touch in reducing reflex responses following heel lancing. This effect was also observed when using linear regression

to investigate accounting for individual sensitivity when exploring the effect of gentle touch on the amplitude of the reflex response.

These results demonstrate that taking individual sensitivity into account via consideration of the ratio of responses or linear regression demonstrates an effect of gentle touch on reflex withdrawal. This conclusion is contrary to the conclusion reached in the original analysis of the effects of gentle touch in Chapter 3, in which the technique of age-matching was used to account for individual variability rather than measuring infant responses to experimental noxious stimuli. This suggests that the methods utilised here of measuring infant noxious-evoked brain activity to gauge individual nociceptive sensitivity are better able to account for individual variability and enable observation of intervention effects on the reflex response than age-matching. This would be expected, as the methods used here measure the feature of interest, nociceptive sensitivity itself, and this would be expected to be a more comprehensive method of accounting for variability in nociceptive sensitivity, as discussed above.

5.5.4 Comparison of the current investigation into the effect of gentle touch with that in Chapter 3

In Study 1, the mean ± standard error of the magnitude of noxious-evoked brain activity in response to the heel lance was 0.97 ± 0.23. In Study 2, prior to rejections, the response to the heel lance was 0.73 ± 0.10 in the no touch group, and 0.67 ± 0.12 in the gentle touch group. In Chapter 3, the response to the lance was 1.12 ± 0.17 in the no touch condition, and 0.72 ± 0.17 in the gentle touch group. The range of mean responses to the lance in the control group, and the

difference in the responses to the lance in the gentle touch group, are likely to be due to individual variation, and highlight the importance of taking this into account, especially in investigations involving small sample sizes.

Regarding reflex withdrawal activity, in Study 2, the median ± standard error of the RMS of reflex withdrawal to heel lancing was 36.7 ± 4.35 μV in the no touch group, and 38.3 ± 12.9 μV in the gentle touch group. This was comparable to the magnitude of the reflexes quantified in Chapter 3; the RMS of the reflex withdrawal was 35.2 ± 7.8 μV in the no touch condition, and 32.8 ± 5.7 μV in the gentle touch condition, but again the slight variation in the responses highlights the influence of individual variability in relatively small sample sizes.

When considering behavioural and physiological responses, in the infants analysed in Chapter 3, the PIPP-R scores in the no touch and gentle group were 7 (6, 11) and 8 (5, 10) respectively, facial expression durations in all infants were 6 (0, 14) seconds and 4 (2, 12) seconds respectively, the proportion of responding infants was 9/14 (64 %) and 12/16 (75 %) respectively, the facial expression duration in just responding infants was 13 (9, 15) seconds and 7 (4, 13) seconds. No significant differences were seen between groups, comparable with the data in this chapter. However, a trend towards a touch-induced reduction on facial expression duration in responding infants was observed in Chapter 3. As it is likely that the studies in these chapters were underpowered to detect an effect of gentle touch on behaviour [13], the PIPP-R and facial expression scores were pooled. No effect of gentle touch was apparent in the pooled data (PIPP-R score comparison p=0.35; facial expression comparison in all infants p=0.87; facial expression comparison in

responding infants p=0.90). Although previous literature has demonstrated that infant massage and touch-based therapies reduce infant clinical pain scores and facial expressions as detailed in Chapter 3 [338 353 354 357 358], this indicates that gentle touch over this shorter stimulation period does not reduce these behavioural or physiological measures of infant pain. As discussed in Chapter 3, it is possible that longer stimulation durations are necessary for effects on behaviour and physiology to be observed.

