Measuring pain in the newborn infant

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Abstract

Hospitalised infants require multiple painful procedures a day as part of their essential medical care. However, identifying and managing pain in non-verbal populations is challenging – the gold standard in adults is self-report of pain, but in infants we must rely on surrogate measures. In this thesis, electroencephalography (EEG), behavioural measures and physiological changes are used to investigate infant pain responses, exploring how responses to noxious stimulation are modulated by analgesics, age and pathology.

It is essential to validate measures of pain in infants. As pain is both an emotional and sensory experience, noxious-evoked brain activity likely provides an important surrogate pain measure. An EEG template of noxious-evoked brain activity was validated for use in an independent group of infants: the noxious-evoked brain activity was only elicited in response to noxious stimulation and not in response to stimulation of other sensory modalities; was correlated with pain-related behaviour; and was sensitive to analgesic modulation by the use of topical local anaesthetic. This provides a novel approach, which can be used to test analgesic efficacy in infants.

Behavioural responses form the cornerstone of clinical infant pain assessment. However, it is not clear whether the youngest, most premature infants are able to mount behavioural responses that can discriminate between noxious and innocuous stimulation. In this thesis, I have investigated the behavioural response to noxious and tactile stimulation in infants from 28-41 weeks corrected gestational age (CGA). The youngest infants demonstrated a lack of behavioural discrimination, being equally likely to mount a behavioural response to a tactile or a noxious stimulus. Responses diverged with increasing age, such that from approximately 32 weeks' gestation, infants were significantly more likely to display facial grimacing to noxious stimulation.

Finally, the impact of pathology on pain experience has not been well studied. I have investigated how early life infection impacts pain-related responses and demonstrate, using a multidimensional approach, that infants with infection display significantly greater noxious-evoked brain activity and are more likely to mount a behavioural response compared with non-infected infants.

In summary, this thesis demonstrates that responses to pain are altered by age and pathology, and provides a novel brain-derived approach to testing the efficacy of analgesic interventions in infants.

Acknowledgements

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Abbreviations

ACC - Anterior cingulate cortex

CGA - Corrected gestational age

CRP - C-reactive protein

CNS - Central nervous system

ECG - Electrocardiography

EEG - Electroencephalography

EMG - Electromyography

fMRI - functional Magnetic Resonance Imaging

GA - Gestational age

HIE - Hypoxic ischaemic encephalopathy

IASP - International Association for the Study of Pain

IV - Intravenous

IVH - Intraventricular haemorrhage

LED - Light emitting diode

LPS - Lipopolysaccharide

MEG - Magnetoencephalography

MRI - Magnetic Resonance Imaging

NICE - National Institute for Health and Care Excellence

NICU - Neonatal Intensive Care Unit

NIRS - Near-Infrared Spectroscopy

PAG - Periaqueductal gray

PIPP - Premature Infant Pain Profile

PIPP-R - Premature Infant Pain Profile - Revised

PMA - Post menstrual age

PNA - Postnatal age

RVM - Rostral ventromedial medulla

RMS - Root mean square

SEM - Standard error of the mean

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Chapter 1

Introduction

1.1. Thesis overview

One of the duties of a doctor is to 'take all possible steps to alleviate pain and distress' (1). The current gold standard for the assessment of pain in patients is self-report but, in the neonatal setting, where the patient is pre-verbal, the measurement, and therefore treatment, of pain remains a challenge. Measurement of pain in this population relies on the use of surrogate markers, such as changes in behaviour and physiology. Interest in the use of brain-derived measures has also been increasing. In this thesis, electroencephalography (EEG), behavioural measures and changes in physiology are used to investigate the responses of neonates to clinically required, noxious procedures and non-tissue damaging experimental noxious stimulation.

Historically, pain in infants has been under recognised and undertreated, with infants routinely undergoing major surgery without the use of analgesics, even into the late 1980s (2). Around this time, the prevailing view that infants were unable to feel pain was seriously challenged and practice began to change, with the provision of analgesia for surgical procedures becoming standard (2). However, the growth of neonatology as a specialty, and the advances in technology which have allowed infants born at the extremes of viability to survive, means that the youngest, sickest infants still experience a high burden of pain as part of their routine care (3). Furthermore, despite significant

technological advances allowing these infants to survive, there have not been concomitant advances in the development of analgesics for use in the neonatal population, and many infants undergo acute, painful procedures without analgesia (3).

The final trimester of pregnancy and first weeks of life represent a time of significant neurodevelopment, during which the infant is particularly vulnerable to neurological insults, and there is mounting evidence to suggest that early life pain can have a long-term detrimental impact on neurodevelopment (4,5). Hence, adequate treatment of pain in this population is not only important to minimise suffering, but to limit long-term negative consequences.

In the absence of verbal report, the assessment of pain in infants currently relies on the use of pain scores (6). Pain scores are often composite measures based on both behavioural and physiological responses. While many pain scores have been validated for clinical use (7–9), they still have a number of limitations. The responses measured are non-specific and are reliant on observation by well-trained observers. Furthermore, many aspects of neonatal care, such as ventilation, sedation and the use of inotropes and muscle relaxants, make the interpretation of both behaviour and physiology difficult. Previous work has also demonstrated that a proportion of infants will show no behavioural response to well described painful stimuli, such as a heel lance, despite evidence of noxious-evoked brain activity (10), suggesting that relying on behavioural measures alone may underestimate some infants' pain. Finally, whether the youngest, sickest, infants are able to mount reliable behavioural and physiological responses is debatable (11). The development of behavioural responses to

noxious input across gestation has not been well described. This is particularly pertinent in the setting of neonatal care, where, as stated previously, it is the youngest infants who often experience the highest number of painful procedures (12) and in whom the potential detrimental impact of under recognised and under-treated pain is greatest (13). One aim of this thesis is to explore the development of behavioural discrimination between tactile and noxious stimulation in infants from 28 – 41 weeks' gestation.

The challenge of assessing pain in the infant population is a barrier not only to treatment of pain clinically, but also to the development and evaluation of analgesics, both pharmacological and non-pharmacological, in the research setting (14). Non-pharmacological measures, such as swaddling, sucrose and kangaroo care, are commonly used in many neonatal units, for the treatment of acute pain, particularly that associated with tissue breaking procedures such as venepuncture. However, evidence for the use of pharmacological analgesics, such as morphine, topical local anaesthetic and paracetamol, which are well established analgesics in adult patients, is often contradictory in the infant population (15–17). The inability to accurately assess pain in the absence of self-report, and the lack of pain-specific surrogate markers, has led to interest in the development of brain-derived measures of pain (14).

Pain is a complex sensory and emotional experience, which is the result of complex peripheral and central processes. In adults, pain has been shown to result in the activation of a complex network of cortical and subcortical brain regions, which includes the somatosensory cortices, insular cortices, thalamus and brainstem (18). These same regions are active in the infant brain in response

to noxious stimulation (19), and brain-derived measures aim to quantify this activity. Brain-derived measures of infant and adult nociception have been developed using near-infrared spectroscopy (NIRS), EEG and functional magnetic resonance imaging (fMRI) (10,19–21). In adults, measures obtained using fMRI have been demonstrated to be able to distinguish painful from non-painful stimulation in the absence of self-report (22). They have also been used to test the efficacy of analgesics (23). In infants, noxious-evoked brain activity recorded using EEG has been used to demonstrate that sucrose does not modulate the magnitude of this activity, suggesting a potential lack of central analgesic efficacy (24). Hence, the use of objective and quantifiable measures of noxious-evoked brain activity are a promising development for analgesic drug-discovery and evaluation in both adults and infants. In Chapter 4, a novel EEG template of nociceptive brain activity is used to quantify the magnitude of noxious-evoked brain activity and to investigate the effect of a commonly used analgesic, topical local anaesthetic.

Brain-derived measures of nociception also have an important role in furthering our understanding of how pathology may disturb nociceptive processing. Work in adults has suggested that infection, and the inflammatory response to it, have the potential to disturb these processes and lead to increased pain sensitivity (25,26). Furthermore, animal work has demonstrated that early life exposure to infection not only increases pain sensitivity in the short term (27), but in the long term can lead to perturbations in nociceptive processing (28,29). Infection is common in the neonatal period, with around 1 in 10 term born infants being identified as at risk of infection (30). The impact of infection on nociceptive

processing in human infants has not been studied, but the potential implications for long-term pathology make it an important area for research. The final results chapter of this thesis focuses the impact of neonatal infection on pain-related brain activity, reflex activity and behaviour.

In this thesis, I explore infant responses to pain. Specifically, I aim to:

1) Characterise the emergence of behavioural discrimination to tactile and noxious stimulation across gestation
2) Validate a template of noxious-evoked brain activity and investigate the analgesic efficacy of local anaesthetic
3) Determine whether early life infection alters an infant's response to pain

Firstly, however, a more detailed review of the literature is given in the Introduction.

1.2. Neonatology

Neonatology focuses on the care of the newborn infant, especially those who are ill or premature. It is a relatively new specialty, only emerging in the middle of the 20th century (31) with Virginia Apgar presenting her eponymous score for the assessment of the new born infant in 1953 (32). However, it was not until the late 1960s that technological advances provided the capacity to monitor and support the sickest neonates (31). Over the last 50 years further advances have transformed the field; in the 1960s, the mortality rate for infants born weighing less than 1000g was over 90% (33), by 1999, the survival rate for infants

weighing 751-1000g was over 85% and over 95% of infants weighing 1000-1500g survived (34).

The number of infants admitted for neonatal care has been increasing (35). In 2015, over 95,000 infants, or 1 in 8 infants born, were admitted to a neonatal unit in England, Scotland and Wales (36). Around 60% of these infants were born at full term (37 weeks onwards) and the rest were born prematurely. Around 60,000 infants, or 1 in 13 infants born, were born prematurely, that is before 37 weeks' gestation, in England and Wales in 2016 (37). Infants born prematurely are further classified to those born extremely preterm (less than 28 weeks), very preterm (28 to 32 weeks) or moderate to late preterm (32 to 37 weeks) (38). Previously, infants born at 28 weeks' gestation were considered 'previable', that is too young to survive. Infants born at 24 weeks now have roughly a 50% chance of survival (39). However, survivors continue to have a high burden of morbidity, with only around 50% of surviving infants born at 22-26 weeks' gestation doing so without severe or moderate neuromotor or sensory disabilities (40). As the number of infants being born prematurely is increasing (41), there is an increasing need to understand and address the causes of neurodevelopmental impairment in prematurity. Furthermore, as part of their essential medical care, infants who are born prematurely necessarily receive multiple pain procedures a day (3). It is essential that we therefore better understand pain in infants. However, while the field of neonatology is relatively new, the field of infant pain research is arguably even more in its infancy.

1.3. History of infant pain

It is now widely accepted that infants are capable of experiencing pain and that managing this pain is an important aspect of their care (6). That this was not the dominant view until the late 1980s is shocking. In their review article, Rodkey and Ridell suggest a number of reasons underlying this 'infant pain denial' (42). They propose that the view of infants as 'primitive beings' with undeveloped brains led to a pervading skepticism regarding infant pain such that any responses to noxious stimulation were disregarded as 'mere reflexes' (42). This skepticism about infant pain coupled with fear of intraoperative bradycardia and hypotension caused by inhaled anaesthetic agents, led to many, particularly premature, infants undergoing surgery with the administration of muscle relaxants alone (43). It is particularly pertinent that while this practice was commonplace among medical professionals, the public and many parents, were unaware (44). Jeffrey Lawson was a premature infant born in 1985 and admitted to the Washington National Children's Hospital. During his admission, he underwent open heart surgery for closure of a patent ductus arteriosus, a not uncommon complication of premature birth. Unbeknownst to his mother, he was 'anaesthetised' with a paralytic agent alone. Following surgery, he developed shock and multi-organ failure and sadly died (45). When his mother questioned the anaesthetist about their practice, the anaesthetist informed her that 'Jeffrey was too sick to tolerate powerful anesthetics' and that 'it had never been demonstrated to her that premature babies feel pain' (44). However, work by Anand and others began to challenge this accepted medical practice. Anand and colleagues demonstrated that infants, both preterm and term, undergoing

surgery mounted a stress response characterised by hyperglycaemia and hyperlactataemia associated with a surge in catecholamines release (46). They demonstrated that these hormonal and metabolic responses to surgery were associated with a high mortality rate (47). In a subsequent randomised controlled trial, Anand and colleagues showed that the addition of fentanyl analgesia to the standard nitrous oxide and muscle relaxant 'anaesthesia' reduced not only this stress response but also the number of postoperative circulatory complications (48). A further randomised controlled trial demonstrated that the use of halothane to anaesthetise infants in addition to nitrous oxide, also diminished this stress response and improved clinical stability (49). These studies, along with the work of Jill Lawson to raise public awareness, led to a step change in practice with the American Academy of Paediatrics advocating the routine administration of 'anaesthesia or analgesia to neonates undergoing surgical procedures' in 1987 (50).

This change in accepted opinion gave rise to a new area of research investigating infant pain, and brought the challenge of measuring and treating pain in non-surgical settings, such as during procedures on the neonatal unit. A major focus of research was in the development of surrogate measures of pain in non-verbal populations, which necessitates a clearer understanding of what is meant by 'pain'.

1.4. Pain versus nociception

The International Association for the Study of Pain (IASP) defines pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (51). Importantly, in the context of neonatal care, it specifies 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.' The difficulty in assessing pain when the patient is unable to communicate verbally however is that pain is a subjective experience and no surrogate marker can adequately replace the gold standard of verbal report.

The IASP definition of pain also highlights the role of early life experience in informing an individual's perception of what is painful. 'Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life.' The emphasis on injury in early life is particularly pertinent to the study of pain in infants admitted for neonatal intensive care, where routine monitoring often requires the use of repeated acute painful procedures. Carbajal and colleagues found that infants were experiencing an average of 12 painful procedures per day for the first 14 days of admission (3). If pain is a purely subjective experience, then it could be argued that it is never possible to measure pain in patients who are unable to communicate. Instead surrogate markers are used to attempt to quantify pain experience, which include measures of brain activity, reflex withdrawal, physiological responses and behaviours.

Nociception is 'the neural process of encoding noxious stimuli'. The definition of nociception comes with the caveat that the output of these processes may range from autonomic responses, such as elevations in blood pressure, to more complex behavioural responses without implying 'pain sensation'. The distinction between pain and nociception is evident in the context of adult pain where pain may be reported in the absence of noxious input (51). In infants, the short and long-term detrimental consequences of exposure to painful procedures arguably mean that whether it is pain or nociception that is measured is irrelevant and that the obligation to limit both is paramount.

1.5. Nociceptive pathways

A nociceptor is a 'sensory receptor that is capable of transducing and encoding noxious stimuli' (51). These are primary sensory neurons that have a peripheral terminal innervating peripheral tissues, a cell body in the dorsal root ganglion (or trigeminal ganglion for those nerves innervating the face) and a central terminal, which synapses on to second order neurons in the CNS (52). Nociceptors are mostly unmyelinated C-fibres but some are thinly myelinated Aδ fibres, Aδ nociceptors respond to mechanical stimuli but some also respond to intense heat and C-fibre nociceptors respond to noxious thermal, mechanical and chemical stimuli (53). Aδ fibres are thought to be responsible for the rapid induction of 'acute sharp pain' which triggers reflex withdrawal (54) and C-fibres are thought to mediate the more delayed, longer lasting 'dull pain' evoked by noxious stimuli (55). Other, larger, myelinated primary sensory afferents, called

A α and A β fibres, conduct information about non-noxious stimuli such as tactile stimulation and vibration. Nociceptive afferents synapse onto second order neurons in the dorsal horn of the spinal cord (52) . These second order neurons include projection neurons, spinal interneurons and neurons involved in descending modulation (56). Projection neurons decussate in the spinal cord before ascending in the spinothalamic tract to transmit nociceptive input to the brain. The other primary sensory afferents, A α and A β fibres, do not project onto second order neurons at the level of the spinal cord, rather, these travel ipsilateral to the site of innervation in the dorsal column-medial lemniscus pathway to the medulla where they synapse onto second order neurons which then decussate. These pathways are shown in Figure 1.1.

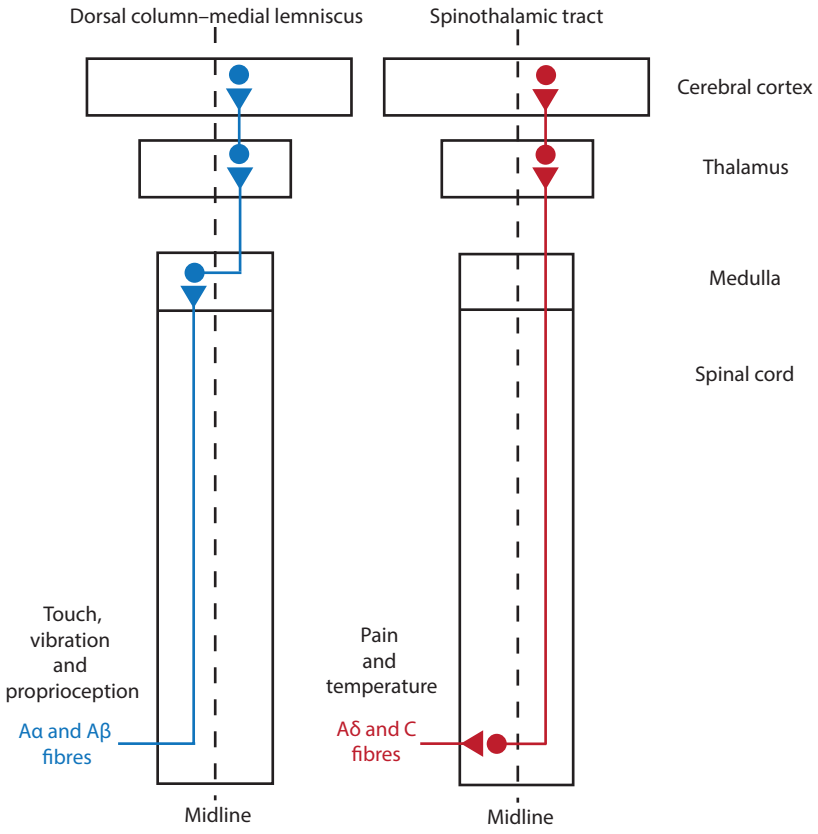

Figure 1.1: Schematic of the pathways involved in the transmission of touch and pain from the periphery to the cortex.

Interneurons may be excitatory or inhibitory and are thought to be involved in modulating the nociceptive input (57). Neurons involved in descending modulation originate largely in the periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) and terminate in the dorsal horn where they may also exert excitatory or inhibitory effects (58). The dorsal horn also receives input from primary sensory afferents and thus plays an important role in integrating sensory information (59). It is the first point of nociceptive processing in the CNS and disruption to the balance of inhibition and excitation in the dorsal horn is thought to be involved in the development of pathological pain states (60).

Nociceptive information is transmitted to the brain via several pathways and the organisation of these pathways is highly complex (61). Ascending projection neurons travelling in the spinothalamic tract convey information to sensory nuclei in the thalamus and thence the cortex, but also project to other areas such as the brainstem, which includes the PAG and the parabrachial nucleus (62). Distinct pathways have been identified that are thought to carry distinct information, with projections to the thalamus and thence to the somatosensory cortex thought to mediate sensory aspects of nociception, and projections to the brainstem and thence brain regions such as the amygdala thought to mediate emotional or affective components of 'pain', although this remains an area of debate (62).

Descending modulation of nociceptive input in the spinal cord is important in nociceptive processing. This modulation can be either inhibitory or facilitatory, but both have likely important roles in protecting the individual. Descending inhibition can prevent an individual being distracted by an injury to allow them to escape from a hazardous situation whereas descending facilitation can draw attention to damaged tissues and promote healing by encouraging the individual to protect that region (63). The balance of inhibition to facilitation is tightly controlled and it is thought that disturbances to the descending pain modulatory systems are altered in adult chronic pain conditions (64). A number of brain regions are implicated in descending modulation but key pathways involve the PAG and the RVM (65). The PAG receives inputs from several cortical areas and brain structures, including the anterior cingulate cortex (ACC)(66), the insular cortex (66), the amygdala (67) and the hypothalamus (68). The main output of the PAG is to the RVM, which then projects to the spinal cord as the main relay for descending modulation (69,70) . Work in animals has demonstrated that in the neonatal period, these pathways are predominantly excitatory, and it is not until later in development that descending inhibition is seen (71). Work in human infants has demonstrated a high level of excitability within the developing spinal cord that is thought to reflect this lack of descending inhibition (72). This excitability is evidenced by low reflex withdrawal thresholds and increased magnitude reflex withdrawal to tactile and noxious stimuli in the youngest infants (72,73) and suggests that infants are more sensitive to external stimuli. Reflex withdrawal decreases in amplitude, duration and latency across the preterm period, and this coincides with the emergence and maturation of noxious-evoked brain activity, suggesting that top-down inhibition may emerge

during the preterm period (73). It is thought that injury in the neonatal period could disrupt the balance of the emerging descending pain modulatory system and may lead to vulnerability towards developing chronic pain states (74,75).

A large number of brain regions have been identified as being involved in nociceptive processing. A meta-analysis performed using adult data obtained from a number of imaging modalities, has demonstrated that the most common of these regions include the primary and secondary somatosensory cortices, the insular cortex, the ACC and prefrontal cortex and the thalamus (18). That so many regions of the brain are involved in the processing of pain is not surprising, since it is a complex, 'sensory and emotional' experience (51). This division between the sensory and affective or emotional aspects of pain is thought to have an underlying anatomical basis from the pathways in the spinal cord to the regions of the brain involved. Hence, the thalamus and somatosensory cortices have been linked with the sensory aspects of nociceptive processing (76–80) while the amygdala, ACC and prefrontal cortex have all been shown to be associated with the affective aspects of pain processing (81–84). Recent work in infants has demonstrated that a similar network of brain regions identified as active in adults are also activated in the infant brain in response to noxious stimulation (19).

1.6. Brain development

Human brain development begins early in gestation and continues into adolescence. Neural progenitor cells begin to emerge during the third week after conception, the fifth gestational week, and form the neural plate, which then fuse by the end of the sixth gestational week to form the neural tube, the precursor to the central nervous system (85,86). Neuron production begins as early as the 7th gestational week (87). Neural cells are produced in the ventricular zone and migrate out to the developing neocortex. The first neurons migrate to a layer known as the preplate, which then splits as new cells migrate, to form the marginal zone and the subplate (88). The marginal zone becomes cortical layer I, and the subplate disappears late in gestation, hence both are transient layers but both play an important role in cortex development. These later migrating neurons form a layer between the outer marginal zone and the inner subplate called the cortical plate, which gives rise to cortical layers II-VI, with the earlier migrating neurons forming the deeper layers of the cortex, and later migrating ones forming the more superficial layers (89).

From the 24th gestational week, synapses begin to form in the cortical plate with the subplate playing an important role in this early synaptogenesis (90). Prior to the formation of thalamocortical connections, afferent axons from the thalamus activate neurons in the subplate (91). The subplate also acts as a 'waiting zone' for thalamic afferents prior to their growth to the cortical plate (92). At this stage, the subplate is approximately four to five times thicker than the cortical plate (92). The crucial role of the subplate in the formation of thalamocortical

connections has been demonstrated by animal studies. Deletion of visual subplate neurons during this 'waiting period' leads to aberrant pathway formation, with axons failing to stop and form cortical connections in the target area in the visual cortex (93,94). The same effect is seen when auditory subplate neurons are ablated (93). The dissolution of the subplate zone begins around the 30th gestational week, coinciding with the formation of direct thalamocortical connections, and progresses at different rates in different regions of the cortex (92). By term, the majority of subplate neurons have apoptosed (92).

The gross morphology of the brain changes rapidly across gestation, with the early preterm brain showing little in the way of cortical folding, and the full-term infant brain closely resembling the gyral pattern of the adult (95). This maturation of folding is demonstrated in Figure 1.2. The advent of fetal MRI has provided the opportunity to be able to describe normal and abnormal fetal brain development in utero (96).

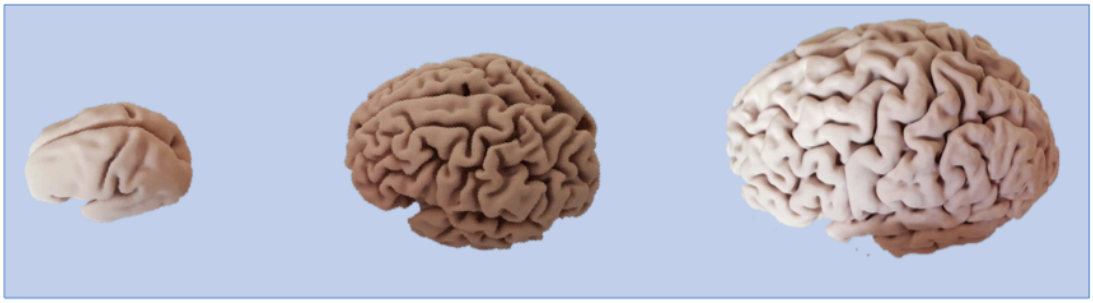

Figure 1.2: Cortical folding.
Photographs of 3D printed models of brains taken from MR images of infants scanned at 29 weeks' (left) and 37 weeks' (middle) gestation and from an adult (right).

The preterm period represents a time of rapid neurodevelopment, and hence represents a time of great vulnerability of the developing brain. A major concern, particularly for infants born extremely prematurely, is that this period of rapid

neurodevelopment is occurring outside of the protective environment of the uterus. It is also these infants, who as part of their routine care, are exposed to a high number of noxious inputs, the long-term detrimental consequences of which are described in the following section.

1.7. Long-term consequences of early life pain and epidemiology of painful procedures

Pain can have detrimental consequences in the short and long term. Infants undergoing painful procedures, such as retinopathy of prematurity screening, have been shown to display physiological instability in the hours after, with increased episodes of oxygen desaturation and apnoea (97,98). In the longer term, greater cumulative exposure to pain and stress in early life has been associated with reduced postnatal body and head growth (99), detrimental changes to white matter microstructure (5), a reduction in cortical thickness (4), increased pain sensitivity (100), poorer cognition and motor function in toddlerhood (101) and altered cognitive and motor development in school-age children (102).

Despite changing perceptions of infant pain, the burden of infant pain in the neonatal setting is still large. Infants, as part of their routine care, undergo a substantial number of painful procedures and the provision of analgesia remains variable (3,103,104). Carbajal et al. found that infants in the first 14 days of admission underwent an average of 12 painful procedures per day and that only

20.8% of these procedures were performed with analgesia (3). One infant underwent 364 painful procedures over the 2 week period (3). While the vast majority of these painful procedures may be relatively minor, such as heel pricks (12), a survey carried out in neonatal units in the UK found that only 45% of units routinely administered analgesia prior to chest drain insertion (104). In adults, chest drain insertion is considered to be a painful procedure, with 46% of patients reporting their pain as 9/10 or 10/10 in one study (105). Furthermore, it is the youngest infants who undergo the most procedures and who are at the highest risk of neurodevelopmental impairment (12). It is also the youngest infants who are least able to mount a response to painful procedures and who are therefore, at the greatest risk of having their pain underestimated (106).

1.8. Infant pain assessment tools

Assessing pain in non-verbal individuals is challenging. Since self-report is the gold standard in pain assessment in verbal patients, pain assessment in non-verbal individuals relies on the use of surrogate measures such as behavioural and physiological responses. A schematic of some of the different surrogate measures used in this thesis is included in Figure 1.3.

