INVESTIGATING PAIN IN THE DEVELOPING HUMAN BRAIN

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A thesis submitted for the degree of
Doctor of Philosophy

Trinity Term 2019

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Abstract

Infants born prematurely or critically ill require a plethora of painful procedures during a period of extensive structural and functional neurodevelopment. Pain is however inconsistently and inadequately managed in neonatal care due to the inherent challenges of pain assessment and a lack of evidence-based analgesics. Noxious stimuli provoke behavioural and cardiorespiratory responses, which are valuable indicators of pain in the absence of verbal report. These measures can however be subjective and limited in specificity and sensitivity. EEG and fMRI can provide objective measures of noxious-evoked brain activity with which to investigate the development of nociceptive processing, its modulation in early life, and efficacy of analgesics.

In this thesis, I used electrophysiological measures to characterise the development of noxious-evoked brain and spinal reflex activity in the preterm period. Nociceptive-specific brain activity increased in magnitude with gestational age, whereas reflex withdrawal activity decreased in amplitude, duration, and latency. The relative proportion of these responses was strongly correlated with gestational age, suggesting a developmental relationship between spinal and supraspinal maturation. FMRI was subsequently used to investigate the relationship between noxious-evoked brain activity and connectivity of the periaqueductal grey (PAG), a core region of endogenous pain modulation and common target of analgesics. Functional connectivity of the PAG to the middle frontal gyrus and anterior cingulate cortex was negatively correlated with noxious-evoked BOLD activity. Developing connectivity of the PAG may begin to influence noxious-evoked brain activity in term neonates and influence the developmental relationship between electrophysiological measures.

Many external and internal factors influence pain. Sex differences in pain sensitivity and nociceptive processing are extensively reported in adults and animals. I therefore used electrophysiology and fMRI to investigate sex differences in nociceptive processing in healthy term infants. Noxious stimulation evoked greater magnitude of electrophysiological activity and greater BOLD activity across multiple brain regions in female compared to male infants.

Morphine is a commonly used analgesic in neonates despite inconclusive evidence of its efficacy. In my final study, noxious-evoked brain activity was employed as a co-primary outcome with clinical pain scoring to investigate the efficacy of oral morphine for procedural pain relief in infants born prematurely. The placebo-controlled trial was terminated early due to safety concerns. Despite significant cardiorespiratory effects, morphine did not attenuate multimodal measures of pain, raising questions concerning the balance of safety and efficacy of this drug.

In summary, this thesis demonstrates that age, connectivity of endogenous pain modulatory brain regions, and sex affect noxious-evoked brain activity, and this valuable biomarker of pain can be used alongside multimodal outcome measures and rigorous physiological monitoring to assess the efficacy of analgesic agents in neonates.

Acknowledgements

I dedicate this body of work to my mother, Anne Moultrie, the bravest woman I know – forever elegant, composed, and determined in the face of adversity.

First and foremost, I would like to thank my husband, Rob. Without your unwavering love, faith and encouragement, I would never have made it through. You are my rock.

Thank you to my amazing family. Thank you to my Dad for his fervent belief in me and his words of wisdom, and my sister Charlotte for her positivity and infectious enthusiasm.

To Rebeccah for being an inspirational supervisor, mentor, and role model. Thank you for taking me under your wing as a naïve young doctor and providing me with guidance, support and so many opportunities to develop as a researcher. Thank you for your confidence and belief in me and for giving me the freedom to explore my interests.

Thank you to Eleri for facilitating these challenging studies in the Neonatal Unit and for providing me with invaluable support during the tougher times over the past few years.

Thank you to all the members of the Paediatric Neuroimaging group for supporting me through the high and the lows. In particular, thank you to Gabi for the much-needed laughs and chats, and thank you to Sezgi for her infectious enthusiasm. Thank you to Caroline for her help and wizardry with all things Matlab. Thank you to Luke for his help with all things MRI and for his entertaining and unbelievably thorough emails.

Thank you to Mike, Jon, and David, the wonderful radiographers who supported these studies. Thank you for your enthusiasm, professionalism and for being unbelievably patient and accommodating. You are an absolute joy to work with.

Last but not least, thank you to the amazing parents who consented for their infants to participate in these studies. I am forever grateful and inspired by your belief in the importance of research.

Funding

The work in this thesis was funded by a *Clinical Doctoral Fellowship* supported by the Wellcome Trust [102176] and the NIHR Biomedical Research Centre, based at Oxford University Hospitals NHS Trust, Oxford.

The clinical trial was funded by the National Institute for Health Research Efficacy and Mechanism Evaluation programme [14/187/01] and the Wellcome Trust [095802 and 102076].

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Abbreviations

ACC – anterior cingulate cortex
AI – anterior insula
BBR – boundary-based registration
BIIP - Behavioural Indicators of Pain
BNFc – British National Formulary for children
BOLD – Blood Oxygen Level Dependent
EEG - electroencephalography
EMG - electromyography
fMRI – functional Magnetic Resonance Imaging
GA – gestational age
IVH – intraventricular haemorrhage
IQR – interquartile range
M3G – morphine-3-glucuronide
M6G – morphine-6-glucuronide
NFCS - Neonatal Facial Coding System
MFG – middle frontal gyrus
NIPPV – non-invasive positive pressure ventilation
NIRS – Near infrared spectroscopy
NRES – National Research Ethics Service
PAG – periaqueductal grey
PIPP – premature infant pain profile
PIPP-R- premature infant pain profile - revised
Poppi – Procedural pain in premature infants
QST – quantitative sensory testing
REC – Research Ethics Committee
RF - radiofrequency
RMS – root mean square
ROP – retinopathy of prematurity
RVM – rostroventral medulla
S1 – primary somatosensory cortex
S2 – secondary somatosensory cortex
SAR – specific absorption rate
T – Tesla
TE – echo time
TR – repetition time
VAS – Visual Analogue Scale

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Chapter 1

1 Introduction

Pain relief is a fundamental right and a basic expectation of all patients receiving medical care (1). However, in the absence of verbal communication, pain management presents a significant challenge. Treating pain in infants is like "going into battle with such inadequate weapons and limited knowledge of the enemy" (2). Until recently, much of our understanding of nociceptive processing in early life has been derived from developmental animal models (3). However, in the last decade, non-invasive neuroimaging methods and novel experimental paradigms have been developed and optimised for use in infants (4–8). These techniques provide the opportunity to improve our mechanistic understanding of nociceptive processing in human infants in vivo. In order to manage pain effectively in neonatal care, we must understand the neurobiological mechanisms involved in processing noxious stimuli, their maturation during preterm development, and their modulation by maturing endogenous mechanisms and analgesic interventions.

The work in this thesis investigates the development of nociceptive brain and spinal reflex activity, the functional connectivity of endogenous pain modulatory brain regions, sex differences in nociceptive processing, and the efficacy and safety of a common but controversial analgesic drug for procedural pain in premature infants. First however, this introductory chapter provides an overview of pain in the context of neonatal care, reviews the neural circuitry required for pain perception and its early development, discusses the methods with which we assess pain, and examines the evidence base for common pain-relieving interventions used in neonatal care.

1.1 Pain in the context of neonatal care

It was not until the 1950s that the care of newborns was formally acknowledged as a discipline of medicine requiring dedicated specialism and expertise (9). Since 1965, the survival of infants with a birthweight of less than 1000g has increased from less than 10% to over 95% (10). This dramatic improvement can be attributed to revolutionary advances in thermoregulation, nutrition, and cardiorespiratory support, including ventilation, administration of surfactant, and antenatal steroids (9). Each of these advances has been underpinned by a substantial body of research into the developmental physiology of premature infants and the pathophysiology of conditions with which they can be afflicted. Pain is no exception. In order to treat it successfully we must understand the developmental neurophysiology underlying nociceptive processing in this unique patient population.

Worldwide, it is now estimated that 15 million infants are born prematurely every year (11). These infants can be classified as extremely premature (<28 weeks' gestation), very premature (28-32 weeks' gestation) or late premature (33-36 weeks' gestation). Technological advances have facilitated a gradual lowering of the gestational age (GA) compatible with extra-uterine life and today infants born as early as 22 weeks' gestation are receiving neonatal care with an approximately 15% chance of survival (12). Increased survival of premature and sick infants has however not come without consequences. Morbidity is generally inversely related to GA at birth, although the impact of even late preterm birth is not insignificant (13). As such, the focus of neonatal research has gradually shifted towards the improvement of quality of life and the prevention of adverse outcomes in surviving infants, in particular the prevention of brain injury and neurodisability. Infants born prematurely in the second or early third trimester of

gestation are at greater risk of developing neurological sequelae and associated disability as they are exposed to developmentally-unexpected stimuli and physiological stresses during a critical period of neurodevelopment. Despite some improvement in neurodevelopmental outcomes, premature infants remain at significant risk of developmental delay and only about 50% of surviving infants born at 22- 26 weeks' gestation live without moderate or severe neuromotor or sensory disability (14). Considering neonatal care has life-long consequences for its survivors, their families and wider society, there is an urgent need to address the modifiable factors that can negatively impact neurodevelopment. Exposure to repeated painful stimuli during this critical period is one such factor. Although pain management is a fundamental component of all medical care, until recently it has been relatively neglected in this patient population as the assessment and treatment of pain in infants present significant challenges, which will be discussed in this chapter.

1.2 Defining pain

In order to discuss the assessment and treatment of pain, we must review its definition. The Subcommittee on Taxonomy of the International Association for the study of Pain (IASP) first provided authoritative guidance in 1979, recognising the multidimensional nature of this complex subjective experience. Pain was, and still is, defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'(15). Historically, this definition has suffered strong criticism and inspired impassioned debate due to its implied reliance on communication. To many the definition irresponsibly called into question the experiences of infants and cognitively impaired children and adults (16,17). In order to address this

potential discrimination, a caveat was added in 2011 clarifying that the inability to self-report does not negate the experience of pain or a requirement for pain-relieving interventions (18). However, ultimately, there are no surrogate markers that are considered equal to the gold standard of verbal report. Pain, the subjective psychological state, is distinguished from the independent but related neurobiological process of nociception, the neural process of encoding of noxious information. The outputs of this process include autonomic and complex behavioural responses and do not necessarily imply pain. However, in the absence of self-report, how else can we measure a conscious experience in another human being? Certainly, there are significant limitations to relying on behavioural measures to assess pain and analgesia in infants and these will be explored.

1.3 History of pain in neonatal care

Pain has not always been recognised in neonates. Until the 1980s, it was generally accepted in both medical and scientific communities that infants lacked the capability of perceiving pain (19). Whilst parents broadly assumed that their infant would receive pain management, neonatologists and anaesthetists were reluctant to provide analgesic and anaesthetic agents due to a lack of definitive proof of the existence of pain in these patients, and certainly none that could justify the feared adverse effects of available drugs (20). Surgeries were therefore performed using paralytic agents (21), the focus being on immobilisation to practically facilitate the procedures rather than the prevention of suffering.

It is interesting to consider the historical background to scepticism regarding pain in infants (for review see (22)). Charles Darwin's celebrated work on evolutionary theory and the expression of emotions in man promoted the concept of infants as primitive beings with under-developed senses and merely reflexive behaviours (23). This theory was supported by the first experimental work on neonatal pain performed by Genzmer, who reported infants displayed no evidence of discomfort in response to pinpricks that penetrated the skin, suggesting newborns were insensitive to pain (22). Subsequent studies in the United States in the early 20th century documented the development of sensorimotor responses in the first month of life and reported an increase in reflexive behavioural reactions with postnatal age (24,25). In the 1920s, Peiper, a German scientist, concluded from his own pinprick experiments that infants displayed a behavioural repertoire of pain responses even from birth with a similar latency to adults and although he conceded that these behaviours appeared reflexive and thus could not be directly equated to adult pain, he also concluded they should not be used to deny the experience of pain (22). In the 1930s, more extreme experiments involving the application of electric shocks were performed, from which the authors determined that these reflexive behaviours were subcortical responses generated by primitive brain areas (26). They argued that the infant responses could not be attributed to pain due to the impossibility of determining their psychological nature. Furthermore, psychologists such as McGraw in the 1950s promoted the theory of the 'decorticate' infant, using histological studies of cortical development and lack of myelination as evidence of limited cortical function in the newborn, negating a potential experience of pain (27). McGraw argued that complete cortical development was necessary to support consciousness and memory of pain and notably equated the responses of a newborn to those of a patient receiving general

anaesthetic. This was further substantiated by clinicians' observations that infants rarely required post-operative pain relief as they appeared to 'tolerate discomfort well' (28).

It was not until the seminal work of Anand and colleagues that standards of analgesic provision were directly challenged. Their studies demonstrated that infants undergoing surgery mounted significant hormonal and metabolic stress responses, which they proposed could be detrimental to neurodevelopment (29). Furthermore, they demonstrated that these responses were attenuated by the addition of fentanyl analgesia to the standard provision of nitrous oxide and muscle relaxants, and this was associated with an improvement in post-operative morbidity and ventilatory support requirements (30). Anand and colleagues subsequently demonstrated that the provision of halothane anaesthesia also significantly improved hormonal stress responses and postoperative cardiorespiratory stability (31). Although these studies did not provide the previously desired unequivocal proof of pain experience in these patients, the utilitarian demonstration of the consequences of untreated pain and the benefits of anaesthetic and analgesic treatments was sufficient to challenge the attitudes to pain and standards of practice at the time. This inspired research into the assessment of pain in infants, and over the next two decades a plethora of neonatal pain assessment tools were developed based on various combinations of behavioural responses and physiological parameters (32). By 1995 there was almost universal agreement amongst anaesthetists in the UK that infants perceive pain. A survey confirmed that the majority of anaesthetists (91% of respondents) were providing systemic opioids to infants for major surgery, unlike in 1988 when this practice was only adopted by a minority (20).

Despite the publication of national guidelines and consensus statements regarding the management of pain (33,34), pain assessment is often neglected in practice, analgesics

remain under-utilised (35–37) and wide variations exist in practice between and even within countries (38–40). These issues are likely due to the epistemic challenges of objectively quantifying pain and analgesia in neonates and the related paucity of analgesic drugs with proven efficacy and safety in this patient population. Investigating the assessment of pain in premature neonates is now a key neonatal research priority (41).

1.4 The development of nociceptive circuits

In this section, I will provide an overview of the neural circuitry required for the perception of pain and summarise our understanding of its development with evidence from both human and animal studies.

1.4.1 Peripheral encoding and signal transmission

Nociceptive pain requires activation of nociceptors. These specialised primary sensory neurons of the peripheral nervous system consist of free nerve endings, which are triggered by mechanical, heat and chemical stimuli. Large diameter thinly myelinated Aδ-fibres rapidly conduct action potentials at a velocity of 12-30m/s and can be subdivided based on their thermal response thresholds (42). Type I fibres have a high heat threshold, are sensitive to mechanical and heat stimuli, and transmit the sharp, localised sensation of 'first pain' to alert the individual to impending tissue injury. Smaller diameter unmyelinated C-fibres are mostly polymodal, propagate activity at a lower velocity (0.5-2m/s) and their depolarisation results in the more diffuse, sustained and dull 'second pain' sensation (42). Activation of single nociceptors however does not necessarily result in

pain, and central mechanisms of summation play a key role in determining pain thresholds (43).

The anatomical connections of the nociceptive peripheral nervous system are present and functional by 20 weeks' gestation, despite being poorly myelinated. During human embryonic development, cutaneous receptors develop regionally from 7.5 weeks gestation, beginning in the perioral area (44) and nociceptive fibres reach the dorsal root ganglion of the spinal cord by 19 weeks' gestation (45). An extremely preterm infant has an equal or even greater density of nociceptors than adult skin (46). Although nociceptors are present, nerve conduction is less efficient. Non-invasive studies performed in human neonates have demonstrated that conduction velocities even at term gestation are approximately half that of adults (47), and do not reach adult levels until 5 years of age (48). However, slower transduction of noxious inputs should not negate the potential to perceive pain.

In the dorsal horn, nociceptive afferents synapse with second order projection neurons that decussate and ascend the spinal cord to transmit sensory information to the thalamus. These neurons include wide-dynamic range neurons that respond to an array of stimulus intensities and also receive input from Aβ primary afferents, and nociceptive-specific neurons, which have a small receptive field with a higher response threshold (49). During development, A fibres first penetrate the dorsal horn (at embryonic days 15-17 in the rat), followed by C fibres (50,51), in order to synapse with second order neurons. In the adult, C-fibres terminate in laminae I and II, depending on whether they are peptidergic or non-peptidergic respectively and Aδ fibres terminate in laminae I and V (52) . However, during foetal development, non-nociceptive A fibres also exuberantly extend beyond their adult position in the dorsal horn in lamina III and IV up to laminae I and II (53). This

overlap between non-nociceptive and nociceptive inputs occurs during the first three postnatal weeks in the rat, following which the fibres regress, and this is thought to contribute to the hyperexcitability of reflexes in rat pups (54). In the adult rodent, noxious stimulation induces expression of c-fos, an immediate early gene marker of neural activity, in the superficial laminae of the dorsal horn (55). However, in the neonatal rat, neural activity can be demonstrated in these laminae in response to both non-nociceptive Aβ fibre and nociceptive afferent stimulation from postnatal day 3 and to a lesser extent by day 10 (56). At what stage this transition occurs in human neonates is not known.

Several distinct tracts are responsible for the transmission of noxious input from the dorsal horn to supraspinal structures, including the spinothalamic, spinomedullary, and spinobulbar tracts. Of these, the spinothalamic tract is the most studied and most strongly associated with pain (57). High-resolution tracers have been used in rats to outline the projections from nociceptive spinal laminae. Lamina I projections ascend to relay thalamic nuclei to transmit sensory discriminative properties of pain, whereas deeper laminae project to the central thalamic nucleus and other regions, such as the globus pallidus, amygdala and hypothalamus and may have a role in alertness and motor components of pain (58). Spinothalamic tracts appear early in development and are structurally identifiable in the rat by embryonic day 18 (59). However, studies of c-fos expression have revealed that these afferent tracts are not functional until at least postnatal day 3 in the rodent, at which time expression is observed in the ventrolateral medulla in response to noxious stimulation (59). Brain regions such as the thalamus, hypothalamus and periaqueductal grey do not express this functional marker until postnatal day 14 (59).

As well as ascending transmission of noxious input, there are local circuits that drive reflex responses. Nociceptive afferents synapse with interneurons in the spinal cord,

which in turn synapse with motor neurons that trigger contraction of flexor muscles, resulting in movement away from a stimulus. This circuitry underlying the protective nociceptive flexion reflex develops early in gestation. Neuromuscular junctions appear between 10-13 weeks' and motor neurons project to the ventral horn between 11- and 19-weeks' gestation (60). Motor afferents are densely packed and undergo considerable re-organisation and pruning to produce a more adult-like distribution by 25 weeks' gestation (60). Unlike in adults, vigorous spinal reflexes can be evoked by both noxious and innocuous mechanical stimulation in premature and term infants, which are exaggerated in magnitude and duration compared to adult responses (61). Their utility in the assessment of pain in infants will be discussed in more detail later in this chapter.