5.5.5 Strengths and limitations

The limitations of investigating responses to single trials, inherent to the analysis of clinically required heel lance responses, are apparent in this chapter; movement artefact during the lance meant that the response must be rejected and the infant could not be included in the analysis, while responses to one or more of numerous applications of experimental noxious stimuli could be rejected without preventing the inclusion of the infant in the analysis. Additionally, responses to experimental noxious stimuli could be averaged to increase the signal to noise ratio, and the distribution of responses to experimental noxious stimuli could be observed to identify outlying responses or responses that greatly skewed the average, which could then be removed in order to attain the best possible characterisation of an individual infant's response. However, with a single trial this is not possible, meaning that noise had a greater influence and there was greater variance associated with the correlation between the two noxious stimuli. The advantages and disadvantages associated with the use of different noxious stimuli are discussed further in the General Discussion.

Another limitation of this study is that in the control data analysed in Study 2, there does not appear to be a large spread of responses to experimental noxious stimuli or heel lances: there appear to be many low responders to experimental stimuli clustered with magnitudes of noxious-evoked brain activity around ~ 0.2 – 0.3, which is comparable to background brain activity (mean ± standard error 0.23 ± 0.098). This lack of spread could make it difficult to see an effect of gentle touch, as the effects of pain relief interventions are expected to be larger when responses are larger, but there are fewer large responders in the control group with whom to compare. Additionally, there appear to be greater levels of noise in the relationship between responses to the two stimuli in the Study 2 data compared to the Study 1 data. As the simulations performed to estimate the required sample size for Study 2 were based on the data from Study 1, it is possible that this led to an underestimate of the required sample size, and in the future more data should be collected in which infants receive the 128 mN experimental noxious stimulus and heel lancing in order to create a homogeneous dataset. However, despite the relatively small sample size analysed here, the results here still demonstrate that taking into account nociceptive sensitivity aids between-subject comparisons, and the demonstration that this is a useful technique even in small sample sizes is crucial for the future applicability of this method.

5.5.6 Future directions

This technique has great promise for future research. Given that the application of experimental noxious stimuli does not distress the infant [210] [222] and is relatively

simple, this method could be used to account for individual nociceptive sensitivity and reduce the sample sizes required to observe intervention effects in future investigations into the efficacy of pain relief interventions. However, while correlation between the response to experimental noxious stimuli and heel lances, both applied to the heel, has been demonstrated here, the correlation between responses to experimental noxious stimuli and the procedure of interest would have to be demonstrated before this method could be used, to determine if responses to experimental stimuli are also correlated with responses to alternative procedures at other body sites. This research would be of interest when considering whether responses to noxious stimuli are always correlated regardless of the site of stimulation, or whether nociceptive processing differs quantitatively at different body locations within individuals, which may alter nociceptive sensitivity and the within-subject relationship between responses to experimental and clinical stimuli following application at different sites.

Of relevance to the work in this thesis would be the investigation of the relationship between responses to experimental noxious stimuli and to immunisations on the thigh, as studied in Chapter 4. Individual nociceptive sensitivity could then be accounted for when investigating the effect of paracetamol for immunisation in an extension of this work.

As responses to experimental noxious stimuli and heel lances are correlated, future work could investigate whether it is possible to predict the magnitude of the response to a clinical procedure from the response to experimental stimuli. It could also be investigated whether the extent of effect of an intervention could be

predicted from responses to experimental noxious stimuli. If, like gentle touch, pharmacological analgesics also have greater effects in infants who have higher nociceptive sensitivity, this would be especially useful when balancing the potential for pain relief with the risk of adverse side effects.

As detailed in the Introduction, in adults verbal reports of pain perception can be used to account for individual variability in imaging experiments (Figure 5.3), rather than exploring the relationship between nociceptive responses to stimuli as investigated here in infants. However, when using machine learning to train models to be able to predict the perception of pain based on brain activity, individual variability is an issue. To overcome this, recent research has explored the relationship between background inter-stimulus brain activity and laser-evoked potentials as recorded by EEG, demonstrating that these are correlated. This relationship can be used to normalise pain-evoked responses or to separate infants based on background EEG features for individualised training paradigms (i.e separate training sets for infants with different background EEG characteristics), both of which lead to a higher prediction accuracy [530] [531]. Although the correlation between two noxious stimuli as investigated in this chapter may be expected to be stronger than the correlation between background brain activity and evoked responses, this approach should be investigated in infants, as use of background brain activity to account for individual variability could eliminate the need for the application of the experimental noxious stimuli.