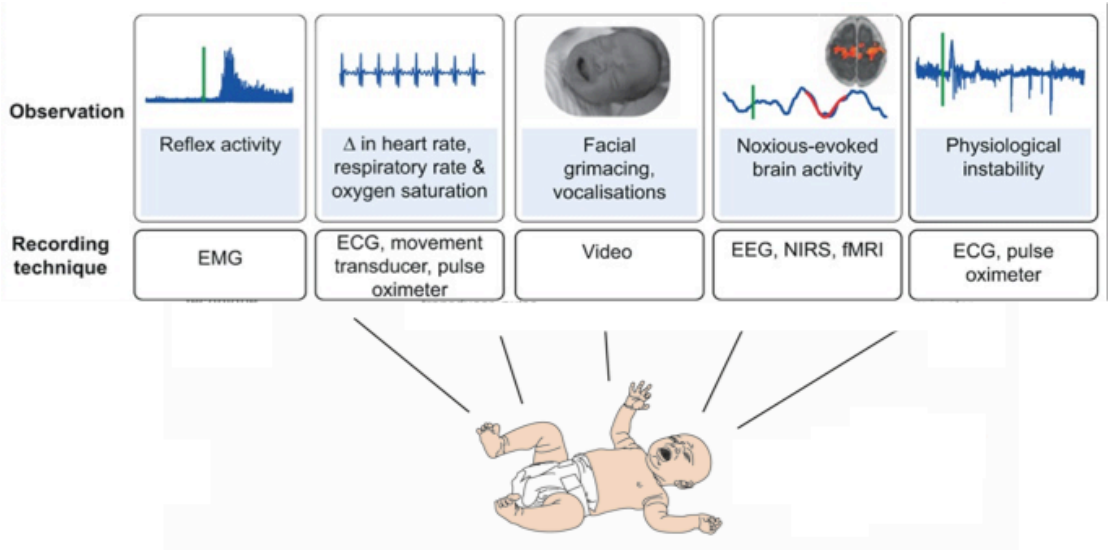

Figure 1.3: Schematic of surrogate measures of pain in infants.

These measures are often incorporated into multidimensional pain assessment tools (107). Over 30 such pain assessment tools exist for use in infancy (107). The most commonly used tools in the neonatal setting are the Neonatal Facial Coding System (NFCS) (108), the Behavioural Indicators of Infant Pain (BIIP) Scale (109), the Neonatal Infant Pain Scale (NIPS) (110) and the Premature Infant Pain Profile (PIPP) (111) which has also been recently revised (PIPP-R) (112).

The NFCS was the first infant pain assessment tool described. It incorporates 10 facial actions, which were adapted from the facial action coding system used in adults, and includes brow bulge, eye squeeze, nasolabial furrow, open lips, horizontal mouth stretch, vertical mouth stretch, pursed lips, taut tongue, chin quiver and tongue protrusion. Grunau and Craig demonstrated that the response in term infants to a heel lance, as measured using these facial actions, differed significantly from the response to the tactile input of a heel rub (108). They also demonstrated that sleep state influenced the infants' facial reactions, with

infants who were awake showing more facial movement than those who were asleep, although the different facial expressions were differentially affected. The NFCS has been used to study pain behaviour away from the bedside as a research tool (113,114) but it has also been demonstrated to be a useful tool for bedside application in preterm infants (7).

The best validated pain assessment tool for neonates is the PIPP, which incorporates both behavioural and physiological responses, as well as contextual factors (the gestational age of the infant and the baseline behavioural state) (111). This score will be used throughout the following Chapters, and so a detailed explanation is given. The PIPP uses three facial actions from the NFCS; brow bulge, eye squeeze and nasolabial furrow as these were found to be the most sensitive and specific facial indicators of pain (111). These facial actions are illustrated in Figure 1.4.

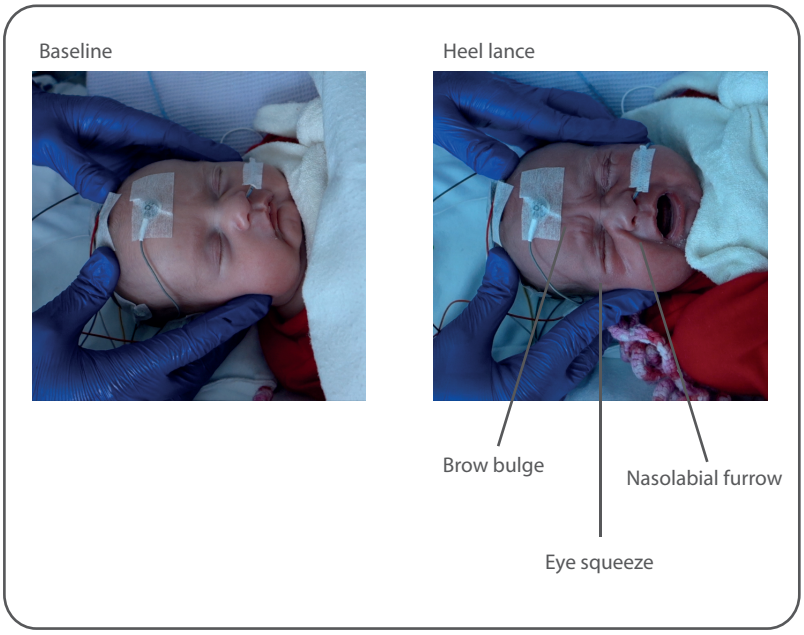

Figure 1.4: Facial actions included in PIPP score. Photographs printed with permission from parents.

Two physiological indicators are used; increase in heart rate and decrease in oxygen saturations. These can be easily and reliably measured in neonates and have been shown to be physiological indicators of pain in both term and preterm infants (115–118). The contextual factors include a weighting for increasing prematurity, as the youngest infants have been repeatedly shown to have diminished behavioural responses to painful stimuli (106,116,119), and a weighting for behavioural state, as it has previously been described that infants in deep sleep show diminished responses (108). This approach of adding weighting factors to clinical pain scores when it is plausible that behavioural pain responses may be muted, implies that there is an implicit understanding that a dissociation between underlying nociceptive activity and behavioural representations of pain may exist.

The infant's baseline behavioural state, heart rate and oxygen saturations are assessed in the 15 second period prior to the stimulus. The maximum heart rate and minimum oxygen saturation recorded in the 30 seconds following the stimulus is then used to calculate the score. The facial actions are scored based on the duration of their presence in the 30 seconds following the stimulus also. A score out of 21 is calculated, with the authors suggesting that a score of 6 or less indicates minimal or no pain, and a score greater than 12 indicates moderate to severe pain (111).

The PIPP has recently been revised (PIPP-R) to make it more feasible for use in the clinical setting and so the infant only receives a score for the contextual variables, age and behavioural state, if they demonstrate a response to the

stimulus (112). While the weightings were introduced to ensure that the infants least likely to respond to a painful stimulus did not have their pain overlooked, it was possible for an extremely premature infant in quiet sleep to obtain a score of 6/21 with no other response, behavioural or physiological, to the stimulus, thus artificially inflating scores for these infants (112) and making interpretation of results in the youngest infants potentially misleading.

In the absence of self-report, pain scores serve a useful role in clinical 'pain' assessment and in the research setting by providing a framework for assessment. One major limitation of pain scores is that they are not specific to pain and differentiate pain from distress poorly (113). Hence, procedures such as a nappy change, which is thought to be distressing but not painful, may, if scored, imply that an infant is in pain (120). In the context of assessing analgesic efficacy for procedures which are thought to be both painful and distressing, such as lumbar puncture, where the positioning and restraint of an infant causes distress before any specific noxious input is received, this makes assessment difficult because while the specific nociceptive input may be altered, the distress associated with the procedure is unlikely to be modified.

A further limitation of pain scores is that they have been demonstrated to be less robust in the youngest, sickest infants (106,121). Furthermore, in the setting of neonatal care, assessment of pain may be further complicated by concurrent use of sedating or paralysing drugs that mask surrogate behavioural markers and cardiovascularly active agents that mask physiological responses.

One major difficulty in determining the validity of infant pain scores is the inherent lack of a gold standard to compare them against, which as discussed in verbal patients is self-report. This has led to interest in developing brain-derived measures to try to provide an objective, quantifiable assessment of the cortical processing underlying pain perception (122).

1.9. Brain-derived measures

Interest in using brain-derived approaches to study pain in humans began in earnest in the early 1990s with the advent of positron emission tomography (PET) and fMRI (18). These early studies demonstrated activation of multiple cortical and sub-cortical regions in adults in response to painful stimulation (123,124). Brain-derived approaches in neonates have tended to focus on technologies that allow for bedside application such as near-infrared spectroscopy (NIRS) and EEG as many infants are too unstable to leave the neonatal unit environment.

1.9.1. Near-infrared spectroscopy (NIRS)

NIRS measures regional changes in cerebral oxygenation. The oxygenated and deoxygenated haemoglobin concentration in human tissue can be measured by determining the level of absorption of near-infrared light. NIRS is easy to use in the neonatal unit setting as the sensors, or optodes, can be placed on the infant's scalp while the infant remains in their cot. NIRS has been used extensively in neonates to measure the cortical response to multi-modal stimulation including

noxious stimulation (10,21,125–130). Slater and colleagues used NIRS to investigate the cortical response to noxious stimulation in infants from 25 to 37 weeks gestational age (21). They demonstrated an increase in total haemoglobin concentration in the somatosensory cortex contralateral to the side of the heel being lanced which could be seen even in the youngest infants (21). They also showed that cortical responses correlated with behaviour, although cortical responses were still recorded in some infants in the absence of a behavioural response (10). At the same time, Bartocci and colleagues demonstrated bilateral increases in cortical oxygenated haemoglobin concentration following venipuncture of the hand in infants aged 28-36 weeks (130). These papers taken together demonstrated that even very premature infants are able to process noxious stimuli at the cortical level. More recently, Bembich and colleagues used multichannel NIRS to investigate the spatial distribution of these cortical responses and observed an increase in oxyhaemoglobin concentration in the most superior portion of the somatosensory and motor cortex (131). A later paper also confirmed that the nociceptive event-related activation increased with increasing PMA (132), which has been demonstrated for noxious-evoked brain activity recorded using EEG (73). NIRS has also been used to assess the effect of various non-pharmacological pain management options on cortical pain responses. Infants who were receiving skin-to-skin contact during venipuncture had significantly smaller increases in oxygenated haemoglobin than infants who were lying in their cot or incubator (133). Bembich and colleagues compared the cortical activation seen in response to a heel lance in two groups of infants, those who were being held by their mothers or those who were receiving oral glucose (134). They demonstrated cortical activation in association with the heel lance

during maternal holding but not in those infants receiving glucose, but no difference in clinical pain scores between the groups. They speculated that the lack of activation in the infants receiving glucose reflected evidence of its interference with cortical processing of pain, while the analgesic effect of maternal holding was mediated by multisensory stimulation. Although further work is needed, these studies demonstrate the utility of using brain-derived measures to assess analgesic efficacy.

1.9.2. Electroencephalography (EEG)

EEG is widely used in the neonatal setting to record cortical electrical activity. It measures changes in electrical field potentials as a function of time with deflections representing changes in voltage and polarity generated by populations of cerebral neurons (135). Electrodes are placed over the surface of the scalp, providing a limited degree of spatial resolution, however EEG shows excellent temporal resolution with changes in the order of milliseconds detectable.

EEG can be recorded during periods of rest, known as background EEG, or in response to specific sensory stimulation, activity referred to as event-related or evoked potentials. Background EEG activity changes rapidly across development and the features associated with normal development and pathological states are well described (136). The normal pattern of EEG in extremely premature infants is discontinuous, that is, demonstrating long periods of background inactivity interspersed with short bursts of activity (137). These periods of inactivity, are

known as inter-burst intervals and in extremely premature infants can last for more than a minute (138). The inter-burst intervals decrease in length as the infant matures, such that by 35 weeks, the EEG shows continuous activity (136,139). Prolongation of this inactivity is associated with poor outcome (140).

The bursts of activity seen in the preterm EEG can be characterised. One common pattern of activity, often observed in premature infants, is as a delta-brush which is a delta wave with fast rhythms superimposed (137). Delta-brushes can be seen spontaneously, in relation to limb movement (141) or in response to stimulation, including noxious stimulation (142,143). It is thought that delta brushes are analogous to spindle bursts seen in the developing rat brain which are also seen in response to spontaneous movement, and are believed to play an important role in the formation and strengthening of new cortical connections (144). In the same way, delta-brushes are believed to play a role in cortical connectivity in the developing human brain (141). Another characteristic pattern of burst activity is a sawtooth, which are oscillations in the theta range (4-8Hz) and are seen physiologically in the occipital (138) and temporal (145) regions in EEG recordings of very premature infants. Other patterns of activity, such as sharp waves, are commonly seen in pathological states, including in preterm infants with intraventricular haemorrhage (146) and periventricular leukomalacia (147).

The EEG of normal, healthy term infants is continuous with sleep wake cycling evident (148). The EEG is reactive to internal and external stimuli (136). EEG is most commonly used in term infants in the setting of hypoxic ischaemic

encephalopathy (HIE). A normal EEG in these infants is highly associated with a favourable outcome at MRI, but a persistently abnormal EEG during therapeutic hypothermia was associated with moderate to severe injury (149). The prognostic value of EEG in preterm and term infants with HIE has led to increasing interest in the development of automated methods to detect and classify abnormalities in background EEG (150).

EEG has been widely used in infants to record event-related potentials, that is, changes in EEG activity associated with specific sensory stimulation (151–154). Visual evoked potentials have been well characterised in infants and abnormal responses have been described in association with pathology, including in term infants with HIE and in infants exposed to methadone in-utero (155–158). Auditory evoked potentials have also been well characterised and are used clinically as part of the newborn hearing screening programme in the UK (159,160). The immature, non-specific neuronal bursts seen in response to stimulation in the preterm EEG, are gradually replaced by specific evoked-potentials as the infant matures to term age (142). The emergence and maturation of these event-related potentials has been used to predict those infants who are at risk of poor neurodevelopmental outcomes (161).

In adults, event-related potentials evoked by laser stimulation, laser-evoked potentials, have been used to measure noxious-evoked brain activity. Laser stimulation selectively activates thermosensitive Aδ and C nociceptors and it can be used to apply stimulation of very brief duration which can be time-locked to the EEG (162,163). The use of laser-evoked potentials would be inappropriate in

the neonatal population, however, a noxious-evoked potential in response to a clinically required heel lance, time-locked to the EEG, has been well described (20,73,142,164). This vertex potential, recorded maximally at the Cz electrode, occurs around 500ms after noxious stimulation of the foot but is not seen in response to non-noxious stimulation (20). It was first described in infants from 35-39 weeks gestational age but starts to appear from around 32 weeks' gestation (73,142). Prior to this gestation, both noxious and non-noxious stimulation evoke non-specific neuronal bursts, delta brushes (142).

Noxious-evoked brain activity has also been demonstrated in response to experimental noxious stimulation and has been shown to correlate well with reflex withdrawal, a measure that has been used in the study of infant pain (165). Furthermore, the magnitude of noxious-evoked activity has been shown to be graded with the intensity of the stimulus (164) and, as mentioned previously, to increase in magnitude across the preterm period (73).

The specific pattern of noxious-evoked brain activity has been used to assess the analgesic efficacy of sucrose, which is widely used to treat procedural pain in neonates. In work by Slater et al., it was demonstrated that the magnitude of noxious-evoked brain activity in infants treated with sucrose was not significantly different than in those infants who received a sterile water placebo despite a significant decrease in the PIPP score (24). This was the first time that an objective, brain-derived measure, was used to assess analgesic efficacy in infants in a randomised control trial and provided an exciting avenue of research.

1.10. Pain relieving measures

Neonates admitted to a neonatal intensive care unit undergo a large number of painful procedures during the course of their admission (3). There are a number of pharmacological and non-pharmacological options available for pain relief (166), however, the evidence for the use of many of these is conflicting (167). The most commonly used pharmacological analgesics are opiates, paracetamol and topical local anaesthetic agents.

Morphine has been used extensively for pain relief and sedation in neonates but the evidence for its analgesic efficacy is limited and there remain significant concerns over the potential for significant adverse events (17,168,169). Despite its long history of use in prolonged pain management, there is no evidence for its use in procedural pain. This is currently under investigation as part of a randomised controlled trial (170).

A recent Cochrane review of the use of paracetamol for prevention or treatment of pain in newborns concluded that there was a paucity of good quality evidence demonstrating analgesic efficacy in procedural pain (15). However, there is increasing evidence to suggest that paracetamol works well for post-operative pain, either as a morphine sparing agent or alone (171). The evidence for the use of topical local anaesthetics in infants is also limited. Some studies have demonstrated an analgesic effect in venipuncture (172,173) and lumbar puncture (174), while others have demonstrated a lack of efficacy for intramuscular injection (175,176), heel-lance (114) and venipuncture (177).

Non-pharmacological measures have gained much attention, partly because of a lack of availability of pharmacological options, and partly because of a perceived lack of significant side effects. The use of sucrose is widespread for procedural pain in many neonatal units, and the Cochrane review published in 2013, concluded that it was safe and effective in reducing procedural pain in infants (178). However, as the work by Slater et al demonstrated, sucrose decreases behavioural responses in the absence of any alteration to the nociceptive input received by the spinal cord or cortex (24). Furthermore, a recently published study in mice demonstrated that repetitive exposure to sucrose in the neonatal period led to decreases in white and grey matter in adulthood (179). No study has looked at the long-term effects of early repeated exposure to sucrose on brain development in humans, but due to the ubiquitous use of sucrose in term and preterm infants, any suggestion of a detrimental impact on neurodevelopment needs addressing.

Kangaroo care, or skin-to-skin, is another non-pharmacological measure, which is being increasingly used to modulate the response to noxious procedures in young infants. It has been shown to decrease behavioural and physiological responses to procedural pain in term infants (180), preterm infants (181) and very preterm infants (182). It also provides the opportunity for parental involvement in neonatal care.

The major limitation of all currently conducted trials of analgesics, both pharmacological and non-pharmacological, is that they primarily rely on the use

of pain scores. Pain scores are limited in their ability to distinguish pain-related distress from non-pain related distress (113), and so, are not the ideal tool to assess the analgesic efficacy of agents which may provide analgesia while not preventing distress, as was discussed earlier. For example, it is feasible that topical local anaesthetics modulate the nociceptive input associated with skin breaking procedures, however, the infant is still distressed by other aspects of the procedure, such as being in an unfamiliar clinical environment and having a limb restrained. Conversely, some non-pharmacological interventions have been shown to have a convincing 'analgesic effect' because they dampen down the behavioural responses recorded (183), however, as in the case of sucrose, it could be that the nociceptive input to the cortex remains unchanged (24). The long-term detrimental consequences of exposure to early life pain have been well described (4,101,102), and if these long-term consequences are related to the magnitude of the nociceptive input reaching the CNS rather than to the infant's behavioural response, then it is important to be able to assess this directly. Clearly, it is important to limit both pain and distress, and so, a multi-dimensional approach to pain relief, which addresses both, is optimal.

The assessment and management of pain in infants poses an important challenge for clinicians and researchers alike. The recent advent of brain-derived approaches provides an opportunity to expand our understanding of this area. In this thesis I am going to investigate infant pain responses using a multidimensional approach.

Chapter 2

General Methods

This thesis describes a set of studies investigating infant pain responses. A number of different modalities are used including electroencephalography (EEG), electromyography (EMG), behavioural measures and changes in physiology, to investigate the responses of infants of a range of gestational ages. A number of different stimuli are used, including clinically required noxious procedures such as heel lance and cannulation, experimental noxious stimulation and non-noxious stimuli. 186 infants were included in the work presented, ranging in age from 28 weeks corrected gestational age (CGA) up to 46 weeks. This chapter describes the General Methods that are used in the Results chapters.

2.1. Neonatal intensive care

This work was conducted in the Neonatal Unit and Maternity wards at the Women's Centre in the John Radcliffe Hospital, Oxford. The Women's Centre has approximately 7,500 deliveries a year and is a regional referral unit for women with high-risk pregnancies. The Neonatal Unit is a level 3 unit, with 48 cots (16 Intensive Care, 12 High Dependency and 20 Special Care). It provides intensive care for infants born in Oxfordshire and across the region. The Neonatal Unit admits around 800 infants a year, with around 450 of these being infants born at

less than 37 weeks, and around 50 being infants born at less than 27 weeks' gestation.

2.2. Ethics and recruitment

2.2.1 Ethical approval

Ethical approval for studies was obtained from the Oxford Research Ethics Committee (REC) of the National Research Ethics Service (NRES). Studies were conducted as part of the ethics application entitled "Investigating pain in the developing human brain" (REC reference 12/SC/0447). The research conformed to the standards set by the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2.2. Recruitment

114 infants were recruited from both the maternity wards and the neonatal unit between January 2015 and December 2017. Additional infants were included in some aspects of the data analysis who were recruited from July 2012. Only parents of infants who were clinically stable were approached for inclusion. Infants were not included in the studies if they had documented neurological malformations, IVH of grade 3 and 4, or maternal substance abuse. None of the infants had received any analgesics or sedatives in the 72 hours prior to the study.

A copy of the parent information sheet and parental consent form are included in the appendix. Parents were given an information sheet and a verbal explanation of the study and were then given an appropriate amount of time to decide whether they wanted their baby to take part. If the study involved the use of experimental noxious stimulation, then the stimulator that was going to be used during the study was shown to the parents. Written, informed consent was obtained prior to any infant taking part in the study. Parents were able to withdraw their baby from the study at any time.

2.3. Stimuli

The responses to a variety of stimuli (visual, auditory, tactile and noxious) were studied in this work and where used, will be described in the relevant chapters. A heel lance and control heel lance were used throughout and a detailed explanation follows.

2.3.1. Heel lance and control heel lance

Heel lances are widely used in neonatal care to obtain capillary blood samples from infants. A lancet is used which has a trigger to release a retractable blade. The lancet is applied to the infant's heel, the blade is released and blood is then collected as drops form on the skin.

All heel lances were performed only when clinically required as part of the infant's medical care. Heel lances were not performed solely for the purpose of a

study. Care was taken to ensure the infants were given appropriate comfort measures, such as swaddling, when performing the heel lance. The foot chosen for heel lancing was based on clinical judgment and not controlled during the experiment. Heel lances were performed on the medial or lateral plantar surface of the heel. In term infants, BD Microtainer Quikheel Infant Lancet (Becton, Dickinson and Company) with a penetration depth of 1.0 mm was used, and in preterm infants, BD Quikheel Preemie Lancet with a penetration depth of 0.85 mm was used. Prior to the lance, a period of background EEG was recorded with the foot held. The control lance was then performed. The lancet was rotated by 90 degrees and held against the infant's foot so that when the lance was released there was no contact with the infant's heel. The control heel lance was always applied first so as not to interfere with blood collection, to avoid tactile stimulation of damaged skin and so that the infant could be returned to their parent(s) for comforting after blood collection was complete. The heel lance was performed at the same site as the control lance. If insufficient blood was obtained with a single heel lance, a second heel lance was performed. If the infant appeared settled and parents were happy, this was also recorded. The foot was not squeezed in the 30 seconds following the heel lance to allow the PIPP-R score to be calculated in response to only the lance procedure rather than the squeezing of the foot. If any droplets of blood formed during this time, these were collected into the sample container.

Release of the lancet blade was time-locked to the EEG and EMG recordings using an event-detection interface and accelerometer which has previously been described (184). For facial expression analysis, a video camera was used to

record the infant's face throughout the procedure for analysis away from the clinical setting and the timings of the lance and control lance were marked on the video using an LED, which flashed when the person performing the lance pressed a foot pedal at the point of stimulation.

2.4. Recording techniques

2.4.1. EEG and EMG recording techniques

Electrophysiological activity was acquired with the SynAmps RT 64-channel headbox and amplifiers (Compumedics Neuroscan), with a bandwidth from DC - 400 Hz and a sampling rate of 2 kHz. CURRYscan7 neuroimaging suite (Compumedics Neuroscan) was used to record the activity. All equipment conformed to the electrical safety standard for medical devices, IEC 60601-1.

EEG was recorded at eight scalp electrodes (Ambu Neuroline disposable Ag/AgCl cup electrodes) in positions Cz, CPz, C3, C4, FCz, Oz, T3 and T4, according to the modified international 10-20 system (see Figure 2.1). The reference electrode was positioned at Fz and the ground was placed on the forehead. EEG conductive paste (Elefix EEG paste, Nihon Kohden) was used to optimise contact with the scalp. Impedances were reduced by gently rubbing the skin with EEG preparation gel (NuPrep gel, D.O. Weaver and Co.) prior to electrode placement.

Bipolar EMG electrodes (Ambu Neuroline 700 solid gel surface electrodes) were placed on the biceps femoris of both legs

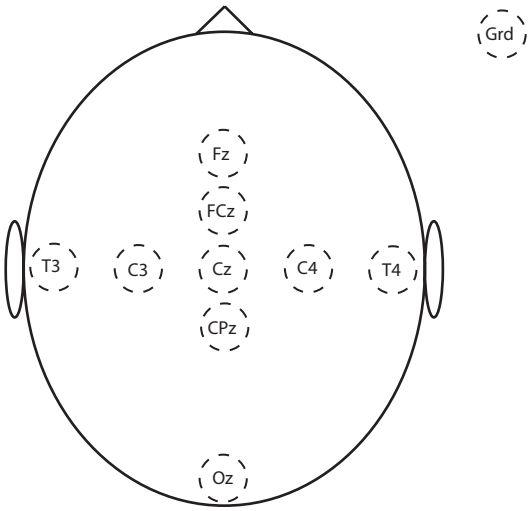

Figure 2.1: Schematic of EEG electrode placement on the scalp

2.4.2. Clinical pain scoring

Clinical pain scores were calculated in response to clinically required noxious procedures, including heel lance and cannulation, and in response to the control heel lance, using the Premature Infant Pain Profile- Revised (PIPP-R) score (112). A video camera was used to record the infant's baseline behavioural state and any change in facial expression evoked by the procedure. An LED was synchronised to flash when the experimenter pressed a foot pedal at the point of stimulation. Physiological recordings were made using a Philips neonatal monitor (IntelliVue MX800 patient monitor, Philips). A pulse oximeter probe was placed on the infant's hand or foot to record oxygen saturation and leads were placed on the chest to record the ECG to calculate the heart rate. The data was recorded in real-time and exported for analysis using ixTrend software (ixellence GmbH).

2.5. Analysis

2.5.1. EEG analysis

EEG analysis on the acquired data was performed post acquisition using MATLAB. First, the unprocessed EEG was filtered with a low-pass filter of 70Hz and a high-pass filter of 0.5Hz and a 50Hz notch filter was applied (demonstrated in Figure 2.2). 1500ms epochs were extracted with 500ms before the stimulus and traces were baseline corrected to the pre-stimulus mean. EEG data epochs were rejected if gross movement artifacts were present.

The magnitude of the response recorded was calculated by projecting a previously characterised template of noxious-evoked brain activity, defined in a population of term infants, onto the EEG data using singular value decomposition (73,142,164,185).

Let X_0 be the template. The singular value decomposition of X_0 is given by:

$$X_0 = U_0 G_0 \Gamma_0^T$$

Given a new data set, X_1, the corresponding weights, U_1, are given by:

$$U_1 = X_1 V_0 \Gamma_0^{-1}$$

Thus, the corresponding weight for each new EEG trace was obtained, which represents the magnitude of the noxious-evoked brain activity.

The template was projected on to the individual EEG trials recorded at the Cz electrode in the 400-700ms post stimulus interval. The magnitude of the response was calculated for each trial.

To achieve maximum correlation with the template and to allow for inter-individual latency variation, Woody filtering of ± 50 - 100ms (as detailed in individual results chapters) was applied to the individual EEG traces. This technique is widely used to correct for the temporal variability of event related potentials and is based on correlation-averaging techniques, where the cross correlation of the template and the single-trial EEG data is maximised (186).