1.4.2 **Supraspinal nociceptive processing**

Historically, it was believed that pain must be a subcortical process as cortical lesions and stimulation during neurosurgical procedures did not appear to alter pain perception (62,63). However, it is now widely accepted that in order to perceive the sensory and emotional qualities of nociceptive pain, noxious input must be processed at the level of the cortex. Thalamocortical connections are therefore fundamental to our ability to consciously perceive pain. By 23-24 weeks' gestation, thalamocortical afferents have accumulated in the subplate, a transient developmental structure with a critical role in corticogenesis (64,65). These afferents migrate out towards targets in the cortical plate. Cortical processing of noxious stimuli is therefore unlikely in infants born at less than 25 weeks' gestation as they lack the thalamocortical connectivity (66). From 26- to 28-weeks' gestation, synaptogenesis begins from the deepest to the most superficial cortical layers (67). Smaller, delayed somatosensory evoked potentials can be recorded from

infants born prematurely from this stage of development (68–70). The expanding cortex rapidly undergoes primary gyrification and sulcation (71), and by 32 weeks' gestation the 6-layered laminar organisation of the cortex is evident (66). Between 31- and 34- weeks' gestation, thalamocortical axons begin to establish synapses with layer IV neurons in the cortical plate (66). After reaching its maximal thickness the subplate then begins to gradually resolve. By term gestation, the subplate underlying the primary somatosensory cortex is no longer clearly identifiable as a distinct layer and disappears within the first postnatal month (72). Resolution is however slower in the prefrontal cortex and this is thought to be due to protracted development of cortico-cortical connectivity and tertiary gyrification (66,67,73). All of the major white matter tracts are in place in the brain of a full-term neonate, although they will continue to undergo significant growth, pruning and re-organisation during the postnatal period (74). The structural presence alone of thalamocortical connectivity is however insufficient to establish a capacity for pain perception.

The supraspinal processing of noxious inputs is complex. There is no dedicated pain cortex akin to the visual or auditory cortex. Instead, a network of cortical and subcortical brain regions is involved in generating the perception of pain. These regions include, but are certainly not limited to the thalamus, somatosensory cortices (S1, S2), insula, anterior cingulate cortex (ACC), prefrontal cortex, brainstem and cerebellum (75,76). Classically, the pain 'neuromatrix' proposed by Melzack has consisted of a lateral sensory-discriminative component, attributed to brain regions such as the somatosensory cortices, and a medial affective-cognitive-evaluative component, attributed to regions such as the anterior cingulate cortex, insula and amygdala (77–79). However, neuroimaging experiments have failed to consistently identify a core set of brain regions uniquely representing the pain experience in human adults. Ultimately, there is no fundamental

network that if ablated would cure all pain (80). A complex array of individual and environmental factors influences whether brain regions are activated or inactivated during pain (81). Therefore, the concept of a rigid matrix has more recently been challenged (82–86) and a dynamic connectome theory has been proposed, which attributes the conscious experience of pain to the co-ordination or synchronisation of activity between a dispersed group of brain regions that are not specific to pain (87–89). Investigating the patterns of brain activity evoked by noxious stimulation during development is likely key to our understanding of pain in infants.

1.4.3 **Descending pain pathways**

The influence of supraspinal structures on nociceptive spinal reflexes was proposed in the Gate-Control theory (90), following on from earlier studies, which demonstrated the exaggeration of nociceptive reflexes following spinal cord transection in the rodent (91). However, it was Reynolds who provided empirical evidence that stimulation of a region of the midbrain can produce profound analgesia (92) and it was subsequently demonstrated that this stimulation selectively attenuates nociceptive reflexes without altering responses to innocuous stimuli (93).

Nociceptor activation is not linearly related to pain experience (94). An identical noxious stimulus administered to two individuals does not produce an identical pain experience. Equally, an identical stimulus administered to a single individual in different contexts does no produce an identical pain experience. Factors such as emotions, expectations, and distraction can dynamically influence our perceptions. Endogenous pain modulation is provided by a network of brain regions that play a critical role in regulating nociceptive

input through a balance of inhibition and facilitation. Inhibition of pain promotes survival by facilitating escape from noxious threats, whilst facilitation of pain oppositely promotes behaviour enabling recovery from injury. The periaqueductal grey (PAG), a region of grey matter surrounding the cerebral aqueduct in the midbrain, is a vital area of convergence of cortical, forebrain and spinal projections and plays a central role in the endogenous modulation of pain. Its intricate connectivity allows both descending control of nociceptive transmission and feedback modulation. The PAG receives input from brain regions including the anterior cingulate cortex (ACC), insular cortex, amygdala, prefrontal cortex and hypothalamus, and transmits descending outputs mainly to the rostroventral medulla (RVM) (95), with smaller projections to noradrenergic cell groups, orbitofrontal cortex, and medial thalamus (96).

The rostroventral medulla (RVM) is an area of the brainstem's pontomedullary reticular region that receives input from the PAG as well as the nucleus cuneiformis, noradrenergic pontine cells, the hypothalamus and prefrontal cortex. This structure has descending projections to laminae I, II and V of the spinal dorsal horn that can amplify or dampen nociceptive transmission and thus alter pain experienced. The RVM projections consist of OFF cells that inhibit nociceptive processing and can be activated by opioid administration (97–99), and ON cells that facilitate the transmission of noxious inputs and are conversely depressed by opioids (98,100,101). Microstimulation of this brain region also produces selective analgesia (102).

In adults, these modulatory brain regions can provide endogenous analgesia exemplified by placebo analgesia (103) and this system is also a target of analgesic drugs such as opiates (104), cannabinoids (105), paracetamol (106), and non-steroidal anti-inflammatory agents (107), and serotonin/norepinephrine reuptake inhibitors (108). Mu-

opioid receptors are found throughout the descending pain modulatory system including the PAG, RVM, insular cortex, amygdala, hypothalamus, and spinal dorsal horn (109–112). In animal studies, microinjection of opioid agonists into the PAG, RVM, insula and amygdala, inhibits behavioural responses to noxious stimulation (102,113–118). Lesioning of the PAG however markedly reduces the effects of systemic morphine (119) and ablation of the RVM or its connections to the dorsal horn prevents analgesia induced by PAG stimulation (120), which demonstrates the important role of this system in opioid-mediated analgesia.

Little is known about the development of descending pathways in human infants. Much of our understanding has been derived from developmental animal models. In rats, primary afferents are present and functional in the periphery as well as in the dorsal horn of the spinal cord from birth, responding to both mechanical and electrical noxious stimuli (121). Although descending projections are also present from birth, they are not functional (122). Weak inhibition of reflexes in response to pinch stimuli can be induced by stimulation of the dorsolateral funiculus from postnatal day 10, and this inhibition increases to adult levels by postnatal day 22 (122). Furthermore, stimulation of the PAG inhibits the tail flick reflex in rats from postnatal day 21 (123). However, prior to day 21, stimulation of the RVM surprisingly facilitates reflexes and spinalisation of newborn pups abolishes this descending excitatory drive resulting in reduced reflexes (124). This is due to the excitatory influence of GABA in the first few of weeks of life in the rat (125). The RVM then undergoes a maturational switch to exert biphasic control of the dorsal horn, with increasing inhibitory control of nociceptive transmission from day 21. Low intensity stimulation of the region facilitates nociceptive transmission and resultant reflexes, whilst greater intensities inhibit responses (125). Glycine receptor mediated inhibition also does not develop until the third postnatal week in rats (126,127).

The sensitivity and exaggerated nature of reflexes in premature infants provides evidence of the hyperexcitability of the spinal cord in early life, which is consistent with a lack of descending inhibition and dominance of facilitation from brainstem modulatory circuits. In Chapter 3 of this thesis, electrophysiology is used to characterise both the development of nociceptive brain and spinally-mediated reflex activity in premature infants. Beyond cautious cross-species interpretation of the animal literature, it is not known at what stage of human development descending modulatory influences mature. Therefore, in Chapter 3 the relationship between brain and reflex responses is investigated across the human preterm period. Secondly, fMRI is used to investigate the emergence of endogenous pain modulation by exploring whether functional connectivity of the PAG to cortical brain regions relates to noxious-evoked brain activity in infants.

Investigating the development of endogenous pain modulation in early life is not only important for our understanding of pain perception and its inter-individual variability, it is also critical to our understanding of the effects of analgesics and the consequences of exposure to repeated noxious stimuli in early life. In rats, administration of opioids during the fourth postnatal week accelerates the development of RVM inhibitory control, whereas antagonism of opioid receptors disrupts the development of this system (128). Animal studies have also demonstrated that pain exposure in early postnatal life can alter RVM signalling in adulthood. In rodents exposed to inflammatory pain as neonates, RVM stimulation later produces greater inhibition of nociceptive reflexes (129). Early pain exposure also leads to increased PAG beta-endorphin levels and reduced PAG opioid receptor expression in later life (130). Therefore, the development of this important system is likely vulnerable to disruption during neonatal care.

1.5 Assessment of pain in neonates

Having outlined the development of neural circuitry underlying pain perception, in the following section the methods that have been developed to assess pain in non-verbal infants are reviewed.

1.5.1 Behavioural responses

The intrinsic salience of noxious stimuli drives dramatic effects on behaviour (88) and behaviour is the only expression through which infants can communicate their distress and discomfort to a caregiver. In the absence of verbal report, it provides the most obvious and easily observable surrogate marker of pain. In the 1980s, Grunau and Craig first systematically examined the facial expressions exhibited by infants and coded the discrete features elicited in response to a noxious clinical procedure (131). The features most commonly identified in response to heel lancing of the foot for blood collection were brow bulge, eye squeeze and nasolabial furrow and their expression was influenced by sleep state (131). The Neonatal Facial Coding System (NFCS) provided the first validated clinical pain assessment tool for use in newborns (131). Body movements, including leg extension and finger splaying have also been investigated as indicators of pain (132–134) but these behaviours are less specific than facial expression (132,133). Autonomic increases in cardiac and respiratory rate or decreases in oxygen saturation can also be associated with acute pain (135). However, these indicators are compromised by the effects of prematurity, illness, and medications and therefore are poor predictors of pain in isolation (133,136). Over 40 different multidimensional pain assessment tools have now been developed for use in neonatal care, incorporating various combinations of facial

features, body movements and changes in physiological parameters (137). The most commonly used scale in clinical practice and clinical trials is the Premature Infant Pain Profile, which is based upon the assessment of the three facial actions mentioned above, changes in heart rate and oxygen saturation, and weighting factors for GA and behavioural state (135).

It has been proposed that pain-related behaviours should be used as the infantile equivalent of self-report and should not be demoted to surrogate measures of pain (16). However, behavioural measures still require us to infer the existence of pain and suffering (138). Unfortunately, behavioural assessment tools have significant limitations. Although they can be sensitive indicators, behaviours included in pain scores are not necessarily specific as they can occur in the context of stress or distress (139). Facial expression changes and rises in physiological parameters such as heart rate can be evoked by innocuous stimuli such as nappy changes (140) or in response to innocuous sensory stimuli such as a control heel lance, in which the skin is not broken (132). Both changes in heart rate and facial expression are less likely to be elicited in very premature infants (141,142) and for this reason, the PIPP score incorporates an arbitrary weighting factor to inflate the scores of more premature infants. This weakens the accuracy and reliability of this tool as a measure of pain as it is driven by the unverifiable assumption that these more premature infants perceive pain equally but are less able to communicate it than older infants. Furthermore, these measures can be compromised by illness (143) or sedating or paralysing agents commonly encountered in neonatal intensive care. Premature infants with severe intraventricular haemorrhages (IVH) also commonly display behavioural signs such as grimacing and restlessness, accompanied by changes in cardiac and respiratory rates (144,145), which could be interpreted as pain using behavioural measures. Ultimately, there is no gold standard such as verbal report against

which these measures can be validated in this population. Nevertheless, behavioural scores remain the only method of pain assessment used clinically in neonatal care.

1.5.2 **Reflexes**

Spinally-mediated reflexes resulting in withdrawal of a limb away from a noxious stimulus are one of the most commonly used surrogate markers of pain in animal studies. The postnatal development of reflexes in rat pups appears to parallel the development of reflexes in premature infants (61,146) and therefore studies of this measure using surface electromyography (EMG) have significantly improved our understanding of nociceptive development and responses to analgesics. Reflexes in newborn rat pups are exaggerated and inappropriate, and noxious stimulation commonly elicits a tail reflex towards the stimulus (147). In extremely premature human infants, noxious stimulation similarly elicits a non-specific and poorly localised response evidenced by bilateral limb withdrawal reflexes and generalised body movements (61,148). The threshold, duration and magnitude of reflex activity reduce with increasing postnatal age in rat pups (61,121) and similarly with increasing GA in human infants (61,148,149). Furthermore, reflex habituation is observed in response to repeated stimulation from 34 weeks' gestation (61). Altogether this developmental refinement of reflex activity may reflect the emerging influence of descending modulation as well as reorganisation of the spinal dorsal horn (53,150). By term gestation, noxious stimulation evokes a dominant reflex ipsilateral to the site of stimulation. However, the magnitude of this activity is correlated with stimulus intensity (148,151) and greater intensities can elicit bilateral limb withdrawal (151).

Reflexes can easily be observed at the cotside and body movements feature in several neonatal pain assessment tools such as the Behavioural Indicators of Pain (BIIP) score (152,153). The utility of reflexes in the assessment of pain is however limited by their lack of nociceptive specificity particularly in premature infants. Nevertheless, the study of reflexes has provided important mechanistic insights into early nociceptive development and could provide a useful, objective outcome measure for the assessment of analgesic efficacy in the peri-term population in combination with other surrogate measures of pain.

1.5.3 **Brain-derived measures of pain**

Ultimately pain is processed at the level of the cortex. Therefore, brain-derived measures of pain using near-infrared spectroscopy (NIRS), electroencephalography (EEG), and functional magnetic resonance imaging (fMRI) have been developed in infants over the past decade.

1.5.3.1 Near-infrared Spectroscopy

NIRS was the first brain-derived measure of nociceptive activity used in infants. This technique allows spectroscopic measurement of cerebral oxygenation through optodes placed on the scalp, which emit and detect infrared light. The amount of light absorbed is determined by the concentration of oxygenated and deoxygenated haemoglobin in cerebral tissue (154). Based on the principle that increased neural activity results in an increase in blood flow that can be detected by an increase in tissue oxygenation (155), NIRS can be used to investigate regional functional activation of the brain. It has been applied in infants to demonstrate functional activity in the visual cortex in response to

visual stimulation (156), in the temporal cortices in response to auditory tones (157), and in the motor cortex during passive limb movements (158,159), and most recently to study nociceptive processing in infants (160,161). Clinical noxious stimuli such as venepuncture and heel lancing evoke an increase in haemoglobin concentration over the somatosensory cortices (160,161). Slater et al., demonstrated that this change is greater in the hemisphere contralateral to the site of stimulation and importantly occurs in infants as premature as 25 weeks' gestation (160). These noxious-evoked cortical responses have since been shown to correlate with facial responses (162) and increase in magnitude with postmenstrual age (163). However, noxious-evoked brain activity can also be observed in some infants in the absence of behavioural indicators of pain (162). This technique can easily be applied at the cotside and therefore has more recently been used to investigate the cortical effects of non-pharmacological pain-relieving interventions in neonatal care. Skin-to-skin contact (164) and oral glucose (165) have been shown to attenuate increases in oxygenated haemoglobin evoked by clinical noxious procedures. Although NIRS has proved useful, it has limited spatial resolution and limited penetration, detecting mainly superficial cortical responses (166).

1.5.3.2 Electroencephalography

Electroencephalography (EEG) is a technique used to record synchronised cortical electrical activity and is commonly employed in neonatal care in the assessment of epilepsy and hypoxic ischaemic encephalopathy (167). Electrodes sited on the scalp detect changes in the voltage and polarity of electrical field potentials over time, which are represented as deflections in the EEG trace. The pattern of EEG activity recorded in infants evolves dramatically during the preterm period, reflecting the extensive structural

and functional maturation of underlying cortical and subcortical structures occurring in this critical period. Extremely premature infants have infrequent bursts of activity interspersed with long periods of quiescence, which reduce in duration with age (168,169). Characteristic bursts of activity in the theta range (4-8Hz) are observed, first in the occipital and then in the temporal cortices (170,171). Patterns of immature brain activity like these characteristically disappear during the preterm period and their persistence has been associated with neurological pathology and is useful in prognostication (172–175). Between 32 and 35 weeks, the EEG signal becomes continuous at rest and sleep states can be differentiated (176). In term infants, the EEG provides evidence of sleep-wake cycling, with longer periods of active sleep than quiet sleep, and it is more specifically reactive to external stimuli (177).

Considering the dramatic evolution of resting state brain activity briefly outlined above, it is perhaps not surprising that sensory stimulation evokes an evolving pattern of neuronal activity during the preterm period. During the third trimester, neuronal bursts known as delta brushes, characterised by high frequency activity (8-25Hz) nested within a slow wave (0.3–1.5 Hz), commonly occur both spontaneously and in response to sensory stimulation (178–181). These non-specific exaggerated neuronal responses are most likely evoked centrally in extremely premature infants and with increasing GA spread to the occipital, frontal and temporal cortices (179). Non-specific widespread bursting activity likely plays a role in activity-dependent refinement of somatosensory pathways (182). This activity is gradually replaced by adult-like evoked potentials, heralding the functional maturation of underlying neural circuitry (183). From approximately 34-35 weeks' gestation there is a developmental switch from non-specific burst activity to somatosensory-evoked, visual-evoked and noxious-evoked potentials (183–185). Auditory- and visual-evoked potentials have been well characterised in

neonates and are clinically useful in the assessment of visual perception and hearing acuity (186–192).

A noxious-evoked potential was first characterised in infants by Slater et al., in 2010 in response to heel lancing, a common clinical painful procedure in neonatal care (7). By time-locking the EEG to the event and using Principal Component Analysis, the authors identified a clear and reproducible evoked potential at the vertex electrodes (Cz and CPz) ~500ms after release of the heel lance blade (7). Importantly, this noxious-evoked potential was not elicited by a control heel lance procedure, whereby the lancet was rotated and did not pierce the skin, and it does not occur following visual, auditory or innocuous tactile stimulation (5). The evoked potential is similar in morphology and topography to noxious-evoked potentials characterised in response to brief laser, electrical and mechanical noxious stimulation in adults (193–195). A very similar noxious-evoked potential has also since been reported in infants in response to other acute needle-related procedures such as vaccination (196) and cannulation (197).

In order to objectively quantify the magnitude of noxious-evoked brain activity across independent infant populations, a mathematical EEG template of noxious-evoked brain activity has been developed (5). The template was characterised using Principal Component Analysis in a population of healthy term infants in response to heel lancing and validated in infants aged 34-42 weeks' gestation. The template can be mathematically applied to an individual's EEG data to automatically calculate the relative magnitude of their response. In healthy term-born infants, the noxious-evoked potential is maximal at the vertex and its magnitude is correlated with stimulus intensity and reflex activity (5). Noxious-evoked potentials are however rarely observed in infants less than 32 weeks' gestation (183,198) and characterising brain activity changes in these infants

is an active area of research. Many factors can influence our responses to noxious stimulation and in infants noxious-evoked brain activity has been shown to be modulated by premature birth (199), mode of delivery (200), acute sepsis (197), and stress levels (201). Sex differences in pain is also a growing area of research, with considerable evidence of increased female sensitivity and differences in nociceptive processing from both adult and animal studies (202–204). Chapter 5 of this thesis investigates whether sex differences in nociceptive processing begin in early life and can be observed in healthy newborn infants.