5.6 Conclusion

In sum, the work in this chapter demonstrates that the magnitude of noxious-evoked brain activity and reflex withdrawal responses to experimental noxious stimuli and heel lances are correlated within-subject, and that this relationship can be used to account for individual nociceptive sensitivity and aid between-subject investigations into the efficacy of pain relief interventions. The observation that reflex withdrawal is reduced by gentle touch when accounting for individual nociceptive sensitivity here but not when using the technique of age-matching in Chapter 3, highlights further that this method may be better able to account for individual differences in nociceptive sensitivity. Additionally, this method can be of use in instances when age-matching is not possible due to the differing distribution of ages in each group, such as in Chapter 4.

Chapter 6: General Discussion

6.1 Thesis summary

There is a paucity of information regarding analgesic efficacy in neonates, but the range of adverse consequences associated with early life pain [2] [3] indicates the importance of its effective management. A range of surrogate measures have been used to assess infant pain, but there are often discrepancies between these indicators [240], and behavioural and physiological measures have provided inconclusive results regarding the efficacy of pain relief interventions, possibly due to confoundment by procedure-associated distress. In this thesis, a multimodal approach to infant pain assessment, with a focus on noxious-evoked brain activity, is utilised to gain a comprehensive understanding of the infant pain experience. Using this approach, the aim of the research described in this thesis was to investigate the efficacy of pain relief interventions, and to improve the methods with which to assess them. In Chapter 3, gentle touch prior to experimental noxious stimuli and heel lancing was investigated. In Chapter 4, immunisation-evoked brain activity was characterised before the effect of paracetamol could be investigated for this procedure. In Chapter 5, a novel method of accounting for individual nociceptive sensitivity, and whether this can aid investigations into the efficacy of pain relief interventions, was explored.

This research is of importance as it has the potential to impact both clinical and research practice. Gentle touch was demonstrated to be effective in reducing noxious-evoked brain activity prior to both experimental noxious stimuli and heel

lancing, and given its lack of adverse effects and simplicity of application, could easily be implemented into clinical practice. The demonstration that immunisation-evoked brain activity can be fit with the previously validated template of noxious-evoked brain activity permits the use of an already validated and objective method of assessing analgesic efficacy, and proffers the possibility that further procedures can be characterised and fit with the template in order to assess a wider range of pain relief interventions. Despite the small sample size, paracetamol appears to cause a trend towards a reduction in immunisation-evoked brain activity, and given that it is administered anyway for the week 8 immunisations as an antipyretic, it seems logical for its administration to be prior, rather than post, immunisation to allow for any analgesic effects. Lastly, the research composing Chapter 3 of this thesis demonstrates how individual nociceptive sensitivity can be accounted for to aid between-subject investigations into analgesic efficacy, and also replicates the finding that gentle touch reduces noxious-evoked brain activity. Experimental noxious stimuli could be applied in future between-subject investigations in order to account for individual variation in nociceptive sensitivity and allow observation of intervention effects.

In the following, the strengths and limitations of the research composing this thesis are discussed. The challenges associated with neonatal recruitment and how they can lead to small sample sizes are detailed, as well as the range of stimuli that have been utilised to investigate noxious-evoked responses in this thesis and the advantages and disadvantages associated with their use. Additionally, the strengths and limitations of the methods used to estimate infant pain as part of a multimodal approach to pain assessment are discussed.

6.2 Research in a vulnerable population

6.2.1 Recruitment challenges

Recruitment for investigations involving neonatal infants can be difficult for several reasons. When investigations are centred on clinically required procedures such as blood tests or immunisations, the frequency with which infants are eligible for research depends on the frequency with which infants require these procedures. While there are infants requiring blood tests almost daily, immunisations are only due in ex-preterm infants on the neonatal unit once or twice per month, limiting the rate of recruitment. With experimental stimuli, a wider pool of infants are available for recruitment, however as this research is not linked to any essential procedure, parents in this understandably busy period of the first few days of their infant's life are often less inclined to give up their time.