These steps are summarised in Figure 2.2.

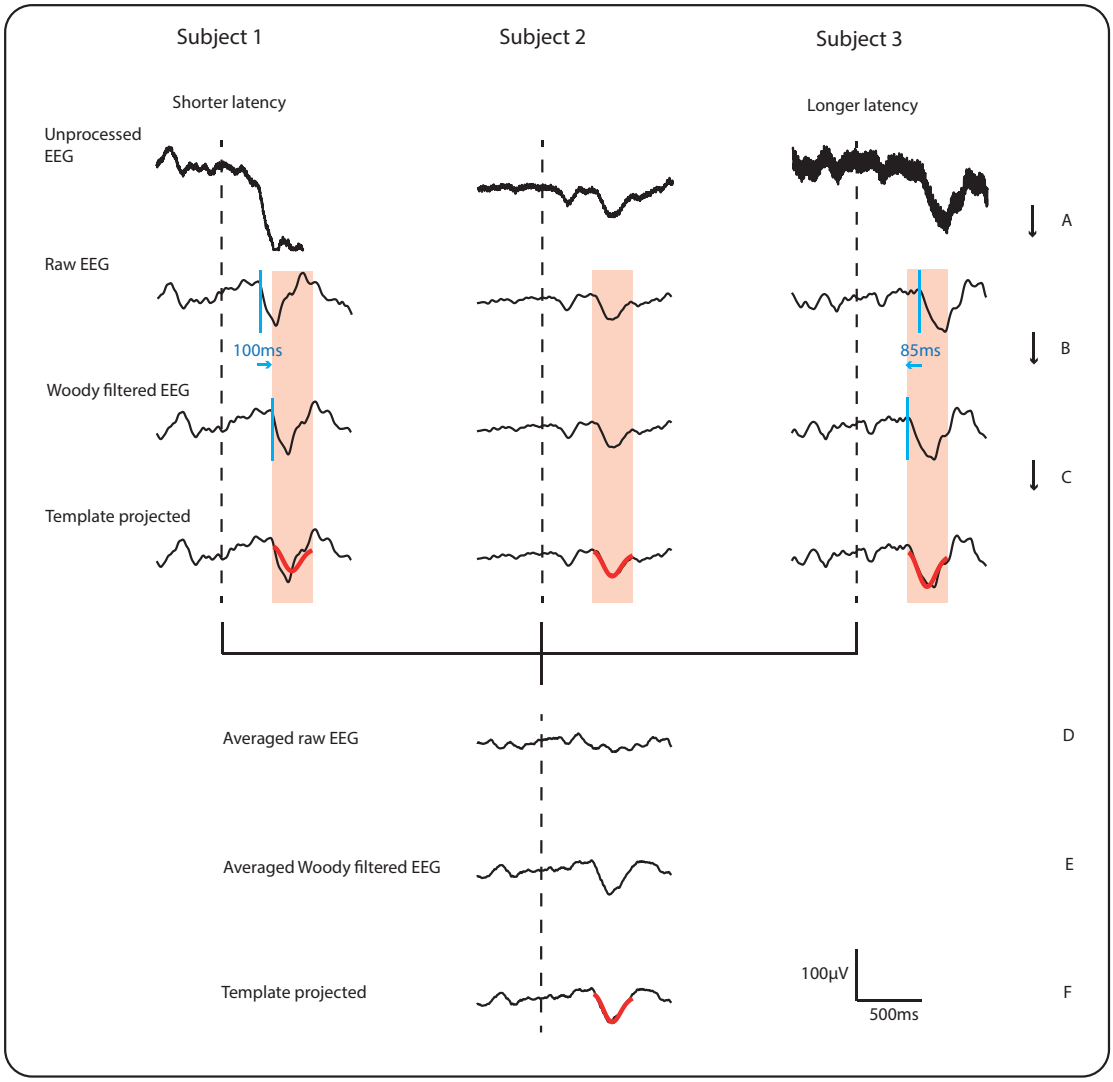

Figure 2.2: EEG analysis pathway
The steps involved in analysing EEG traces are shown in single trials obtained from 3 subjects recorded in response to a heel lance. (A) The unprocessed EEG is filtered to remove high and low frequency noise (low-pass filter of 70Hz, high-pass filter of 0.5Hz and a 50Hz notch filter with harmonics). (B) Woody filtering is applied to the individual trials to maximise correlation with the template. Here, three examples are shown, an individual trial where a shorter latency to evoked response is seen (Subject 1), an individual trial where the response is seen in the 400-700ms time window (Subject 2) and an individual trial where a longer latency to evoked response is seen (Subject 3). (C) The template is projected on to the Woody filtered EEG data to calculate the corresponding weight of the response. (D) The averaged raw EEG of the three trials is shown. The evoked potential is obscured due to the inter-individual latency variation demonstrated in the single trials. (E) When Woody filtering is applied to correct for this latency variation, the evoked potential is seen. (F) The template is then projected on to the Woody filtered average.

2.5.2. EMG analysis

EMG signals were filtered between 10 - 500 Hz with a notch filter at 50 Hz (and harmonics). Epochs were extracted from 500ms before to 1000ms after the stimulus and rectified. The data was divided into 250ms windows and in each window the root mean square (RMS) signal was calculated. The post stimulus mean was calculated as the mean across the four windows after the stimulus (0 - 1000ms) (24).

2.5.3. Facial expression analysis

Analysis of the facial expression was undertaken post acquisition by experienced observers and scored using the Premature Infant Pain Profile - Revised (PIPP-R) (113). Observers were blinded to the stimulus type when scoring the videos.

The baseline behavioural state was scored in the 15 seconds preceding the procedure. A score between 0 and 3 was given according to whether the infant was active and awake, quiet and awake, active and asleep or quiet and asleep respectively.

The presence of three facial expressions (brow bulge, eye squeeze and nasolabial furrow) was assessed in the 30 seconds following both the procedure. Each facial expression was taken individually and the duration for which it was present was timed using a stopwatch. If the infant stopped displaying the facial expression, the timer was paused, and if the expression was seen again, during the 30-

second period, the timer was restarted to give a cumulative time. This time was then used to calculate a score between 0 and 3. This was repeated for each of the facial expressions to give a total facial PIPP-R score of between 0 and 9.

The duration of facial expression activity was also calculated and defined as the duration during which any of the three facial expressions were observed, whether together or individually, to a maximum of 30 seconds.

2.5.4. Physiological response analysis

The physiological response to procedures was also calculated according to the PIPP-R score. The physiological data was downloaded from ixTrend and imported into MATLAB. An in-house script was used to calculate the baseline average oxygen saturations and heart rate in the 15 second preceding stimulation, and the minimum oxygen saturations and maximum heart rate in the 30 seconds following. Scores between 0 and 3 were then calculated for both.

Chapter 3

The development of behavioural discrimination across gestation

3.1. Introduction

Infants primarily communicate their needs through behaviours, such as crying, body movements or change in facial expression. These behaviours are vital for survival, and the measurement of noxious-evoked facial expression change forms the cornerstone of pain assessment and, consequently, treatment in hospitalised infants (187). However, how discriminative these behavioural responses are in the youngest infants remains questionable. The intensity and duration of pain-related facial expressions in term-born infants have been shown to discriminate between responses to noxious and non-noxious tactile stimulation (8) but a lack of facial expression discrimination is reported in the most prematurely-born infants (188).

Similarly, a lack of discrimination is observed in both the magnitude of spinal reflex withdrawal activity and the patterns of evoked brain activity following tactile and noxious stimulation in very premature infants (165),(142). Indeed, in preterm infants, tactile stimulation can evoke reflex withdrawal that is indistinguishable from noxious stimulation (165), and both types of stimulation evoke non-modality specific bursts of brain activity prior to approximately 34 weeks' gestation (142). In contrast, in term-aged infants, nociceptive reflex activity is more refined (73), and tactile stimulation evokes significantly smaller reflexes than noxious stimulation (165). Likewise, after

approximately 34-35 weeks' gestation, distinct patterns of brain activity are evoked by noxious stimulation, which are not observed following tactile stimulation or other sensory modalities (142,185). It is possible that discrimination of facial expression responses to tactile and noxious stimulation develop following the same trajectory.

The aim of this study was to establish the developmental trajectory of pain-related facial expression in infants from 28 – 41 weeks' gestational age and to establish how this relates to facial expression changes evoked by tactile stimulation. It was hypothesised that in the youngest premature infants, facial expression change would not differ in response to tactile or noxious stimulation, and, consistent with the emergence of noxious-evoked brain activity, divergent behavioural responses would not emerge until approximately 34 weeks' gestation. To test this hypothesis, the facial expression responses to tactile and noxious stimulation were analysed in 105 infants from 28 – 41 weeks corrected gestational age.

3.2. Methods

3.2.1. Participating infants

105 infants were recruited from the Neonatal Unit and Maternity Wards of the John Radcliffe Hospital, Oxford. Infants were born between 23 and 41 weeks' gestation and were between 28 and 41 weeks gestational age at the time of study. Further infant demographics are given in Table 3.1.

Demographic characterisation of the infants studied	
Total infants	105
GA at birth (weeks)	31.1 (28.1 – 37.0)
GA at time of study (weeks)	35.4 (32.3 – 38.9)
Postnatal age at time of study (days)	10 (4-29)
Birth weight (g)	1610 (940 – 2680)
Weight at study (g)	1946 (1400 – 3215)
Male infants (%)	59 (56)
Multiple gestation infants (%)	33 (31)
Spontaneous vaginal deliveries (%)	34 (32)
Assisted/caesarean deliveries (%)	71 (68)
Apgar score at 5 min	10 (8-10)
Infants admitted to NICU (%)	77 (73)
Estimated cumulative prior pain exposure	14 (4 – 41)
Infants ventilated during admission (%)	38 (36)
Days of ventilation	0 (0-2.5)
Infants who had previously receiving received morphine during admission (%)	26 (25)
Infants with Grade 1 or 2 IVH (%)	9 (9)
Infants with a history of previous surgery (%)	3 (3)
Infants with a previous diagnosis of postnatal infection (%)	38 (36)
Infants with a history of necrotizing enterocolitis (%)	3 (3)

Table 3.1: Demographic characterisation of the infants included in Chapter 3.
Median is displayed with (lower quartile, upper quartile).

3.2.2. Experimental procedures

3.2.2.1. Heel lancing and control heel lance

All infants were studied while undergoing a medically required heel lance for blood collection. Prior to the heel lance procedure, all infants had a control heel lance performed. For further details see General Methods.

3.2.3. Analysis

3.2.3.1. Facial expression scores

Facial expression responses were recorded using a video camera and scored away from the bedside. Facial expression responses were scored using the facial component of the PIPP-R score as detailed in the General Methods. The score for the facial component of the PIPP-R was used as well as the presence of any of the three facial expression responses and the total duration of facial expression change.

Observers were blinded to the stimulus type when scoring the videos. Inter-rater and intra-rater reliability were calculated using intra-class correlation. This was performed for 20% of the videos, which were rescored by the first observer and 35% of the videos, which were rescored by a second independent observer. Videos were selected at random for re-scoring. Intra-rater reliability was 0.95 and inter-rater reliability was 0.96.

3.2.4. Statistical Methods

Generalised linear regressions with logit link functions were used to describe the proportion of infants with facial expression responses, across gestational age. For this, proportions were first calculated in two-week intervals (starting at 28 – 30 weeks) with intervals overlapping by one week. McNemar's test was used to identify the age at which the proportions of responders to lance and control were significantly different. Comparison of the duration of facial expression response to the tactile and noxious stimulation was carried out using Wilcoxon signed rank tests, and the duration of the response to tactile stimulation was correlated with the response to noxious stimulation using Pearson's correlation coefficient.

It was calculated that a minimum of 6 infants were required in each group in order to be able to detect a significant difference if all infants responded to lance and not to control using the McNemar's test. This was inflated to a minimum group size per two-week interval of 10 infants in order to account for missing data and variation in responses.

3.3. Results

3.3.1. Behavioural responses to tactile and noxious stimulation are dependent on gestational age

Overall, 24% of infants mounted a facial expression response to tactile stimulation and 69% mounted a facial expression response to noxious stimulation. However, the likelihood that the stimuli evoked a facial expression response was age dependent - the proportion of infants who mounted a facial expression response to the noxious stimulation (clinically required heel lance) increased significantly with gestational age ($p = 0.0005$, generalised linear regression, coefficient $\beta = 0.16$, Figure 3.1). Conversely, the proportion of infants who mounted a facial expression response to the non-noxious tactile stimulation (control heel lance) decreased significantly with gestational age ($p = 0.0005$, generalised linear regression, coefficient $\beta = 0.16$, Figure 3.1). There was no significant difference in the baseline behavioural state score, scored as part of the PIPP-R, (see General Methods), indicating that the sleep state of the infants prior to the application of each stimulus modality was comparable between the groups ($p = 0.91$, paired t-test, average baseline state before tactile stimulation: 2.16 ± 1.08 (mean ± standard deviation), before noxious stimulation: 2.15 ± 1.13).

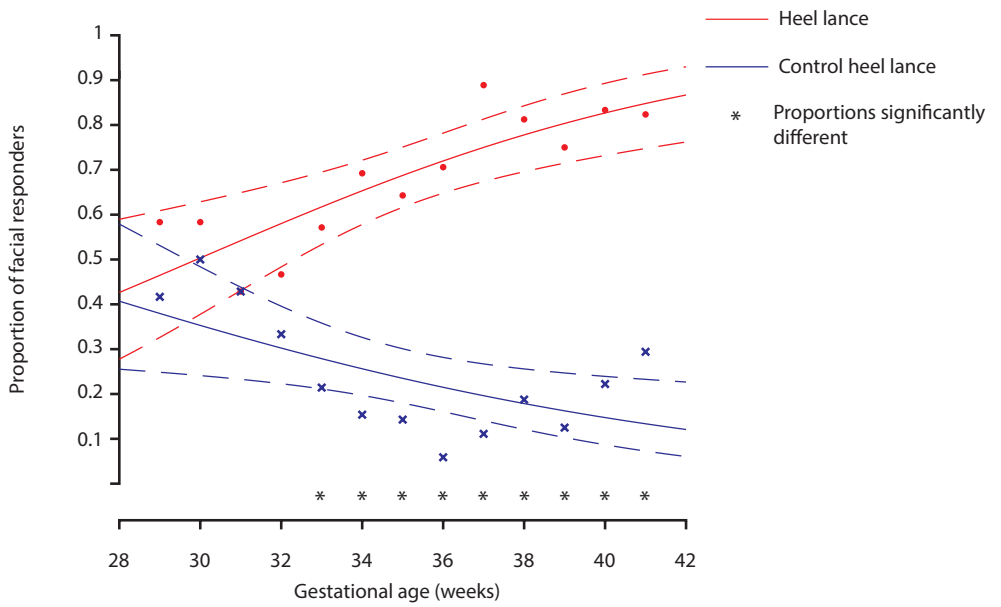

Figure 3.1: Behavioural responses to tactile and noxious stimulation and their relationship to gestational age.
The proportion of infants who mount a facial expression response, of any duration, to tactile, control heel lance stimulation (blue) and noxious, heel lance stimulation (red) are shown according to gestational age. Proportions were calculated in two-week intervals, with intervals overlapping by 1 week. The fit from a generalised linear model (solid lines) and 90% confidence intervals (dashed lines) are overlaid. * indicate ages at which the proportion of responders to tactile and noxious stimulation were significantly different.

A similar pattern was observed when the analysis was repeated using the score obtained in the facial component of the PIPP-R score to classify facial responders. Infants were classified as mounting a behavioural response if they displayed facial expression change of sufficient duration to obtain a score of 1 or more (minimum of 3 seconds). Overall, 13.3 % of infants had a non-zero facial expression score to tactile stimulation and 50.5 % had a non-zero score to noxious stimulation. The scores changed with gestational age, with the proportion of infants receiving a non-zero score to noxious stimulation increasing with increasing gestational age ($p = 0.023$, generalised linear regression, coefficient $\beta = 0.09$, Figure 3.2) and the proportion of infants receiving a non-zero score to tactile stimulation decreasing with increasing

gestational age (p=0.025, generalised linear regression, coefficient β = -0.014, Figure 3.2).

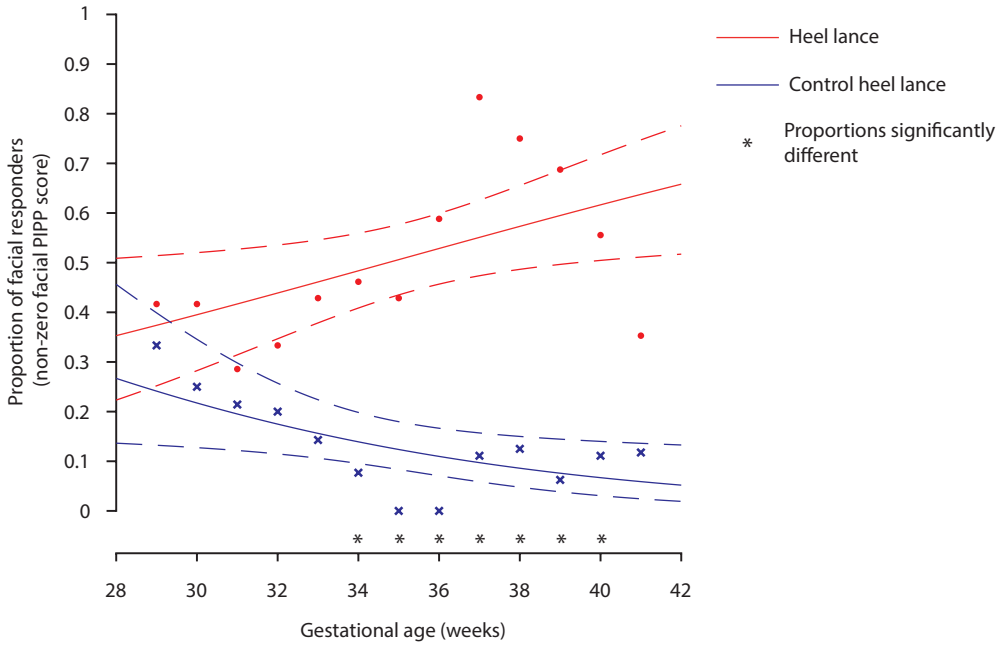

Figure 3.2: Behavioural responses measured using the facial component of the PIPP score and their relationship to gestational age.
The proportion of infants who mount a facial expression response, of sufficient duration to receive a score on the facial component of the PIPP-R score, to tactile, control heel lance stimulation (blue) and noxious, heel lance stimulation (red) are shown according to gestational age. The fit from a generalised linear model (solid lines) and 90% confidence intervals (dashed lines) are overlaid. * indicate ages at which the proportion of responders to tactile and noxious stimulation were significantly different.

3.3.2. Facial expression responses to tactile and noxious stimulation diverge from 32.4 weeks' gestation

The proportion of infants with a facial expression response following tactile and noxious stimulation diverged with increasing age. The proportion of infants displaying a

differential facial response to lance was compared in two-week intervals overlapping by one week. The proportion of infants displaying a differential facial response to lance became significant in the 33-34+6 week interval (p=0.023, McNemar's test). Further analysis was conducted to identify the minimum age at which significance was reached using a two-week interval increasing in increments of 1 day. Using this approach, significance was reached in the interval beginning at 32+3 (32.4) weeks (p=0.023, McNemar's test) thus, infants displayed facial expression discrimination between noxious and tactile stimuli from 32.4 weeks. Prior to 32.4 weeks' gestation, there was no significant difference in the proportion of infants responding to lance or control, hence there was equal likelihood that a facial expression response would be elicited following either tactile or nociceptive input (Figure 3.1).

When the analysis was repeated using the score obtained in the facial component of the PIPP-R score to classify facial responders the age interval at which a significant difference in the proportion of infants responding to noxious stimulation compared with tactile stimulation was identified was slightly older, 34-35+6 (p=0.041, McNemar's test) with the minimum age defined as 33+3 (33.4) weeks (p=0.041, McNemar's test)(Figure 3.2). This difference occurred because the requirement for a facial expression response to be of a minimum of 3 seconds duration in order for it to be classified as a response, meant that more younger infants were classified as facial non-responders to the lance, since the duration of response to lance in these infants was generally shorter, as highlighted by the next result.

3.3.3. The duration of facial response does not discriminate between tactile and noxious stimulation in the youngest infants

When considering infants below 32.4 weeks' gestation, the duration of the evoked facial expression activity recorded following nociceptive or tactile input was not significantly different (p=0.13, Wilcoxon signed-rank, Figure 3.3), whereas, in infants greater than 32.4 weeks, the duration of noxious-evoked facial activity was significantly greater than the response following the tactile stimulation (p<0.0001, Wilcoxon signed-rank, Figure 3.3).

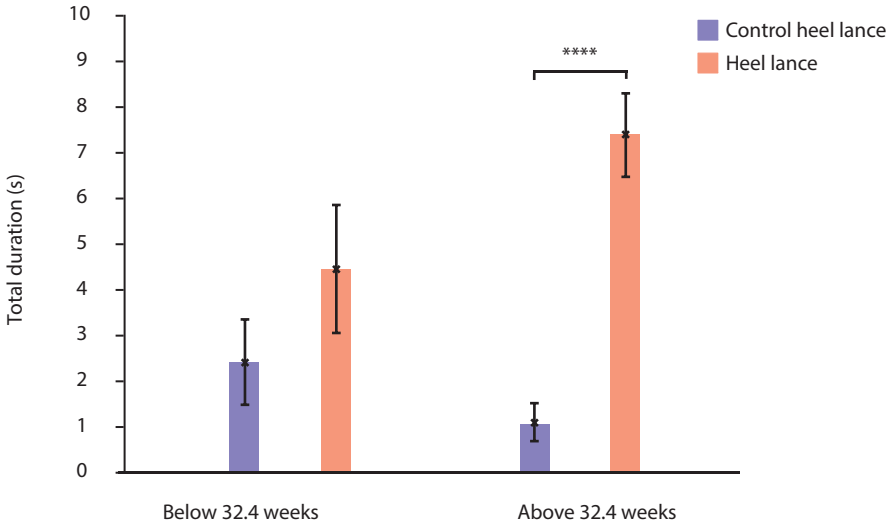

Figure 3.3: The duration of facial expression responses to tactile and noxious stimulation above and below 32.4 weeks.
The average total duration of facial expression response to tactile (blue) and noxious (red) stimulation are shown with mean ± SEM for infants below 32.4 weeks and above 32.4 weeks. **** $P < 0.0001$.

3.3.4. When they occur, facial expression responses to tactile stimulation are highly correlated with the response to noxious stimulation

24 % of all infants mounted a facial expression response of any duration following tactile stimulation. The highest proportion of responders, 42%, were in infants less than 32 weeks CGA, although there were infants of all ages who responded to the control lance. In these infants, the duration of the facial expression activity following tactile stimulation was highly correlated with the duration of the response to the noxious stimulation ($p < 0.001$, $r = 0.73$, $\beta = 0.82$, $N = 25$, Figure 3.4.).

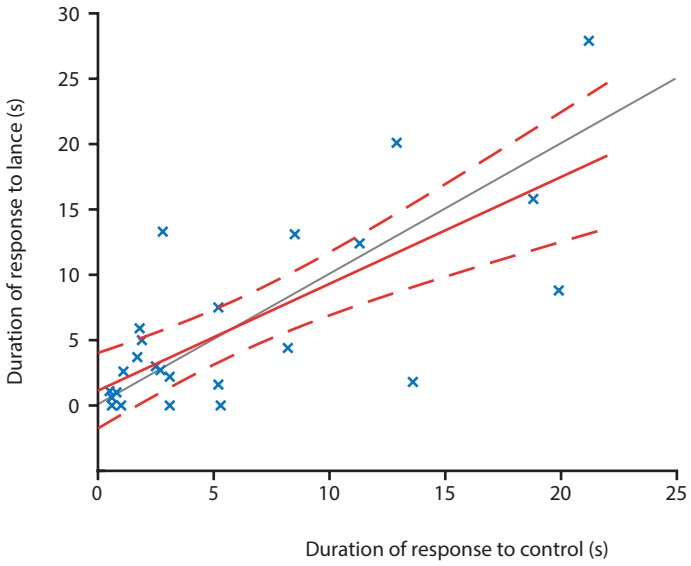

Figure 3.4: The facial response to tactile stimulation, when it occurs, is highly correlated with the response to noxious stimulation.
Considering only those infants who mounted a facial expression response to the tactile stimulus, the duration of the response was significantly correlated with the response to the noxious stimulus ($p < 0.001$, $r = 0.73$). Solid red line indicates linear regression, and dashed lines the 95 % confidence intervals. Light grey line indicates $y = x$.

3.4. Discussion

In this chapter, it has been demonstrated that behavioural responses to noxious and tactile stimulation in infants are dependent on gestational age. The youngest infants were equally likely to respond behaviourally to tactile or nociceptive stimulation and the duration of their responses was not significantly different. Above 32.4 weeks, behavioural discrimination between tactile and noxious stimulation was seen; infants were significantly more likely to display a behavioural response to noxious stimulation than to tactile stimulation, and the duration of the response to noxious stimulation was significantly longer. A correlation between the duration of response to tactile stimulation and noxious stimulation was observed in those infants responding to the tactile stimulus.

3.4.1. Behavioural discrimination to tactile and noxious stimulation emerges at approximately 32.4 weeks corrected gestational age

It has been demonstrated here that behavioural discrimination to tactile and noxious stimulation emerges from around 32.4 weeks gestational age. In the youngest infants, a facial expression response was equally likely following either tactile or nociceptive stimulation, whereas by term age, infants were approximately four times more likely to display a facial expression response following nociceptive input compared with tactile stimulation. The development of discriminative patterns of noxious-evoked facial expression activity observed here follows a similar trajectory to nociceptive brain activity and reflex withdrawal (142,165). Patterns of noxious-evoked brain activity that are distinguishable from that evoked by tactile stimulation begin to emerge from

around 32 weeks' gestation, and are more likely to occur than the immature pattern of non-specific neuronal bursts seen earlier in gestation, from around 34-35 weeks' gestation (73,142). Concurrently, while nociceptive reflex specificity is still not fully mature in term infants, the magnitude, duration and latency of the reflex activity reduces with increasing gestational age (73) and, unlike in premature infants, tactile-evoked reflex withdrawal is significantly smaller than noxious-evoked reflex activity in term infants (165). An important avenue of further work would be to record all three measures; brain activity, reflex withdrawal and noxious-evoked facial expression activity in a single cohort to determine how, in an individual, the emergence of discriminative activity occurs. Nevertheless, the results from this study suggest that the maturation of facial expression responses coincides with the emergence of modality-specific brain activity and the refinement of the reflex withdrawal response.

Previous work has suggested that behavioural discrimination may emerge earlier in gestation (189). Johnston et al. demonstrated that infants from 26-31 weeks gestational age displayed a differential behavioural response to a sham heel lance procedure compared to a medically required heel lance. However, the sham condition was the nurse touching the infant's foot which is arguably a less salient stimulus than the heel lance. Here, a control heel lance procedure was used to ensure that the tactile stimulus was well matched to the noxious stimulus, and provided an acute sensory experience when the lancet was depressed, albeit without the nociceptive input. Without matching the stimuli in this way, it is not possible to determine whether the differential responses are due to the different intensities of the stimuli rather than the additional nociceptive input. Furthermore, it is more plausible that behavioural discrimination would emerge with a similar time course to the maturation of brain activity and hence, unlikely that

behavioural discrimination would be seen this early. Indeed, in the previous studies looking at brain activity and reflex withdrawal, the same experimental paradigm used here was employed (142,165).