Although the infant pain experience cannot be entirely represented by a brief pattern of electrical activity, this measure does provide evidence of specific cortical processing of noxious input in infants, which can be objectively quantified. Despite the limited spatial resolution of EEG, noxious-evoked brain activity is a valuable measure that can be used to improve our mechanistic understanding of the development of nociceptive processing and as a surrogate biomarker of pain and analgesia in the absence of verbal report.

1.5.3.3 Functional magnetic resonance imaging

Functional magnetic resonance imaging (fMRI) has revolutionised the study of pain neurophysiology. This non-invasive and non-ionising imaging technique indirectly records neural activity by measuring BOLD (Blood Oxygen Level Dependent) signal changes, enabling the localisation of functional activity. Since its application to the study of pain in 1991 (205), it has facilitated the investigation of brain regions involved in supraspinal nociceptive processing and their connectivity (206), the study of the functional differences underlying clinical pain states (207), and exploration of the

biological, cognitive and environmental factors (208) and analgesic drugs (209) that can modulate our perception of pain.

Event-related fMRI classically requires the cooperation of a subject to perform a set of specific tasks during the acquisition of images in order to enable localisation of regions of brain activity underlying the cognitive process under investigation. fMRI was first applied in infants to investigate visual processing (210). Seven infants aged between 6 weeks and 36 months were sedated and imaged during the presentation of a flickering light, producing activity in the occipital cortex (210). Subsequent studies continued to principally investigate the visual system of infants (see review (211)) as well as responses to auditory stimulation (212–217). In order to induce cooperation and minimise subject movement, scans were mostly performed under sedative agents including propofol (218), pentobarbital (219,220), chloral hydrate (210,221–225) and midazolam (226). However, due to concerns over the effects of sedation and the desire to investigate the responses of healthy infants, many researchers began to rely on encouraging natural sleep of infants in the scanner (213–215). In order to investigate the development of sensorimotor processing, novel paradigms were designed to induce passive limb movements using tailor-made stimulation devices (222,227–229), and these studies overall revealed functional activation of the primary somatosensory cortices, supplementary motor and premotor areas and cerebellum, with less lateralisation than adults.

Investigating nociceptive processing using fMRI in infants presents significant challenges. Most studies of noxious-evoked brain activity using modalities such as EEG or NIRS have recorded responses to clinical procedures such as heel lancing. Such procedures would however be inappropriate and impractical in the scanner and would not permit repetition of stimulation necessary to improve signal-to-noise. Furthermore,

stimulation devices used within the scanning environment cannot contain ferromagnetic material. The ideal experimental paradigm would stimulate nociceptors to induce nociceptive brain activity, without causing overt distress or eliciting excessive movement. The PinPrick device is a non-tissue damaging mechanical noxious stimulator available in a range of intensities, principally used for quantitative sensory testing (QST) in adults with chronic pain (230). This mechanical noxious stimulus predominantly activates type 1 Aδ nociceptive afferents (231), and in adults evokes a classic biphasic negative-positive EEG potential detected maximally at the vertex (232). It also evokes BOLD activity in brain regions commonly associated with pain processing (233). Hartley et al., applied PinPrick stimulation of various grades (32mN, 64mN, 128mN) to term infants and demonstrated that this device also evokes nociceptive-specific EEG activity and reflex activity similar, but of smaller magnitude, to heel lancing, crucially without evidence of behavioural distress and therefore was acceptable to parents (151). Williams et al., subsequently demonstrated the feasibility of applying PinPrick stimulation to study noxious-evoked brain activity in infants using fMRI (8). In a single infant imaged at 1.5T, they observed functional activations in the somatosensory cortices, supplementary motor area, precuneus and frontal cortex (8). In this study they also applied somatosensory stimulation using brush and von Frey hairs to 19 term infants and demonstrated intensity encoding of functional brain activity and a general reduction in responses in infants that received chloral hydrate sedation (8). Goksan et al., subsequently applied a range of Pinprick intensities (32mN, 64mN, 128mN) to 10 healthy non-sedated term infants and 10 healthy adults in a 3T scanner (4). The authors demonstrated that this low-intensity noxious stimulation evoked widespread cortical and subcortical activity in term neonates, which largely overlapped with the brain regions activated by the same stimulus in adults (4). Regions of the brain active in both populations included the primary somatosensory

cortices, anterior cingulate cortex, thalamus and insular cortices (4). The authors however noted that two brain regions, the amygdala and orbitofrontal cortex, were not active in infants, which could suggest differences in affective processing of noxious stimuli in newborn infants with limited experience of noxious stimulation (4). Although caution should be exercised in applying reverse inference to interpret the results of the study, the overlap in functional activity between infants and adults could suggest that the infant pain experience is broadly similar to that of adults. Whether functional brain activity in the infant is directly translated to a conscious perception similar to that which we associate with pain is ultimately impossible to determine.

The human brain undergoes dramatic structural and compositional changes during early development and thus tailored approaches to the acquisition and analysis of neuroimaging data in infants have been developed and will be reviewed in the methods chapter of this thesis (Chapter 2). fMRI provides the most convincing evidence to date of the similarity between infant and adult nociceptive processing and opens an avenue of research for investigating the development of nociceptive brain activity and the effects of demographic, contextual and environmental factors that modulate it in early life. Adult fMRI studies have revealed that attention (234), distraction (235), emotion (236), the placebo effect (103,237) and many analgesic drugs (238,239) engage top-down modulatory networks to profoundly alter the experience of pain (94). fMRI has provided evidence that the PAG is a key site for higher cortical control of pain modulation in humans and increased activity in this brain region correlates with reduced pain intensity ratings (240). fMRI has also allowed investigation of the functional connectivity of this brain region to cortical areas implicated in pain modulation. Activity and connectivity of the brain at rest appears to influence subsequent responses to noxious stimulation (241). Ploner et al., studied connectivity in the periods prior to application of noxious

stimulation and observed that connectivity between the PAG and anterior insula is predictive of whether subjects report a stimulus as painful (242). fMRI can now be applied to explore the connectivity of the PAG in human infants and how this relates to functional brain activity (243). Understanding the function of this system in early life is important as it contributes to inter-individual variability in pain sensitivity, it has been implicated in the development of chronic pain (208), and this circuitry may be altered by repeated pain and opiate exposure in neonatal care (244–248).

1.6 Management of pain in neonates

Ultimately, the assessment of pain in infants is purposeless if we do not have safe and effective analgesic interventions with which we can treat it.

1.6.1 Non-pharmacological pain-relieving interventions

Non-pharmacological pain-relieving interventions have been widely promoted and adopted in neonatal care due to their attenuation of behavioural responses to painful procedures without significant associated side effects (249). These interventions include skin-to-skin contact, facilitated tucking, swaddling, non-nutritive sucking, breastfeeding and administration of sweet solutions such as sucrose. Sucrose is the most commonly studied of these interventions, with more than 75 publications investigating its analgesic effect (249). Although this substantial body of literature provides evidence of reductions in composite pain scores to heel lancing, venepuncture and intramuscular injections, investigations of brain activity have been unable to convincingly demonstrate a concurrent effect of this intervention on central nociceptive processing. Bucher et al., conducted a randomised controlled trial of infants receiving sucrose or placebo for heel

lancing and although they observed significant reductions in heart rate response and crying, there was no effect on cerebral blood volume detected by NIRS (250). A subsequent electrophysiological study, focusing on frontal asymmetries in brain activity, reported attenuation of frontal brain activity in infants who received sucrose prior to noxious heel stroking, and this was believed to reflect reduced affective processing of noxious input (251,252). However, in 2010, a randomised placebo-controlled trial employing a more specific EEG measure of nociceptive processing (previously described), demonstrated no significant effect of sucrose on nociceptive brain activity recorded at the vertex, despite attenuation of behavioural responses, evidenced by PIPP scoring (253). Overall, it appears that sucrose selectively attenuates behaviour without dampening supraspinal nociceptive input. In rats, the effect of sucrose on behavioural nociceptive responses persists following midbrain transection but is inhibited by the administration of opiate-receptor antagonists (254), suggesting its behavioural effect may be mediated by the brainstem (255,256).

Sucrose is a simple intervention, which is widely believed to be effective and harmless, and as such has been heavily endorsed by many as a standard of care (33). However, there is now emerging evidence that this intervention may have long-term adverse effects if it is judiciously administered to premature infants for every minor procedure at doses that range widely between 0.05 to 2 mL of 12% to 50% solution (249). A study of very premature infants reported that the number of doses of sucrose administered in the first postnatal week was associated with worse neuro-behavioural outcomes relating to motor development and alertness at term-equivalent age (257). These infants were receiving sucrose up to 3 times per invasive procedure. The effect reported could not be attributed to the number of painful procedures as it was not observed in the control group of infants who received water. A study in animals has since provided supportive evidence of

structural neurodevelopmental effects (258). Tremblay et al., observed that repeated administration of oral sucrose during the first postnatal week in mice is associated with reduced hippocampal and cerebellar grey matter and white matter volumes in adulthood (258). Furthermore, an MRI study of premature infants has most recently reported an association between pain and glucose exposure, and reduced structural and functional brain development (259). The authors interpret their findings as evidence to suggest that sucrose fails to attenuate the long-term neurodevelopmental effects of repetitive pain exposure. Although there is good evidence of attenuation of behavioural responses by many non-pharmacological interventions, consistently using these interventions in preference to pharmacological agents could leave infants vulnerable and exposed to excessive nociceptive input contributing to long-term neurodevelopmental effects of premature birth.

1.6.2 **Analgesic drugs**

Pain management guidelines have broadly been based upon expert committee opinions, due to the limited availability of evidence from clinical trials of analgesics (260). Pharmacological analgesics remain under-utilised in practice as a result of concerns over their side effects and the limited and often conflicting evidence of their efficacy (261). Considering that both nociceptive and metabolic pathways are immature in premature infants compared to those of older children and adults, it is unsurprising that analgesic mechanisms, dosing and efficacy may differ significantly in this patient population.

Opiates were introduced into neonatal intensive care in the late 1980s for postoperative analgesia and sedation during mechanical ventilation (262), years before clinical trials

were undertaken to determine their safety and efficacy in neonates. Studies in the 1990s subsequently demonstrated that opiates, such as morphine improved ventilator synchrony (263), behavioural measures of comfort (264,265), and metabolic stress responses (266). Morphine, the archetypal opioid, became one of the most frequently used analgesic drugs in neonatal care (267), although evidence for its analgesic efficacy remains controversial.

Opiates bind μ-, δ-, and κ- opioid receptors distributed at spinal and supraspinal sites such as the PAG, RVM and limbic system, modulating ascending transmission of nociceptive inputs and ultimately decreasing pain perception (110). Although descending modulatory pain pathways may be immature in premature infants, morphine could be effective in reducing nociceptive responses even in early life due to its actions at the dorsal horn (268). Animal models have been used to investigate the developmental neurobiology and analgesic efficacy of opioids. In adults, opioids selectively suppress the activity of Aδ and C nociceptive afferents (269). However, the distribution and expression of μ opioid receptors change significantly during early development (270–272). In the first postnatal week in rats, there is a marked widespread increase in the density of these receptors in the dorsal horn, followed by a reduction to adult levels by the third postnatal week (271) when expression becomes constrained to C and Aδ fibres (272) as in the adult (273). Therefore, the actions of opioids are less specific in the newborn rodent, non-discriminately affecting both non-nociceptive Aβ mechanoreceptors and nociceptive fibres. For this reason, in young animals morphine has been shown to be more effective in attenuating flexion withdrawal reflexes elicited by mechanical stimulation than in older animals (274,275). It is however oppositely less effective in prolonging withdrawal latencies to noxious thermal stimulation in the first few weeks of life (274,276) as this activity is largely mediated by C-fibres (275).

Despite the common use of morphine, there is surprisingly no clear concentration-response curve for this drug in infants or children, likely due to significant pharmacokinetic and pharmacodynamic variability (277), which is an active area of study. Like many drugs used in neonatal care, dosing recommendations for morphine have broadly been based upon sparse pharmacokinetic data or derived from childhood and adult studies (261). Morphine undergoes hepatic glucuronidation into two active metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Its clearance in infants is therefore affected by the maturation of glucuronidation (278), which reaches adult levels in the first few postnatal months (279,280). A study investigating age-related differences in post-surgical morphine requirements demonstrated that term infants in the first week of life required significantly less morphine than older infants when their pain was assessed regularly by nurses using the Visual Analogue Scale (VAS) (281). Pharmacokinetic modelling has subsequently confirmed that morphine doses should be calculated not only dependent on body weight but also with respect to postnatal age. Specifically, infants less than 10 days of age should receive reduced doses as their drug clearance can be 50% less than older infants (282).

Despite the continued use of morphine in clinical practice in ventilated infants and the investigation of its effects in multiple large-scale clinical trials, its analgesic efficacy in neonates remains controversial (283). Studies have been conducted to assess its efficacy in the context of post-surgical pain (284) as well as across a variety of acutely painful clinical procedures such as heel lancing (267,285), tracheal suctioning (286–288) and peripherally-inserted central venous cannulation (289). Several studies have reported a significant analgesic effect of morphine (285,287–289), whilst others have found no evidence of analgesia (267,286). Scott et al., used the NFCS to assess pain responses evoked by heel lancing in premature infants receiving intravenous morphine infusions

(285). Although they demonstrated attenuation of facial responses by morphine, a high rate (20-30μg/kg/hr) was notably used, beyond the intravenous infusion range now recommended in the British National Formulary for children (BNFc (290)), and pain responses did not correlate with serum morphine concentrations (285). In the NOPAIN trial, Anand et al., also reported a significant reduction in Premature Infant Pain Profile scores in response to tracheal suctioning in infants receiving high doses of continuous intravenous morphine (287). A subsequent large-scale multicentre trial (NEOPAIN) investigated whether management of pain and stress with intravenous morphine infusions could reduce rates of death, severe IVH and periventricular leukomalacia in ventilated premature infants (288). Morphine infusions unfortunately did not reduce this composite outcome, and instead an increase was observed in the most premature infants potentially due to hypotension and only modest reductions in PIPP scores were reported (288). Using a similar trial protocol derived from this trial, Simons et al., however observed no effect of morphine infusion on three different pain scores employed and no effect on neurological outcomes (286). Due to the considerable heterogeneity in study procedures, drug dosing and outcome measures, it is challenging to draw conclusions regarding the efficacy of this drug from the conflicting evidence available to date.

The limited sensitivity and specificity of largely behavioural measures used in analgesic trials may be limiting our ability to determine the efficacy of analgesic drugs. Studies of morphine have employed many different behavioural or composite pain scores, including the Premature Infant Pain Profile (267,286–288,291), the Neonatal Facial Coding System (285), the Modified Infant Pain Scale (284), the Neonatal Infant Pain Scale (286), and the Douleur Aigue Nouveau-ne (267). Some studies have even used non-validated methods of pain assessment such as adrenaline levels (292) or the duration of brow bulge (289), a component of the facial expression response used in several scales. Although facial

responses can be closely associated with perceived emotions in older children and adults, in the neonate they are essentially complex brainstem reflexes, which may not necessarily reflect underlying noxious input reaching the brain or the degree of pain perceived (162). They can be observed in the foetus in the absence of noxious stimulation (293), evoked by noxious stimulation in infants with significant brain damage (294), and elicited in healthy infants by non-noxious stimulation (140). As previously discussed, painful stimuli evoke a variety of quantifiable responses including changes in facial expression, heart rate, reflex activity and brain activity. In adults, noxious-evoked EEG potentials correlate well with pain perception (295,296) and these responses are attenuated by analgesic drugs such as opiates (297–299). A multimodal approach incorporating all of these available measures therefore could provide a more complete representation of infant pain and improve the evaluation of analgesia (300). In Chapter 5 of this thesis, the efficacy and safety of oral administration of morphine for procedural pain relief was investigated in a randomised controlled trial. The study employed the multimodal measures of pain described in this chapter, as well as 24-hours of physiological monitoring pre- and post-administration in order to provide a holistic view of the effects of this controversial drug.

As is the case for all drugs, the benefits of morphine must outweigh its unwanted adverse effects to ethically justify its administration. Opiates are unfortunately associated with a risk of respiratory depression, which is mediated by activation of opioid receptors in the pre-Bötzinger complex of the medulla (301). Fear of respiratory depression has impacted the use of morphine in neonatal practice. Some studies have reported a dose-dependent relationship with apnoeas (302,303). Morphine is predominantly administered to infants who are being mechanically ventilated or post-surgery, and there is some evidence that it may prolong the duration of ventilation in these infants (304). Enders et al., also

investigated its effects during non-invasive continuous positive pressure ventilation and reported that higher doses were associated with an increase in apnoeas (303). They additionally observed that degree of prematurity and low birthweight increase the risk of morphine-induced apnoeas. Taddio et al., have also reviewed a cohort of non-ventilated infants who received morphine for peripheral insertion of a central catheter and they observed an increase in apnoeas that was however non-significant when subjects who had received overdoses were excluded (305).

There have been concerns that neonatal exposure to opiates may also have long-term neurodevelopmental consequences (see review (306)). Opioid receptors have a significant role in many fundamental neurodevelopmental processes including neuronal migration, differentiation and maturation (307). Furthermore, considering the over-expression of μ-opioid receptors in early development, extended periods of administration of opioid agonists to very premature neonates could have significant neurodevelopmental consequences due to the non-discriminate suppression of non-nociceptive sensory input during a critical period for activity-dependent development. Results from both animal models and clinical studies are contradictory (306). Some studies have reported that chronic opioid exposure in the absence of pain can result in impairments in cognitive function, fear behaviour and cortical regions of apoptosis (308–312). However, other animal studies have demonstrated a protective effect of morphine when administered in the presence of pain (130,312,313). In infants born prematurely, it is challenging to disentangle the effects of morphine from the many potential factors, such as increased pain exposure, hypotension, hypoxaemia, and illness severity, that could contribute to neurodevelopmental impairment. Some studies have reported a negative impact of morphine on structural brain development in terms of cortical volume but also demonstrated that this does not appear to be associated with a functional impact

on neuro-behavioural (314) or cognitive outcomes in childhood (315). Other studies have however reported contradictory results. Zwicker et al., conducted a prospective follow-up study of very premature infants and after adjusting for multiple clinical confounders, they reported that chronic morphine exposure in very premature neonates is independently associated with reduced cerebellar growth and poor motor and cognitive outcomes (316). To date, the evidence for the long-term effects of chronic opiate exposure in neonates is inconclusive. Administration in the context of pain and appropriate dosing are likely key to preventing any potential neurodevelopmental effects of this intervention, whilst reducing the negative impact of repeated pain exposure in early life.

1.7 Summary of chapter

Pain is challenging to define in non-verbal subjects but is important to assess and treat in neonatal care. By term gestation, the basic connectivity of the peripheral and central nervous system underlying pain perception is established, relaying noxious information from the nociceptor to the sensory and affective regions of the brain via the spinal cord. Animal studies however suggest that endogenous pain modulatory circuitry may be immature at term and is likely excitatory rather than inhibitory in premature infants. Clinical pain assessment tools have been developed for use in neonates based on behavioural and cardiorespiratory responses, but these have significant limitations and have provided conflicting evidence of the efficacy of common analgesics. Modern neuroimaging methods, such as EEG and fMRI, allow the study of nociceptive brain activity, which can improve our understanding of the development of nociceptive processing in early life and provide valuable objective outcome measures for much-needed clinical trials of analgesics in infants.