For studies involving clinical procedures, communication with the clinical team is key. Responses to blood tests were investigated in Chapters 3 and 5, and these blood tests are required for several reasons; while some blood tests are required at specific times (for jaundice levels and infection markers), the timing of other blood tests (the day 5 Guthrie test and arterial blood gas tests) are more flexible, and in these instances, the clinical team must be aware to leave the blood tests so that there can be a chance to recruit for research. This can sometimes be an issue, as information about leaving blood tests can be lost in clinical care handovers, and the clinical team sometimes prefers to perform blood tests before the start of the

working day. For the immunisations investigated in Chapter 4, a great deal of communication was required to determine with the clinical team when the infant was ready for immunisation, and that the parents had already been informed that their infant is due immunisations, and for this decision to be made with enough time to allow parents to be approached and consider participation in the research study. Lastly, once parents had expressed interest in taking part in research, several participants were lost due to transfer or discharge (as seen in Figure 2.1, General Methods).

Due to the infrequency with which infants require the clinical procedures investigated, and the difficulties associated with neonatal recruitment, sample sizes for neonatal investigations involving the recording of brain activity are often small. For these reasons, efforts are made to minimise the numbers required as seen in this thesis; within-subject investigations are performed using experimental stimuli to control for individual variability, and for between-subject investigations, the technique of age-matching was employed in Chapter 3, and in Chapter 5 an alternative, taking into account individual nociceptive sensitivity, was explored. Appreciation of the challenges associated with neonatal recruitment demonstrates the importance of this new method of taking individual sensitivity into account in order to reduce the required sample sizes.

6.2.2 Choice of pain relief interventions

Given the vulnerability of the population of interest, the interventions investigated here have been carefully considered; research involving the administration of

interventions in infants, especially those of pharmacological nature, is stringently regulated. Gentle touch was chosen as a non-pharmacological intervention, as along with the purported benefits of reduced pain perception following CT-optimal touch in the adult literature [346] [376] and reduced indicators of infant pain following infant massage [354] [358] [338], gentle touch is not associated with any risk of adverse effects and the application of gentle touch was encompassed within the existing ethics. Paracetamol, a pharmacological intervention, was investigated opportunistically as it was being administered as an antipyretic in line with clinical guidelines, and therefore did not involve administration purely for research. Investigation of both of these interventions began in an exploratory manner, but can form an important basis on which future larger-scale work, including randomised controlled trials, could be performed to thoroughly investigate the effects of gentle touch and paracetamol, as will be discussed below.

A factor to consider in the investigations of pain relief interventions here is the use of standard clinical care involving comfort measures. As was deemed clinically appropriate, infants could receive swaddling, a dummy, a gloved finger to suck on, or be held by their parents. Given the evidence of non-pharmacological comfort methods in reducing pain behaviours [337] [338] and brain activity [336] [335], this is important to note and could confound the effect of interventions. Sucking a dummy or finger is especially likely to influence behavioural indicators such as facial expressions. However, infants received these comfort measures across all investigations in a balanced manner, and so they are not likely to have had an effect in group comparisons. Another factor that can influence nociceptive responses and which could also not be controlled is behavioural state [216] [247] [187],

however, any potential effects of behavioural state on brain activity or reflex withdrawal are also likely to be balanced across groups, and the PIPP-R accounts for behavioural state by assigning extra points to infants in the quiet sleep state compared with the active sleep, quiet awake, or active awake state.