The biological processes underlying the emergence of modality-specific brain activity and potentially behavioural discrimination, at this gestation, has been previously posited to be related to the development of thalamocortical connections that occurs around this time (90,142,190). Between 31 and 34 weeks of gestation, thalamocortical axons form synapses in the cortical plate and between 35 and 37 weeks, interhemispheric connections strengthen (190). These major changes in the organisation of cortical pathways are thus thought to underlie the major changes in sensory processing that occur at this gestation. Future work would benefit from an imaging component to allow structural development and behavioural responses to be correlated.

3.4.2. Infants who do not respond to lance are represented at all gestational ages

The proportion of facial non-responders to the lance described here, 31% overall, 40% in preterm infants, is in keeping with previously published work (10,191) and with the proportions reported in Chapter 4. Johnston et al. found a lower proportion of preterm non-responders, 20%, in their work (192), however, they classified non-responders as those infants failing to score in all aspects of the PIPP score, taking into account both the behavioural and physiological response, so it is likely that some infants who do not mount a behavioural response will have a physiological response. Furthermore, infants in that study had their foot squeezed during the 30 seconds following the lance during

which the score is calculated, so it is not be possible to differentiate between the response to the specific nociceptive input of the lance and the response to the heel squeeze. Here, the infant's heel was not squeezed during the 30 seconds following the control or lance to ensure that the facial response recorded was in response to the procedure itself. The similarly high proportion of facial non-responders described in other work following the same experimental protocol used here, suggests that the additional responders may well be responding to the squeezing of the heel (10,191). Squeezing the heel in order to obtain a blood sample is an integral part of the heel lance procedure and it is important to consider the impact of this part of the procedure in the clinical setting. In the research setting, however, it is important to minimise these potential confounding factors in order to more clearly understand the underlying processes.

A similar proportion of infants of all gestations did not mount a facial response to the lance. Interestingly, previous work has also demonstrated that, despite a lack of behavioural response, a number of these infants still demonstrate significant noxious-evoked brain activity (21) suggesting that in some infants, there is a disconnect between behavioural responses and brain activity. In these infants, the use of behavioural measures may well underestimate their experience of pain. The difficulty in relying on behavioural measures alone was highlighted in work looking at sucrose as an analgesic for heel lancing in infants, here they showed that sucrose decreased behavioural scores without impacting brain activity (24).

The reasons why an infant might not respond behaviourally to a painful procedure have been investigated. Previous work has suggested that younger infants and those who are

sick are the least likely to mount a behavioural response (119,192). A possible reason underlying this observation is that the youngest, sickest infants lack the muscle strength or energy reserves required to mount a behavioural response. Worryingly, it is these infants who are likely to carry the highest burden of painful procedures and so, be most at risk of long-term consequences of undertreated pain (12). The work by Johnston et al. also found that infants who had recently undergone a painful procedure were less likely to mount a behavioural response, they suggested that this lack of response represented the recently stressed infant becoming exhausted (119). This observation supports the theory initially postulated by Als in his Synactive Theory of Development, where he posits that under extreme stress a preterm infant will become disorganised and will no longer be able to mount coherent responses to external stimuli (193). This, taken together with the evidence that infants who do not respond behaviourally may still demonstrate brain activity, highlights the importance of utilising multi-dimensional measures of pain, including brain-based measures, as described in Chapter 4.

3.4.3. The response to tactile stimulation and noxious stimulation were highly correlated in some infants

Overall, 24% of infants responded to tactile stimulation, and in those that did respond, it was observed that there was a high degree of correlation seen between the duration of facial expression activity following tactile and following noxious stimulation. There are a number of possible explanations for this finding. The first is that in these infants, the behavioural response seen is a reflection of the level of arousal caused by the stimulus

rather than the nociceptive component and what is being measured is this arousal response. High levels of behavioural response have been recorded in infants undergoing non-noxious, but arguably, very salient stimuli such as temperature measuring and nappy change (120). The control heel lance procedure, as discussed earlier, is well matched to the lance in terms of its intensity and the high degree of correlation in the duration of the responses may reflect this.

Alternatively, if the behavioural response is truly reflective of the nociceptive input, it may be that, in these infants, the tactile stimulus is aversive or even noxious. One possibility is that the control heel lance may have been applied on areas of skin that were previously lanced and that the responses recorded reflect some degree of hypersensitivity in this previously damaged skin (194). Another possibility is that these infants are displaying allodynic responses whereby a non-noxious stimulus becomes noxious. One possible explanation for this is the presence of infection, which is commonly seen in the neonatal setting and allodynia has been described in animal models of infection (195). In Chapter 5, the impact of infection on the responses to pain is investigated.

Another possible explanation for this observation is that, while the control heel lance is not in itself painful, some infants may be displaying a conditioned response associated with the heel lance procedure. Conditioned responses to the non-noxious component of procedures have been described previously both in terms of level of arousal and in terms of physiological responses (196). Importantly, the tactile stimulation described here, while not thought to be noxious, is a mechanical stimulus and unlikely to be pleasant. It is therefore important to distinguish this 'procedural' touch from the

affective touch of a parent, which has been demonstrated to be beneficial to infants (181,182). Furthermore, if these infants are displaying increased tactile sensitivity, it is plausible that they may be more vulnerable to the adverse effects of both noxious procedures (97,98) and also routine handling (197–199).

3.4.4. Strengths and limitations

The purpose of this study was to investigate the emergence of behavioural discrimination to noxious stimulation, but pain is a complex, subjective sensory and emotional experience and its assessment in non-verbal infants is difficult. The use of purely behavioural measures in pain assessment, and indeed treatment, therefore has limitations and has been questioned (14,200). Although recent work has argued that behavioural measures are more informative of pain and distress than physiological measures (201), the majority of clinically validated pain scores are composite measures relying on the use of behavioural and physiological responses (111), and it is important to consider the emergence of behavioural discrimination in the context of other measures. It is compelling therefore, that the emergence of behavioural discrimination seems to follow a similar trajectory to that of nociceptive brain activity and reflex responses, but it would also be interesting to consider whether there is a similar trajectory to physiological responses. While previous work has demonstrated the maturation of brain activity and emergence of noxious-evoked brain activity with a similar timeline, noxious-evoked brain activity was not recorded in most infants here, so it is not possible to determine whether those infants who display facial expression discrimination between the stimuli also have mature patterns of evoked brain activity. This would be of particular interest in the period from approximately 32 – 35 weeks'

gestation as discrimination starts to emerge.

In this study, it is not possible to exclude an order effect as all infants received the stimuli in the same order, with the control procedure performed before the clinical heel lance. This was done for clinical reasons and to avoid the possibility of creating hypersensitivity to the control lance as result of the tissue damage caused by a preceding lance (194). It is not possible to exclude an order effect therefore, such that the response to the heel lance is intensified or diminished by the previous tactile stimulus. Work by Holsti et al demonstrated that behavioural responses to a heel lance were heightened in infants who had previously received clustered care (202), particularly those who were older than 30 weeks' gestation. However, the clustered care included a number of nursing procedures such as nappy change, which has previously been shown to be a sufficiently stressful event that a significant proportion of preterm infants will be considered to be in 'pain' using behavioural pain scores (203). However, here, there was no difference in the baseline behavioural state of infants before the control lance when compared to the heel lance suggesting that the control lance did not alter the behavioural state of the infant.

3.5. Conclusion

In summary, I have demonstrated that behavioural discrimination between tactile and noxious stimulation emerges at approximately 32 weeks' gestation. The implication of these findings in clinical practice is profound, both in terms of clinical trials of analgesics and individual infant pain assessment. Given that infants below 32 weeks' gestation do not behaviourally discriminate between tactile and noxious events, using pain-related behaviour alone to assess the analgesic efficacy of pharmacological interventions may not provide an accurate picture. Indeed, even if pain were to be entirely alleviated by an analgesic compound, changes in facial expression may still be evoked by non-noxious handling procedures giving the false impression that effective pharmacological interventions do not provide analgesia. While the majority of clinically validated pain scores are composite measures relying on the use of behavioural and physiological responses (111), recent work has argued that behavioural measures are more informative of pain and distress than physiological measures (201); however, it will be important to consider the emergence of behavioural discrimination in the context of these measures. Moreover, whilst using multiple measures is likely to provide the best assessment of analgesic efficacy (14), in the very youngest infants brain activity, reflex withdrawal and facial expression do not differ in response to tactile or noxious stimulation. The urgent need for improved methodology to assess analgesic efficacy is clear, but how this can be measured in the most premature infants is not established.

Chapter 4

Validating a template of noxious-evoked brain activity

4.1. Introduction

The measurement of pain intensity in non-verbal populations necessarily requires a degree of inference, based on quantifiable surrogate pain-related measures. Interest in the use of brain-derived approaches has increased in recent years. Given that pain is a perception that involves higher-level cortical activity, measurement of noxious-evoked brain activity provides an opportunity to assess more directly the cortical processing of pain. EEG is one method of assessing cortical activity and has been widely used in infants.

Specific noxious-evoked brain activity recorded using EEG was first described in infants by Slater et al (20). Noxious-evoked brain activity has been used to assess analgesic efficacy (24) and to investigate the developmental changes that occur across gestation during early human development (142). The magnitude of noxious-evoked brain activity has also been shown to relate to reflex withdrawal and to be graded with stimulus intensity (164). Previously, this noxious-evoked brain activity has been redefined in a subset of the infants within each study (24,164). The necessity to take this approach arose because the pattern of noxious-evoked brain activity had not been independently validated across datasets. The need to define activity for each separate study poses a number of difficulties. The first is that it is inappropriate to define a response in the group of infants and then to retest it in the same study population.

However, the alternative, defining the activity in a separate subset, uses vital participants to characterise the activity who cannot then be used to test the hypothesis of interest. In infant pain research, particularly when investigating clinical procedures, the number of potential participants is limited and so, it is important to maximise the study population. Furthermore, defining the activity each time means that it cannot then be translated to other groups. Designing a pre-defined template is important since it means that analysis can be more robust as the activity has not been characterised and evaluated in the same population. Furthermore, it allows the same template of brain activity to be used in different populations.

The template of noxious-evoked brain activity used here was defined in a group of term infants and identified by comparing the brain activity recorded in response to noxious and non-noxious stimulation (185). In order for the template of noxious-evoked brain activity to be used more widely, it is important to validate it. In this chapter, I validate this template in a number of settings. In the first study, the template is used to quantify the magnitude of noxious-evoked brain activity in background EEG and in response to control heel lance and heel lance stimulation. The template is then used to investigate the correlation between noxious-evoked brain activity and pain-related behaviour. In the second study, the template is used to assess evoked-brain activity in response to stimulation of different sensory modalities, using tactile, visual, auditory and noxious stimuli. In the third study, the effect of topical local anaesthetic on noxious-evoked brain activity elicited by experimental noxious stimulation is investigated. The fourth study is a pilot study aimed at determining whether the same techniques used to define the template of noxious-evoked brain activity can be applied to a different stimulus, cannulation, and whether topical local anaesthetic modulates the response seen.

4.2. Study Aims and Methodological Overview

This study has four core aims, which are described below.

Study 1:

The aim of the first study was to demonstrate that the template of noxious-evoked brain activity could be used in an independent sample to discriminate noxious-evoked brain activity from that elicited by a non-noxious stimulus and background EEG activity. Further, the study aimed to determine whether noxious-evoked brain activity was correlated with pain-related behaviour in infants at 34-42 weeks corrected gestational age (CGA).

Study 2:

The aim of the second study was to use the template of nociceptive brain activity to assess the response to stimulation of different sensory modalities.

To achieve this EEG activity was recorded in 14 term infants during background stimulation and in response to different sensory stimuli: auditory, visual, tactile and experimental noxious. Trains of approximately 10 stimuli were used. One infant did not receive tactile stimulation and one infant did not receive visual stimulation due to technical difficulties. Responses to a total of 132 auditory, 129 visual, 123 tactile and 140 experimental noxious stimuli were analysed.

Complete recordings were obtained in 12 infants. The sample size of 12 infants was determined by performing a power calculation using the data obtained in the characterisation of the template. It was anticipated that the magnitude of the noxious-evoked brain activity in response to sensory stimuli other than the noxious stimulation would be equivalent to the magnitude of the noxious-evoked brain activity observed in the background data. It was calculated that a sample size of 12 infants would be required to observe a difference in the noxious-evoked brain activity of this order with a power of 95% at a two-sided significance level.

Study 3:

The aim of the third study was to test whether the application of topical local anaesthetic, for a clinical procedure, modulated the noxious-evoked brain activity measured using the template.

In this study, 12 term born infants who were attending an outpatient clinic appointment requiring a venous blood sample were recruited and studied. Parents were offered pain-relieving measures for infants including the use of topical local anaesthetic. Topical local anaesthetic cream [tetracaine (4%, w/w), Ametop Gel; Smith and Nephew Healthcare] was applied to the dorsal surface of both hands and one foot if parents agreed. The cream was removed after about 30 minutes (as per the BNFc recommendations for use of topical local anaesthetic for venipuncture). EEG was recorded in background and in response to the application of trains of around 10 experimental noxious stimuli to each foot (treated and untreated) prior to the clinical

procedure. The order in which the stimuli were presented (treated or untreated skin) was randomly selected.

The sample size of 12 infants was determined by performing a power calculation using the data obtained in the characterisation of the template. It was anticipated that local anaesthetic would reduce the magnitude of the noxious-evoked brain activity to a magnitude equivalent to that observed in the background data. It was calculated that a sample size of 12 infants would be required to observe a reduction in the noxious-evoked brain activity of this order with a power of 95% at a two-sided significance level.

Study 4:

The aim of the fourth study was to determine whether the template could be used to measure noxious-evoked brain activity to a previously unstudied, clinically required noxious stimulus, insertion of a cannula to obtain a venous blood sample.

26 term born infants who were attending an outpatient clinic appointment requiring a venous blood sample (obtained via cannulation) were recruited and studied. 13 infants were recruited and studied before a change to clinical practice included the use of topical local anaesthetic cream. 13 infants were recruited and studied after this change was introduced. Where topical local anaesthetic cream [tetracaine (4%, w/w), Ametop Gel; Smith and Nephew Healthcare] was used it was applied to the dorsal surface of both hands with parental consent. The cream was removed after about 45 minutes (as per the BNFc recommendations for use of topical local anaesthetic for cannulation).

EEG, facial expression and physiological data were recorded while infants underwent cannulation for blood collection (sample obtained using a 24G neonatal cannula).

This was a pilot study and since the magnitude of the evoked response to cannulation was unknown, it was not possible to perform a power calculation. This study could be used to inform future studies.

4.3. Methods

4.3.1. Participating infants

Demographic characterisation of the infants included in the studies				
	Study 1: Behavioural correlation	Study 2: Multimodal stimulation	Study 3: Analgesic modulation	Study 4: Cannulation
Number of infants	32	14	12	26
GA at study	37.3 (36.5, 39.3)	39.8(37.8, 41.3)	42.1 (41.1, 43.6)	42.7 (41.6, 43.4)
GA at birth	36.4 (31.9, 39.1)	39.6 (37.2, 41.2)	38.7 (38.0, 39.8)	39.0 (37.8, 40)
PNA at study (days)	3 (1, 25)	2 (1,2)	24.5 (21.5, 27.3)	27 (24.3, 29)
Birthweight	2818 (1772, 3402)	3127 (2532, 3649)	3330 (3125, 3488)	3418 (3081, 3560)
Number of males	18	3	5	14
Applied stimuli	Control heel lance, heel lance	Experimental noxious, visual, tactile, auditory	Experimental noxious	Cannulation for venepuncture

Table 4.1: Demographic characterisation of the infants included in the studies in Chapter 4.
Median is displayed with (lower quartile, upper quartile).

4.3.2. Stimulation techniques

4.3.2.1. Control heel lance and heel lance

Infants received a heel lance only when clinically required. Full details of the procedures are described in the General Methods in Chapter 2.

4.3.2.2. Experimental noxious stimulation

Acute experimental noxious stimulation was applied using the 'PinPrick' (MRCSystems). Pinprick stimulators were developed in adults for quantitative sensory testing in patients with neuropathic pain (204). They are weighted devices that deliver a pre-

specified force when applied perpendicularly to the skin and do not break or damage the skin. Different forces are available, ranging from 8mN to 512mN. Previous work in infants has demonstrated that the EEG and EMG response to experimental noxious stimulation at 32, 64 and 128mN is graded with increasing intensity but lower than that evoked by a heel lance (164). In adults, a force of 128mN has been described as mildly painful (205) and has previously been shown to elicit noxious-evoked brain activity in infants without causing a significant increase in clinical pain scores (164). A force of 128mN was used in these studies.

In Study 2, the stimuli were applied to the infant's heel. In Study 3, the stimuli were applied to the dorsal surface of the infant's foot. In Study 2, the experimental noxious stimuli were time-locked to the EEG recordings using 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. The video recordings were reviewed after acquisition, and the time of stimulation was manually event-marked as the point where the barrel of the stimulator was first depressed. In Study 3, the experimental noxious stimuli were time-locked using a contact trigger device (MRC Systems), which was directly linked to the EEG recordings, allowing the point of stimulation to be marked on the recordings during acquisition.

4.3.2.3. Experimental tactile stimulation

Tactile stimulation was applied to the infant's heel using a modified tendon hammer with a built-in force transducer (Brüel & Kjær). A trigger pulse at the point of stimulation event-marked the EEG.

4.3.2.4. Visual stimulation

Visual stimulation was delivered to the infants using a flash of light from a light-emitting diode (LED) light (Maxima-84 Hybrid, Manfrotto). The light was held approximately 50cm behind the infant's head and at an angle of around 45° up from the cot, pointing toward the infant. Stimuli were initiated by a push button that also event-marked the EEG recordings within 1ms of the point of stimulation. Infant gaze or sleep state was not controlled during the study.

4.3.2.5. Auditory stimulation

Auditory stimulation was delivered to the infant using single tones of 500 Hz frequency and 100ms duration at a volume of about 80 dB. Tones were delivered using an MP3 player (ZEN StyleM300, Creative) and speakers (X-mini MAX II Portable Speakers,Xmi). The speakers were positioned either side of the infant's head about 5 cm away from each ear. An output trigger pulse from the MP3 player event-marked the EEG recordings within 1ms of the point of stimulation.

4.3.2.6. Cannulation

Infants were cannulated on the dorsum of either hand using a 24G cannula (Neoflon™ Straight IV Cannula, Becton Dickinson UK Ltd) to obtain a venous blood sample. The point of stimulation was time-locked to the EEG recordings using a high-speed camera that was directly linked to the recordings at the time of acquisition. The video recordings were reviewed after acquisition, and the time of stimulation was manually

event-marked as the point where the bevel of the needle was first seen to touch the skin (see Figure 4.1).

Figure 4.1: Images from high-speed video camera during cannulation.
Images from high-speed video camera demonstrating image prior to cannula insertion (t=-2000ms), the point of skin touch (t=0) and cannula causing indentation of the skin (t=900ms). Image quality reduced during still capture, clearer during the time of marking.

Videos were scored by one experienced scorer and then rescored at a later time. Only videos where event-marks were within 50ms of each other were included in the analysis.

4.3.3. Analysis

4.3.3.1. EEG analysis

Epochs with movement artifact in the baseline or gross movement artifact were rejected. The template was then projected, as described in the general methods, using singular value decomposition on to the individual EEG trials recorded at the Cz electrode in the 400-700ms post stimulus interval.

In Studies 1, 2 and 3, Woody filtering of 50ms was applied to allow for inter-individual latency variation. In Study 4, Woody filtering of 100ms was applied to also account for extra variation introduced by the use of manual time locking.

For Study 1, significant noxious-evoked brain activity was defined as being greater than or equal to a magnitude of 0.48. This pre-determined threshold was set at 80% of the distribution of background weights (the magnitude of the template response within the background data) in a previously studied group of infants.

For Study 2, the EEG recorded during multimodal stimulation at other electrode sites, FCz, Cpz, Oz, C3, C4, T3, T4, were averaged without applying Woody filtering to produce a topographic representation of the brain activity recorded.

In Study 5, cluster analysis (206) was used to identify noxious-evoked activity in response to noxious stimulation that was significantly different from the activity recorded during a baseline period at the Cz electrode. A *t* statistic was calculated of the difference in the brain activity evoked by noxious stimulation and during baseline. A significant difference in activity was defined to have occurred when the *t* statistic was above a predefined threshold of the 97.5 percentile of the *t* distribution. The start of a cluster was the earliest time when the above-threshold *t* statistic was reached and the cluster ended when the *t* statistic fell below threshold. The cluster-based test statistic was calculated from 1000 random permutations of the data, and the threshold for cluster significance was set as the 97.5 percentile of the permuted data. Principal component analysis was then utilised to obtain a representative waveform of the

noxious-evoked brain activity, which could be compared to the template of nociceptive brain activity.

4.3.3.2. PIPP-R scoring

In Study 1, facial expression change was quantified using the facial component of the PIPP-R score. The facial PIPP score was calculated using the pre-defined time intervals specified in the PIPP-R. The duration of facial expression was calculated using the 3 facial expression components of the score, with the total duration of any facial expression used.

In Study 4, the total PIPP-R score was calculated.

4.3.4. Statistical methods

In Study 1, linear mixed effects modeling was used to compare brain activity elicited in response to non-noxious and noxious stimulation. The correlation between noxious-evoked brain activity and facial PIPP and duration of facial expression were calculated using linear correlations and Pearson's correlation coefficients.

In Studies 2 and 3, linear mixed effects modeling were used for comparison of brain activity elicited in response to stimulation of the different sensory modalities and in response to stimulation of treated and untreated skin. Stimulus modality and pre-treatment with local anaesthetic were taken as fixed effects and individual subjects were taken as random effects.

In Study 4, a linear correlation was used to compare the morphology of the principal component identified through principal component analysis with the nociceptive template. A linear regression model was used to compare the brain activity in response to cannulation of treated and untreated skin and to determine the effect of corrected gestational age on the magnitude of noxious evoked brain activity. For the two groups of infants undergoing cannulation, an unpaired t-test was used to compare the PIPP-R scores to cannulation of infants with treated and infants with untreated skin.

4.4. Results

The main results presented in this chapter have been published in Hartley, Duff, Green et al., 2017 (185).

4.4.1. Study 1

4.4.1.1. The measurement of noxious-specific brain activity

Noxious-evoked brain activity has previously been demonstrated to occur in response to noxious stimulation but not in response to non-noxious tactile stimulation (20). In this study, the ability of the template to discriminate brain activity evoked by noxious heel lance from that evoked by the non-noxious control procedure and during a background period was tested.

EEG was recorded in a sample of 32 infants aged 34-42 weeks CGA during a period of background, in response to a control heel lance and in response to a clinically required heel lance. The magnitude of noxious evoked-brain activity was quantified using the template. Noxious-evoked brain activity elicited by noxious stimulation, the heel lance, was significantly greater than the response elicited by the non-noxious control heel lance ($p=0.04$) and from the background activity ($p<0.001$). There was no significant difference in the brain activity elicited by control when compared to background activity ($p>0.05$)(Figure 4.2).

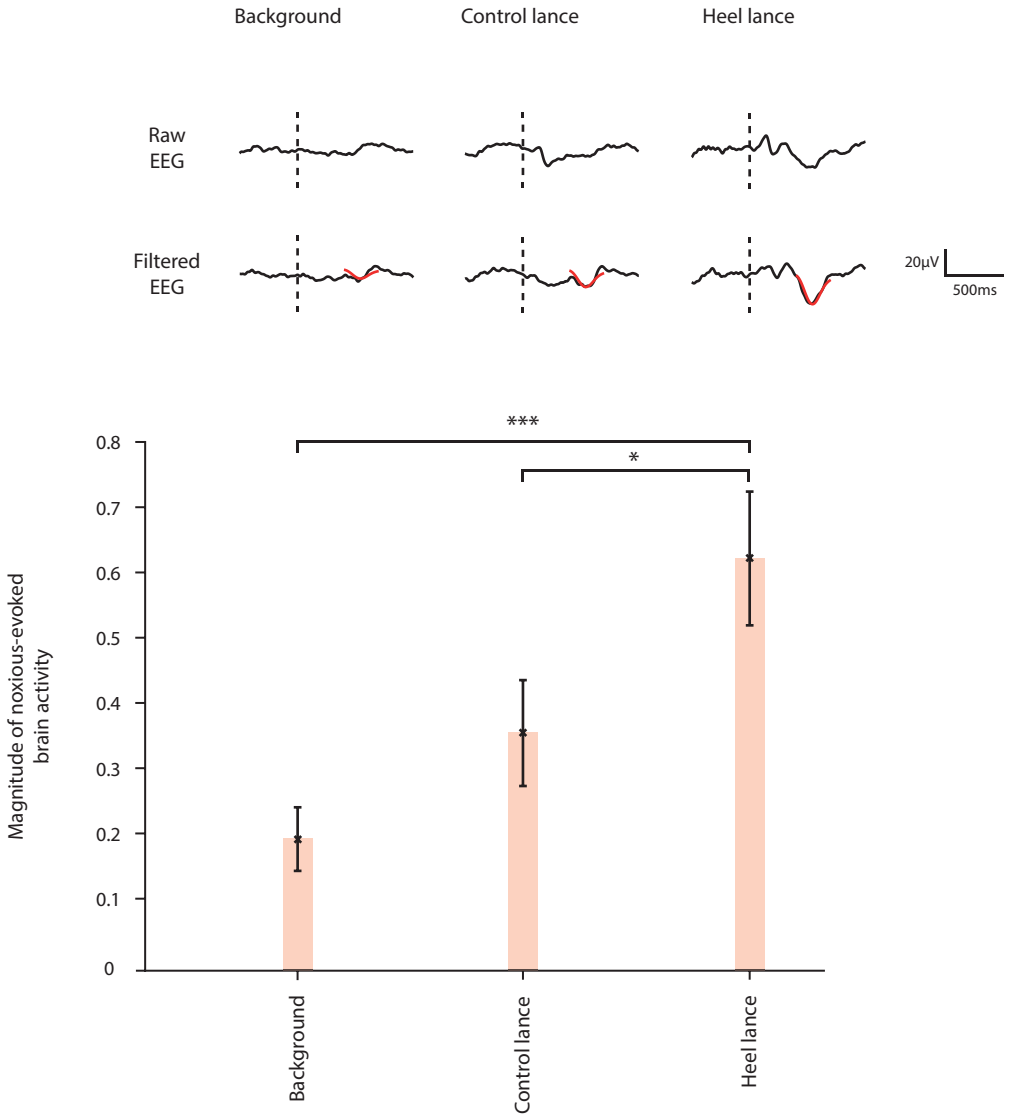

Figure 4.2: Comparison of noxious-evoked brain activity in background and in response to non-noxious and noxious stimulation.
The average background EEG activity at the Cz electrode in 32 infants (from 34-42 weeks CGA) and the average evoked activity after a control heel lance (non-noxious stimulation) and a clinically required heel lance (noxious stimulation) is shown. The raw EEG is shown in the top panel and the EEG with Woody filtering applied is shown below with the template overlaid in red. The magnitude of noxious-evoked brain activity is displayed with mean ± SEM. *** $P < 0.001$ * $P < 0.05$.

4.4.1.2. Noxious-evoked brain activity correlates with pain-related behaviour

Facial expression currently forms the cornerstone of many infant pain assessment tools (108,111) and so, the relationship between noxious-evoked brain activity and pain-related behaviour, assessed using facial expression change, was investigated. The facial expression response to a clinically required heel lance was recorded in the same group of 32 infants and scored using the facial component of the PIPP-R score. The magnitude

of noxious-evoked brain activity was significantly correlated with pain-related behaviour, measured using the facial component of the PIPP-R score (p=0.047, r=0.35) (Figure 4.3). However, as has been previously reported, a proportion of infants, 11/32 (34.3%), did not mount a facial expression response that was of sufficient duration to receive a score of 1 or more. 7/32 (21.9%) infants mounted no facial expression change to the heel lance. Of the infants who had a facial PIPP score of 0, 36% still demonstrated a significant increase in noxious evoked brain activity. Of the infants who mounted no facial expression change, 42% demonstrated a significant increase in brain activity.