1.8 Overview of thesis

This thesis aims to 1) explore the development of brain and spinal nociceptive activity and emergence of pain modulation in human infants, 2) to investigate sex differences in nociceptive processing, and 3) to apply multimodal measures of nociceptive activity and physiology to assess the analgesic efficacy and safety of morphine, a common but controversial drug used in neonatal care.

Chapter 2 describes the general methods used in this thesis to investigate noxious-evoked behaviour, reflex and brain activity.

In Chapter 3, electrophysiology is first used to characterise the development of nociceptive brain and spinally-mediated reflex activity in premature infants and to investigate the relationship between these responses across the human preterm period. Secondly, fMRI is used to investigate the emergence of endogenous pain modulation by exploring whether functional connectivity of the PAG to cortical brain regions relates to noxious-evoked brain activity in infants.

In **Chapter 4,** electrophysiology and fMRI are used to investigate sex differences in nociceptive processing, which have been extensively described in adults and animals.

In **Chapter 5,** given the paucity of evidence-based analgesic drugs available to manage pain in neonatal care, multimodal measures of pain-related responses are applied to test a controversial analgesic agent. Clinical pain scores, noxious-evoked brain and reflex activity and detailed cardiorespiratory monitoring are used to assess analgesic efficacy and safety of oral morphine for acute procedural pain in non-ventilated infants in a randomised placebo-controlled trial. Finally, **Chapter 6** concludes this thesis with a discussion of the significance of the reported findings and avenues of further work.

Chapter 2

2 General Methods

This chapter describes the methods used in the experimental chapters of this thesis to investigate noxious evoked responses in infants. Data were acquired using EEG, EMG, fMRI, and video and physiological monitoring.

2.1 Ethics and recruitment

The studies described in Chapters 3 and 4 were conducted under the ethics application I co-wrote entitled "Investigating pain in the developing human brain" (REC reference 12/SC/0447), which was granted approval by the Oxford Research Ethics Committee (REC) of the National Research Ethics Service (NRES). Ethical approval for the clinical trial in Chapter 5 was obtained from the Medicines and Healthcare products Regulatory Agency and the Northampton Research Ethics Committee (15/EM/0310) and the trial was registered with the European Clinical Trials Database (2014-003237-25).

2.1.1 EEG Recruitment

All studies were conducted at the John Radcliffe Hospital, Oxford and conformed to the standards of the Declaration of Helsinki and Good Clinical Practice guidelines. Term infants were recruited from the postnatal wards and premature infants from the Newborn Care Unit, which is a tertiary centre with over 800 admissions per year.

Infants were not eligible for inclusion in any of the studies if they were clinically unstable, had received analgesics or sedatives in the previous 72 hours, had a clinical neurological diagnosis including IVH grade 3 or 4, or there was a history of maternal substance abuse.

All parents were provided with a parent information sheet describing the rationale, aims, procedures and risks involved (see Appendix). Both EEG and MRI studies were recruited for using a common information sheet, which describes both investigations (Appendix I). After a period of consideration, discussion, and opportunity for questions, written informed consent was obtained from the parent with legal responsibility for the infant. Parents were clearly informed of their right to withdraw from the study at any stage without prejudice or impact on the clinical care of their infant.

Infants recruited for an EEG in Study 1 of Chapter 3 are a separate cohort from the peri-term infants studied with EEG in Study 1 of Chapter 4. Infants recruited for MRI scans were included in study 2 of Chapter 3 or study 2 of Chapter 4, with some overlap of participants when they satisfied the inclusion criteria of both studies. Twins were studied when consent was provided by parents, however only one of the infants was included per study to avoid bias. The infants recruited to the clinical trial in Chapter 5 are furthermore an independent study population, and both twin infants studied were included in this case.

2.1.2 **MRI recruitment**

Recruiting newborn infants for MRI scans is particularly challenging. Scanning sessions are reserved 1-2 months in advance and newborn infants can only be recruited from the postnatal ward one or two days prior to the booked session. I worked closely with the midwifery staff on the postnatal wards to try to ensure I only approached healthy mothers

of eligible infants who were interested in hearing about the opportunity to participate in research. After an explanation and discussion of the study, parents were shown the three levels of ear protection provided to infants during scanning and given the opportunity to apply the low-intensity experimental noxious stimulus that would be used to their own hand. Following successful recruitment, unexpected illness of the infant or mother, exhaustion of the mother, or early discharge of the patient the night prior to the scanning session resulted in some unavoidable withdrawals (Fig 2.1). I recruited a total of 43 infants, 6 of whom were recruited for pilot scans in order to optimise the acquisition parameters.

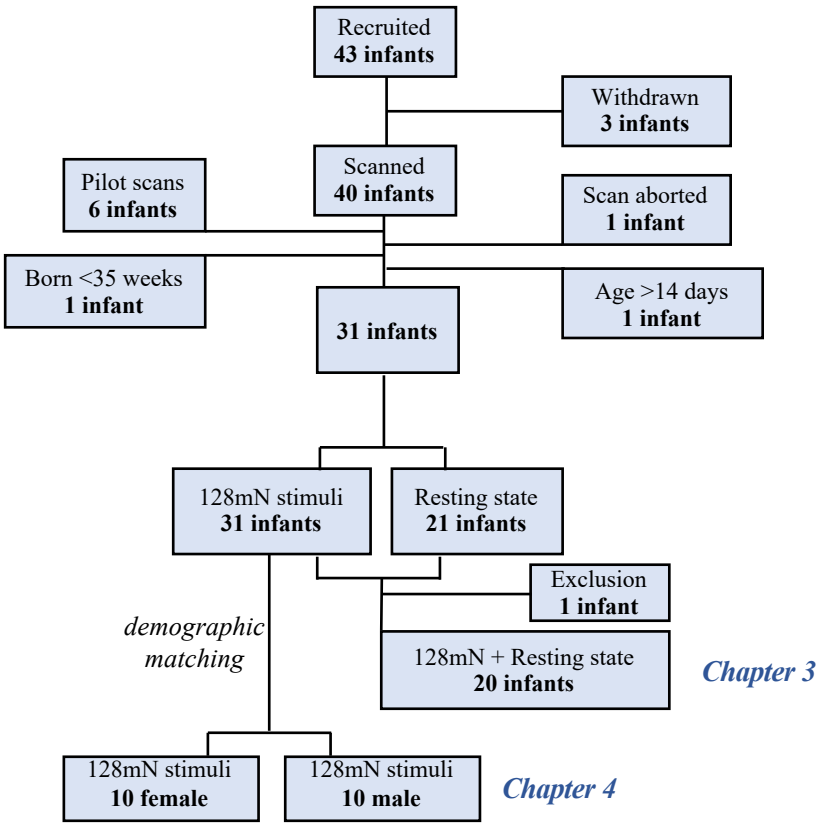

Figure 2.1: MRI recruitment flow diagram.

2.1.3 Clinical trial recruitment

Pain is an emotive subject for parents. They have an expectation that adequate pain relief is provided for their infant during painful procedures on the neonatal unit. It was therefore challenging to bring to their attention a painful procedure that their infant would undergo, an ophthalmological examination for retinopathy of prematurity (ROP), for which we cannot presently provide adequate pain relief (317). In order to ensure sensitive and accurate delivery of information about the trial we discussed our recruitment strategy with parent representatives. After commencing the trial, it became evident that many parents were unaware that their infant required ROP examinations. We therefore improved parent education by designing and distributing an information leaflet explaining the condition and procedure. We worked closely with the nursing staff to appropriately time our approach of the parents. We communicated information about the trial to eligible parents during multiple brief visits, in order not to overwhelm them and allowing them time to consider their questions and make a carefully considered decision about the participation of their infant.

2.2 Noxious stimuli

2.2.1 Clinical heel lance and control heel lance

In all electrophysiological studies reported in this thesis, noxious evoked responses were recorded in infants undergoing heel lancing for the collection of capillary blood samples, a common clinical procedure. Heel lances were only performed when formally requested for clinical purposes by the neonatal team. Prior to the procedure, infants were settled and swaddled for comfort in a blanket. Care was taken to ensure reflexive movements of their lower limbs were not obstructed. All heel lances were performed by the author or a clinical research nurse on the medial or lateral plantar surface of the heel using a BD

Quikheel Preemie Lancet (Becton, Dickinson and Company; penetration depth: 0.85 mm). The choice of foot was left to the clinical judgement of the individual performing the procedure. Prior to the clinical heel lance, a control heel lance was performed during which the lancet device was rotated by 90^{o} and held against the infant's heel at the intended site of heel lancing, and subsequent depression of the trigger released the blade to the air without slicing the infant's heel (Fig 2.2). The infant's foot was held loosely for the next 30 seconds to allow the recording of behaviour and physiological parameters for calculation of a clinical pain score. The clinical heel lance was then performed, following which the infant's foot was again held loosely for 30 seconds without squeezing for calculation of a clinical pain score (Fig 2.2). Any droplets of blood produced during this time were collected using the sampling bottle.

The release of the heel lance blade was automatically marked on the EEG recordings using an accelerometer device attached to the lancet (6). The release of the blade was also signalled on the video recordings of behavioural responses using an LED light, which flashed when the individual performing the heel lance simultaneously depressed a foot pedal.

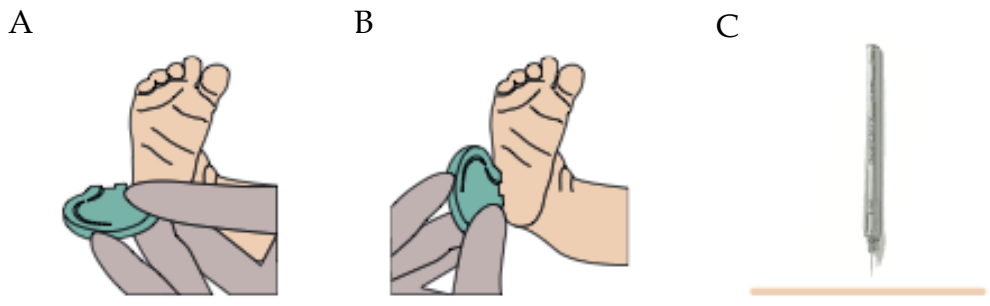

Figure 2.2: Noxious stimuli. A. *Control heel lance:* The lancet is held rotated against the lateral aspect of the foot, releasing the blade into the air. **B**. *Clinical heel lancing:* The lancet is depressed against the lateral plantar surface of the heel. **C**. *Experimental noxious stimulus:* The 128mN PinPrick is held perpendicularly to the the skin and depressed in a fluid action lasting ~1 second.

2.2.2 **Experimental noxious stimuli**

In Chapters 3 and 4, noxious-evoked brain activity was also recorded using fMRI. Clinical heel lancing is not feasible within the magnetic environment of the scanner therefore low intensity experimental noxious stimulation was applied in these studies with an MRI-compatible device. The PinPrick stimulator (MRC Systems), developed originally for QST in neuropathic patients (230), is a weighted flat-tipped device that exerts a specified mechanical force when applied perpendicularly to the skin's surface. It primarily activates type 1 A∂ nociceptors within the upper epidermal layers (231) evoking electrophysiological (232) and BOLD activity similar to noxious experimental laser stimulation in adults. Application of this force to an infant's heel does not cause behavioural distress and is acceptable to parents (151). It produces a noxious-evoked potential similar in morphology but significantly smaller in magnitude to the potential evoked by heel lancing (151), and evokes BOLD activity in most brain areas active in response to a painful stimulus in adults (4,8). In previous studies, the stimulus was applied to the plantar surface of the infant's foot consistent with the clinical heel lancing procedure. MRI data were however acquired in a Siemens Magnetom Verio 3 T scanner. Due to the narrower bore diameter of the new Siemens Prisma 3T scanner, which replaced this system, in the studies in this thesis the PinPrick stimulus was applied to the dorsal aspect of the foot, as spatial constraints made it impossible to hold the device perpendicularly above an infant's raised heel in order to provide a consistent force (Fig 2.2).

Noxious-evoked BOLD data was elicited by a train of 10 applications of the 128mN PinPrick stimulus, with a minimum inter-stimulus interval of 25 seconds. This flexible interval allowed time for the infant to relax their leg back to a resting position following

reflexive withdrawal and to settle prior to the next stimulus application. In some infants, a train of ten stimuli was also applied to the infant's foot using a 64mN PinPrick stimulus. This stimulus did not however produce significant brain activity on group analysis. Therefore, the data collected in response to a 64mN stimulus was not used to investigate noxious-evoked brain activity in any of the chapters of this thesis.

2.3 Outcome measures

2.3.1 EEG

2.3.1.1 Acquisition of EEG data

Noxious-evoked brain activity was acquired in response to heel lancing using EEG in all three experimental chapters of this thesis. EEG electrodes (Ambu Neuroline disposable Ag/AgCl cup electrodes) were sited on the infant's head at positions Cz, CPz, C3, C4, FCz, Oz, T3 and T4 according to the modified international 10-20 system, with a reference electrode at Fz and a ground electrode on the forehead (Fig 2.3). The electrode sites were prepared with EEG gel (NuPrep gel, D.O. Weaver and Co.) and electrodes applied with conductive paste (Elefix EEG paste, Nihon Kohden) to increase contact and reduce impedances. Electrophysiological data were 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 and recorded using the CURRYscan7 neuroimaging suite (Compumedics Neuroscan).

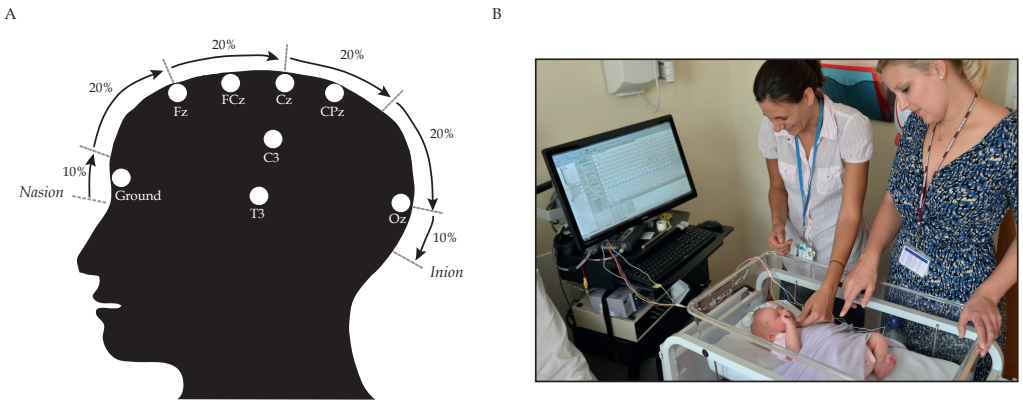

Figure 2.3: EEG data acquisition. A. Schematic of electrode placement. **B**. EEG equipment at the cotside of a recruited infant.

2.3.1.2 Analysis of EEG data

EEG data was analysed post-acquisition using MATLAB. The signal was filtered between 0.5 and 70Hz, with a Notch filter at 50Hz to minimise interference from electrical devices and maximise capture of physiological signal (Fig 2.4). Epochs were extracted from 500ms pre-stimulus to 1000ms post-stimulus. Post-stimulus signal was baseline corrected to the pre-stimulus mean.

In a previous study of term infants, nociceptive-specific brain activity evoked by heel lancing was identified using Principal Component Analysis in the period 400–700ms post-stimulus (151). The nociceptive-specific potential was the second principal component as this had a significantly greater weight in the 400-700ms period following heel lancing, than in this period following a non-noxious control or in background EEG activity collected at rest. This principal component, defined in a sample of healthy term infants, was therefore used as a template of nociceptive-specific activity in Chapter 3 of this thesis. Using singular value decomposition, the template was projected onto the filtered, epoched data acquired in premature and term infants (Fig 2.4). Each EEG trace was Woody-filtered to find the maximum correlation between the signal and nociceptive-

specific component, allowing a maximum jitter ±50ms in the period 400-700ms post-stimulus (Fig 2.4). This compensated for variation in latency of the noxious-specific response and is a method commonly used in the analysis of evoked potentials (318).

Following the publication of the electrophysiological study reported in Chapter 3 of this thesis (319), a further study was performed formally characterising the template of nociceptive brain activity in a larger group of infants and testing its specificity (5). The study demonstrated that, unlike noxious-evoked activity, the pattern of electrophysiological activity evoked by visual, auditory, and tactile stimulation quantified using the template was not significantly greater than background activity. The study furthermore validated the use of the template in infants from 34 weeks' gestation and demonstrated a reduction of its magnitude following application of a topical anaesthetic, providing evidence of analgesic modulation. The studies in Chapters 4 and 5 therefore employ this published template of noxious-evoked activity in EEG analyses (Fig 2.4).

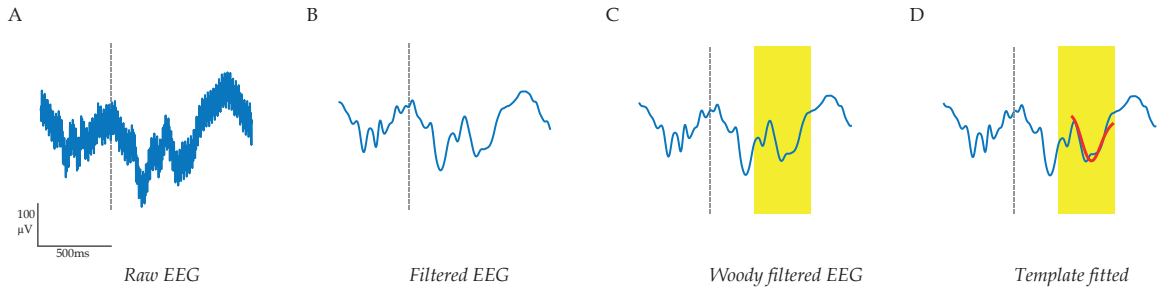

Figure 2.4: EEG analysis. The raw epoched EEG trace (**A**) is filtered and the post-stimulus period is baseline corrected using the pre-stimulus mean (**B**). Woody filtering is applied in the 400-700ms period post-stimulus (highlighted in yellow), with a jitter of 50ms (**C**), and the template (red) is fitted to the EEG trace in this window using singular value decomposition (**D**). Dashed lines indicate the point of noxious stimulation.

2.3.2 EMG

2.3.2.1 Acquisition of EMG data

Bipolar EMG electrodes (Ambu Neuroline 700 solid gel surface electrodes) were sited on the biceps femoris of each of the legs to record reflex limb withdrawal. Data were acquired, as for EEG data, with the SynAmps RT 64-channel headbox and amplifiers and recorded using the CURRYscan7 neuroimaging suite (Compumedics Neuroscan).

2.3.2.2 Analysis of EMG data

The raw EMG signal was filtered between 10 and 500 Hz with a notch filter at 50 Hz. The signal between 2000ms pre- to 4000ms post-stimulus was extracted, rectified, and divided into 250ms segments (Fig 2.5 A). The root mean square (RMS) was calculated for each of the 250ms segments post-stimulus. In Chapters 4 and 5, the measure of noxious-evoked reflex activity reported is the mean RMS in the first second post-stimulus, as in previous studies (151,253). However, in Chapter 3, a novel algorithm is described and used to mathematically define a start and end point of the reflexes, in order to characterise the development of the amplitude, duration and latency of this response in premature and term infants (Fig 2.5 C).