6.3 Noxious stimuli

A range of stimuli were utilised in this thesis, each associated with different strengths and limitations. The investigation of responses to experimental stimuli is of prime utility as these low intensity stimuli have been well characterised, can be automatically time-locked to the brain activity recordings, and can be applied on multiple occasions to enable within-subject comparisons and the averaging of responses to improve signal to noise ratio. Additionally, a wider pool of infants can be recruited, as the application of these stimuli is not dependent on clinical requirement. However, experimental stimuli are not clinically relevant, and therefore the interventions investigated in this thesis, gentle touch and paracetamol, were also tested specifically for the clinical procedures for which they may be effective: heel lances and immunisation.

Heel lances are also well characterised and automatically time-locked, but in contrast to the possibility for multiple applications of experimental stimuli, are generally clinically required only once. Therefore, within-subject comparisons are not possible. Additionally, the single trial nature of recording responses to heel lancing results in greater loss of data; in Chapters 3 and 5, data from several studies had to be rejected as there was movement artefact during the heel lance,

while rejection of responses to experimental stimuli in these chapters was less of an issue as the remaining responses to experimental stimuli could still be included in the analysis.

There is as of yet no method for time-locking immunisations to the brain activity recordings without the use of a high-speed camera, and the higher uncertainty regarding the moment of stimulation is therefore a limitation associated with studying immunisation-evoked responses, as discussed in Chapter 4. However, the characterisation of immunisation-evoked brain activity and assessment of suitability of fitting the template is important and necessary to be able to investigate the efficacy of paracetamol specifically for the clinically relevant procedure of immunisation, for which paracetamol is administered anyway as an antipyretic. An advantage of studying immunisations is that most infants received 3 immunisations at week 8, and this therefore meant that responses could be averaged to increase the signal to noise ratio, and data from one or two immunisations could still be included in the analysis even if movement artefact or lack of clear video footage prevented the analysis of the remaining immunisation-evoked responses.

As is also detailed in Chapter 4, a limitation associated with studying immunisation-evoked responses is that the clinical staff member performing the immunisations was not consistent between studies (unlike the research members who were applying experimental noxious stimuli and heel lances in a uniform manner), and therefore administration speed and force varied between studies, which could have influenced the response to immunisations. One last limitation

associated with studying immunisations is the lack of control procedure – a control heel lance was compared to the real heel lance to ensure that the activity being characterised is nociceptive-related during characterisation of the template and in Chapters 3 and 5, however there is no non-noxious control condition with which immunisation-evoked activity can be compared. Given the advantages and disadvantages associated with the application of these stimuli, a strength of the experimental design in Chapters 3 and 4 of this thesis is the inclusion of investigating the effect of gentle touch and paracetamol on multiple stimuli; experimental noxious stimuli as well as heel lances and immunisation respectively.

6.4 Multimodal assessment of infant pain

The use of measures of brain activity, reflex withdrawal, facial expressions, heart rate, and oxygen saturations enables a more comprehensive estimate of the infant pain experience than investigation of each of these measures alone. As the brain is where the experience of pain arises [10], measurement of noxious-evoked brain activity may be able to provide a more specific indication of the infant pain experience than behavioural or physiological indicators. Additionally, measurement of brain activity provides an objective method with which to quantify infant nociceptive processing, as the template of noxious-evoked brain activity is fit to the evoked activity by an algorithm with no requirement for decision-making. However, one limitation in the analysis of this evoked activity is the role of human decision-making in the rejection process – while in some cases it is clear that the EEG trace is due to movement, in other cases it can be difficult to decide. In the future, an algorithm built to recognise and perform rejections would be of great use

to ensure that the criteria used for rejections are consistent and not open to human bias.

Reflex withdrawal is correlated with noxious-evoked brain activity in infants [210], however reflex withdrawal can occur without pain perception and so this method of pain estimation may therefore be less closely aligned with the pain experience. Like the analysis of brain activity, the methods used to assess the magnitude of reflex withdrawal were also objective; the RMS was calculated or an algorithm was used to find the start and end points and the amplitude.