The relationship between the magnitude of noxious-evoked brain activity and duration of facial expression showed a similar trend, but did not reach significance (p=0.16, r=0.25) (Figure 4.4), which is likely due to the relatively small sample size.

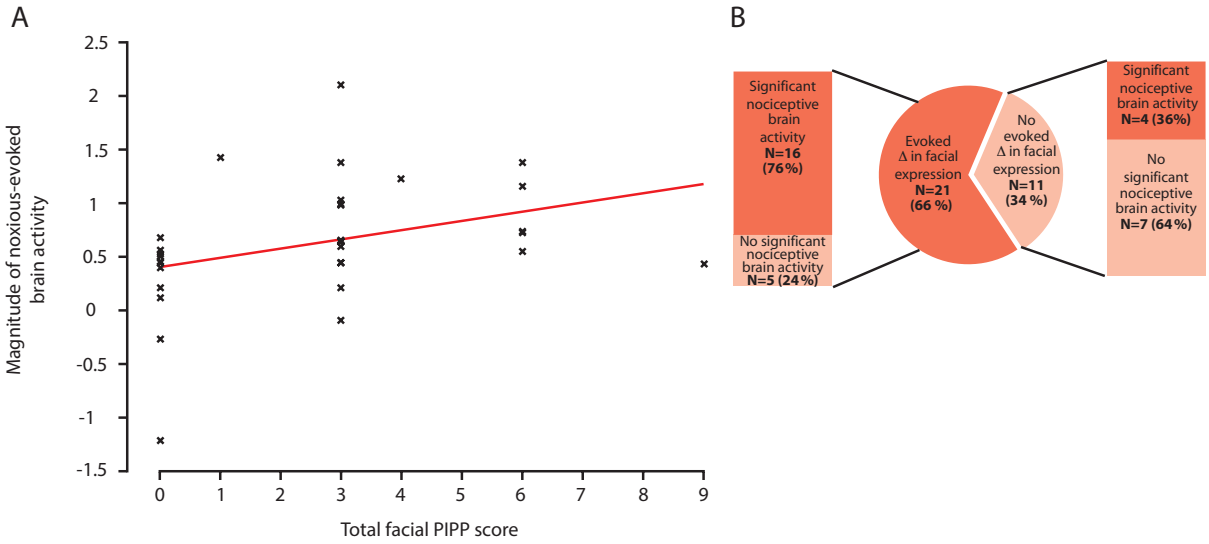

Figure 4.3: Relationship between noxious-evoked brain activity and pain-related behaviour. **(A)** The magnitude of noxious-evoked brain activity is correlated with pain-related behaviour assessed using the total facial PIPP score. **(B)** The proportion of infants with and without an evoked change in facial expression is shown along with the proportion of infants in whom significant brain activity was recorded (see methods).

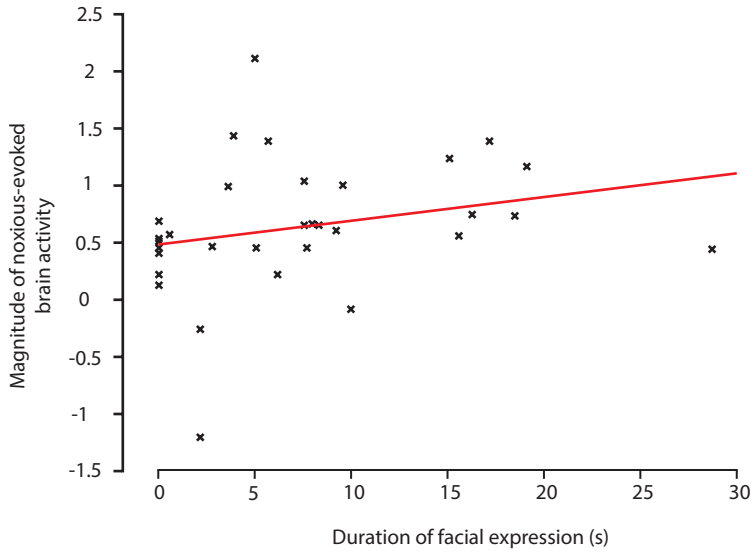

Figure 4.4: Correlation between noxious-evoked brain activity and duration of facial expression. The magnitude of noxious-evoked brain activity and its relationship to pain-related behaviour assessed using duration of facial expression is shown.

4.4.2. Study 2

4.4.2.1. Noxious-evoked brain activity that was significantly different from background was only recorded in response to experimental noxious stimulation

An important step in validating the template was to determine if noxious-evoked brain activity, measured using the template, was only evoked by noxious stimulation and not by stimulation of other sensory modalities. EEG was recorded in 14 term infants during background and in response to stimulation of different sensory modalities: visual, tactile, auditory and noxious. The template was then projected onto the Woody filtered EEG.

Only the brain activity evoked by noxious stimulation was significantly different from background ($p<0.001$) (Figure 4.5). Visual, tactile and auditory stimulation did not evoke brain activity that was significantly different from background ($p>0.05$). Furthermore, the magnitude of noxious evoked brain activity elicited by noxious stimulation was significantly different from all other modalities ($p<0.001$).

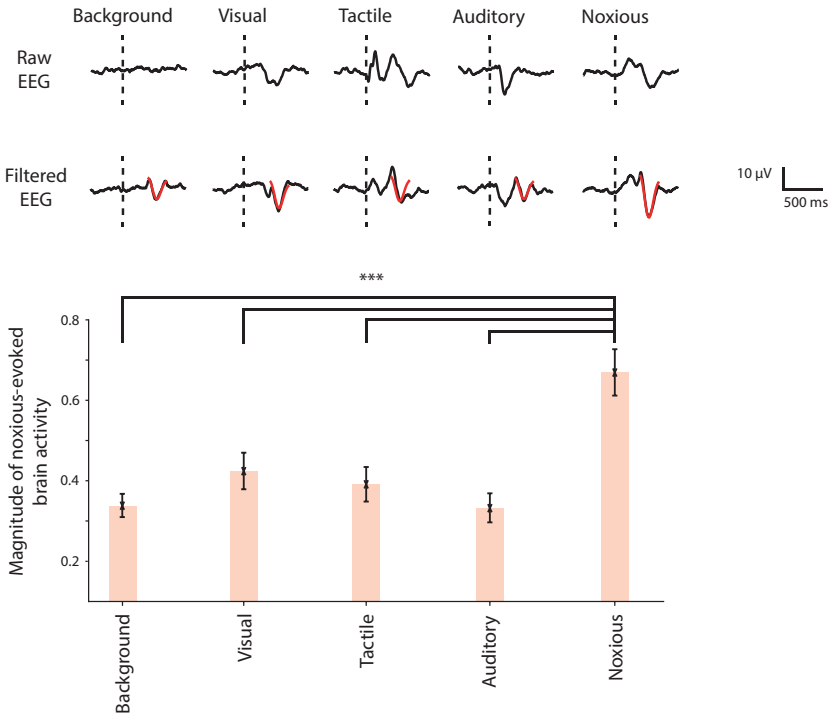

Figure 4.5: Comparison of noxious-evoked brain activity elicited by multi-modal stimulation. The average background EEG activity at the Cz electrode in 14 term infants and the average evoked activity after visual tactile, auditory and experimental noxious stimulation is shown. The raw EEG (without Woody filtering) is shown in the top panel and the filtered EEG is shown beneath with the template overlaid in red. The magnitude of noxious-evoked brain activity is displayed with mean ± SEM. *** $P < 0.001$.

EEG was also recorded at other electrode sites and so the occurrence of modality-specific evoked potentials at other locations was investigated. The average unfiltered EEG in response to all sensory stimuli at the different electrode sites is shown (Figure 4.6).

An evoked potential could be seen in raw EEG recorded at the Oz electrode in response to visual stimulation which is similar in morphology to that described in the literature (207). An earlier evoked potential was recorded in the Cz electrode in response to auditory and tactile stimulation, which has been suggested in infants to represent an arousal response (20). This earlier potential is not clearly seen in response to the

noxious stimulation provided by the experimental noxious stimulus used here. This may be because the experimental noxious stimulus has a contact area of only 0.25mm in diameter and so, is activating only a small number of tactile fibres, compared to the tendon hammer used to provide the tactile stimulation.

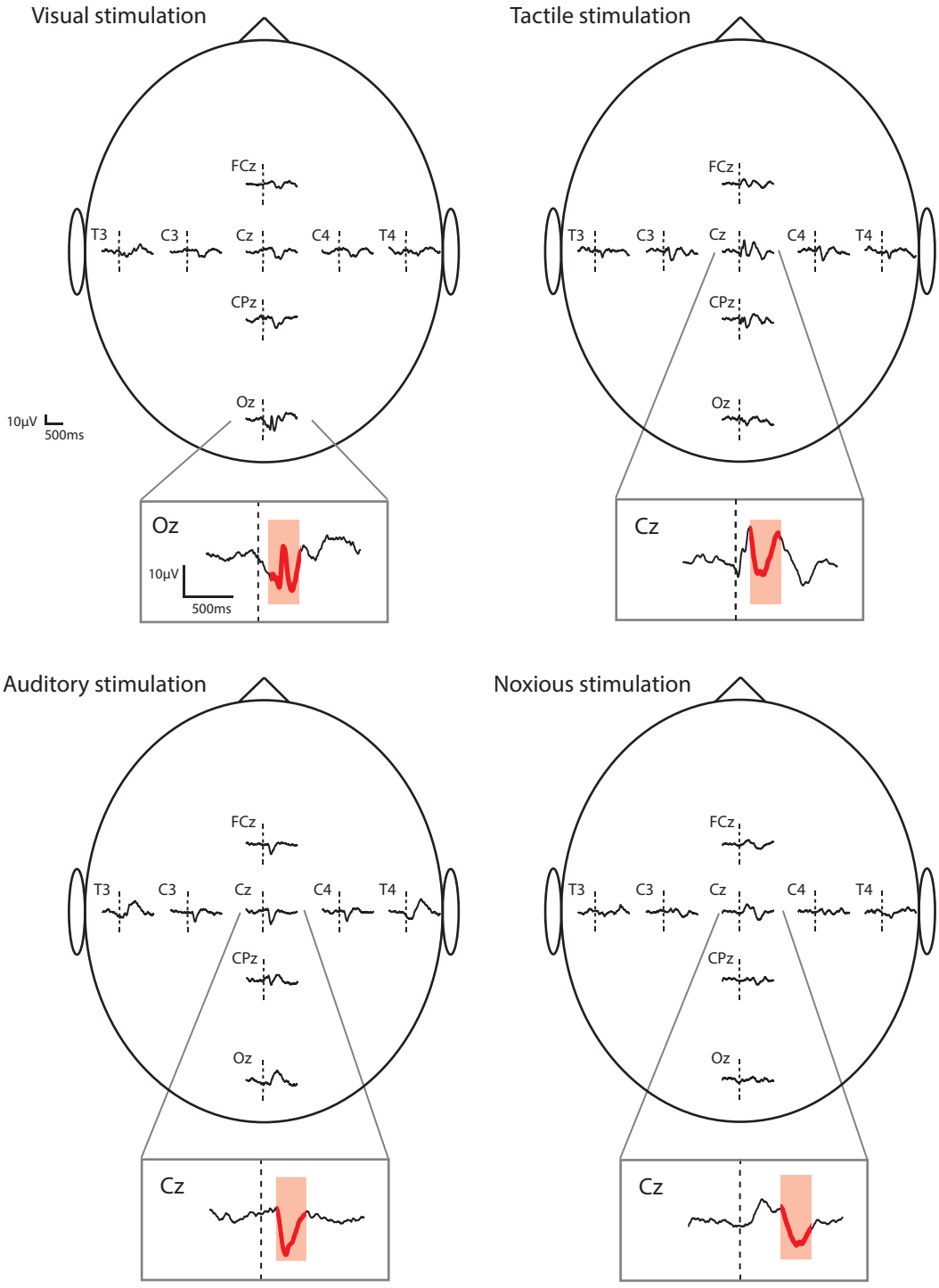

Figure 4.6: Average EEG recorded at different electrode sites in response to the different sensory stimuli.
The unfiltered average EEG for all 12 infants recorded in response to visual, tactile, auditory and noxious stimulation is displayed for 8 electrode sites (FCz, Cz, CPz, Oz, C3, C4, T3, T4). The grey boxes represent the electrode of interest and the red highlighted portion of the trace demonstrates the evoked activity.

4.4.3. Study 3

4.4.3.1. Topical local anaesthetic reduces the magnitude of noxious-evoked brain activity

The template was further validated by demonstrating that the noxious-evoked brain activity recorded was sensitive to modulation. Topical local anaesthetics reversibly block nerve conduction in the skin by targeting superficial free nerve endings (208), by blocking nerve conduction they limit the transmission of nociception from the periphery which should be reflected in a decrease in noxious-evoked brain activity. To test whether this decrease in noxious-evoked brain activity was observed using the template, EEG was recorded in 12 term-born infants during experimental noxious stimulation of skin, to which topical local anaesthetic had or had not been applied, and the noxious-evoked brain activity measured. The magnitude of noxious-evoked brain activity elicited by stimulation of untreated skin was significantly greater than background activity ($p<0.001$) (Figure 4.7). The magnitude of noxious-evoked brain activity elicited by stimulation of treated skin was not significantly different from background activity ($p=0.26$) and was significantly lower than that elicited by stimulation of treated skin ($p=0.002$). One infant received stimulation only of the untreated skin but then became too unsettled to continue. In all but one of the remaining 11 infants, the magnitude of noxious-evoked brain activity elicited by stimulation of treated skin was lower than that recorded in response to stimulation of untreated skin (Figure 4.8).

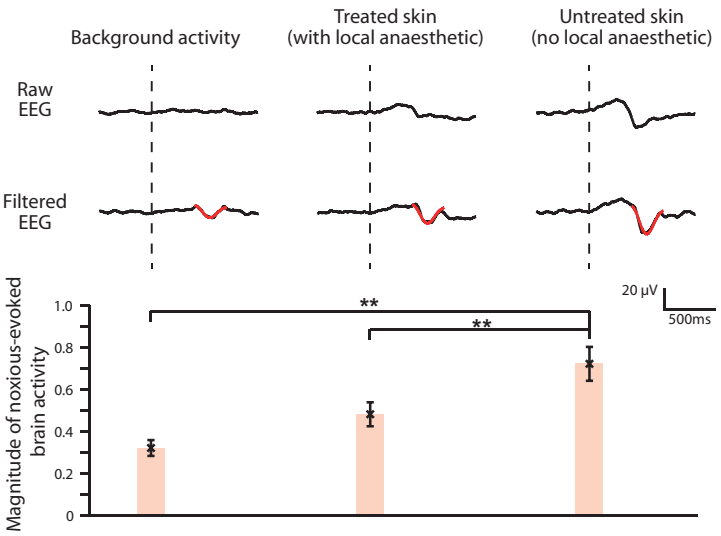

Figure 4.7: The effect of topical local anaesthetic on noxious-evoked brain activity.
The average background EEG activity and noxious-evoked brain activity evoked by stimulation of treated and untreated skin recorded at the Cz electrode in 12 infants is shown. The raw EEG (without Woody filtering) is shown in the top panel and the filtered EEG is shown with the template overlaid in red. The magnitude of the noxious-evoked brain activity in displayed with mean ± SEM. ** $P < 0.01$.

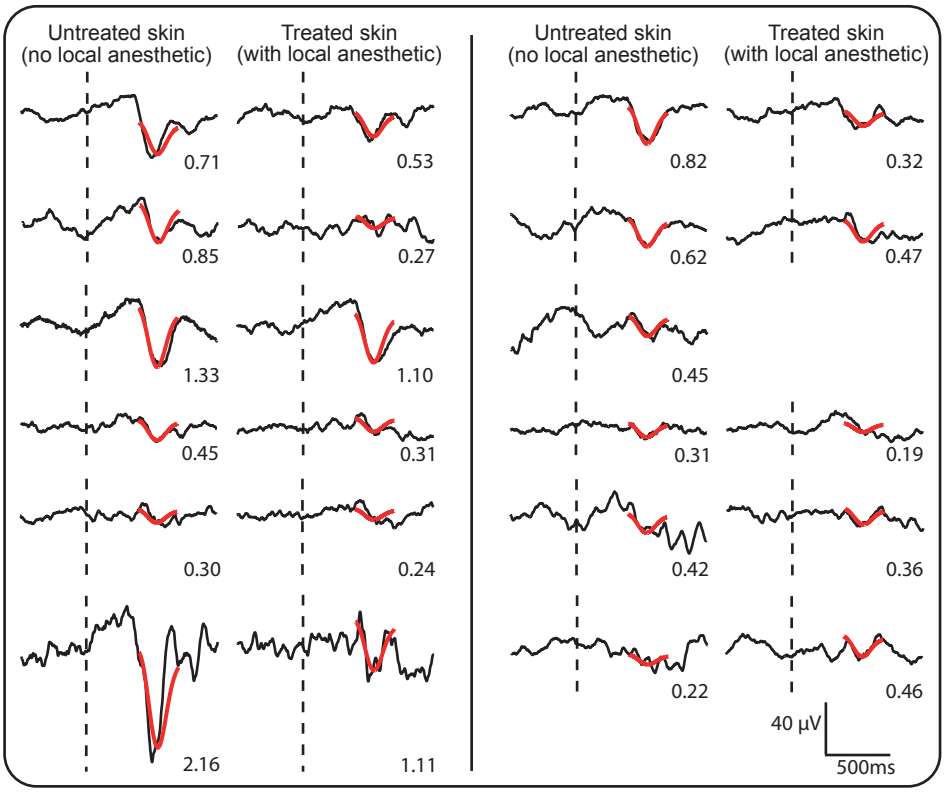

Figure 4.8: Noxious-evoked brain activity in individual infants.
The average filtered EEG recorded for each individual infant during stimulation of untreated and treated skin is shown. The template is overlaid in red and the average magnitude of noxious-evoked brain activity is displayed.

4.4.4. Study 4

4.4.4.1. Characterisation of noxious-evoked brain activity in response to cannulation

The final study aimed to determine whether the same techniques used to define the template of noxious-evoked brain activity in term infants in response to heel lance and experimental noxious stimulation could be applied to characterise the activity seen in response to a previously unstudied stimulus, cannulation, and how this pattern of brain activity compared to the template. The effect of topical local anaesthetic was then investigated.

EEG was recorded during insertion of a cannula, to obtain a blood sample, in a group of 13 term-born infants at around 3-4 weeks of age. EEG recordings, which could be reliably time-locked to the high-speed video recording, were obtained in 12 of these infants. The pattern of brain activity elicited by cannulation was identified by comparing the pattern of brain activity elicited by insertion of the cannula with background activity at the Cz electrode. Woody filtering of up to 100ms was used to account for both the inter-individual latency variation and for variation introduced by the manual time-locking of the video to the EEG (described in Methods). Noxious-evoked activity was identified that was significantly different from baseline in the time window 328 to 583ms after stimulation ($p=0.001$, cluster-corrected non-parametric test) (Figure 4.9).

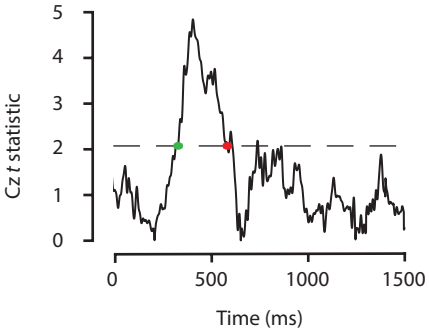

Figure 4.9: Time course of the *t* statistic.
The time course of the *t* statistic in the 1000ms period after stimulation at the Cz electrode comparing the response to cannulation with background activity is shown. The dashed line represents the *t* statistic threshold. The green mark indicates the time point at which the *t* statistic threshold was reached and the red mark indicates the time point at which the *t* statistic fell below threshold.

Principal component analysis was applied in the time window 300 to 600ms in order to obtain a waveform of the noxious-evoked brain activity that could be compared to the template of nociceptive brain activity. The first principal component did not differ between background activity and that evoked by cannulation. The second principal component identified showed a trend suggestive of a difference between background activity and the brain activity evoked by insertion of the cannula ($p=0.054$). The first two principal components accounted for 88% of the variance so the other principal components were not considered. The waveform identified in the second principal component was highly correlated with the template of nociceptive activity ($p<0.001$, $r=0.88$) (Figure 4.10). This high degree of correlation suggested that the pattern of noxious-evoked brain activity was highly conserved between the different noxious stimuli and that the template could also be used to quantify the response to cannulation but projected on to the data in an earlier time window to take into account the shorter latency observed. The template of noxious-evoked brain activity was therefore projected on to the EEG recorded in response to cannulation in the modified 300 to 600ms time window.

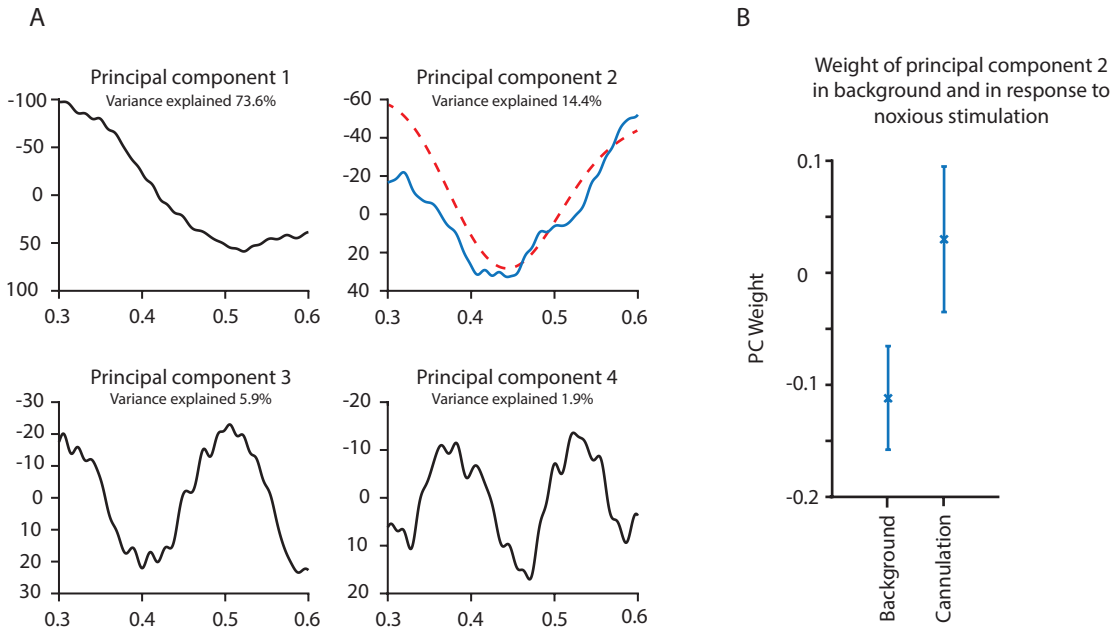

Figure 4.10: Principal component analysis.
(A) The first four principal components identified by principal component analysis in the 300-600ms window of interest are shown. The waveform of the template of noxious-evoked brain activity (red dashed line) is overlaid with the waveform of the second principal component (blue line). **(B)** The weight of second principal component was compared between the background activity and the brain activity evoked by cannulation.

4.4.4.2. Effect of topical local anaesthetic

The effect of topical local anaesthetic on the noxious-evoked brain activity recorded in response to insertion of a cannula to obtain a venous blood sample was investigated. 13 term-born infants were studied during the insertion of a cannula into skin treated with topical local anaesthetic. 11 infants had EEG recordings obtained which could be reliably time-locked to the high-speed video recording of the event. These were compared to the 12 infants who were studied during cannulation of untreated skin. The template of nociceptive brain activity was projected onto the EEG data obtained in the 300 to 600ms time window to quantify the magnitude of the response. There was no significant difference between the magnitude of noxious-evoked brain activity,

corrected for gestational age, measured in response to cannulation of treated or untreated skin (p=0.65, linear regression model)(Figure 4.11).

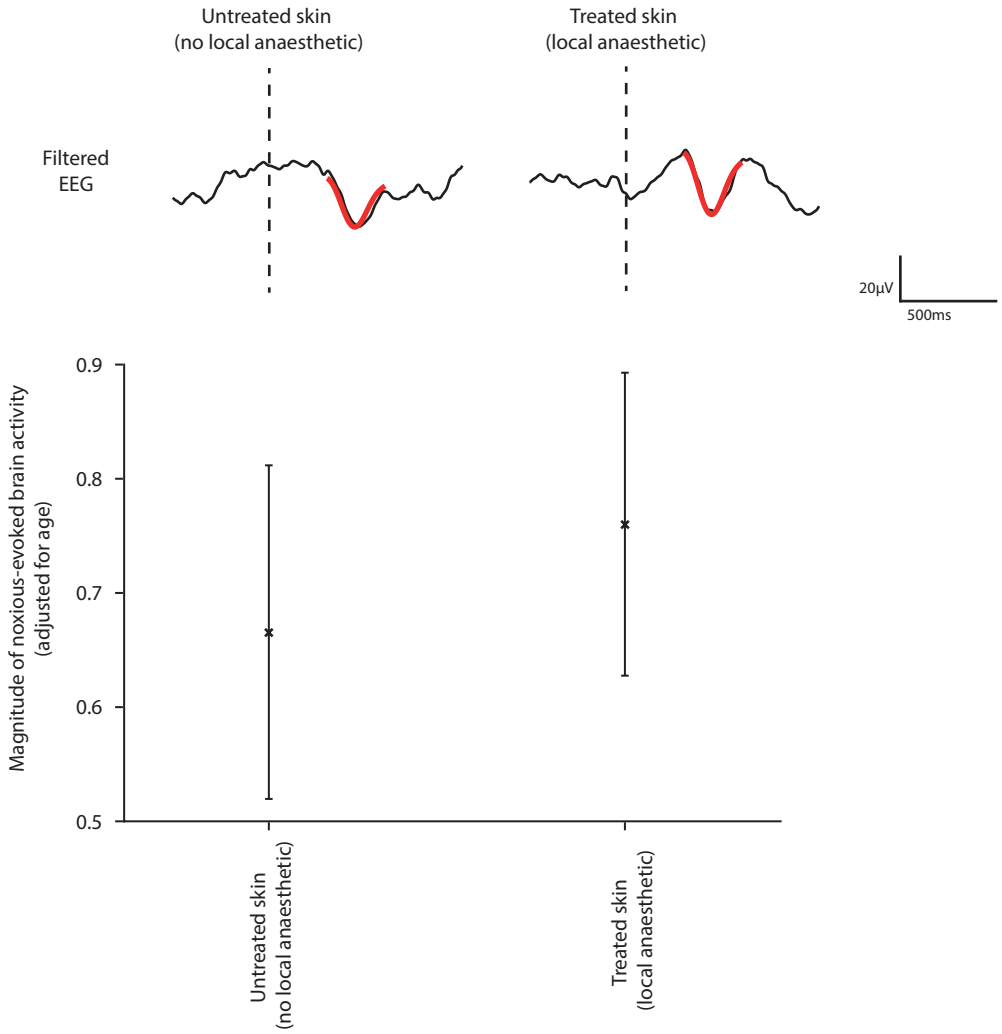

Figure 4.11: The effect of topical local anaesthetic on noxious-evoked brain activity elicited by cannulation.
The average filtered EEG for cannulation of untreated and treated skin is shown. The template is overlaid in red in the 300-600ms time window. The magnitude of noxious-evoked brain activity is displayed with mean ± SEM.