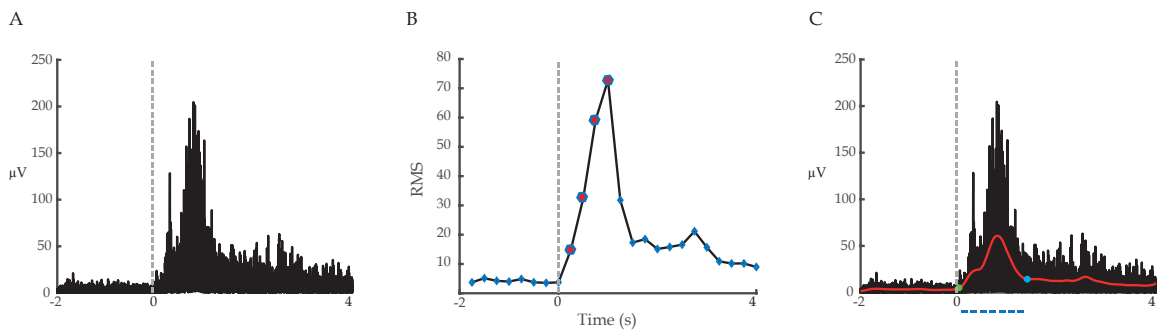

Figure 2.5: EMG analysis. A. The EMG trace was filtered and rectified. **B**. The RMS calculated and plotted in 250ms time bins. The first 4 values post-stimulus (red) were averaged to provide the RMS as a measure of reflex magnitude. **C**. Duration of reflex was calculated from a mathematically defined start (green dot) and end point (blue dot), described in detail in Chapter 3. Dashed lines indicate the point of noxious stimulation.

2.3.3 **Functional MRI**

2.3.3.1 MRI Acquisition

All MRI data were acquired on a Siemens Prisma 3T scanner at the Wellcome Centre for Integrative Neuroimaging (Oxford, UK). Infants recruited for MRI studies were transported from the postnatal ward to the Centre by a clinically-trained investigator (the author) with two assisting research team members and emergency resuscitation equipment. One or both parents also accompanied the infant and feeding was encouraged prior to transport or on arrival at the Centre. For safety, infants were clinically examined, checked for ferromagnetic material, and fitted with two levels of auditory protection, including ear- putty (23dB attenuation) and ear-muffs (Minimuffs, Natus Medical Inc., Galway, Ireland; 7dB attenuation) prior to entering the scanner environment. Significant acoustic noise is produced by the scanner that could cause hearing loss in the absence of protection. The interaction of the static magnetic field and rapidly changing currents within the gradient coils generates Lorentz forces, which cause vibrational movement of the coils and the structures encasing them (320). The 3T scanner used in this thesis

produces noise levels of up to 97dB within the scanner bore during standard acquisition sequences (Noise Survey of 3T Prisma scanner in FMRIB, Oxford). To put this in context, infants cared for in incubators on the neonatal unit are exposed to sound pressure levels ranging between 55 and 67dB (321) and the simple closure of incubator drawers can generate pressure levels as high as 98dB (322). Considering the scanner environment could expose infants to these higher pressures for a longer period of time and hearing loss is related to duration of exposure (323), ear-defenders (Em's 4 Bubs Baby Earmuffs, Em's 4 Kids, Brisbane, Australia; 23dB attenuation) were also fitted once the infant was settled into the scanning cradle to provide a third level of ear protection. Although employing multiple levels of ear protection unfortunately does not provide an exact summed total effect of the individual levels of protection, it is superior to single levels (324). Parents were allowed to accompany their infant into the scanning environment conditional on safety screening and wearing ear protection (MRI-safe ear defenders, JSP Big Blue, 30 dB attenuation).

Infants were soothed and settled prior to scanning to maximise successful data acquisition. In the scanner, they were swaddled (whilst leaving access to the feet for stimulation) and placed in a customised foam cradle, which we devised to elevate the body and head to an optimal position in the centre of the Siemens adult 32-channel receive head coil in order to minimise artefact (Fig 2.6). The cradle was lined with a vacuum mattress to restrict motion of the upper body and extra foam pads were added depending on the infant's head circumference to minimise head motion. Heart rate and oxygen saturation levels were monitored throughout scanning by the clinically-trained investigator (Fibre Optic Pulse Ox- imeter; Nonin Medical, Plymouth, Minnesota), and scans were paused for assessment of the infant if either of the physiological parameters fell outside their normal ranges.

In MRI studies, there is a theoretical risk of heating and tissue damage due to the power deposition of radiofrequency fields applied to the subject during scanning. The specific absorption rate (SAR) is a surrogate measure of power deposition, which was estimated for each sequence prior to acquisition for safety. Infants however experience a lower SAR than adults exposed to similar sequences and therefore scanner estimations that employ standard adult algorithms are reassuringly conservative (325). The infant's temperature was also measured pre- and post- scanning in order to detect any heating effect or identify infants failing to maintain their temperature within normal limits during transport or scanning. A temperature change of more than 0.5 degrees was not observed in any of the infants scanned.

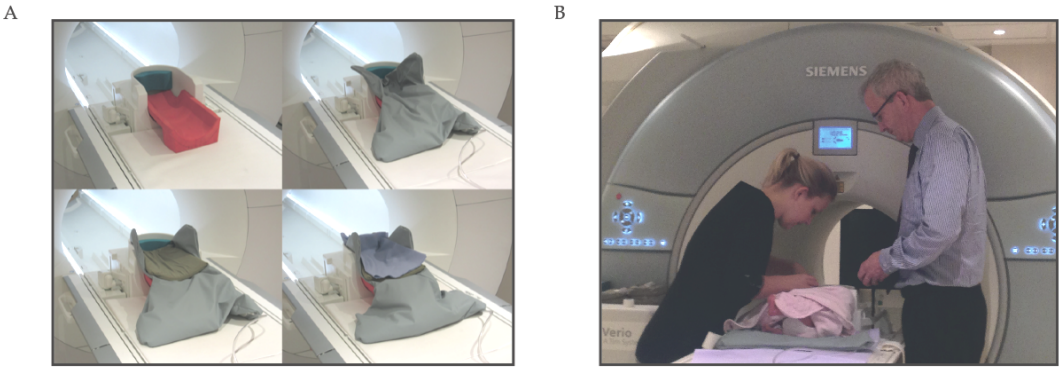

Figure 2.6: MRI acquisition. A. The custom foam cradle (red) is slotted into the adult 32 channel head coil and overlaid with a vacuum mattress (grey) and soft padding for comfort and to limit upper body and head movements. **B**. The swaddled infant is placed in the cradle and three-level ear protection is fitting and checked.

A T2-weighted structural scan was first acquired with the following parameters: TSE (factor 11), 150^{o} flip angle, TE= 89 ms, TR= 14,740 ms, parallel imaging GRAPPA 3, 192x192 in-plane matrix size, 126 slices, 1mm^3 voxels. As a result of the high water content of the brain in infants under 6 months of age, there is inversion of the MR signal such that T2 weighted images in the infant resemble adult T1 images (326). The T2 weighted images provide better contrast for the delineation of anatomical boundaries

(327). The images acquired were immediately reviewed and repeated if movement artefacts were observed. To facilitate post-acquisition distortion correction, a fieldmap image was acquired prior to the functional data with the following parameters: gradient echo 2D Fourier Transform readout, dual echo TE1/TE2 = 4.92/7.38 ms, TR 550ms, TA 1min 40s, 46^{o} flip angle, 90 x 90 in-plane matrix, 56 slices, 2mm^{3} voxels.

Functional data were then acquired during a train of 10 stimuli using the following parameters: T2* gradient echo EPI, 70^{o} flip angle, TE 50 ms, TR 1300ms, multiband 4, 90 x 90 in-plane matrix, 56 slices, 2mm^{3} voxels. To improve image registrations, a single-band reference (SBref) image was acquired at the beginning of the sequence. Each time the investigator depressed the PinPrick stimulus against the dorsum of the foot, a second observing investigator simultaneously depressed a button on a remote that registered the timing of the stimulus using the Neurobehavioural Systems software (Presentation, www.neurobs.com). 500 volumes of resting state data were also collected using the same parameters as the stimulus-evoked data.

Several parameters were optimised for neonatal MRI acquisition. The functional EPI sequence had an echo time of 50ms as a previous study demonstrated that this time between radiofrequency (RF) pulse and acquisition of signal maximises sensitivity to BOLD in term infants scanned at 3T (328). A reduced voxel size of 2mm was chosen compared to previous studies (4) as this offers greater spatial resolution for detecting activity in smaller brain regions such as the PAG and reduces susceptibility-related signal loss (329). The resultant loss of temporal resolution was compensated for by the use of a multiband acquisition protocol, in which a single RF pulse selectively excites multiple slices of the brain simultaneously (330–332) and aliasing is removed by parallel processing. This technique is now being widely adopted as shortening of the repetition

time (TR) and acquisition time is desirable in infant MRI. Several acceleration factors were tested in pilot scans. A slice acceleration factor of four was chosen as in a small pilot study this yielded equal or slightly better signal-to-noise ratio (SNR) when compared to data from a previously published study (4) acquired with single-band EPI and a larger voxel size of $3mm^3$ (temporal SNR range 65-90).

2.3.3.2 MRI Analysis

All subject-level MRI data were originally pre-processed by the author using a neonatal analysis pipeline consisting of tools from FSL's Feat, which has previously been used in neonatal studies (4,328). However, the development of a novel pipeline then began locally. This pipeline was being optimised for the analysis of neonatal resting state fMRI collected by the Developing Human Connectome Project (333). During the development of this pipeline, modifications were made to optimise it for the analysis of stimulus-evoked data collected in neonates and data from a sub-group of infants recruited and studied for this thesis were used to test and validate this modified pipeline (334). The author re-analysed all fMRI data using the optimised pipeline during its development. However, a final version of the pipeline was subsequently released, which included motion-by-susceptibility distortion correction and another student used this final optimised version to again pre-process all data acquired for this thesis. This final version of pre-processed subject-level data was therefore used in the analyses in Chapters 3 and 4 of this thesis and the final pipeline is described below.

2.3.3.2.1 Structural image pre-processing

For each infant, the best T2 structural image (acquired with the least motion artefact) was processed in the first stage of the pipeline using the MIRTK Draw-EM (Developing brain

Region Annotation With Expectation Maximisation) tool v1.1 (335). This tool extracts and segments the brain into grey and white matter and performs a bias field correction.

2.3.3.2.2 Motion and distortion correction

Motion is a significant issue in MRI but particularly so in the context of neonates and when applying noxious stimuli that evoke reflexes. FEAT's MCFLIRT (Motion correction with FMRIB's linear image registration tool) is usually used (336), which applies a rigid body transformation to register each acquired volume to a reference volume, and distortions are most commonly corrected using a static fieldmap. However, all volumes were registered to a reference volume selected for having the least motion contamination, estimated using DVARS (337). Subject motion can also occur during the acquisition of a volume. Head movement creates susceptibility-induced field distortions that can be modelled (338) and corrected for using EDDY (339,340), a tool developed originally for diffusion imaging. EDDY was therefore applied in the pipeline to correct for both inter- and intra-volume motion by performing slice-to-volume correction and estimated dynamic distortion correction. The phase difference fieldmap image was unwrapped and converted to rad/s as standard using standard FSL tools and used to distortion-correct both the multiband functional data using EDDY and the single-band reference later using boundary-based registration (BBR), described in the next section. In the validation study of the pipeline, Baxter et al., tested this method of motion correction against the traditional FEAT pipeline (4,334). The novel application of EDDY to functional data resulted in a significant reduction in the effects of motion and distortion as well as increased signal sensitivity (334).

2.3.3.2.3 Spatial normalisation

A multi-stage registration process was used to transform each infant's functional data to a group standard 40-week template brain via their single-band functional reference volume, their anatomical structural image, and a standard template brain corresponding to their gestational age at study (Fig 2.7). The template brains used were derived by Makropoulos et al., through detailed segmentation of 420 T2 scans of infants aged 27-45 weeks (341). A 40-week gestation standard brain was used to allow comparison of activity from individual infants in a common space and this gestational week has previously been used in neonatal studies (4,243). The infant's structural T2 was registered to an age-matched template brain and then to a common 40-week standard template brain using ANTs's SyN (Advanced Normalisation Tools's Symmetric image Normalisation method) (342). The pipeline concatenates the transformations to minimise resampling.

To register the multiband data to the infant's structural scan, the single-band reference was used as an intermediate. Considering the functional data had already been motion- and distortion-corrected using EDDY, a distortion-corrected single-band image was needed. Using boundary-based registration (BBR)(343) and the border between grey and the grey-white matter boundary delineated during segmentation of the T2 image, the single-band reference was registered to the structural image. This step also used the fieldmap to distortion-correct the single-band reference. The motion/distortion corrected multiband data was registered to the distortion-corrected single-band reference through a 6 DOF rigid body transform performed using FLIRT (FMRIB's Linear Image Registration Tool) and this transformation was then combined with a rigid body transform between the corrected single-band reference and the infant's structural scan to allow registration of the multiband data to the structural scan (Fig 2.7).

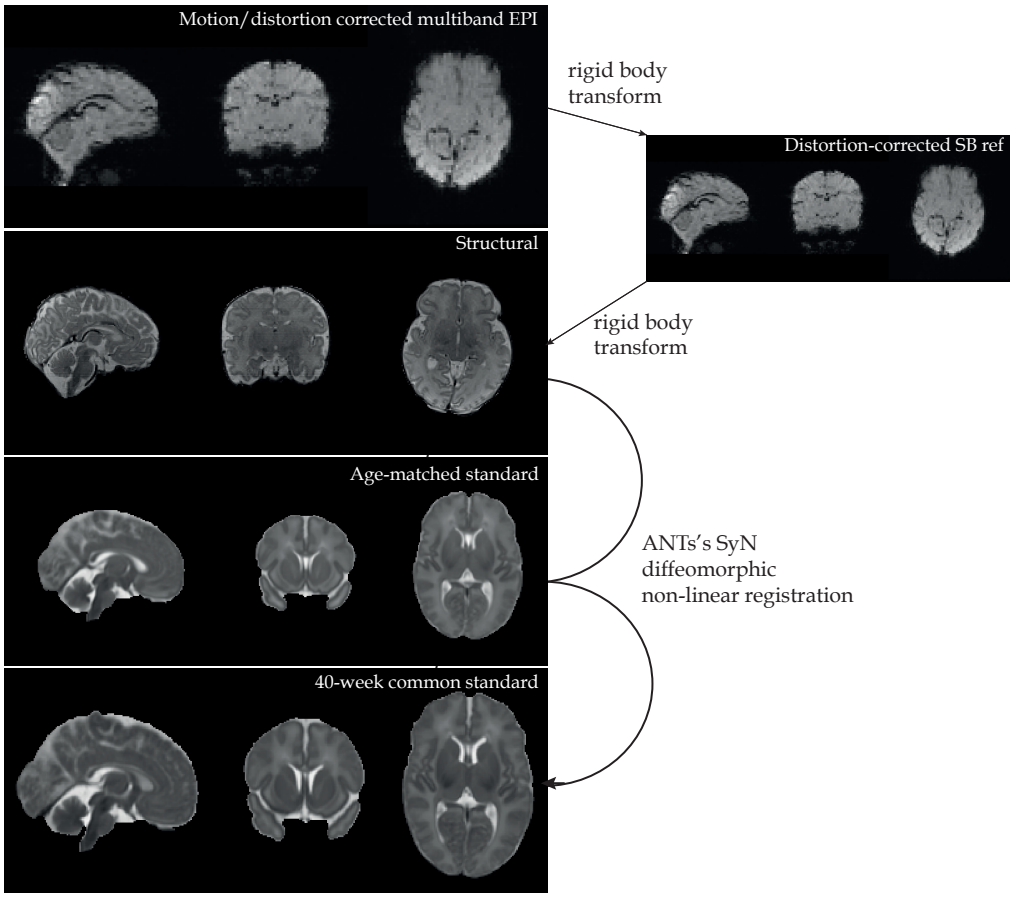

Figure 2.7: Multistage registration of functional data. The motion and distortion corrected multiband data is registered to the 40-week common standard template brain via the infant's structural and week-to-week warps.

2.3.3.2.4 Denoising

Temporal filtering was applied to the functional data to remove slow temporal drift. Given the inter-stimulus interval ranged between 25 and 60 seconds, a high-pass filter cut-off of 100s (0.01Hz) was used for stimulus-evoked data. However, a filter cut-off of 200s (0.005Hz) was applied to resting state data, considering the power of resting state network activity lies mostly between 0.01-0.1Hz (344,345). The MELODIC (Multivariate Exploratory Linear Optimised Decomposition into Independent Components) tool (346) was used to decompose timecourses and spatial maps from functional data using independent component analysis. A trained version of FIX (FMRIB's ICA-based

Xnoiseifier) (347,348) was used to automatically classify components as signal or noise (Fig 2.8). Components were visually inspected to verify labels using criteria outlined by Griffanti et al., (349) prior to using FIX to remove components rejected as noise, as well as 24 motion-related confound regressors, defined during motion correction.

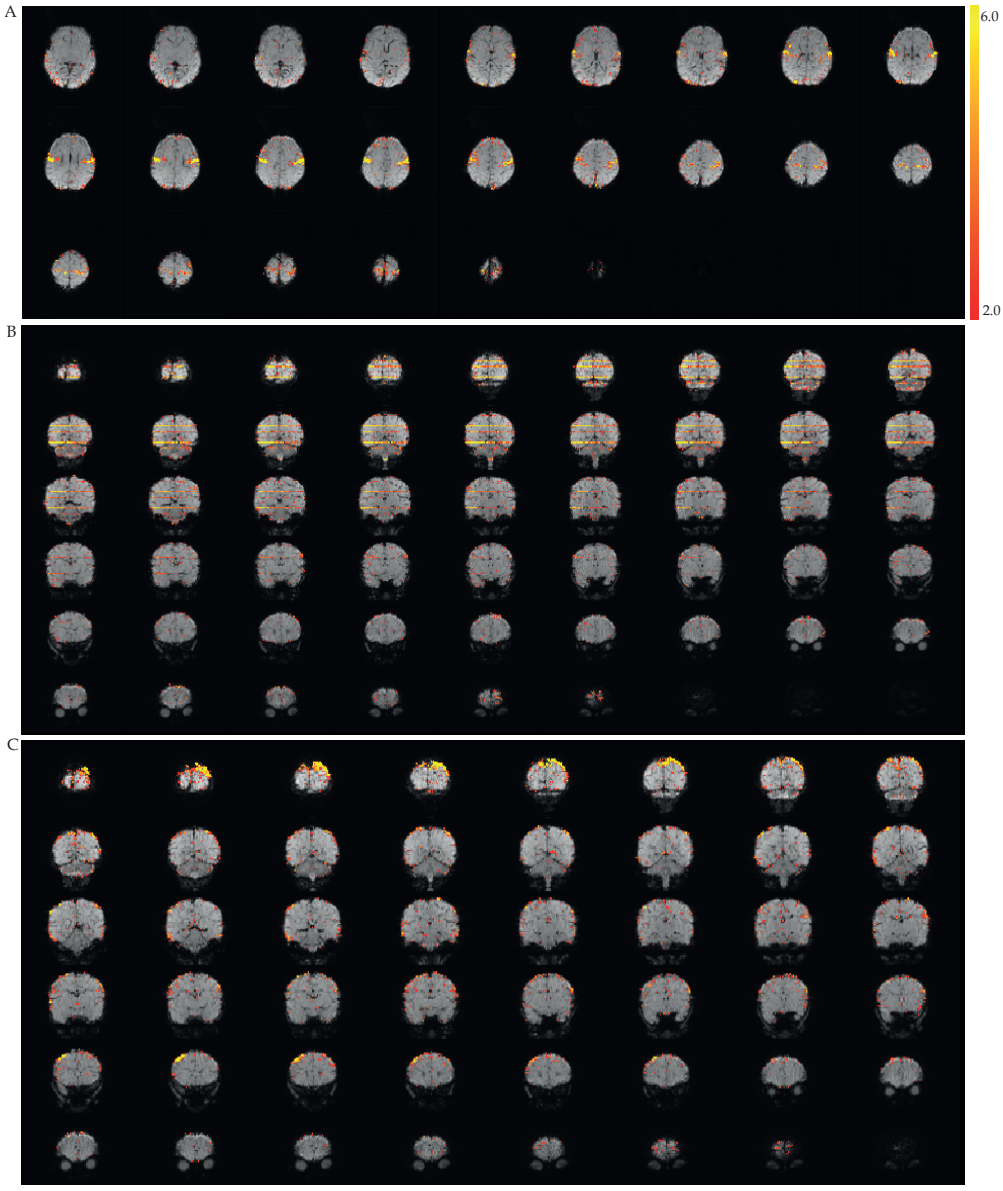

Figure 2.8: Examples of spatial ICA components. A. An anatomically well-defined ICA component identified as signal. **B**. An ICA component representing multiband artefact and rejected as noise. **C**. An ICA component demonstrating ringing around the brain, which was rejected as noise.