Behavioural and physiological changes also do not occur specifically in relation to pain [9 8 201 203]. Calculation of the autonomic heart rate and oxygen saturation changes for PIPP-R scoring is objective, however in contrast, the measurement of facial expression changes and behavioural state is subjective and can be difficult, especially when infant movement, lack of clear video recordings, and dummies or clinical apparatus such as nasogastric tubes or high flow therapy obscure facial view. Facial scoring can be challenging as infant facial expressions are graded rather than simply 'on' or 'off', and it can be difficult to determine exactly when an infant stopped demonstrating an eye squeeze or frown. Additionally, comfort measures, such as dummy or finger sucking, can greatly influence facial expressions, especially the nasolabial furrow which ceases during sucking, and this can influence PIPP-R scores.

Additionally, facial expressions that may not be related to pain, such as during yawning or sneezing, must also be counted. Another difficulty in PIPP-R scoring is

the determination of the behavioural state (quiet asleep, active asleep, quiet awake, active awake) in the 15 seconds of baseline period, as infants may demonstrate varying indicators of behavioural state even in this relatively short period, and eye closure/opening may not provide a direct reflection of the level of arousal of the infant. As a considerable number of points (0 -3) are awarded for behavioural state, this can influence the resulting PIPP-R score, as seen in Chapter 4 when investigating the effect of paracetamol on small numbers of infants receiving immunisations.

Current research is developing automated pain assessment algorithms that can extract facial features associated with pain [532 533], and this will be of great utility in removing possible human bias and ensuring that consistent criteria are used for facial scoring, as well as being less time intensive for clinical and research use. Additionally, in Chapter 3 the use of facial EMG in detecting subtle facial expressions of positive and negative affect, such as smiling or frowning, was discussed in the context of an effect of CT fibre stimulation [374 375], and it would be useful to see if this technique could be used in infants to investigate subtle facial expressions and intervention effects.

In the research described in this thesis regarding the effect of gentle touch and paracetamol, effects were seen on noxious-evoked brain activity but not on behaviour. As explained previously, the lack of effect on behaviour may be due to the limited duration of application with regards to gentle touch, and due to procedure-associated distress with regards to paracetamol for immunisation. While it is important to reduce infant distress, the processing of early life pain in the brain

can cause long term adverse consequences on neurodevelopment [178] [3], and methods which suggest a reduction in this processing are therefore of great merit.

6.5 Future directions

Following the demonstration that immunisation-evoked brain activity can be fit with the previously characterised template of noxious-evoked brain activity, further acute procedures, such as lumbar puncture or tongue-tie frenectomy, could also be investigated for suitability of fit with the template. This validated and objective method could then be used to assess the analgesic efficacy of further interventions specific to further procedures.

In this thesis, evoked brain activity was analysed in the millisecond period after stimulation at the vertex electrode Cz, so that the previously characterised and validated template of noxious-evoked brain activity could be used to measure evoked activity and assess analgesic efficacy. However, measuring evoked activity at one location does not reflect the full extent of nociceptive processing across the brain, and there are a wide range of parameters that could be altered to look for signatures of nociceptive processing that could reflect a different aspect of the nociceptive processing or pain experience, including looking over longer time windows, looking at different electrodes and the spatial distribution of the response, and looking at changes in the frequency domain. To look at spatial topography, a larger number of electrodes would be needed for better coverage, and this must be balanced with the aim of minimal handling and set-up time during infant studies.

Work in adults has utilised EEG to characterise changes in the frequency domain following both acute and sustained noxious stimuli (including injections, thermal, and laser stimulation), demonstrating changes in frequency bands [60 534 535 536 537], and machine learning approaches indicate that pain perception can be classified based on changes in these EEG power bands [538 539 540], demonstrating the existence of characterisable pain-related changes in the frequency domain that could be used to create a signature of pain-evoked brain activity. In infants, an increase in gamma oscillations, consistent with the adult response, has been observed following heel lancing [230], and analysis of longer time periods in the frequency domain is likely to be a suitable approach for characterising longer clinically required painful procedures, such as ROP eye screening, intubation, endotracheal tube suctioning for ventilated infants, or post-operative pain, which occur over minutes or even hours, and cannot be characterised by looking at evoked activity in the millisecond range. It would be interesting to use changes in the frequency domain to investigate responses to the longer, full process of immunisation including needle entry into muscle and fluid injection, rather than just the first contact of the needle with the skin as was investigated in Chapter 4.