The total PIPP-R scores for cannulation were calculated for both groups of infants (untreated, n=12, treated, n=13). One infant was excluded from the untreated group as they were crying in the baseline period. There was no significant difference in the PIPP-R scores between the groups (p=0.19) (Figure 4.12). The mean PIPP-R score for

cannulation of untreated skin was 10.9±3.6 (mean ± SD) and 8.8±4.1 for cannulation of treated skin.

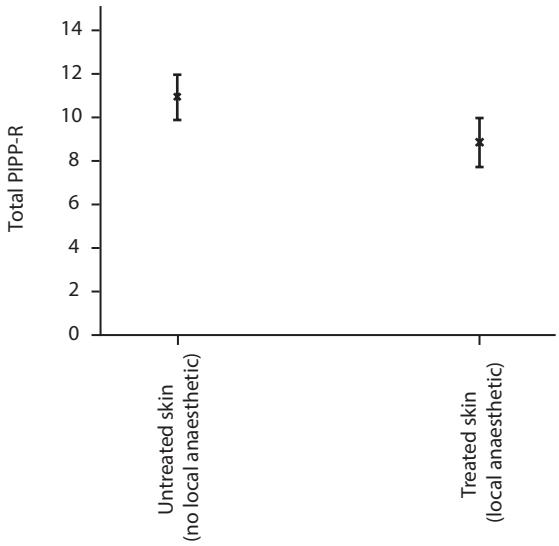

Figure 4.12: Comparison of total PIPP-R score recorded in response to cannulation of untreated and treated skin.
The total PIPP-R score is displayed with mean ± SEM.

4.4.4.3. Noxious-evoked brain activity is correlated with gestational age

Noxious-evoked brain activity to heel lance has previously been shown to correlate with gestational age in infants aged from 32 to 42 weeks CGA (73). Here I demonstrate that the noxious-evoked brain activity is significantly correlated with gestational age in infants at an older age as well, from 40 up to 46 weeks CGA ($p=0.0045$, $r = 0.57$)(Figure 4.13). This significant correlation is not present in the background activity. The correlation between noxious-evoked brain activity, as measured by the template, and gestational age supports the use of the template to measure the response to this novel, clinically important stimulus.

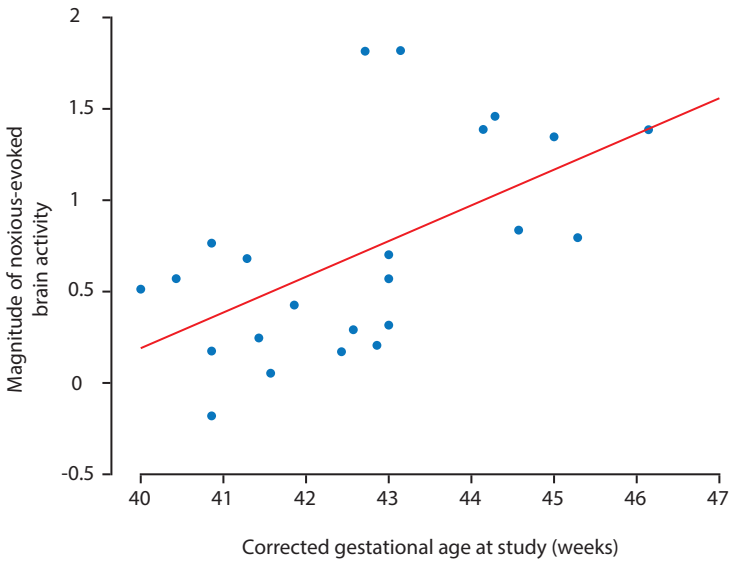

Figure 4.13: The relationship between noxious-evoked brain activity and corrected gestational age.
The magnitude of noxious-evoked brain activity is correlated with the corrected gestational age of the infant at the time of study.

4.5. Discussion

This chapter demonstrates the validation of a template of noxious-evoked brain activity across a wide range of studies. The template is used to demonstrate the correlation between noxious-evoked brain activity and pain related behaviour. The template is also shown to distinguish brain activity evoked by noxious stimulation from that evoked by stimulation of other sensory modalities and to be sensitive to modulation. Finally, the response to cannulation, which has not previously been studied using noxious-evoked brain activity, is shown to correlate closely with brain-activity defined by the template.

4.5.1. Noxious-evoked brain activity correlates with pain-related behaviour

Pain-related behaviour currently forms the cornerstone of most pain scores used in infants both clinically and in the research setting (107). The use of behavioural measures is therefore important. It is demonstrated here that a novel template of nociceptive brain activity developed in infants, correlates with pain-related behaviour. While the use of behavioural measures is important, these measures do have limitations. Previous work has reported that a substantial number of infants will mount no behavioural response to a well-described painful stimulus, such as a heel lance despite demonstrating an evoked brain responses (21). This work by Slater et al. recording both brain activity and behavioural responses has suggested that a reliance on behavioural scores alone will underestimate the infant's experience of pain (21). These previous findings were replicated here, with a number of infants displaying evoked brain activity to a heel lance without mounting a behavioural response. While the absence of a behavioural response does not preclude the possibility that an infant is

experiencing pain, the presence of a behavioural response does not confirm it either. A major limitation of behavioural pain scores is that they are unable to differentiate pain and distress, for example, infants undergoing a non-noxious but potentially distressing diaper change, may display behaviours which are not dissimilar from those displayed by infants undergoing painful procedures such as heel lancing or venipuncture (120). This is particularly the case in the most premature infants who may lack this ability to behaviourally discriminate between non-noxious and noxious touch, as demonstrated in the previous chapter. However, it also needs to be emphasised that the presence of noxious-evoked brain activity does not confirm that an infant is experiencing pain either. Pain is a complex sensory and emotional experience, which is the result of complex peripheral and central processes, and noxious-evoked brain activity reflects only one aspect of this complex process.

4.5.2. Objective quantification of noxious-evoked brain activity

The template of nociceptive brain activity was characterised using the response to a heel lance and to an experimentally noxious stimulus (185). Here it is demonstrated that this noxious-evoked brain activity is only seen in response to noxious stimulation and not in response to stimulation of other sensory modalities or in background activity. It is also demonstrated that the same approach used to define the template, the use of cluster analysis and PCA, can be used to characterise the response to another more complex noxious clinical procedure: the insertion of a peripheral venous cannula. The response to cannulation characterised using these techniques was highly correlated with the original template. The latency of the response seen was decreased in the older

group of infants studied during cannulation, occurring in the 300-600ms time window. There are number of possible explanations for this, including the increased maturity of the infants, the different location of the stimulus, hand as compared to foot, the different stimulus used and the different technique of marking the point of stimulation. The applicability of the template to this previously unstudied procedure underscores its potential as a standardised, translatable marker of noxious-evoked brain activity.

Noxious-evoked brain activity recorded at the Cz electrode has previously been used to quantify the response to noxious stimulation of the foot (73,164). Similar vertex potentials have been recorded in adults undergoing noxious stimulation of the hand (209). Here it is demonstrated that noxious stimulation of the hand in infants also elicits a vertex potential. Previous work in adults using magnetencephalography has demonstrated that there is also somatotopic representation of noxious-evoked activity dependent on the site of stimulation (210) with activity seen in areas of the primary somatosensory cortex representing the different body regions. EEG lacks the spatial resolution of MEG and MRI, but it would be interesting to investigate further whether there is also a lateralised response seen depending on the site of stimulation. This would certainly be an important avenue of further work, particularly if similar techniques are used to characterise the response to other clinically important noxious procedures, such as lumbar puncture and intubation, where noxious-evoked brain activity might reasonably be expected to occur at different locations as well as at the vertex.

The template was defined in a group of term infants between 37 and 42 weeks corrected gestation and has also been shown to be applicable to infants down to 34

weeks CGA (185). Significant neurodevelopment occurs in the final trimester of pregnancy and in the weeks after birth and changes to the magnitude and latency of brain responses have been well described (151,152,154,211). Here it is demonstrated that the template of nociceptive brain activity can also quantify the response seen in older infants, up to 46 weeks' CGA, although with a decreased latency. Decreasing latency to evoked potentials with development in infancy is well described for other evoked potentials (151,152,154,211) and a decreasing latency to the cortical haemodynamic response to noxious stimulation across gestation has also been described (21). That this template can be used across a range of gestational ages in order to quantify the EEG response to a number of clinically required noxious procedures increases its usefulness going forward, particularly its potential for use in testing analgesics in this population (14).

4.5.3. The effect of local anaesthetic

Topical local anaesthetics are widely used in paediatric practice to reduce pain from needle procedures such as cannulation and venipuncture (212). In infants, evidence for their analgesic efficacy is conflicting, with some studies suggesting that they are effective for venipuncture (172,173), while others show a lack of analgesic efficacy for this same procedure (177). While topical local anaesthetics have been shown to be effective in reducing the pain associated with needle insertion during lumbar puncture (174), no benefit has been described for their use in ameliorating pain responses associated with intramuscular injection (175,176). A major limitation of this previous work is that these studies rely on surrogate markers of the pain experience, such as changes in physiology and behaviour, as the gold standard, subjective report, is not

available in infants. Here it is demonstrated that topical local anaesthetic significantly decreases the noxious-evoked brain activity associated with stimulation with an experimentally noxious stimulus. Furthermore, in 10 of 11 infants where brain activity was recorded in response to stimulation of both treated and untreated skin, the magnitude of brain activity measured was lower for stimulation of treated skin. In contrast, a change in noxious-evoked brain activity was not demonstrated when topical local anaesthetic was used before cannulation in this pilot study.

It is possible to see from the individual responses, in Figure 4.8, that the magnitude of the noxious-evoked response shows a great deal of variation between individuals. A major advantage of using an experimental noxious stimulus is that, since it does not evoke a behavioural response and is not tissue damaging, it can be performed a number of times in the same individual. This is particularly beneficial when testing different interventions, topical local anaesthetic for example, as the response in untreated skin can be compared to that in treated skin to allow for each individual to act as their own control. This ability to control for the wide degree of inter-individual variation in response size enables effects to be seen in relatively small sample sizes. Conversely, a major limitation of using a clinical procedure, such as cannulation, is that it would be unethical to perform the procedure more than once for the sake of research so it is not possible to have this intra-individual control. This is a possible explanation for the failure to demonstrate a decrease in noxious-evoked brain activity when topical local anaesthetic was applied prior to cannulation. It could be that, by chance, the individuals in whom the skin was pre-treated were those in whom a greater baseline response would have been recorded. A potential solution to this problem would be to introduce the use of an experimental noxious stimulus before cannulation in order to act as a

gauge of the responsiveness of an individual to allow variation in response size to be controlled for.

The experimental noxious stimulus used in Study 3 is of low intensity, evidenced both by the lower magnitude of brain activity evoked as compared to a skin breaking procedure such as a heel lance, and also by the lack of behavioural response seen (164). This lower intensity may explain why the effect of topical local anaesthetic seen was so convincing in the experimental setting and not when cannulation was investigated. Topical local anaesthetic only acts superficially so it could be that when the more penetrating stimulus of a cannula was used, the noxious stimulus penetrated into deeper tissues, which were not anaesthetised.

No significant difference was detected in the PIPP-R scores recorded after cannulation of treated or untreated skin, which is in keeping with some previously published work (177). Cannulation requires restraint of the infant's limb in unfamiliar surroundings, both of which are likely to be a cause of distress to the infant. The lack of a difference reported here and previously, may suggest that what is being measured by the PIPP-R score is in fact, distress rather than pain, and delineating which, is a challenge. Interestingly, there is the suggestion that the infants who were treated with local anaesthetic had a lower average PIPP-R score (8.8 vs 10.9) than those who were not. One possible explanation for this is that, the topical local anaesthetic was decreasing the pain experienced by these infants, and that this is reflected in the PIPP-R score better than the brain activity for the reasons discussed. Another possible explanation is that infants who received treatment with topical local anaesthetic cream needed to wait between their initial examination and blood taking procedure in order to allow time for

the cream to work and so, had received less handling in the period immediately before blood was taken. Previous work, in premature infants has demonstrated that handling prior to noxious procedures heightens pain responses (202).

A further difference is that infants who were waiting for the topical local anaesthetic to take effect were often fed in the intervening period and so, were more likely to have been fed shortly before the procedure. The analgesic effect of breastfeeding during painful procedures in older infants was recently described in a Cochrane review (213), but the impact of time since last feed has not been investigated. Since hunger is likely to cause an infant to be more unsettled and is a major reason why an infant might cry, it is feasible that having been recently fed may decrease behavioural pain responses, although a greater number of infants would need to be studied to determine if this is a real trend or a factor of small group numbers. This is another factor which would be important to investigate, particularly in the context of pain in the neonatal unit, where timing procedures with feeds is sometimes possible and may be a straightforward way of limiting distress from procedures.

4.5.4. Strengths and limitations

This set of studies validates a novel template of noxious-evoked brain activity in a number of settings. The use of experimental noxious stimuli and heel lance as stimuli for recording noxious-evoked brain activity in infants is well described (20,73,164). The demonstration that this novel template can be used for both stimuli is important since both have their advantages and limitations. The use of experimental noxious stimulation is beneficial since its low intensity means that it is possible for multiple stimulations to be applied in an individual, and, as with the local anaesthetic study,

allows an individual to act as his or her own control. However, this low intensity means that it does not accurately reflect the experience of a clinical, tissue-breaking procedure, such as heel lancing or cannulation, suggesting that results observed cannot always be replicated in the clinical setting, as occurred here. The lack of a behavioural response also means that this important aspect of pain assessment cannot be used. In order to assess analgesic efficacy therefore, it is important to be able to use clinical procedures. Here a major limitation is that, ethically, these can only be performed once, requiring much larger sample sizes to observe an effect.

Pain is a complex, sensory and emotional experience in which, even in infants, context is important. There are a number of factors that may therefore influence the experience of pain, which are difficult to control for in small sample sizes. Furthermore, ensuring first and foremost that an infant is not experiencing undue pain or distress means that it is not ethical to control for all potential confounding factors, for example, the use of pacifiers has been demonstrated to reduce behavioural pain scores (214) and many parents choose to offer these to their infants during painful procedures while others do not. Other factors, such as mode of delivery, have been suggested to modulate the infant's pain response (215) but again, in sample sizes of 10-20 it is not possible to control for this.

It is important to consider the limitations of the template itself. While the template has been validated in infants from 34-42 weeks gestational age, it has not been validated for use in infants outside this age range, and so could not be used in these infants without further validation. In infants younger than 34 weeks' gestation, it is unlikely that the template described here would be applicable since, as discussed previously, the

noxious-specific evoked potential that the template characterises does not appear to emerge until around 34 weeks' gestation (142). However, it is possible in older infants that the pattern of activity would be conserved but would simply demonstrate a shorter latency as is seen for other sensory evoked potentials (151). This could be investigated further by using a similar approach to the cannulation study described above to determine the change in latency in older infants. Additionally, the template was characterised in response to heel lance and experimental noxious stimulation, both of which are acute noxious events. It can't therefore be used to assess the response to more prolonged painful events, such as retinopathy of prematurity screening, or pain in the more chronic setting, such as post-operatively. Time-frequency analysis might be a useful way to explore this problem, for example, in adult studies it has been shown that a decrease in alpha activity and an increase in beta activity are observed in tonic pain (216). Furthermore, EEG lacks the spatial resolution to be able to determine the source of the signal but the addition of an imaging modality might be useful to address this. Recent work by Arichi et al. has demonstrated the potential of simultaneous EEG-fMRI to help localise the source of EEG activity in infants (217). A similar approach could be used here to elucidate the origin of the noxious-evoked brain activity.

4.6. Conclusion

In this chapter, it has been demonstrated that a novel template of nociceptive brain activity can be used to quantify noxious-evoked brain activity. The template shows correlation with pain-related behaviour, the most commonly used current measure of infant pain. Noxious-evoked brain activity is only recorded in response to noxious stimulation and not in response to stimulation of other sensory modalities. The noxious-evoked brain activity measured using the template is modulated by the use of topical local anaesthetic in the research setting. Furthermore, the same methodology used to define the template of nociceptive brain activity in response to heel lance and experimental noxious stimulation, can be used to characterise the response to other clinical procedures. This template is likely to provide a useful, objective measure of noxious-evoked brain activity that can be utilised in the assessment of infant pain in the research setting. However, it is important to emphasise that noxious-evoked brain activity and the template validated here to quantify it, only represents one aspect of the response of an infant to a noxious input. The difficulty in assessing pain in infants without a true 'gold standard' for comparison necessarily limits what can be inferred from any surrogate measure, including the template presented here. Hence, a multi-dimensional approach should be used.

Chapter 5

The impact of early onset neonatal infection on pain responses in the newborn infant

5.1. Introduction

Early onset neonatal bacterial infection is defined as infection occurring in the first 72 hours of life. Infection can lead to sepsis, that is, life-threatening organ dysfunction caused by the host's response to infection (218), which is an important cause of morbidity and mortality in neonates (219). Early detection and treatment of infection is therefore important, consequently, in 2012, the National Institute for Clinical Excellence (NICE) produced a guideline that standardised the management of this condition in the UK and changed the way that newborns were investigated and treated (220). Early onset neonatal infection is usually acquired vertically, shortly before or during delivery, and is most often due to bacteria present in the maternal genital tract (221). The likelihood of transmission of bacteria and infection is increased in cases of preterm or prolonged rupture of membranes (222), which form a physical barrier to the transmission of infection, and also by the presence of known pathogens, such as group B streptococcus, colonising the maternal genital tract (223).

One of the main difficulties in identifying neonatal infection is that, in neonates, infection often presents subtly with non-specific signs such as raised respiratory rate, which can also be seen in other non-infectious conditions such as transient tachypnea of

the newborn (224). The NICE guidelines use the presence of these known risk factors in pregnancy and delivery, and clinical indicators in the neonate, to identify those who are at increased risk of infection. Infants are then stratified into those who need to be monitored more closely or those who need to be investigated and commenced on empiric antibiotic treatment. Examples of the risk factors and clinical indicators used are summarised in Table 5.1.

Risk factors	**Clinical indicators**
Invasive group B streptococcal infection in a previous baby	Altered behavior or responsiveness
Maternal group B streptococcal colonization	Feeding difficulties (for example, feed refusal)
Prelabour rupture of membranes	Signs of respiratory distress
Preterm birth following spontaneous labour	Respiratory distress starting more than 4 hours after birth*
Intrapartum fever high than 38^{o}C, or confirmed or suspected chorioamnionitis	Hypoxia
	Jaundice within 24 hours of birth
Parenteral antibiotic treatment given to the woman for confirmed bacterial infection (such as septicaemia) at any time during labour, or in the 24-hour periods before and after the birth*	Temperature abnormality (<36^{o}C or >38^{o}C) unexplained by environment
	Seizures*
	Signs of shock*
Suspected or confirmed infection in another baby in the case of multiple pregnancy*	Need for mechanical ventilation in a term baby*

Table 5.1: Examples of the risk factors for, and clinical indicators of, early onset neonatal infection. Adapted from NICE guideline (220). * indicates a 'red flag' feature which if present suggests need for commencement of antibiotic therapy.

Generally, infants who are identified as having only one risk factor or clinical indicator of infection will be observed and have their vital signs monitored for a period of time. Infants who have two or more risk factors or clinical indicators, or any 'red flags', will be commenced on intravenous antibiotics and have blood samples taken for blood culture and for measurement of C-reactive protein (CRP). CRP is an acute phase protein whose production by hepatocytes is stimulated by pro-inflammatory cytokines, which are released in response to infection, inflammation and injury (225). There is a time lag in the production of CRP in the inflammatory response to infection, with CRP first detectable several hours after the initial insult, and levels peaking after around 36 hours (226). CRP does not cross the placenta, so the levels measured in the infant reflect the infant's own immune response and not that of the mother (227). Hence, NICE guidelines recommend that CRP be measured when the infant is commenced on antibiotics and 18-24 hours later (220). Based on the result of these investigations, as well as the clinical assessment, antibiotics are continued or discontinued. This timeline is summarised in Figure 5.1.

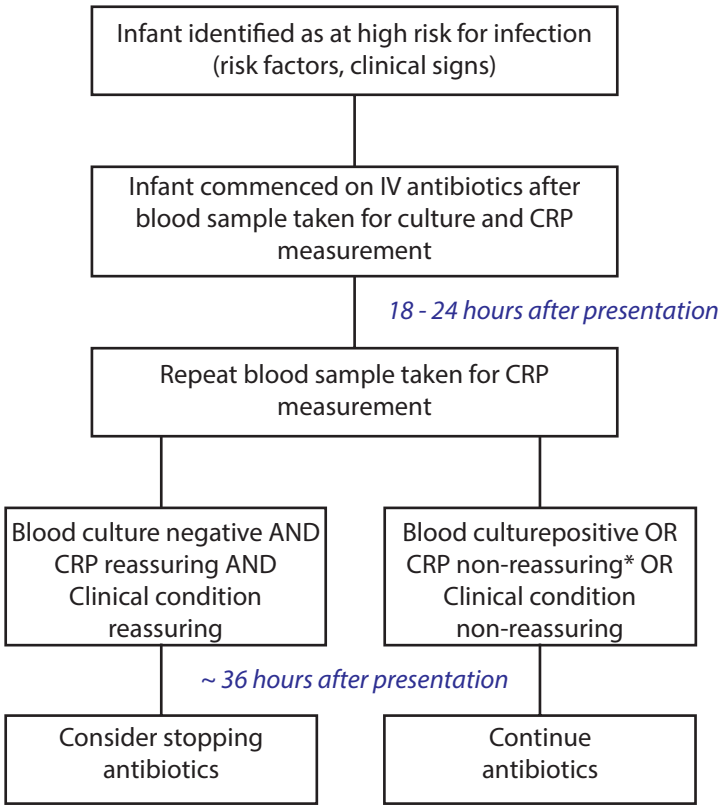

Figure 5.1: Timeline of investigations for early onset neonatal infection recommended in NICE guideline.
* CRP of > 10mg/L is generally considered non-reassuring.

The current NICE guidance results in many more infants being screened for infection than are found to be infected and require a longer course of antibiotics; approximately 1 in 10 live born infants are screened for infection (30) and around 1 in 3 of these infants, approximately 1 in 30 live born infants, receive a minimum of 5 days antibiotic treatment (228). The proportion of infants who are found to have a positive blood culture is very much smaller still, occurring in approximately 1 in 1000 live births (229).

Certain patterns of behavior have been described in 'sick' animals and people, particularly in the context of infection, comprising lethargy, depression, anorexia, reduction in grooming and hyperalgesia (230–232). These patterns of behaviour are thought to represent a concerted defense mechanism by the individual to conserve resources and to overcome disease (230). A similar pattern of behaviour is seen in the 'sick' neonate with poor feeding, irritability or inconsolability and lethargy all being described as important clinical indicators of serious disease (233,234). This 'sickness behaviour' is elicited by the release of pro-inflammatory cytokines that are released in response to infection (235). In animal studies, 'sickness behaviour' can be induced by administration of pro-inflammatory cytokines in the absence of infection (236) and blocked by prior treatment with cytokine receptor anatagonists (237). How the centrally mediated changes seen in 'sickness behaviour' are stimulated by peripheral production of cytokines is still under investigation, but increasing evidence suggests that the vagus nerve is responsible (reviewed in Dantzer et al., 2000 (238)).

Hyperalgesia has been argued to be an important component of sickness behaviour (231). In rat studies, peripheral administration of lipopolysaccharide (LPS), a

component of the outer membrane of gram-negative bacteria and potent activator of the innate immune system, produced both fever and an increase in nociceptive responsiveness, as measured by a decrease in tail flick latency to a noxious heat stimulus (27). This increase in nociceptive responsiveness is thought to be mediated by cytokines, since it is blocked by administration of cytokine receptor antagonists (239). Hyperalgesia in response to immune activation has also been described in adult studies. In healthy adult volunteers, injection of LPS has been shown to increase levels of inflammatory cytokines and to decrease both somatic and visceral pain thresholds, providing further evidence for the importance role of the immune system in pain modulation (26,240). Injection of LPS has also been shown to increase pain sensitivity with a paralleled decrease in activity in brain regions known to be involved in descending pain inhibition (241), suggesting a central mechanism of action. It has also been demonstrated that low grade infection or inflammation, as measured by subclinical elevations in CRP, is associated with increased pain sensitivity (242,243).

In infants, little is known about the impact of infection on pain responses. Recent work has demonstrated that increased levels of stress, as measured by salivary cortisol, increase nociceptive cortical brain responses to a painful stimulus (244). In the context of infection, activation of the HPA-axis is stimulated by pro-inflammatory cytokine release (245), thus, it seems likely that infection too would increase nociceptive cortical brain responses, which would also be in keeping with the findings from animal and adult studies. Infants with infection often undergo a number of painful procedures over the course of their treatment, including venipuncture and heel lancing, and some will require lumbar punctures. If infection increases pain responses then provision of analgesia for these procedures would be more important, especially since behavioural

responses may be dampened if the infant displays lethargy (233,234). Furthermore, work by Taddio et al. has suggested that even a single painful procedure in the neonatal period, circumcision, can lead to increased pain responses later in infancy (246), which are diminished by the provision of adequate analgesia (247), highlighting the importance of adequately assessing and treating early life pain.

In this study, responses to a clinically required heel lance were recorded in a group of infants who were receiving intravenous antibiotics for suspected early onset infection. Infants were studied 18 – 24 hours after commencing antibiotics when they required a heel lance to obtain a blood sample to measure CRP levels. Noxious-evoked brain activity, reflex withdrawal and PIPP-R scores were measured to characterise the response to the noxious heel lance. It was hypothesised that infants with infection would display increased noxious-evoked brain activity to the heel lance. Behaviourally, infants with infection can display both irritability and lethargy. Irritable infants will be hyper responsive to innocuous stimuli and may cry inconsolably, in contrast, lethargic infants may be poorly responsive to even quite salient inputs, hence predicting a general trend in responses is difficult. However, since behaviour has previously been reported to correlate with evoked brain activity (185), and noxious evoked brain activity was hypothesised to be increased, one might expect that behavioural responses would also be increased.

5.2. Methods

5.2.1. Participating infants

Eligible infants were between 36 and 42 weeks CGA at the time of study. All infants had been commenced on IV antibiotics because of risk factors for, or clinical indicators of, early onset neonatal infection (see Table 5.1). Infants were studied approximately 18-24 hours after commencing IV antibiotics, and all infants were less than 72 hours old at the time of study. After the study was complete, infants were classified as infected or not based on blood culture results, CRP levels and the need to complete a minimum of 5 days IV antibiotics. No infant had a positive blood culture result. 11 infants received 5 days IV antibiotics, however, two of these had normal CRPs and so were excluded. The 9 infected infants were then age matched with 9 uninfected controls (see Figure 5.2).