The FSL motion outliers tool was used to produce motion outlier plots and review the effects of motion correction (Fig 2.9).

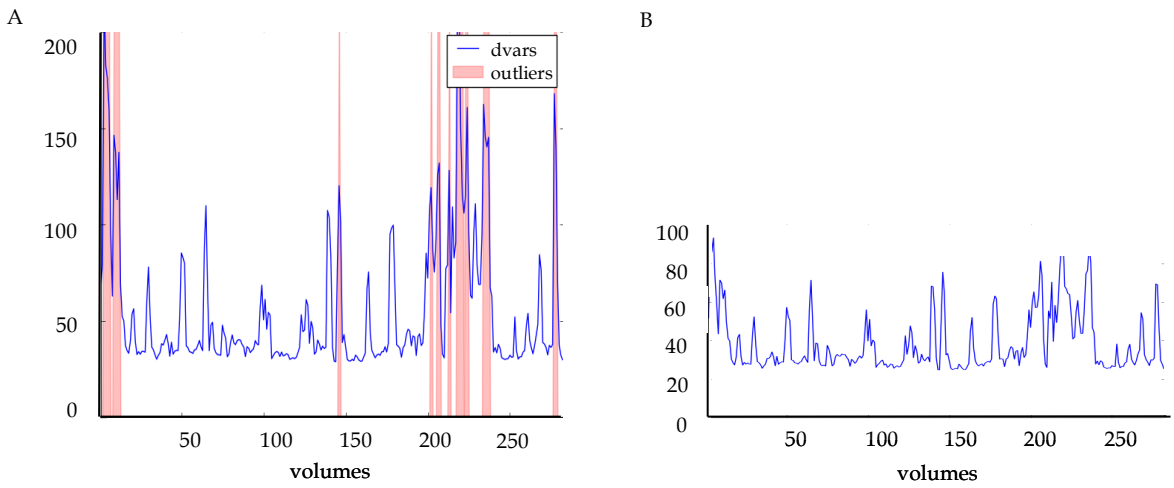

Figure 2.9: The effects of motion correction. A. An example of a subject's motion during acquisition of functional data measured by dvars using FSL motion outliers prior to motion correction. Motion outliers (defined by default settings) are represented by the pink bands. **B**. Following motion correction and ICA denoising the subject's estimated motion plot contained no outliers.

In order to improve SNR, the denoised functional data were spatially smoothed with a Gaussian kernel with a full width at half maximum (FWHM) of 3mm using FSL's SUSAN (Smoothing over Univalue Segment Assimilating Nucleus) (350). SUSAN performs non-linear smoothing by sampling each voxel's immediate environment in order to avoid smoothing across tissue boundaries. Many neonatal studies have used larger smoothing kernels between 5 and 10mm (4,222,351,352). However, this minimal degree of smoothing, equivalent to 1.5 times the voxel size of the data, was used here following demonstration that this value provides the optimal balance between increased sensitivity to signal and artefactual blurring of activity across tissue boundaries observed with larger kernels (334). Lastly, in order to allow comparison of activity between infants in GLM analyses, grand mean scaling was applied to normalise each data set by a

common scaling factor, setting the median background activity across all volumes to a common value of 10,000, which is standard in FSL.

2.3.3.2.5 Subject-level analysis

GLM analyses were performed using FEAT v6.00 to identify clusters of voxels with significant noxious-evoked BOLD activity. An experimental model representing stimulus timings was convolved to a haemodynamic response function. The morphology of the neonatal haemodynamic response function has previously been characterised by Arichi et al. and differs significantly from the canonical adult waveform (353). In term neonates, the positive peak of the response is delayed and reduced in amplitude and is followed by a greater and more prolonged negative undershoot. Modelling the HRF appropriately is important as it can significantly impact the results of functional studies. An FSL tool called FLOBS (FMRIB's Linear Optimal Basis Sets) has been developed, which can be used to generate an optimal basis set that best fits the data through principal component analysis (354). However, in the validation of this pipeline, Baxter et al., recently compared FLOBS to a double gamma waveform based on the parameters described by Arichi et al. There is a trade-off between model complexity and sensitivity to noise. Due to neonatal data being inherently more noisy than adult data, FLOBS performed worse than the double gamma waveform as a result of overfitting (334). Therefore, the double gamma waveform was used in this thesis. The convolved model was temporally filtered to the same degree as the functional data. FEAT was then used to fit the model to the functional data with FILM (FMRIB's Improved Linear Model) prewhitening (355).

All group-level analyses are described in their respective results chapters.

2.3.4 **Clinical pain scoring**

Clinical pain scoring, based on behavioural and physiological responses, was only used in Chapter 5, in which the results of a randomised placebo-controlled trial of oral morphine in premature infants are reported. The Premature Infant Pain Profile – revised (PIPP-R) score (356) was used to quantify responses to a clinically-required heel lance and a routine ophthalmological examination for ROP. The score was calculated retrospectively using video footage of the face and cardiorespiratory parameters recorded 15 seconds pre- and 30 seconds post-procedure. An LED light was positioned within the field of view of the camera and was activated by the experimenter performing the heel lance by depressing a foot pedal at the point of release of the lancet blade. Cardiorespiratory parameters were recorded using a Philips neonatal monitor (IntelliVue MX800 patient monitor, Philips), pulse oximeter probe, and standard neonatal ECG leads sited on the chest. These data were recorded in real-time and exported for analysis using ixTrend software (ixellence GmbH).

The video footage was retrospectively reviewed by a scorer, a trained and experienced neonatal nurse, who was blinded to the identity of the infant and stimulus type (control, heel lance, or ROP examination). A score (0-3) was given for the baseline behavioural state of the infant and a score (0-3) was allocated for each facial expression (brow bulge, eye squeeze, and nasolabial furrow) based on their duration post-procedure. Matlab code developed in-house was used to calculate the change in heart rate and oxygen saturation during this period, and a score of 0-3 was allocated according to the change in each parameter. Lastly, a score was given based on gestational age. The total score was calculated using the table in Appendix III. Scores for gestational age and behavioural state were only included in the total if physiological or behavioural variables scored a non-zero value.

Chapter 3

3 The development of noxious-evoked brain and reflex activity and the emergence of endogenous pain modulation in human infants

3.1 Introduction

Activation of nociceptors does not linearly correlate with the subjective experience of pain (15). Brain regions within the descending pain modulatory network can amplify or dampen pain perception, nociceptive reflexes, and behavioural responses through a balance of facilitation and inhibition of noxious inputs at the level of the spinal dorsal horn. Most of our current understanding of the maturation of supraspinal descending control is based upon developmental studies in rodents using the hindlimb withdrawal reflex as a measure of spinal nociceptive processing. Rat pups withdraw from noxious stimuli from birth and display exaggerated, uncoordinated reflexes with large disorganised receptive fields (357,358). This results from the balance of descending control onto neonatal nociceptive dorsal horn circuits, which is excitatory rather than inhibitory in the first three postnatal weeks (150). Subsequently there is a switch in the polarity of control, with evidence of biphasic facilitation and inhibition in the adult. Supraspinal influences are critical to the development of spinal nociceptive processing, as spinalisation of neonates results in permanent disorganisation of their reflexes (122,359) and continuous opioid receptor antagonism in the fourth postnatal week prevents the normal development of tonic inhibitory control of spinal nociceptive reflexes (128). Little is known however about the development of endogenous pain modulation in early life in humans. Although electrophysiological techniques have been used to independently study the occurrence of noxious-evoked brain activity in premature infants (183) and the characteristics of spinal reflex activity in premature infants

(61,148,149,360), the developmental relationship between these measures of spinal and supraspinal nociceptive processing across the preterm period has not previously been investigated.

Rodent studies of the development of the endogenous pain modulatory system have focused primarily on pharmacological and neurophysiological manipulations of two key brain regions conserved across many species, the periaqueductal grey (PAG) and rostroventral medulla (RVM). The PAG and RVM form the axis through which supraspinal input is transmitted to the dorsal horn and this pathway essentially gates noxious input to the central nervous system. Although electrical stimulation of the PAG produces potent analgesia in the adult rat (92,93) and adult human (361), stimulation of this midbrain structure in the young rat is not anti-nociceptive and does not inhibit reflexes until postnatal day 21 (123). Lesions of the PAG in adult rats abolish the inhibitory effects of stimulating cortical brain regions involved in descending inhibition (362–364) and reduce the effects of morphine analgesia (119). It is not known however at what stage of human development the PAG becomes structurally and functionally connected to cortical pain modulatory brain regions and begins to exert predominantly inhibitory tonic control and variable dynamic control of noxious inputs.

In adult humans, neuroimaging studies have demonstrated that the functional connectivity of the PAG has a fundamental role in opioid-induced and placebo-mediated analgesia, as well as pain modulation by distraction (235), attention (240), and mood (365). Furthermore, alterations in the functional connectivity of this structure to cortical modulatory brain regions have been reported in many chronic pain states, including fibromyalgia (366), back pain (367) and migraine (368). Importantly, functional

connectivity of the PAG to the anterior insula in the seconds prior to a noxious stimulus has been shown to reflect the likelihood of a stimulus being perceived as painful (242). Recently, we have used functional MRI in healthy term infants to investigate the pre-stimulus connectivity of the descending pain modulatory network (including the PAG, RVM, anterior cingulate cortices, anterior insular cortices, middle frontal gyri, and amydala) in term infants and demonstrated that increased overall mean connectivity between brain regions of this network in the ~10 seconds prior to a noxious stimulus is associated with lower noxious-evoked BOLD activity (243). This may suggest that the descending pain modulatory network begins to influence pain-related brain activity at term in humans. It is not known however whether this connectivity is state-dependent, occurring specifically pre-stimulus, or whether this reflects a more stable resting state connectivity modulating the transmission of noxious inputs in infants.

3.2 Aims

In Study 1, EEG and surface EMG were used to record noxious-evoked responses to a clinical procedure in infants aged between 28- and 42- weeks' gestation. The aim of the study was to characterise the development of nociceptive brain activity and spinally-mediated reflex activity and investigate the relationship between these responses across the human preterm period.

Considering the PAG is a critical region in pain modulation where ascending and descending nociceptive activity converge and has been widely studied in both rodents and humans, Study 2 investigated the pre-stimulus and resting functional connectivity of this brain region in human infants. Functional MRI was used to acquire both noxious-evoked BOLD and resting state data in healthy infants aged 35-42 weeks' gestation. The aims of

this study were 1) to investigate whether pre-stimulus connectivity of the PAG to cortical brain regions involved in descending pain modulation negatively (in the seconds immediately prior to stimulation) predicts BOLD activity evoked by noxious stimulation, and 2) to investigate whether such a relationship between PAG functional connectivity and noxious-evoked brain activity is also present in longer periods of resting state brain activity acquired in a separate sequence to the stimulus paradigm.

3.3 Study 1

3.3.1 Methods

3.3.1.1 Participants

40 infants aged 28-42 weeks' corrected gestation requiring a clinical heel lance were recruited from the Newborn Care Unit and postnatal wards of the John Radcliffe Hospital, Oxford (see Table 3.1). Infants were ineligible if they had neurological or genetic diagnoses, or a history of meningitis, culture-positive sepsis, or maternal substance abuse. Clinical cranial ultrasound reports were reviewed for infants born at <32 weeks' gestation or weighing <1500g at birth. Only infants with normal scans or a small, uncomplicated, unilateral, isolated subependymal haemorrhage (grade 1 IVH) were included. At the time of study, infants were less than 34 days old, haemodynamically stable, off mechanical ventilation, and had not received analgesics/sedatives in the week prior to study.

Gestational age at birth (weeks)		34.4 (29.6–40.6)
Gestational age at study(weeks)		36.4 (33.3–40.9)
Postnatal age at time of study (days)		12.1 (11.1)
Birth weight (g)		2194 (1,538–3,627)
Weight at study (g)		2325 (1,620–3,627)
Male infants (%)		20 (50)
Multiple gestation infants (%)		7 (18)
Mode of delivery (%)	Vaginal	18 (45)
	Assisted/caesarian	22 (55)
Apgar score	1 min	7.2 (2.6)
	5 min	9.1 (1.4)
Infants admitted to NICU (%)		23 (58)
Infants ventilated during admission (%)		9 (23)
Days of ventilation		4.8 (6.4)
Estimated prior pain exposure		8 (3.8–28)
Infants with grade I IVH (%)		3 (7)
Infants with history of previous surgery (%)		2 (5)
Infants with previous postnatal infection (%)		30 (75)
Infants with previous necrotizing enterocolitis (%)		2 (5)

Table 3.1: Study 1 participant demographics.

Median (IQR) and mean (SD) are shown unless otherwise indicated.

Prematurity and pain exposure in early life have been shown to affect noxious-evoked brain activity (199). In order to account for each infant's cumulative prior pain exposure, electronic and paper clinical records were reviewed, and the number of oropharyngeal or endotracheal aspirations and tissue-damaging procedures performed for blood taking, such as heel lances, venepuncture, and intravenous cannulations, from birth to time of study were documented. The frequency of these procedures is reliably recorded by the clinical team and they are amongst the most common painful clinical procedures experienced by neonates during the first 2 weeks of life (369).

3.3.1.2 Experimental procedures

In order to record noxious-evoked brain and reflex activity, EEG and EMG electrodes were sited, and data recorded as described in Chapter 2. However, in 10 infants (studied early on), EEG recordings were acquired with reference to FPz therefore these data were re-referenced to Fz post-acquisition. For 8 infants, a reduced electrode montage was also applied due to clinical constraints. Activity was however always recorded at Cz, CPz, C3, and C4 electrodes.

A heel lance control and heel lance were performed for each infant and these procedures were time-locked to the EEG and EMG recordings using the event-detection interface and accelerometer described in Chapter 2. However, in 10 cases the events were time-locked using a microphone secured to the lancet, with audio recording directly linked to the electrophysiological recordings. In order to assess the effect of this change in methodology, we compared the latency of the evoked responses recorded using audio time-locking and accelerometer time-locking in aged-matched infants. There was no

significant difference in latency between the methods (Wilcoxon signed rank test, p=0.95).

3.3.2 Analysis

3.3.2.1 Nociceptive-specific brain activity

In order to determine a threshold for nociceptive brain activity, 10 epochs of EEG activity at rest per infant were extracted from data acquired prior to the clinical procedure. These traces were Woody filtered, with a maximum jitter of ± 50ms, in the region of 400 - 700ms, by identifying the maximum correlation with the component of nociceptive-specific brain activity. The nociceptive-specific component was projected onto the background data to calculate a weight for each trace. The distribution of these values was used to represent background noise levels within the data. A nociceptive-specific response was only defined to have occurred if the weight of the potential evoked by heel lancing was greater than 90 % of the distribution of background weights, which equated to a weight greater than 0.14.

3.3.2.2 EMG

In order to accurately identify the start and end times of reflex activity, a novel algorithm was developed. EMG data was low pass filtered at 5 Hz and smoothed by averaging the signal across moving 250ms windows. The start of the reflex withdrawal was identified as the point at which the magnitude of the differential of the smoothed signal crossed a threshold (set at five times the standard deviation of the baseline or 0.0045) for at least 100ms. The end point of the reflex was defined as the point at which the differential of the signal again fell below a threshold (set at one standard deviation of the baseline or 0.0025) for at least 250ms (319). In order to account for a persistent change in muscle

tone that can occur following a reflex, the differential was used, allowing activity post-reflex to settle at a level slightly elevated above the original baseline. Reflex duration was defined as the difference between start and end points. Reflex amplitude was defined by the area under the smoothed signal between the start and end points. The peak latency was defined as the time of the maximum height of the smoothed curve post-stimulus.

3.3.2.3 Comparison of brain activity and reflex activity

The maturation of noxious-evoked brain and reflex activity was investigated using the relative proportion of the two signals across the gestational age range. For each individual, each reflex characteristic (duration, amplitude, or peak latency) was divided by its maximum value across the population, and the weight of their nociceptive-specific brain activity was divided by the maximum weight across the population. Evoked potentials below the threshold for nociceptive-specific activity were zeroed, as this activity was indistinguishable from noise. The relative proportion of signals was calculated as the difference in the proportion of nociceptive-specific brain activity and the proportion of reflex activity (duration, amplitude or latency), resulting in a value between −1 and 1. Thus −1 indicated an infant with maximal reflex withdrawal (in terms of duration, amplitude or latency) and no nociceptive-specific brain activity. Conversely, 1 indicated maximal nociceptive-specific brain activity with no reflex activity.

Statistical analyses were performed in MATLAB R2016b (MathWorks). Linear regression analyses examining the development of each measure were conducted with gestational age at study, postnatal age, estimated cumulative prior pain exposure, and previous diagnosis of postnatal infection included as independent variables. Q-Q plots were used to verify the normality of residuals.

3.3.3 Results

The main results of Study 1 were published in Current Biology (319).

3.3.3.1 Characterising the development of noxious-evoked brain activity

Noxious-evoked brain activity in response to a clinically-required heel lance and heel lance control procedure were recorded in 40 infants aged between 28- and 42-weeks' corrected gestation. EEG background activity at rest prior to the procedure was also recorded in all infants and used to determine a threshold for nociceptive-specific brain activity (see Methods). Artefact-free recordings were obtained in 36 infants in response to heel lancing and 32 infants in response to the control. A template of noxious-evoked brain activity previously defined in a population of term infants was projected onto the filtered EEG data for each condition. (Fig 3.1). Nociceptive-specific brain activity in response to heel lancing, above the threshold defined in background activity, was identified at the vertex electrode (Cz) in 19 infants aged between 32- and 42- weeks' gestation. Nociceptive-specific brain activity in response to heel lancing was not identified in any of the infants aged less than 32 weeks' gestation. Instead, non-modality-specific responses called delta brushes were detected in these infants (Fig 3.2).