For both of the pain relief interventions investigated in this thesis, gentle touch and paracetamol, it would be of great value to conduct larger-scale investigations that build on this exploratory work, for example randomised controlled trials including the measurement of noxious-evoked brain activity as part of a multimodal approach to pain assessment, to confirm the effect of these interventions. Randomised controlled trials are the gold standard for assessing the efficacy of interventions due to the random allocation of participants, the blinded

administration of interventions and blinded data analysis, and the comprehensive power calculations to estimate required sample sizes, all of which aim to eliminate bias from the investigation process. However, it is not lucrative to conduct randomised controlled trials until pilot data has been collected to suggest the efficacy of a treatment, demonstrating the importance of the exploratory work detailed in this thesis.

It would also be interesting to investigate whether gentle touch is effective in premature infants who undergo more painful procedures while in hospital, to closely monitor the effect of different rates and durations of gentle touch on infant brain activity and behavioural pain responses, and to see if parental or carer touch is as, if not more, effective than brush stimulation. Investigating the type, intensity, and duration of procedures for which this intervention is effective is also important. Regarding paracetamol, its efficacy for a larger range of procedures, both acute and chronic and of different intensities, would also be interesting to investigate, as well as performing dose escalation studies to determine the optimal dose. If these interventions are confirmed to be effective, in the future national and international clinical guidelines could be changed to recommend the use of gentle touch, as well as paracetamol, prior to relevant painful procedures.

It would also be interesting to use fMRI to investigate infant brain activity. Adult studies have demonstrated that paracetamol reduces pain-evoked brain activity as measured by fMRI [475 476] and infant and adult studies have demonstrated that CT-optimal touch activates brain regions involved in social processing [373 369 370 384 383]; it would be interesting to see whether the patterns of regions activated by the two

interventions could provide insight into the methods by which these pharmacological and non-pharmacological interventions alter the pain experience.

Regarding the demonstration in Chapter 5 that accounting for individual variation in nociceptive sensitivity can aid investigations into the efficacy of pain relief interventions, in future research, experimental noxious stimuli could be applied to account for individual sensitivity prior to investigating the effect of alternative interventions. It would also be of interest to investigate whether the magnitude of the response to experimental noxious stimuli could predict the magnitude of the response to a following clinical procedure, or even the magnitude of the reduction that would be induced by a pain relief intervention.

Lastly, while the measures used to assess infant pain here are non-invasive, in instances where infants are receiving clinically required heel lances, analysis of the blood samples could be performed. It could be interesting to investigate whether pharmacological or non-pharmacological pain relief interventions reduce cortisol levels (an indicator of infant stress [225]), or whether gentle touch increases oxytocin or endogenous opioid levels, as these are both proposed mechanisms for its pain-relieving influence [327 364 415 326]. It would also be of interest to investigate whether genotyping and identification of gene variants believed to be pain-related in adults [524] have an influence from birth. The COMT genotype has recently been demonstrated to influence the effect of early life pain on alterations in brain structure at school age [183], and it would be interesting to see if pain-related variants are correlated with nociceptive sensitivity in neonates, and whether these variants explain individual variation in infant responses to noxious stimuli.

The research in this thesis has clear future clinical applications for pain relief interventions in infants. However, first further knowledge regarding factors such as the optimal duration and location of gentle touch, and the optimal dosage of paracetamol, as well as the clinical procedures for which they are both effective in providing pain relief, must be investigated. Once further evidence, including that from performing well-controlled randomised trials, has demonstrated their efficacy in reducing pain-related brain activity and other measures of infant pain, clinical guidelines could be updated to recommend their use prior to the procedures for which they are effective.