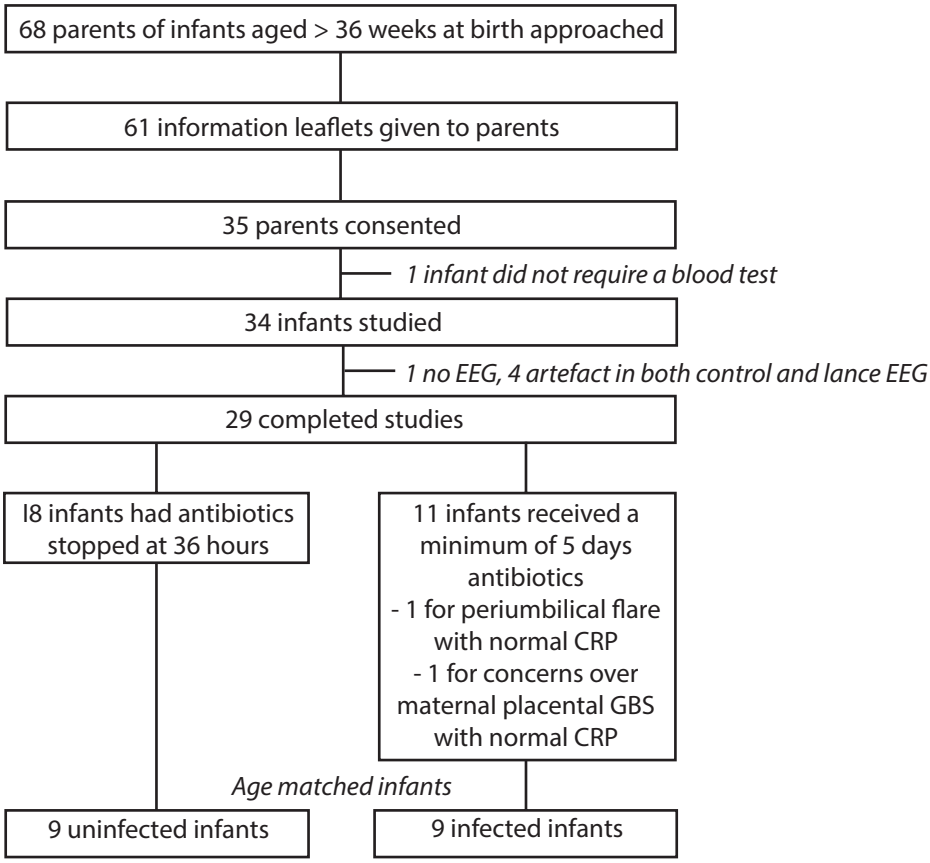

Figure 5.2: Flow diagram of recruitment for study.

Demographic characterisation of the infants included in the final analysis		
	Infected	Uninfected
Number of infants	9	9
GA (weeks)	40.1 (39.9-41)	40.6 (39-40.7)
CGA (weeks)	40.3 (40-41.1)	40.9 (39.2-40.9)
Postnatal age (hours)	33 (32-34)	36 (31-41)
Birth weight (g)	3925 (3380-4295)	3975 (3590-4135)
Number of males	4	6

Table 5.2: Demographic characterisation of the infants included in the final analysis.
Median is displayed with (lower quartile, upper quartile).

5.2.2. Experimental protocol

EEG, EMG, ECG, oxygen saturations and facial expression recordings were made of all infants, as described in General Methods.

All infants were studied while undergoing a clinically required heel lance and prior to the heel lance all infants had a control heel lance procedure performed.

5.2.3. Analysis

5.2.3.1. EEG analysis

EEG analysis was conducted as detailed in the general methods. For this study, Woody filtering of 100ms was applied to take into account the increased latency seen in infected infants (Figure 5.3). Increased latency has also been described in adult patients and animal models of sepsis (248–250).

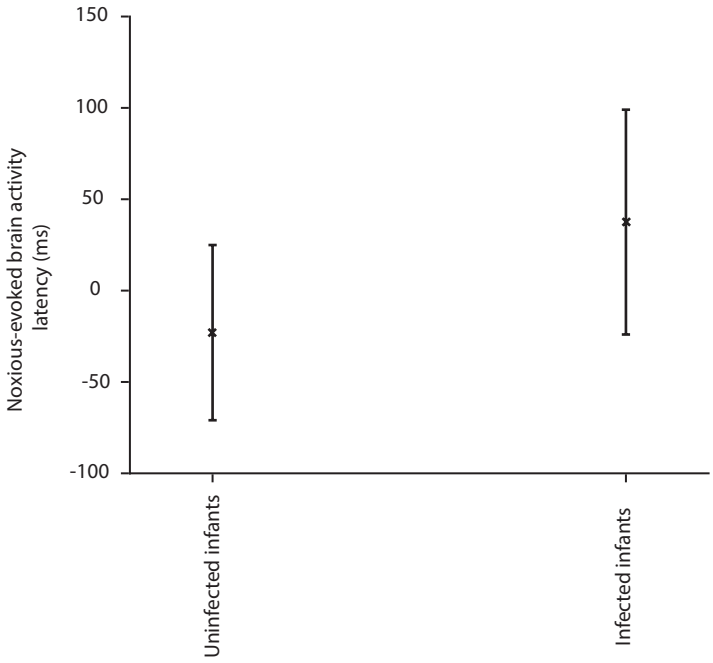

Figure 5.3: Latency to noxious-evoked brain activity in infected and uninfected infants.
Median is displayed ± standard error (calculated using bootstrap sampling).

5.2.3.2. EMG analysis

EMG analysis was conducted using RMS as detailed in the General Methods (Chapter 2).

5.2.4. Statistical Methods

Linear mixed effects modelling, with subject taken as a factor, was used to calculate the difference in magnitude of noxious-evoked brain activity in background, control and lance. As the groups were age-matched, a paired t-test was used to calculate the difference in magnitude of noxious-evoked brain activity to lance between infected infants and uninfected controls. McNemar's test was used to calculate the difference between the proportions of facial responders to heel lance in infected infants and uninfected controls. A paired t-test was used to calculate the difference in the total

PIPP-R scores to lance in the infected and uninfected controls. One infected infant was excluded from this analysis due to the lack of oxygen saturation data. Wilcoxon sign-rank test was used to calculate the difference in medians between the EMG RMS.

5.3. Results

5.3.1. Heel lance elicits noxious-evoked brain activity

In previous work and in Chapter 4 of this thesis, it has been shown that noxious-evoked brain activity is elicited by noxious stimulation and not by non-noxious touch (185). In this independent sample of infants, it was demonstrated again that noxious evoked brain activity is seen only in response to noxious stimulation of the heel lance and not in response to the non-noxious control, emphasising the validity of the measure. The average magnitude of noxious-evoked brain activity elicited by heel lance across all 18 infants was significantly different from that seen in the background EEG (p=0.007, linear mixed effects). The magnitude of noxious-evoked brain activity elicited by control heel lance was not significantly different from background (p=0.4)(Figure 5.4).

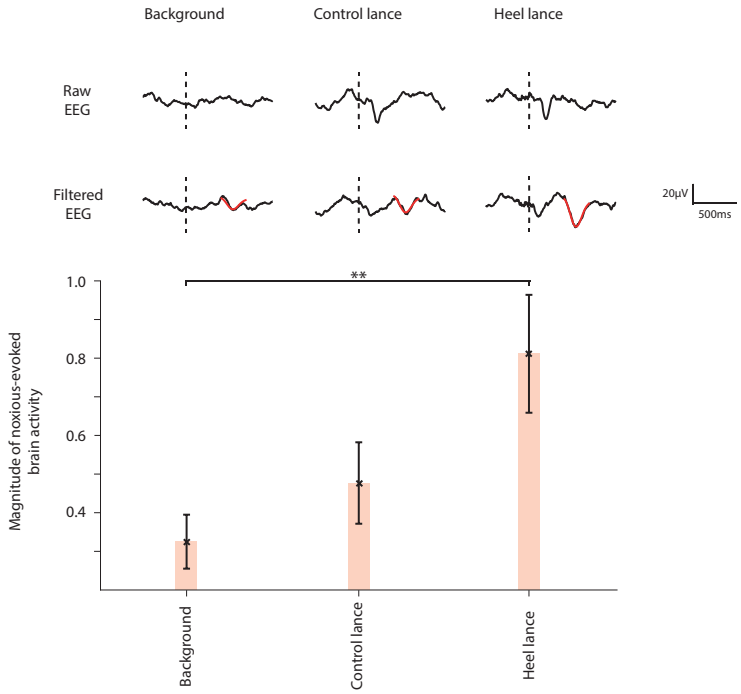

Figure 5.4: Comparison of noxious-evoked brain activity in background and in response to non-noxious and noxious stimulation in all infants.
The average background EEG activity at the Cz electrode in all 18 infants and the average evoked activity after a control heel lance (non-noxious stimulation) and a clinically required heel lance (noxious stimulation) is shown. The raw EEG is shown in the top panel and the EEG with Woody filtering applied is shown below with the template overlaid in red. The magnitude of noxious-evoked brain activity is displayed with mean ± SEM. ** $P < 0.01$.

5.3.2. Noxious-evoked brain activity to lance is significantly greater in infected infants compared to uninfected controls

The magnitude of noxious-evoked brain activity has previously been shown to increase with increasing gestational age (73). Infants of a wide range of gestational ages were included in the study, so to account for this effect in the analysis, infected infants were paired with age-matched controls. When infected infants were compared to age-matched, uninfected controls, the magnitude of noxious-evoked brain activity to lance was significantly greater in the infected infants (p=0.048, paired t-test)(Figure 5.5).

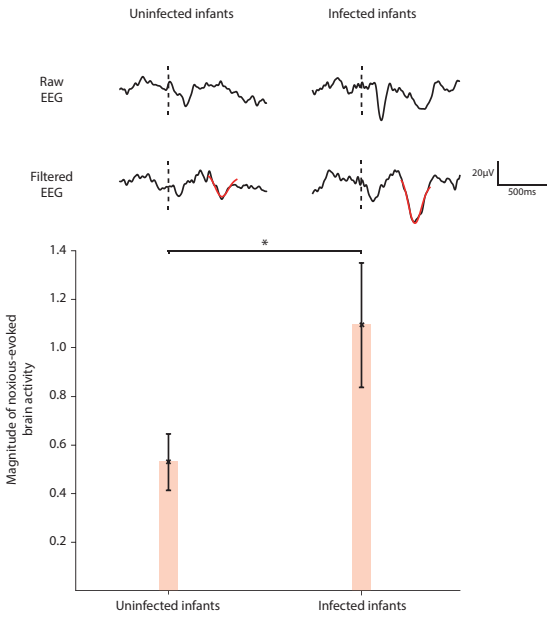

Figure 5.5: Comparison of noxious-evoked brain activity evoked by heel lance in infected and uninfected infants.
The average evoked activity recorded after a clinically required heel lance at the Cz electrode in 9 infected and 9 uninfected infants is shown. The raw EEG is shown in the top panel and the EEG with Woody filtering applied is shown below with the template overlaid in red. The magnitude of noxious-evoked brain activity is displayed with mean ± SEM. * $P < 0.05$.

The noxious-evoked brain activity elicited in the individual infants is demonstrated in Figure 5.6.

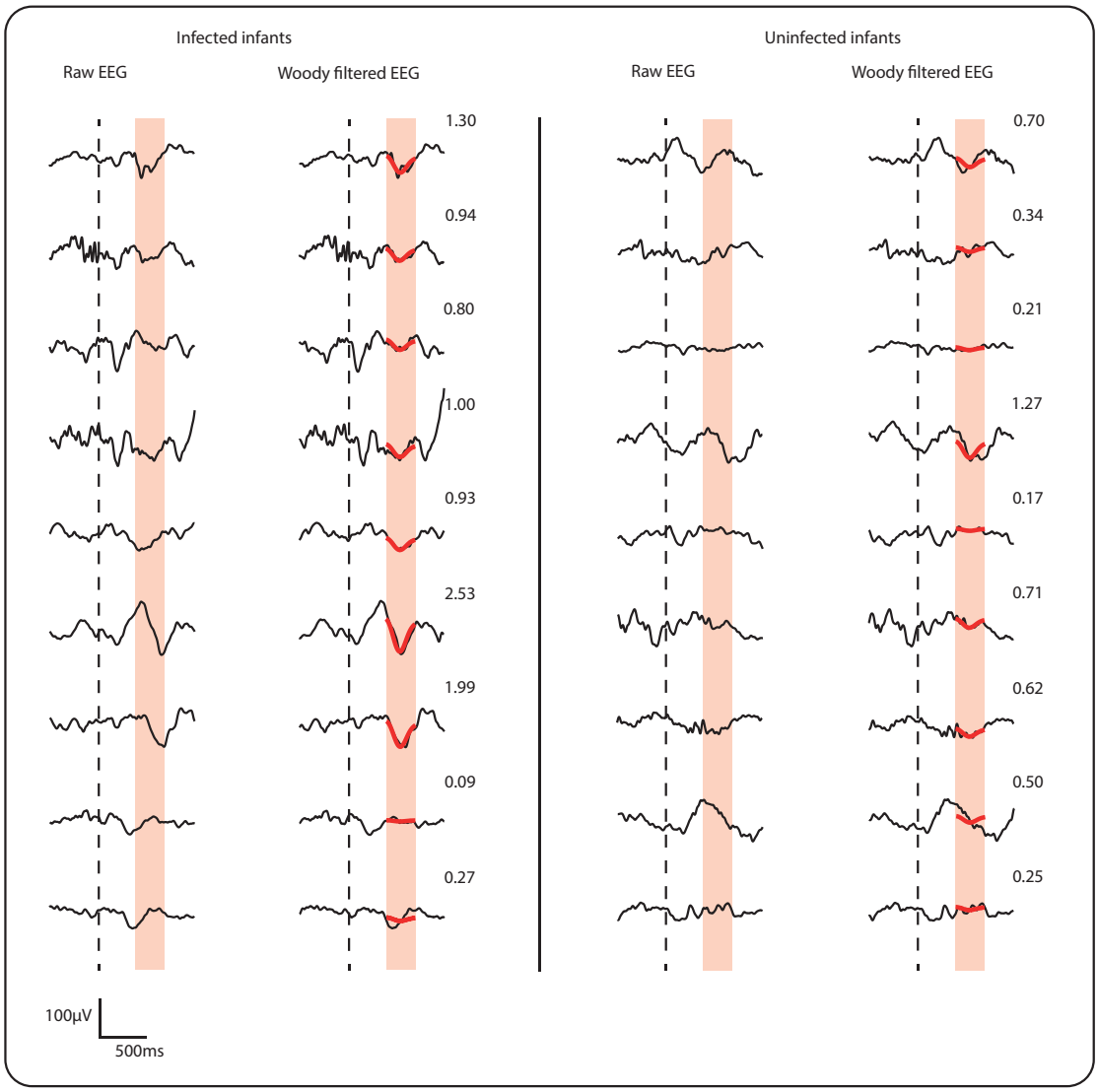

Figure 5.6: Noxious-evoked brain activity in individual infants.
The raw EEG (without Woody filtering) and the Woody filtered EEG (shifted by a maximum of ±100ms) for each individual infant recorded in response to the heel lance is shown. The time window of interest (400-700ms) is highlighted in the red shaded area. The template is overlaid on the Woody filtered traces in red. The magnitude of noxious-evoked brain activity in the individual traces is indicated. The inter-individual latency variation can be appreciated by comparing the location of the evoked potential in the raw EEG from the individual trials compared to that seen in the Woody filtered EEG. Infants are ordered according to the age-matched pairs used in the analysis.

5.3.3. There was no significant difference in the total PIPP-R scores to heel lance in infected infants compared to uninfected infants

Clinical pain assessment in infants is difficult but current recommendations suggest the use of age-appropriate clinical pain scores, such as the PIPP-R score (251). Here, in order to determine whether infection altered clinical pain scores in infants, the total PIPP-R in response to heel lance was calculated in the two groups. In their original work on PIPP scoring, the authors suggested that scores of 6 points or less generally indicated minimal or no pain and scores of 12 and above represented moderate to severe pain (111). One infected infant was excluded from the analysis as oxygen saturations were not available to calculate a total PIPP-R. There was no significant difference in the average scores calculated in the two groups ($p=0.56$) (Figure 5.6). The mean PIPP-R score calculated for infected infants was 5.8 ± 2.7 (mean ± standard deviation) and 4.3 ± 4.3 for uninfected infants.

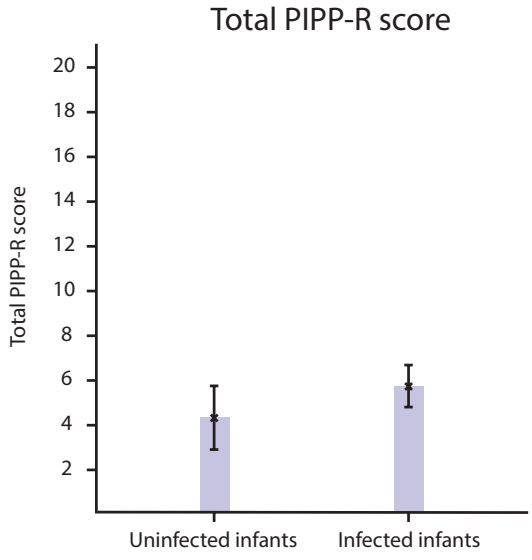

Figure 5.7: Comparison of total PIPP-R score recorded in response to heel lance in infected and uninfected infants.
The average total PIPP-R scored recorded in response to a clinically required heel lance in 8 infected and 9 uninfected infants is shown. The total PIPP-R score is displayed with mean ± SEM.

Interestingly, when considering physiological responses, only 1/17 infants for whom oxygen saturations were recorded, scored for a change in oxygen saturations, with a decrease of 4%. The average baseline oxygen saturations in all infants were 97.7% ± 2.4%. The average change in oxygen saturations was -0.95% ± 1.2%. No infant was receiving respiratory support at the time of study. A change in heart rate sufficient to score was recorded in 13/18 infants. The average baseline heart rate was 137.6bpm ± 14.7bpm and average change was an increase of 11bpm ± 7.9bpm. Further analysis of heart rate is described below.

5.3.4. Infants with infection are more likely to display a facial response to lance than uninfected infants

Facial response was calculated using the facial component of the PIPP-R score. If infants displayed any of the three facial expressions (brow bulge, eye squeeze, nasolabial furrow) for any amount of time in the 30 seconds following the heel lance, they were classified as a facial responder. If no facial response was observed, infants were classified as facial non-responders. Using this binary classification, significantly more infected infants displayed a facial response to lance than uninfected infants (9/9 infected infants, 2/9 uninfected infants, p=0.023, McNemar's test)(Figure 5.7). There was no significant difference in the baseline behavioural state score of the two groups of infants prior to the heel lance, that is, whether the infants were active and awake, quiet and awake, active and asleep or quiet and asleep (p = 0.12, paired t-test, average

baseline behavioural state score in uninfected infants 2.33 ± 1.12, in infected infants 1.33 ± 1.41).

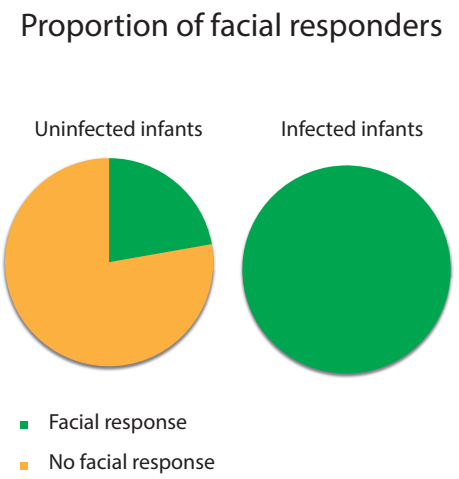

Figure 5.8: Proportion of facial responders to lance.
The proportion of infected and uninfected infants who display a facial response (green) or who do not display a facial response (yellow) to lance is shown.

Taking all infants together, 39% displayed no facial response to the heel lance. This proportion of facial non-responders is very similar to proportions previously reported (10,185). When considering those infants who displayed a facial response of sufficient duration to score in the facial component of the PIPP-R score, that is, for a minimum of 3 seconds, 2 uninfected infants had a facial PIPP-R score, and 7 infected infants scored. The difference in these proportions showed a clear trend towards more infected infants mounting a facial response (p=0.07, McNemar's test). Moreover, the score obtained in the facial component of the PIPP-R score, was significantly higher in response to the lance in the infected infants than in the uninfected control different between the groups (infected infants = 3.1, uninfected infants = 1.2, p=0.04). In terms of the duration of facial response to a heel lance, when present, the duration was not significantly different between the groups (median duration of facial response in infected infants= 13.6s (3 – 15.6s), median duration in uninfected infants = 17.3s (13.2 -21.3s), p=0.4).

2/9 infected infants displayed a facial response to control heel lance, compared to 1/9 uninfected infants (p=1). There was no significant difference in the baseline behavioural state scores of the infants prior to the control lance (p = 0.46, paired t-test, average baseline behavioural state score in uninfected infants 1.67 ± 1.12 (mean ± standard deviation), in infected infants 2.11 ± 1.36). However, it was anecdotally observed that the infected infants were more likely to wake up following the control lance and were therefore more likely to be awake prior to the lance, although there was no statistically significant difference between the behavioural state scores (p = 0.12, paired t-test, average baseline behavioural state score in uninfected infants 2.33 ± 1.12, in infected infants 1.33 ± 1.41). The baseline behavioural state does not appear to affect whether the infant responded facially to the lance or not, since all infected infants responded to the lance despite being in a range of behavioural states.

5.3.5. The physiological and reflex response in infected infants does not differentiate between lance and control

The changes in heart rate evoked by control heel lance and heel lance stimulation were calculated according to the PIPP-R score. In uninfected infants, the heel lance evoked a significant increase in heart rate (p=0.004, one sample t-test), which was significantly greater than the heart rate change evoked by the control heel lance (heel lance 10.1bpm ± 7.6bpm, control heel lance 2.2bpm ± 6.1bpm, p=0.0038). The change in heart rate evoked by the control heel lance was not significant (p=0.31, one sample t-test). In infected infants, the change in heart rate evoked by both the heel lance and the control

heel lance were significant (change evoked by heel lance, p=0.0027, one sample t-test, change evoked by control heel lance p=0.012, one sample t-test). The change evoked by the heel lance was not significantly different to that evoked by the control lance (heel lance 12bpm± 8.4bpm, control heel lance 9.3bpm ± 8.6bpm, p=0.5)(Figure 5.8). The evoked change in heart rate to heel lance is similar to that which has been previously reported (15.6bpm) (185) and the change in heart rate evoked by the control heel lance in uninfected infants is also in keeping with previously published data (2.2bpm) (185). The difference in the heart rate response evoked by the control lance in the two groups, infected infants and uninfected controls, did not reach significance (uninfected infants 2.2bpm, infected infants 9.3bpm, p=0.059, paired t-test) but there was a trend to suggest that infected infants display an exaggerated heart rate response to this normally innocuous stimulus.

Similarly, when comparing the reflex withdrawal response to the two stimuli, uninfected infants displayed a reflex to control lance that was significantly smaller than that evoked by heel lance (EMG RMS control heel lance median 7.8 (3.1-13.4), heel lance 29.6 (18.3-38.1), p=0.012) whereas infected infants displayed a reflex response to both, which was not significantly different (control heel lance 19.9 (15.0-26.1), heel lance 43.5 (25.6-63.2), p=0.19).

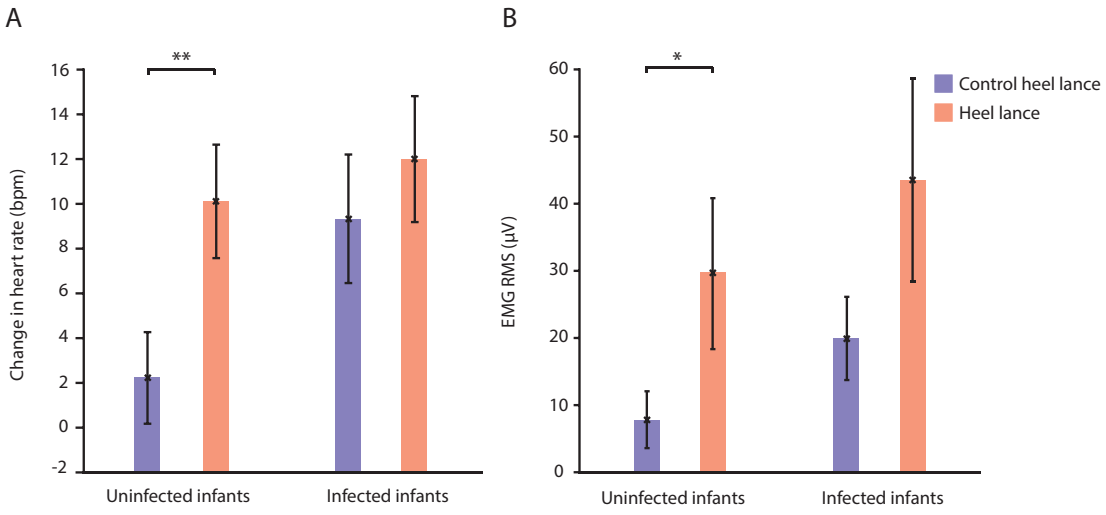

Figure 5.9: The physiological and reflex response to heel lance and control lance in infected and uninfected infants.
(A) The change in heart rate in response to control heel lance (blue) and heel lance (red) is shown for infected and uninfected infants. The mean is displayed with ± SEM. ** P <0.01. **(B)** The magnitude of the reflex withdrawal response, measured using RMS, to the control heel lance (blue) and the heel lance (red) is shown for uninfected and infected infants. The median is displayed with ± standard error (calculated using bootstrapping). * P <0.05.

5.4. Discussion

In summary, it has been demonstrated that early onset neonatal infection alters pain responses in newborn infants. Infected infants show increased noxious-evoked brain activity to a heel lance and are significantly more likely to mount a behavioural response than uninfected controls, although clinical pain scores are unchanged. Infected infants also display heightened physiological and reflex responses to the tactile stimulus of a control heel lance.

5.4.1. Infection increases pain sensitivity in newborn infants

In this chapter, it has been demonstrated that infants with infection have significantly greater noxious-evoked brain activity to a heel lance than age-matched, uninfected control infants. Evidence from animal and adult studies has demonstrated a link between infection and heightened pain sensitivity (26,27,240), however, this relationship has not before been demonstrated in infants. There are several potential mechanisms, suggested by *in vivo* and *in vitro* rat studies and human fMRI studies, by which infection could increase pain sensitivity. Peripherally, immune activation and the release of pro-inflammatory cytokines has been shown to increase the excitability of peripheral nerves (252,253) and to act at the level of dorsal root ganglia (254). At the spinal cord level, inflammation has been shown to enhance spinal pain transmission via the activation of spinal glia (255) . This increased peripheral to central transmission of nociceptive information, in the context of infection, is supported by fMRI findings in adults of up-regulation of activity in brain regions involved in processing of somatic and visceral pain in LPS treated individuals compared to controls (26). Interestingly,

enhanced peripheral transmission of nociceptive information may also be accompanied by decreased descending inhibition; further fMRI work in adults has demonstrated decreased activity in descending inhibitory pathways with peripheral injection of inflammatory mediators (241). Thus, it is likely that the increased pain sensitivity seen in infection and demonstrated here is facilitated at many levels within the pain-processing pathway.

The average weight of noxious-evoked brain activity in infected infants was 1.09, compared to 0.52 in the uninfected controls, with an average of 0.81 for the group as a whole. The template used to analyse the noxious-evoked brain activity was scaled such that the average weight in response to a heel lance in the group of term infants in whom it was characterised was 1 (185). Hence, while the average for the group studied here, as a whole is comparable to previously published work, the average weight in uninfected controls appears relatively low in comparison. A number of factors may explain this discrepancy. Given the significant impact of infection demonstrated here, it is possible that a number of the infants included in the original template were infected, such that the average weight of a response to a heel lance in a 'healthy' term born term infant may in fact be closer to the 0.52 recorded here in the uninfected control infants. Other possible factors are gestational age, since noxious-evoked brain activity is known to increase in magnitude with increasing gestational age (73), and the impact of prior painful procedures, since exposure to repeated painful procedures has been shown to increase pain responses in term infants in the first days of life (192). The infants included in this study were well matched with the infants included in the template in terms of gestational age, so this is unlikely to be having an effect (median CGA of infants here 40.7 (39.5, 41.1) (lower quartile, upper quartile); median CGA of infants in the

template 39.9 (37.9, 41.7)). However, the infants included in this study had all had a minimal number of prior painful procedures, whereas the infants included in the template may have had more. Additionally, the infants studied here, although well matched with the infants included in the template in terms of gestational age, were very young in terms of postnatal age (PNA). The median PNA of infants studied here was 1 day (1, 1) (lower quartile, upper quartile) compared to 3 days (2, 5) in the template. Evidence from rodent studies suggests that hormones essential for delivery exert an analgesic effect in newborn pups that is absent by day 2 (256). Thus, this observed 'newborn analgesia' may still be affecting the noxious-evoked brain activity seen in the young infants studied here but absent from those infants included in the template who were studied after this time. Another important factor in this 'newborn analgesia' could be the route of delivery. Work by Bergqvist et al., demonstrated decreased behavioural responses to a painful procedure in infants born by vaginal delivery compared to those born by caesarean section (215). In such a small sample size, it is not possible to correct for all these factors, but future work focusing on their effects would be beneficial.