In infants aged 32 weeks' gestation or more, the mean magnitude of noxious-evoked brain activity in response to heel lancing was significantly greater than the magnitude of the response evoked by the control or identified in background EEG data recorded at rest (ANOVA, $p<0.0001$; Fig 3.1).

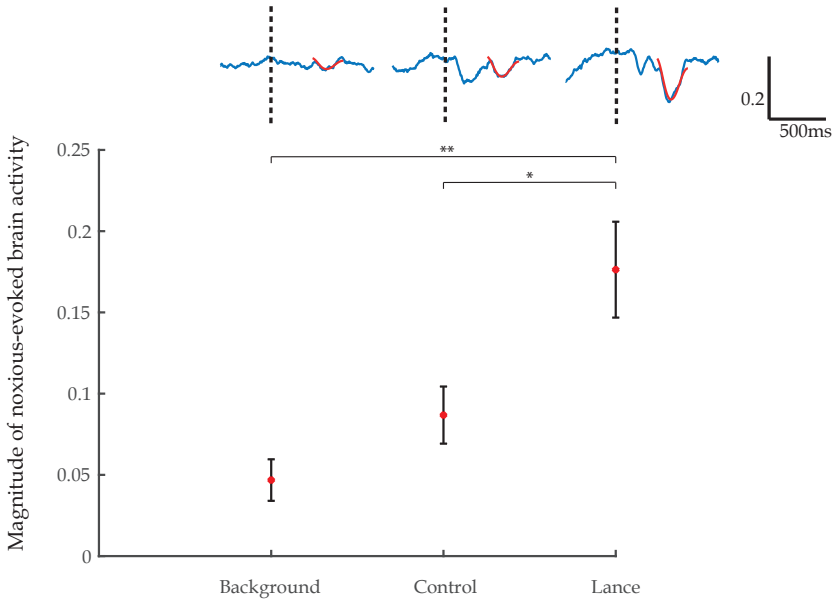

Figure 3.1: Noxious-evoked brain activity in infants aged ≥32 weeks' gestation. The mean Woody-filtered EEG traces for the background (n=29), control (n=25) and heel lance (n=29) are shown overlaid with the noxious-evoked brain activity template (in red). The mean (SEM) magnitude of the noxious-evoked brain activity for each condition is shown. The mean magnitude of noxious-evoked brain activity is significantly greater following the heel lance compared to the control (p=0.013) and significantly greater than background activity (p=0.0001).

Consistent with a previous study (183), nociceptive-specific brain activity was more reliably elicited in infants of 35 weeks' gestation or more. Nociceptive-specific brain activity occurred in 61% (14/23) of infants aged ≥ 35 weeks' gestation compared to 38% (5/13) of infants aged less than <35 weeks' gestation. The magnitude of nociceptive-specific activity identified in infants ≥ 35 weeks' gestation was significantly greater than the magnitude of nociceptive-specific activity in infants <35 weeks' gestation (unpaired t-test, p=0.023). Furthermore, the magnitude of nociceptive-specific activity at Cz significantly increased with gestational age (regression coefficient ß=0.024, n=19, p=0.03; Fig 3.2), irrespective of postnatal age, estimated pain exposure and history of postnatal infection. There was however no significant increase in the magnitude of the

activity evoked by the control lance (p>0.05) or the magnitude of activity in background recordings with gestational age (p>0.05).

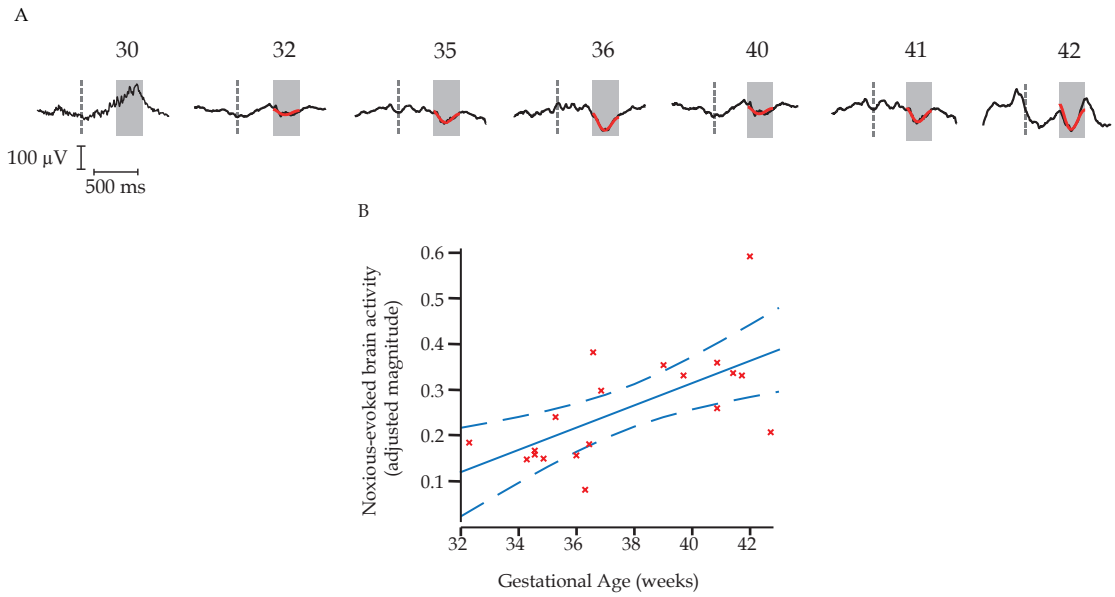

Figure 3.2: Development of noxious-evoked brain activity. A. Examples of EEG traces from infants across the gestational age range. The mean Woody-filtered traces are overlaid with the noxious-evoked brain activity template (red). The point of heel lancing is indicated by a dashed grey line. **B.** The magnitude of noxious-evoked activity increases significantly with gestational age (r^2= 0.35). The regression was adjusted for postnatal age, estimated cumulative prior pain exposure, and previous diagnosis of postnatal infection. Dashed lines indicate 95% confidence intervals.

Nociceptive-specific activity was identified at electrodes other than Cz. In infants with a nociceptive-specific response at Cz (n=19), the mean magnitude of responses at C3, C4, and CPz electrodes were also greater than the background threshold (Fig 3A). The magnitude of responses at these electrodes did not however significantly increase with gestational age. In infants without nociceptive-specific activity at Cz (n=17), the mean activity at C3, C4 and CPz electrodes were not greater than the background threshold (Figure 3.3).

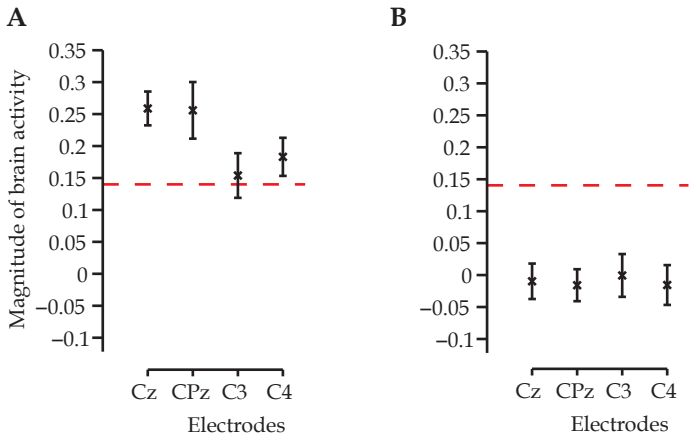

Figure 3.3: Noxious-evoked activity across electrodes. A. Noxious-evoked activity across central electrodes in infants with noxious-specific activity at the Cz electrode and **B.** in infants without noxious-specific activity at the Cz electrode. The background threshold for noxious-evoked activity is represented by a dashed red line.

3.3.3.2 Characterising the development of reflex withdrawal activity

Reflex activity of the ipsilateral limb was recorded in 40 infants aged 28-42 weeks' gestation using surface EMG. In 32 of the 35 infants (with artefact-free data), significant reflex activity, with a mathematically-defined start and end point was identified in response to heel lancing (Fig 3.4).

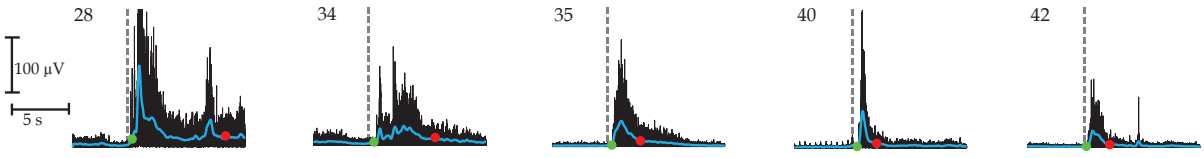

Figure 3.4: Examples of reflex activity across the preterm period. The mathematically defined start and end point of reflex activity is identified by a green and red dot respectively.

With increasing gestational age, reflex activity evoked by the noxious stimulus significantly decreased in duration (ß=0.29, n=32, p=0.039; Fig 3.5A), amplitude (ß=0.02, n=32, p=0.001; Fig 3.5B), and peak latency (ß=0.15, n=32, p=0.002; Figure

3.5C). This represented a reduction in reflex amplitude of 20 μV per week, a decrease in duration of 0.3 s per week, and a reduction in latency to peak of 0.15s per week.

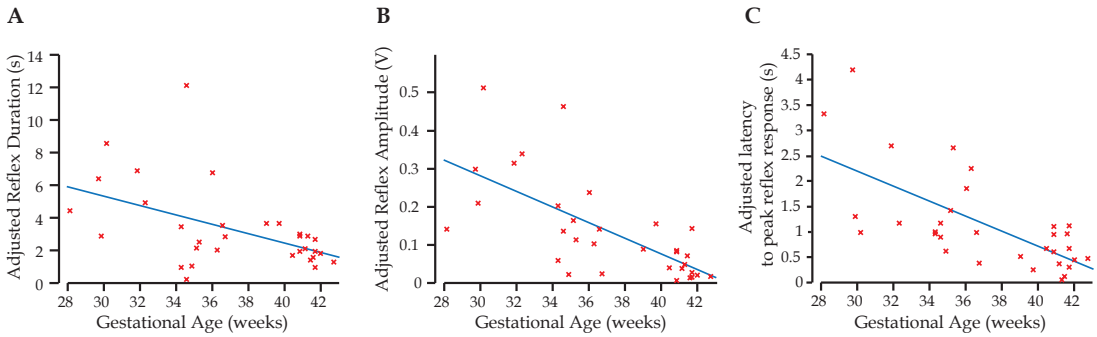

Figure 3.5: Development of reflex withdrawal. The duration (r^2= 0.23) (**A**), amplitude (r^2= 0.33) (**B**), and latency to peak (r^2= 0.37) (**C**) of ipsilateral limb reflex activity across the preterm period (n = 32). Each measure was adjusted for postnatal age, estimated cumulative prior pain exposure, and a history of postnatal infection.

Reflex activity in response to the non-noxious stimulus was less than activity evoked by heel lancing (Fig 3.6). Only 10 of the 28 infants (with artefact-free control data) displayed significant reflex activity in response to the non-noxious stimulus, identified by a mathematically defined start and end point (see methods). There was no significant change in the duration, amplitude or latency of the peak of reflex activity with gestational age (duration: p=0.73; amplitude p=0.98; latency=0.87).

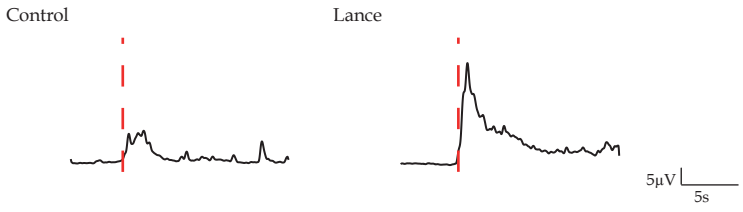

Figure 3.6: Reflex withdrawal to control and lance. Average reflex activity to the control procedure (n=28) and in response to heel lancing (n=35).

3.3.3.3 The balance of supraspinal and spinal cord activity

In 29 infants with both EEG and EMG recordings, the relationship between noxious-evoked brain and spinal reflex activity was investigated (Fig 3.7). The relative proportion of the magnitude of nociceptive-specific brain activity to the duration of reflex activity significantly increased with gestational age (ß=0.054, n=29, p=0.024; Fig 3.8). The relative proportion of the magnitude of nociceptive-specific brain activity to the amplitude of reflex activity also significantly increased with gestational age (ß=0.074, n=29, p=0.0039; Fig 3.8). The relative proportion of the magnitude of nociceptive-specific brain activity to the latency of the peak reflex activity significantly increased with gestational age (ß=0.059, n=29, p=0.024; Fig 3.8). Thus, the most mature infants in the study population had the greatest magnitudes of nociceptive-specific brain activity and the smallest, shortest and earliest reflex activity. This occurred irrespective of postnatal age, estimated prior pain exposure, and history of postnatal infection, which were accounted for in the models and had no significant effect.

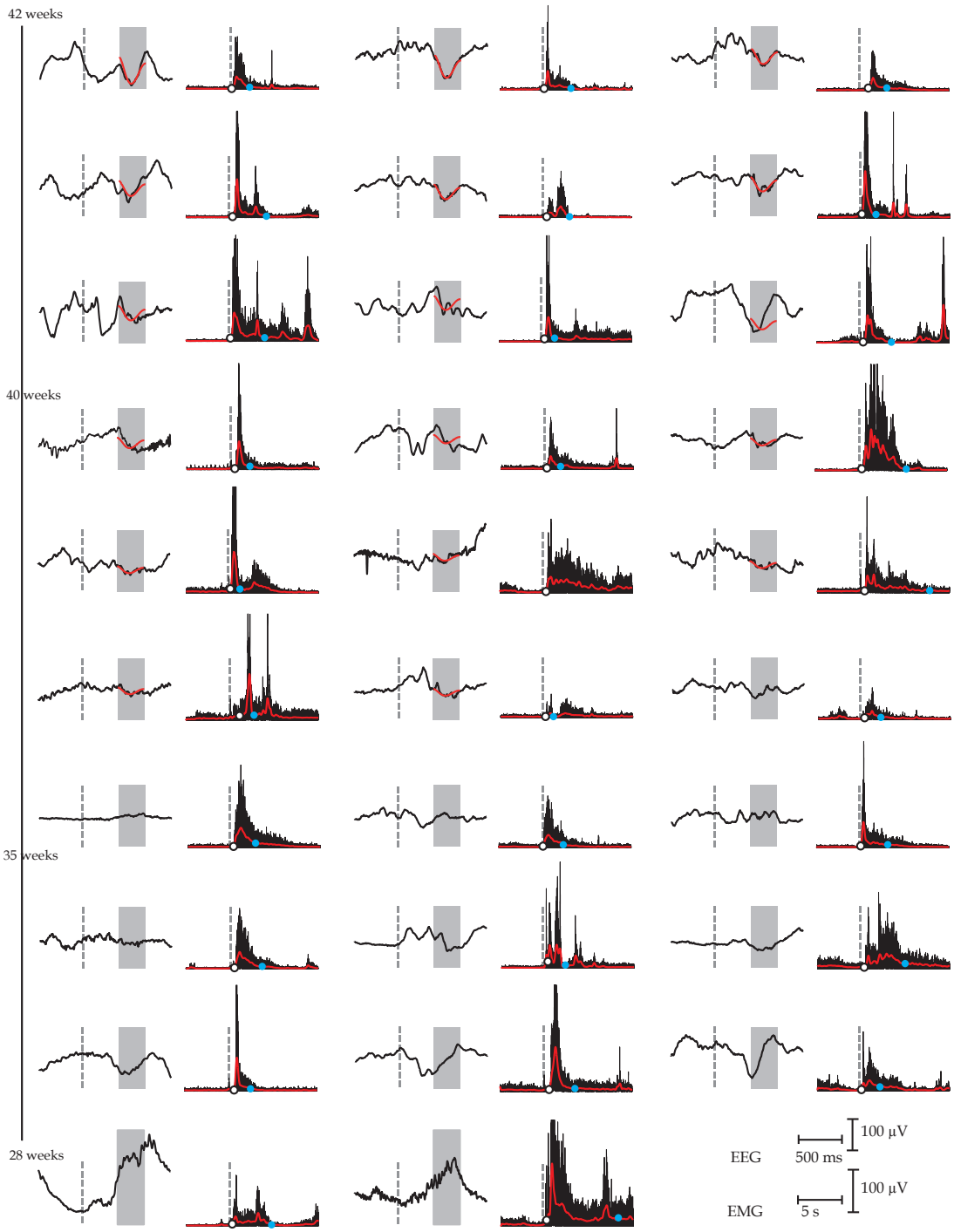

Figure 3.7: Paired EEG and EMG responses to noxious stimulation. EEG and EMG traces for each infant in order of gestational age, beginning in the top left with the oldest infant. The dashed line indicates the point of heel lancing. The mean Woody-filtered EEG traces are overlaid with the fitted template of noxious-evoked brain activity (red). The mathematically defined start and end point of reflex activity is identified by a white and blue dot respectively.

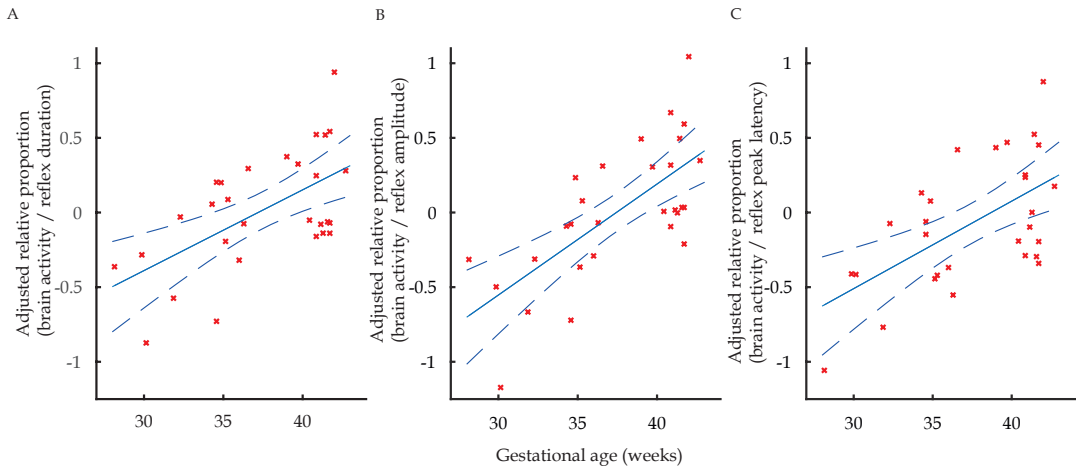

Figure 3.8: Relationship between nociceptive-specific brain activity and reflex withdrawal activity. The relative proportion of noxious-evoked brain and spinal reflex activity for each infant is plotted against gestational age (n = 29). A relative proportion of -1 indicating maximal reflex withdrawal duration (r^2= 0.25) (**A**), maximal amplitude (r^2= 0.31) (**B**) or maximal latency (r^2= 0.28) (**C**) within the study population with no concomitant nociceptive-specific brain activity and 1 indicating maximal nociceptive-specific brain activity (within the study population) and no concomitant reflex withdrawal.