The research in the final results chapter of this thesis provides the suggestion that it may be possible to predict infant nociceptive sensitivity, and the efficacy of an intervention, from responses to experimental nociceptive stimuli. Once this work has been replicated, and the relationship between nociceptive sensitivity and the magnitude of effect of an intervention has been confirmed with additional pain relief interventions as well as gentle touch, this work could have important clinical implications in allowing the prediction of infant pain and pain relief on an individual level, and the opportunity for a more informed decision to be made regarding the balance of pain relief versus the adverse effects associated with some analgesics. In the future, a reduced electrode montage could be applied in the clinical setting to record noxious-evoked brain activity (at electrode Cz) more efficiently following experimental noxious stimuli in order to gauge infant nociceptive sensitivity. If future research could determine a reliable behavioural or physiological correlate of this noxious-evoked brain activity, the characterisation

of infant nociceptive sensitivity in the clinical setting could be even further simplified.

6.6 Concluding remarks

In sum, the work described in this thesis is of both medical and research relevance. The non-pharmacological intervention of gentle touch has been demonstrated to reduce noxious-evoked brain activity prior to experimental noxious stimuli and clinically required heel lances in three investigations involving independent groups of infants, and investigation of the pharmacological intervention paracetamol also suggests analgesic efficacy. This research is of clinical relevance and sets the basis for further investigations into these pain relief interventions, which is of utmost importance given the lack of validated effective pain management options in this vulnerable population.

Regarding the advancement of research methods, immunisation-evoked brain activity has been characterised and found to be suitable for fitting with the template of noxious-evoked brain activity, allowing the efficacy of interventions to be assessed specifically for immunisation needle-evoked brain activity. Additionally, accounting for individual nociceptive sensitivity by applying experimental noxious stimuli has been demonstrated to aid the investigation into the efficacy of gentle touch, and in future research this method could be used to account for individual variation when investigating the efficacy of other pain relief interventions; aiding future research practice by reducing the required sample sizes. This research

provides important foundations to inform future investigations into the efficacy of pain relief interventions and the methods used to assess them.

Chapter 7: Appendices

(i) Patient Information Leaflet (PIL)



Your child is 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 infant have to take part?
No, it is your decision whether or not your infant takes part. If you decide to allow your infant 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.

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 infant'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.

Clinical procedures will be completed in the routine way. The study will not interfere with your infant'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.

As we are interested in how your infant's response to pain changes as they grow, we may ask if we can study your infant 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.

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.

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 infant pulls away during the stimulation (and clinical procedure if relevant).

Vital sign monitoring: Small adhesive electrodes may be placed on your infant'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 infant's foot to measure changes in blood oxygen levels.

Videoing your infant: We may also video your infant 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 ask you if you are happy for us to use these images for teaching, publicity and/or scientific journals. If you choose not to allow us to use the images in this way, this will not affect your child's care.

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

Obtaining video footage of your infant is non-invasive and does not present any risk to your infant. EEG, EMG and ECG have been used clinically for over 20 years without any adverse effects. 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.

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 infant?

We will collect clinical information about your infant, 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 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 so that all information is anonymised.

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 XXXX. The findings may also be used for teaching or academic research presentations. No videos/images will be published without specific consent. No identifying information will be presented about you or your child. Anonymised data may be shared with other researchers doing similar work. Data will be stored for a minimum of 3 years after the results of the study are published.

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.

8. Who is funding this research?
This study has been funded by The Wellcome Trust.

9. 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.

10. 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 you are 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 Dr Rebeccah Slater (details below) or the University of Oxford Clinical Trials and Research Governance (CTRG) office (tel: 01865 572224, email: ctrg@admin.ox.ac.uk).

11. Contact for further information

Dr Rebeccah Slater (Study Lead) *Associate Professor of Paediatric Neuroimaging* *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.

(ii) Consent form

Consent Form



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

Infant's name:

Study Title: Investigating pain in the developing human brain

Chief Investigator: Dr 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) **(v8.0, dated 27/07/2018)**, 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

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