5.4.2. Clinical pain scores were not changed by infection

Clinically, pain assessment in infants is reliant on the use of clinical pain scores (251). When assessing the response to heel lance using a clinically validated pain score, the PIPP-R, there was no significant difference in the scores between the two groups. Both groups of infants had relatively low PIPP-R scores to the heel lance compared to previously published work (183,257) such that, according to the original PIPP paper, both groups of infants would be classified as displaying minimal or no pain (111). These relatively low scores could be explained by the factors discussed above, and also by the

experimental protocol employed. The infant's foot was not squeezed for 30 seconds after the heel lance was administered, the time during which the PIPP-R score was calculated, in order to ensure that the response measured was to the noxious input of the lance and not contaminated with the response to the squeezing of the foot. In other work employing a similar experimental protocol, where the PIPP-R score was recorded before the foot was squeezed, the PIPP-R score calculated was similar (244).

Observation of behavioural responses is an important element of many clinical pain scores. Behaviour and behavioural responses have been noted to be altered in the presence of illness and infection, with sick infants variously described as irritable and restless or lethargic (233,234,258). When considering only the behavioural component of the PIPP-R score, it was demonstrated that infants with infection were irritable; they were significantly more likely to mount a facial response to heel lance compared to uninfected controls and the average facial PIPP score was higher in infected infants compared to uninfected controls (3.1 vs 1.2, $p=0.04$). The duration of the facial response, when present, did not differ between the groups, but this may be due to the very small numbers of uninfected infants mounting a facial response. It would be interesting to see whether, with a larger sample, those uninfected infants who do mount a behavioural response maintain this response for longer than the infected infants. This would fit with the clinical findings of lethargy in infected infants. Thus, infected infants may be both irritable, more likely to mount a behavioural response, but also lethargic, unable to maintain a vigorous response in an attempt to conserve energy in keeping with the sickness behaviour previously described (230).

Clinical pain scores did not differ between infected and uninfected infants, despite significant differences in the facial response component. The difference in facial response provides evidence for the clinical observations of irritability, or hyper responsiveness, in infants with infection. Interestingly, the oxygen saturation component of the PIPP-R score was not particularly informative here, with only one infant receiving a score for a decrease in oxygen saturation. This suggests that term infants have sufficient respiratory reserve that their oxygen saturations are not affected by the response to a noxious stimulus. Seo et al. recorded oxygen saturations in a group of term infants undergoing heel lance and reported average baseline oxygen saturations of 98.8% ± 1.46%, dropping to 98.1% ± 11.98% at the time of heel puncture, and to 93.2% ± 7.88% during sampling (183). The greater drop in oxygen saturations reported there during sampling could reflect an exaggerated response to the more prolonged aversive input associated with squeezing the foot in order to obtain a blood sample. That the greatest decrease in oxygen saturations were associated with the squeezing of the foot which is thought to be distressing rather than painful, suggest that that changes in oxygen saturations might not be a useful indicator of pain in this population.

5.4.3. Lack of physiological and reflex discrimination between noxious and tactile stimulation are a feature of early onset infection

It is demonstrated that infants with infection display physiological and reflex withdrawal responses to noxious heel lance and non-noxious control heel lance that are indistinguishable, in contrast to the distinct responses seen in uninfected controls. The increased responses to tactile stimulation in infected infants are suggestive of allodynia.

Allodynia is defined by the International Association for the Study of Pain as 'pain due to a stimulus that does not normally provoke pain' in contrast to hyperalgesia which is 'an increased response to a stimulus which is normally painful'. The definition of allodynia has been updated to 'pain in response to a non-nociceptive stimulus', that is a stimulus which is not capable of activating nociceptors (51). This definition is useful in adult patients where verbal report of pain is available, however, in non-verbal populations it is perhaps less applicable. An alternative definition of allodynia that is used in animal models and is perhaps more useful in non-verbal populations is 'increased distress or reactivity to a stimulus that is normally innocuous' (259). Allodynic rats display decreased thresholds for evocation of tactile withdrawal response when their hindpaws were stimulated with von Frey hairs of increasing force (260). They also demonstrate behavioural and cardiovascular responses to light stroking of the skin which elicits no response in control animals (260). This is analogous to the increased physiological and reflex responses seen here in infected infants to the usually innocuous tactile stimulation of the control heel lance. Central administration of both LPS and IL-1beta can produce allodynia in rat models (261,262) suggesting that the same inflammatory mediators could be involved in the development of both hyperalgesia and allodynia. The mechanisms underlying development of allodynia are unclear but are thought to be related to a phenotypic switch in the function of Aβ fibres which are usually activated by tactile stimulation (263). The possibility that infection causes infants to become allodynic has important implications in the care of these infants.

5.4.4. Strengths and limitations

Implementation of the NICE guidance for recognition, diagnosis and management of early onset neonatal bacterial infection has led to a very standardised approach to this condition in the UK (220). This clear pathway provides an excellent experimental paradigm to investigate the impact of infection on pain responses in infants. This is highlighted by the uniformity of the group of infants that were studied here. The infants had a very small range of postnatal ages, even in hours, studied at 33.5 hours (31.3-39.5) (lower quartile, upper quartile). All infants had undergone a limited number of prior painful procedures, minimising the impact of this potential confounding factor. All infants were studied during the same procedure, a clinically required heel lance, the use of which as a noxious stimulus to study noxious-evoked brain activity is well described (20,73,185). Furthermore, the use of multi-dimensional recordings from all infants, incorporating EEG, EMG, physiological and behavioural data, allowed pain responses to be clearly characterised. Another strength of this experimental paradigm was that, at the point of study, it was not known whether the infant was infected or not, thus minimising any potential bias from the investigators.

One limitation is the relatively small number of infants included in the study. The requirement for the repeat CRP to be carried out within 18-24 hours of the infant commencing antibiotics meant that there was a relatively short time window in which to approach parents which was logistically difficult. For future work it would be important to recruit more infants to ensure that the results seen here are representative of the population as a whole. However, the infants recruited to the study seem be an accurate reflection of the population of infants started on antibiotics, with

the proportion of infected infants studied here, around 1 in 3, being similar to proportions reported in the literature (228,264).

The range of CRPs recorded in the infected infants was large, 11.3-61.8, and although absolute values of CRP are not necessarily indicative of the severity of infection, it is likely that the population of infected infants contains a spectrum of severity of disease. How the severity of infection affects responses would be interesting to investigate. In order to explore this further, a much larger group of infected infants would have to be studied. No infant studied here had either a positive blood culture or a positive lumbar puncture result, both of which would indicate a more severe infection, but in a larger sample, these could be studied.

A further limitation of the study is the sole use of a clinical procedure, such as a heel lance, which, most often, is only required once. In the use of experimentally noxious stimuli it is often possible to present the subject with trains of stimuli, thus reducing the impact of noise or artifact in a single trace. However, the benefit of using a clinically relevant procedure such as a heel lance is that it gives the opportunity to study the multi-dimensional response to noxious stimulation, no behavioural response is evoked by experimental noxious stimulation (164).

5.5. Conclusion

Early onset neonatal infection is an important clinical condition and pain management in this vulnerable group of patients presents a significant challenge. Here, I demonstrate that infected infants have increased pain sensitivity. This is important because these infants may undergo a number of painful procedures during the course of their treatment including further heel lance, repeat cannulation and lumbar puncture, highlighting the need to offer pain-relieving measures. How long these altered responses persist for is an important clinical question. Studies in rats suggest that immune challenge in early life can increase pain sensitivity into adolescence and beyond (28,265,266). It could be that infection in early life sets up maladaptive processes in the modulation of pain that persist well beyond infancy and into adulthood (267).

Functional MRI has been used in adult studies to investigate the mechanism underlying increased pain sensitivity in infection and inflammation (26,241). fMRI has also been used in infants to investigate noxious-evoked brain activity (19). The ability of fMRI to provide spatial resolution would provide a useful adjunct to EEG to elucidate the mechanisms underlying increased pain sensitivity in infants. Future work would therefore benefit from having this imaging component.

While early onset neonatal infection in term infants is an important condition, it is relatively rare compared to the high burden of late onset disease seen in preterm infants admitted to a neonatal intensive care unit (268). Whether the same altered responses are seen in preterm infants is an important area of further work particularly

as the long term impact of both sepsis and pain on neurodevelopment in this vulnerable group are well described (4,99,199,269,270).

Chapter 6

Discussion

6.1. Thesis summary

The aim of this thesis was to investigate infant pain responses using a multi-dimensional approach. Adequately assessing and treating infant pain is not only important to prevent short-term distress and physiological instability (97,98), but also, to prevent long-term sequelae (4,5,102). In the absence of verbal report, robust, reliable and reproducible surrogate measures must be used to assess pain. In this thesis, one such surrogate measure, a template of noxious-evoked brain activity was validated. This template of noxious-evoked brain activity was shown to be correlated with another commonly used and well-validated surrogate measure of pain – facial expression response. It was also demonstrated that noxious-evoked brain activity, as measured by the template, was only evoked in response to noxious stimulation and not by stimulation of other sensory modalities. Finally, it was demonstrated that the template was sensitive to modulation by the use of topical local anaesthetic. That the template shows specificity to noxious-evoked brain activity and is sensitive to modulation by analgesics makes it a promising option for use in the testing of analgesics, where currently work relies heavily on the use of short-term behavioural and physiological responses. Having an objective, quantifiable and translatable measure of pain is useful not only to test analgesics, but also to further our understanding of normal pain processing in infants and to investigate how pathology affects and disrupts this. Here, it has been demonstrated, using the template of noxious-evoked brain activity, that early

onset neonatal infection increases pain sensitivity in infants. Behavioural measures are also subtly altered by infection as well as physiological and reflex responses. Other work has demonstrated that there is a disconnect between behaviour and brain activity under certain conditions (244), and although here, behavioural responses and brain activity both seem to be increased, the interaction between behaviour and brain activity needs to be investigated further. This is particularly important in the clinical setting, where behaviour is easily observed, but brain activity is not available.

Behavioural responses form the cornerstone of infant pain assessment. This is partly because they are easily observable, both in a clinical and a research setting, and partly because observations of facial expressions form a large part of human communication. The work presented here has demonstrated that even young infants, from around 32 weeks, are able to demonstrate behavioural discrimination between tactile and noxious stimulation. However, several situations have been observed where the use of behavioural measures is limited. Below 32 weeks, infants did not demonstrate behavioural discrimination between tactile and noxious stimulation, either by the presence of a facial response or by its duration. It was also demonstrated that around 20% of infants aged 34-42 weeks displayed no facial response to a heel lance, and yet 36% of these infants still demonstrated a significant increase in noxious-evoked brain activity. This disconnect between behaviour and brain activity was observed by Slater et al, where sucrose was found to decrease the proportion of infants demonstrating a facial response to a heel lance but not to alter brain activity, and highlights the importance of multidimensional measures of pain (24).

The same approach that was used to define the template of noxious-evoked brain activity in response to heel lance or experimental noxious stimulation of the foot was used to characterise noxious-evoked brain activity elicited in response to cannulation of the hand. This pattern of noxious-evoked activity was well correlated with the template, although was seen to occur with a shorter latency. That the pattern of brain activity was so well conserved between the different stimuli and stimulation sites suggests that it may be translatable to other clinically important acute noxious procedures, such as IM injection, although this would need to be investigated. A template of noxious-evoked brain activity that can be used to quantify the response to different clinical procedures, and so to test the different analgesic agents used in different procedures, would be really useful. While the real benefit of noxious-evoked brain activity is likely to be in the research setting, more work needs to be done in order to optimise measures available to assess pain in the clinical setting.

6.2. Limitations and further work

There is an increasing body of evidence to suggest that there are many factors that modify the response to painful stimuli. The effect of increasing gestational age on brain activity and reflexes has been well described previously (73) and was demonstrated here again when investigating the response to cannulation. Others have demonstrated an impact of the mode of delivery (215) and sleep state (108) and also prior pain experience (271). There are likely to be more factors that influence pain responses on a population level. The work presented here on the impact of infection on pain responses, for example, suggests that the presence of infection should potentially be controlled for.

In small groups of infants, such as those included here, it is not possible to correct, or adjust for, all these factors. Furthermore, more information is needed about the degree to which these factors can influence pain responses, and also the duration of their effect. Mode of delivery, for example, is thought to only influence pain responses for a short period, while the stress hormone response to the process of birth is ongoing (215). However, as the field of infant pain research grows, it is likely that these factors, and others, will have to be taken into account.

A high degree of variation in responsiveness to painful stimuli is seen across individuals. The wide range of magnitudes of the noxious-evoked brain activity demonstrates this variation. For example, in the study looking at the effect of topical local anaesthetic, during stimulation of untreated skin by experimental noxious stimulation, the magnitude of the average infant response ranged from 0.22 to 2.16. In this study, because experimental noxious stimulation was used, each infant was able to act as its own control and so, the large variation in responses between individuals did not mask the effect of the topical local anaesthetic. For the clinical procedure, cannulation, where an individual was cannulated only once, with or without topical local anaesthetic, this within subject control was not available. It was therefore not possible, in the small number of infants studied, to ascertain whether an effect of topical local anaesthetic was being masked by the random 'allocation' of more responsive individuals to treatment with topical local anaesthetic. One way to address this problem would be to conduct a larger scale, randomised control trial for the use of topical local anaesthetic. While other studies have been done, the evidence for the use of topical local anaesthetic in infants remains conflicting (16,114,173,177), and, as discussed previously, this may be in part due to the difficulties in delineating pain from distress

using clinical pain scores. This is a question that would benefit from an objective measure of nociception, such as that provided by the template. Another possible way to control for the high degree of inter-individual variation in smaller scale studies would be to have a standard way to assess an individual's response that could be applied prior to any intervention. Experimental noxious stimulation could be used in this setting. Prior to a clinical procedure, infants could receive experimental noxious stimulation that could be used to gauge their level of responsiveness, this could then be controlled for when assessing the impact of an intervention on the clinical procedure, ensuring that infants who were more or less responsive were equally represented between the groups.

In this thesis, a striking effect of infection on pain responses is demonstrated. However, the duration of this effect was not investigated, nor were the mechanisms underlying this observation. Future work could focus on following infants diagnosed with infection to determine whether the increased pain sensitivity described here persists beyond the period of infection, and if so, for how long. Infants undergo a relatively large number of routine painful procedures throughout their first year, providing the opportunity to study pain responses to clinical required procedures. Infants could be assessed during routine heel lancing for newborn screening on day 5, and again during routine immunisations at 2, 3, 4 and 12 months. One might expect the increased pain sensitivity to last at least during the acute inflammatory response to infection, as much of the work in adults and animal studies has demonstrated the effect of acute inflammatory mediators (27,241). However, other work in animals suggests that the effect may last much longer, with early life immune challenge being linked with increased pain sensitivity into adolescence and beyond (28,266).

The use of fMRI would be a useful adjunct to help elucidate the underlying mechanisms of increased pain sensitivity at a cortical and subcortical level. In adults, fMRI studies have demonstrated increased activation of brain regions involved in pain processing but also decreasing descending inhibition (26,241). The relevance of these findings to infants in whom descending inhibition is thought to be immature is unclear.

Infection is a particularly relevant example of a pathological process seen in the neonatal unit setting because of its prevalence, but the impact of other pathological processes would also be interesting for future study. Jaundice, for example, is a common neonatal problem that is known, at high levels, to be neurotoxic, and at lower levels is noted to cause an infant to be sleepy or drowsy, but the impact of jaundice on pain responses has not been studied. Furthermore, these infants undergo repeated painful heel lances in order to monitor their jaundice levels. Interestingly, recent work looking at the neurobehaviour of jaundiced infants noted that while they appeared drowsy initially, if they were stimulated they became more irritable and difficult to console (272). This is analogous to the increased probability of a facial response seen in the infected infants studied here. The detrimental impact of early life pain and the importance of early life experience in perturbing normal nociceptive processing have been described in both human and animal studies, and with chronic pain affecting around 20% of European adults (273), understanding how common pathologies interact with pain processing in early life is an important area of research that has the potential to address a major clinical problem in adulthood.

6.3. Concluding remarks

'The management of pain must be considered an important component of the health care provided to all neonates, regardless of their gestational age or severity of illness' (6).

The first step in adequately managing pain is accurately assessing its presence and severity. The next step is providing effective analgesia, which is well tolerated and has an acceptable side effect profile. Significant advances in the management of infant pain have occurred over the last 30 years, starting with the acceptance that infants, including those born prematurely, are able to feel pain, and the recognition that this pain requires treatment. Despite significant changes in clinical practice, however, infants admitted to a neonatal unit still experience a high burden of painful procedures during their routine care and the long-term consequences of this early life pain are now beginning to be seen. The assessment of infant pain remains a challenge, with reliance on subjective, non-specific measures. The treatment of infant pain is limited by restricted analgesic options many of which have conflicting evidence to support their use. Hence, it is important to develop measures that work both in the clinical setting, to provide accurate assessment of pain, and in the research setting, to aid the development and evaluation of analgesic options.

In this thesis, a template of noxious-evoked brain activity has been validated and used to investigate the efficacy of topical local anaesthetic and the impact of pathology. The relationship between behaviour and brain activity has been explored and the development of behavioural discrimination across gestation has been described. The

lack of behavioural discrimination demonstrated in the youngest infants raises questions about the validity of using behavioural measures in the very premature, and highlights the need for greater understanding of how pain processing matures across development. This work demonstrates that a template of noxious-evoked brain activity could provide a useful, objective measure for clinical trials in the development and assessment of analgesics, which is an important step in advancing the treatment of infant pain.

7

Appendices

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Information Sheet for Parents/Participants

You and your baby are invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear 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?

Babies and young children in hospital are exposed to painful procedures as part of their routine medical treatment. As they are unable to say whether or not these procedures hurt, it is difficult to assess the amount of pain they feel and to be sure that they are receiving the right medicines. Although we know that babies and young children register pain in their brains, other ways need to be used to assess the amount of pain they feel. We have discovered a method of assessing pain activity in babies' brains while they are having their routine blood tests and other clinical procedures. This brain activity can be monitored in different ways. One way is with electrodes gently placed on the scalp to record the activity that arises in specific parts of the brain during painful procedures. We can also look at this activity by imaging your baby's brain in a head scanner. We wish to understand more about this brain activity and use it to test how much pain babies are experiencing.

We will investigate how the brain activity related to pain changes with age and whether we can extend the technique to measure pain in babies and young children who come to hospital. Our aim is to provide a gentle but accurate way of measuring pain so that better pain medicines can be developed for babies and young children.

3. Does my baby have to take part?

No, it is up to you to decide whether or not your baby will take part. If you do decide to allow your baby to take part you will be asked to sign a consent form. If you decide you do not want to take part in the study, we fully understand and assure you that this will have no adverse effect on your baby's normal clinical care.

4. What is involved in the study?

In this study we would like to understand how babies respond when they experience different sensory stimuli, such as painful and non-painful touch, auditory and visual stimulation. We will do this by using techniques that measure your baby's brain activity and also by videoing your baby's face

Touch stimulation will involve gently touching your baby. We will touch your baby with a small hand-held device with a soft, circular rubber bung (similar to an eraser on a pencil) or a punctate (non-skin penetrating) stimulator.

Babies recruited into the study that require a blood test / immunization / blood sampling / cannulation / suctioning will have these clinical tests completed in the routine way. The doctors and nurses caring for the baby will decide which clinical procedures are necessary and when they need to be done. No extra painful procedures or extra blood tests will be done for the purpose of this study. The study will not interfere with your baby's clinical care, nor will there be any delay if an emergency procedure is required. **No painful procedures will be carried out solely for research purposes. All painful procedures will be clinically required.**

As we are interested in how your baby's response to pain changes as they grow, we may ask to study your baby more than once during their stay in hospital/clinic. We will ask you whether you are happy for your baby to be studied more than once,

Measuring brain activity

Three techniques are used to monitor the way in which babies respond to pain and other sensory stimuli. The study that your baby will be participating in will involve one or more of these techniques:

Electroencephalography (EEG): This is a portable imaging system that is routinely used on the neonatal unit, on children's wards and clinics. It involves gently placing electrodes on the baby's head to measure brain activity.

Near-infrared spectroscopy, (NIRS): A technique involving sensors placed on the head, which can detect changes in blood oxygenation. It is also portable, and can be performed at the cot side and/ or clinic.

Magnetic resonance imaging, (MRI): MRI is used in clinical care as well as in research because it allows a safe, non-invasive way of imaging the brain. This technique measures changes in blood oxygenation. The study will involve babies being placed inside an MRI scanner at the FMRIB Centre that is part of the John Radcliffe Hospital.

All studies have a dedicated team of clinicians, radiographers and research personnel that will ensure the safety of your baby at all times. Parents may accompany their child during the study, if they wish to do so.

Other measures

Electromyography (EMG) is a safe non-invasive technique used to record muscle activity. Small adhesive electrodes will be placed on the skin over the muscle in order to measure whether your baby reacts and pulls away during the blood test, other clinical procedure or sensory stimulation.

If not already in place for clinical reasons, two small adhesive electrodes will be placed on your baby's chest to measure any change in heart rate, and a small probe will be wrapped around your baby's foot in order to measure any change in blood oxygen levels.

Videoing your baby

We also video your baby during our studies. We do this so after the study, we can assess facial expression changes, which we use to calculate a pain score. Pain scores are a widely used means of measuring pain in babies and young children. Recorded images by video and/or photograph will not be used for public use and only for study analysis. We may ask you if you are happy for us to use images recorded by video and/or photograph, for teaching, publicity and/or scientific journals, but this will be consented for separately from the study, and would be optional.

5. What are the known risks of the study?

Obtaining video footage of your baby is non-invasive and does not present any risk to your baby. EEG, NIRS, EMG and ECG have been used on the neonatal unit, wards and clinics for almost 20 years without any adverse effects.

MRI has been used to image both premature and term babies and does not present any risk to your baby. MRI is not a portable technique and needs to be conducted in the MRI suite located within the hospital grounds. This will therefore involve transporting your baby to and from the unit, accompanied by a clinical member of the neonatal staff.

6. What are the possible benefits of taking part?

There are no direct benefits for your baby, for taking part in this study. This study is designed to gather information that will help to develop better ways to care for premature and unwell babies in the future.

7. What information will be collected about my baby?

We will collect basic clinical information about your baby's delivery, condition at birth and their progress and medical treatment while they are/were in the neonatal/maternity/paediatric unit. For example, their gestational age at birth and on the day of the study, their need for support with breathing or any medication they are/were receiving. This information helps us to determine which factors may influence the way a baby copes with pain.

All information and videos that are collected about/of your baby during the course of the research will be kept strictly confidential. Each baby 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.

8. What if something goes wrong?

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 (tel 01865 234537, email: rebeccah.slater@paediatrics.ox.ac.uk) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or ctrg@admin.ox.ac.uk

9. What will happen to the results?

Test information, relevant medical information, and video clips will be anonymised and stored under lock and key, to be accessed only by the research team within restricted/secured areas of the John Radcliffe Hospital. The anonymised data including video files will be stored safely for the duration of the five-year research project.

The results from all babies will be analysed individually and then combined to provide information about this cohort of babies. Longitudinal studies, (repeated at different ages), will be done on some babies. There will be no identifying information about any baby, when the data is presented in any forum. The findings from this study will be published in order to disseminate this information to all professionals who care for preterm or unwell babies. The findings may also be used for teaching or academic research presentations in order to allow the results to be shared with the wider scientific community. No videos/images will be published without separate consent.

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.

10. Who is organising and funding the research?

This study has been funded by The Wellcome Trust.

11. Withdrawal from the project

You are free to withdraw your baby from the study at any time without having to give a reason. If your baby becomes distressed the research procedures will stop. The clinically required procedure will go ahead subject to a review by a suitably qualified clinician. If you decide to withdraw your baby from the study, we will ask your permission to use the images/data that have already been recorded. All information regarding their medical records will be treated as strictly confidential.

12. Who has reviewed the study?

All research in the NHS is also looked at by an independent group of people, called a Research Ethics Committee, to protect participants' interests. This study has been reviewed and given favourable opinion by South Central Oxford C Research Ethics Committee.

13. Comments or concerns during the study

If you have any comments or concerns you may discuss these with the investigator. Alternatively, the Patient Advice and Liaison Service (PALS) is an independent and confidential service for patients, carers and relatives. If you have concerns about the way the research project is being conducted you can also contact the PALS service on 01865 221473/743324 or email PALSJR@ouh.nhs.uk or you can write to the Chief Executive, Oxford University Hospitals NHS Trust, Headley Way, Headington, Oxford OX3 9DU.

14. Contact for further information.

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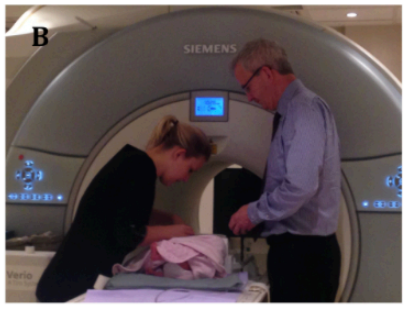

Pictures show examples of an EEG (A) and an MRI study (B).

Thank you for reading this information sheet.

Oxford University Hospitals NHS
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CONSENT FORM

Patient Identification Number for this study:

Title of project: Investigating pain in the developing human brain

Name of Principal Investigator: **Dr Rebeccah Slater**

Please read each statement and initial in each box

1 I confirm that I have read and understood the information sheet dated **15/09/2016 (version 7)**, for the above study, and have had the opportunity to ask questions and have had these answered satisfactorily ☐

2 I understand that my baby's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my baby's medical care or legal rights being affected. ☐

3 I understand that relevant sections of my baby'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 baby's taking part in this research. I give permission for these individuals to access to my baby's records. ☐

4 I consent to my baby/infant being videoed during the study. I understand that recorded images will not be used for public use, only analysis. No identifiable information, including videos recordings and imaging, will be used in any publications/presentations. Only anonymised data will be published or presented at meetings. ☐

5 If I have been approached about my baby's participation in an MRI study; I understand that the scan is a research scan that is not useful for medical diagnosis, and that scans are not routinely looked at by a doctor. If a concern is raised about a possible abnormality on my baby's scan, I will only be informed if a doctor thinks it is medically important such that the finding has clear implications for my baby's current or future health. ☐

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6 I would be happy for my baby to be studied on one occasion for the NIRS / EEG / MRI Study. *(please delete as appropriate)* ☐

7 I would be happy for my baby to be studied on more than one occasion, up to a maximum of 5 occasions for the NIRS / EEG / MRI Study. *(please delete as appropriate)* ☐

8 I would be happy for the collected data to be used for teaching or academic research presentations. ☐

9 I agree to take part in the above study ☐

_____	_____	_____
Name of Parent	*Date*	*Signature*
_____	_____	_____
Name of Person taking Consent	*Date*	*Signature*

1 form for Patient;
1 to be kept as part of the study documentation,
1 to be kept with hospital notes

Chapter 8

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