3.3.4 Summary of Results

- Noxious-evoked brain activity increased in magnitude across the preterm period.
- Noxious-evoked reflex withdrawal reduced in amplitude, duration and latency across the preterm period.
- Refinement of reflex activity in terms of amplitude, duration and latency occurred proportionately with the maturation of nociceptive brain activity.

3.4 Study 2

3.4.1 Methods

3.4.1.1 Participants

In order to investigate endogenous pain modulation in infants, 21 healthy infants aged 35-42 weeks' gestation were recruited for a functional MRI scan within 10 days of birth during which both stimulus-evoked and resting state data were acquired (Table 3.2). All infants were healthy, clinically-stable inpatients on the postnatal ward and had no history of admission to the Newborn Care Unit or congenital or neurological pathology.

Demographic	
Gestational age at birth (weeks)	38.3 (35.3 – 41.4)
Gestational age at study (weeks)	38.4 (35.9 – 41.7)
Postnatal age (days)	2 (1 -10)
Birthweight (g)	3441.9 (703.3)
Sex	12 male, 9 female
APGAR at 5 minutes	9.9 (0.4)
Skin-breaking procedures	3 (0 -19)

Table 3.2: Study 2 participant demographics.

3.4.1.2 Experimental procedures

Infants were transported from the postnatal ward to the Wellcome Centre for Integrative Neuroimaging, and following examination and application of safety equipment, MRI data were acquired in the Siemens Prisma 3T scanner as described in Chapter 2. In order to record noxious-evoked brain activity, a 128mN pinprick stimulator was applied ten times to the left foot of each infant with a minimum inter-stimulus interval of 25 seconds. 500 volumes of resting state data were also acquired (see Chapter 2). The order of acquisition of stimulus-evoked and resting state data was randomised to control

for an effect of scan order. Two infants unfortunately became unsettled during the scanning session and data collected were therefore incomplete and could not be included.

3.4.2 Analysis

This study employs analytical methods developed in Goksan, Baxter, Moultrie et al., 2018 (243).

3.4.2.1 Noxious-evoked BOLD activity

Functional MRI data were pre-processed according to the methods described in Chapter 2 using a modified version of the Developing Human Connectome Project pipeline for stimulus-evoked data optimised for neonates (334). Four infants were excluded from further analyses after pre-processing of the data; two subjects had predominantly negative noxious-evoked BOLD activity, one subject had excessive motion that resulted in movement of the brain outside of the field of view, and one subject was excluded due to excessive noise in the PAG timeseries despite ICA denoising.

The group mean stimulus-evoked positive response was estimated by permutation testing using FSL's Randomise (370) with 5000 permutations, a cluster-defining threshold of 3.1 and a 5% family-wise error rate (FWER) correction. A mask of noxious-evoked BOLD activity was defined from the significant voxels ($z > 3.1$, $p < 0.05$) identified on group analysis and used to calculate the mean post-stimulus percentage change in BOLD for each infant using the *Featquery* function.

3.4.2.2 Defining descending pain modulatory brain regions

In order to investigate the pre-stimulus connectivity of the PAG to cortical brain regions involved in descending inhibition, core regions most frequently reported in adults – the

anterior cingulate, anterior insula, amygdala, and dorsolateral prefrontal cortex (95) - were identified using a neonatal atlas. With the exception of the PAG, anatomical masks of brain regions were derived from the M-CRIB atlas (371) (see Fig 3.9). The amygdala was defined directly from the atlas. The dorsolateral prefrontal cortex is not specifically defined in the atlas, therefore a mask of the medial frontal gyrus (MFG) was created from combination of the rostral middle frontal and caudal middle frontal regions delineated in the atlas. The anterior insula mask was created from voxels in the insula mask located anterior to the central sulcus. The anterior cingulate cortex mask comprised a combination of the caudal anterior cingulate and rostral anterior cingulate regions. The PAG was defined using a previously derived mask of the region (243), based on an adult mask (372), which was transformed to the standard 40-week brain template used in this study and edited to ensure its location within anatomical landmarks identified on the high definition T2 image (Fig 3.9).

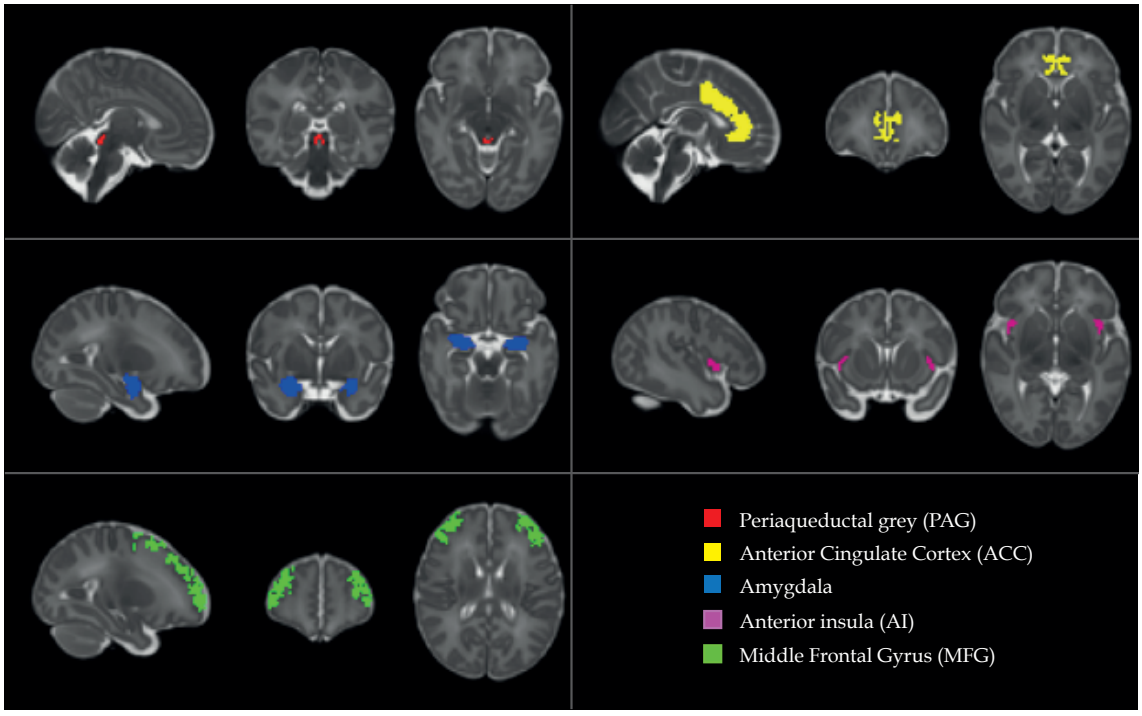

Figure 3.9: Anatomical masks. Anatomical masks of descending pain modulatory brain regions overlaid on a 40-week standard brain template.

3.4.2.3 Pre-stimulus Functional Connectivity

For each infant, the anatomical masks were registered from 40-week template standard space to the infant's structural space in order to allow precise removal of voxels classified as CSF in the segmentation stage of the analysis pipeline. The masks were thresholded and transformed to the infant's functional space and thresholded again at 0.5. The mean timeseries was calculated for each masked region of interest using the *fslmeants* function and demeaned (Fig 3.10). The six volumes immediately prior to each stimulus application were identified, giving a pre-stimulus period of ~9 seconds. In a previous study, we used the preceding three volumes (243). However, due to the shorter TR of the acquisition sequence used in this study, it was possible to double the number of pre-stimulus volumes, whilst allowing for recovery of neural activity following the previous stimulus (353). Thus 10 sets of six data points were identified in each region of interest's mean timeseries. Several infants only had 9 pre-stimulus periods as the first stimulus was applied before six volumes had been acquired. For each infant, the correlation between the identified pre-stimulus data points for each pair of regions of interest was calculated to estimate the connectivity between regions. The stability of pre-stimulus connectivity across stimuli was determined using linear mixed effects models for each region pair in R statistical software, with stimulus number as the predictor and subject as a random effect, and p values determined using likelihood ration tests. To investigate the relationship between pre-stimulus PAG connectivity and noxious-evoked BOLD activity, linear regression models were run with noxious-evoked percentage change in BOLD as the response variable, and pre-stimulus functional connectivity between regions, gestational age at study and postnatal age as explanatory variables.

3.4.2.4 Resting state connectivity

As for pre-stimulus connectivity, the anatomical masks of regions of interest were transformed to the infant's functional space via structural space and the mean timeseries extracted and demeaned (Fig 3.10). For each infant, the correlation between the mean timeseries for each pair of regions of interest was calculated to estimate the resting state functional connectivity between regions. Linear regression models were run with the noxious-evoked percentage change in BOLD as the response variable, and resting state functional connectivity between regions and gestational age as explanatory variables. The parameter estimates and p-values for these models are reported.

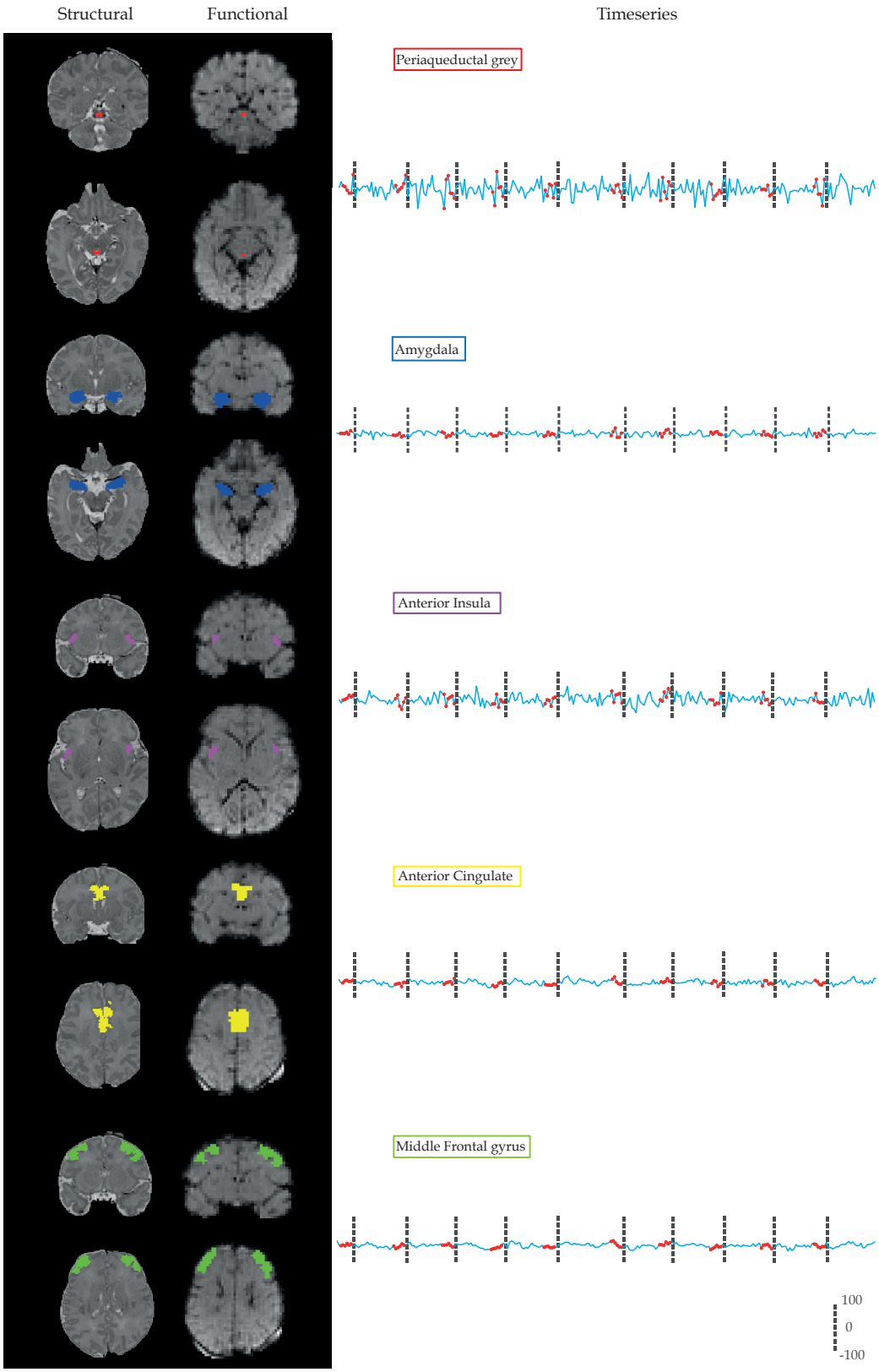

Figure 3.10: Registration of regions of interest. Examples of the registration of anatomical masks to subject structural space (**A**) and functional space (**B**) shown in coronal and axial views. Examples of the mean timeseries extracted for each region (**C**). Red circles indicate pre-stimulus volumes. The point of stimulation is marked by a dashed grey line.

3.4.3 Results

Functional MRI data were successfully acquired at rest and in response to low-intensity experimental noxious stimulation of the foot in 15 healthy infants aged 35-42 weeks' gestation.

3.4.3.1 Characterising noxious evoked brain activity

Non-parametric analysis of the mean group BOLD activity produced eight clusters of significant activity in response to noxious stimulation, spanning multiple brain regions. Group mean noxious-evoked brain activity was very clearly localised within grey matter regions (Fig 3.11) and local maxima were identified in brain regions commonly reported in pain fMRI studies in adults. These areas included the postcentral and precentral gyri (somatosensory and motor cortices), bilateral insular cortices, and bilateral thalami (Table 3.3).

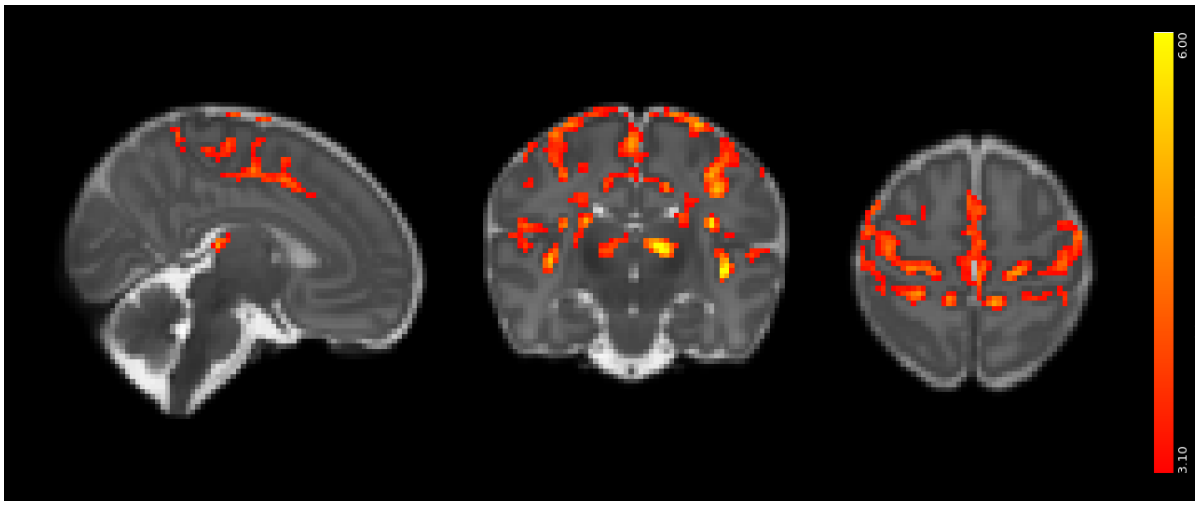

Figure 3.11. Noxious-evoked BOLD activity.
Group noxious-evoked BOLD activity (n=15; cluster threshold z=3.1, 5000 permutations, FWER-corrected $p<0.05$) is overlaid on a standard template brain for infants at 40 weeks' gestation.

CLUSTER	SIZE	REGION	HEMISPHERE	PEAK	COORDINATES		
1	4284	Precentral gyrus	Ipsilateral	5.57	-26.0	-14.0	30.0
		Insula	Contralateral	5.34	26.5	-18.5	12.0
		Postcentral gyrus	Contralateral	5.3	17.5	-29.0	45.0
		Precentral gyrus	Contralateral	5.23	22.0	-15.5	37.5
2	658	Insula	Ipsilateral	6.43	-24.5	-20.0	9.0
		Postcentral gyrus	Ipsilateral	4.99	-20.0	-26.0	24.0
		Superior temporal	Ipsilateral	4.86	-24.5	-27.5	16.5
3	175	Subcortical matter (edge of thalamus)	Contralateral	5.52	13.0	-12.5	24.0
4	124	Subcortical matter (edge of thalamus)	Ipsilateral	4.32	-12.5	-17.0	24.0
5	89	Superior parietal	Ipsilateral	4.82	-11.0	-57.5	34.5
		Inferior parietal	Ipsilateral	3.28	-15.5	-56.0	21.0
6	84	Thalamus	Ipsilateral	7.23	-6.5	17.0	16.5
7	77	Cuneus	Contralateral	4.32	17.5	-44.0	21.0
8	75	Thalamus	Contralateral	5.22	7.0	-15.5	18.0

Table 3.3: Localisation of noxious-evoked BOLD activity. Regions of the brain with local maxima of significant noxious-evoked BOLD activity.

3.4.3.2 Noxious-evoked brain activity is inversely correlated with pre-stimulus connectivity of the PAG

The correlation of functional data points acquired in the ~10 seconds (6 volumes) preceding each noxious stimulation was used to investigate the pre-stimulus connectivity of the PAG to the middle frontal gyri (MFG), anterior cingulate cortices (ACC), anterior insular cortices (AI), and amygdala respectively. The pre-stimulus functional connectivity for each pair of regions was not significantly different across applications of the noxious stimulus (linear mixed effect models - PAG-MFG connectivity: χ^2=1.08, p=0.29; PAG-ACC connectivity: χ^2=0.75, p=0.38; PAG-AI connectivity: χ^2=1.84,

p=0.17; PAG-Amygdala connectivity: χ^2=0.70, p=0.40). The mean pre-stimulus connectivity between the PAG and each region was only positive in 7 of the 15 infants, suggesting that on average the PAG is weakly connected. However, the mean pre-stimulus functional connectivity between the PAG and MFG was significantly inversely correlated with the mean percentage change in BOLD activity evoked by noxious stimulation (linear regression: ß=-1.6 ±0.5, p=0.0083; Fig 3.12). This suggests that infants with greater connectivity between these regions have less noxious-evoked brain activity. The mean pre-stimulus functional connectivity between the PAG and ACC was also significantly inversely correlated with the mean percentage change in BOLD activity evoked by noxious stimulation (linear regression: ß=-1.3 ±0.6, p=0.038; Fig 3.12). Connectivity between the PAG and amygdala, and PAG and AI were negatively correlated with noxious-evoked activity but not significantly (PAG-amygdala: ß=-0.71, p=0.37; PAG-AI: ß=-0.72, p=0.17; Fig 3.12). All models were adjusted for infant gestational and postnatal age at study. Correction for multiple testing could be considered and if a Bonferroni-Holm post-hoc correction was applied only connectivity between the PAG and MFG would retain significance.

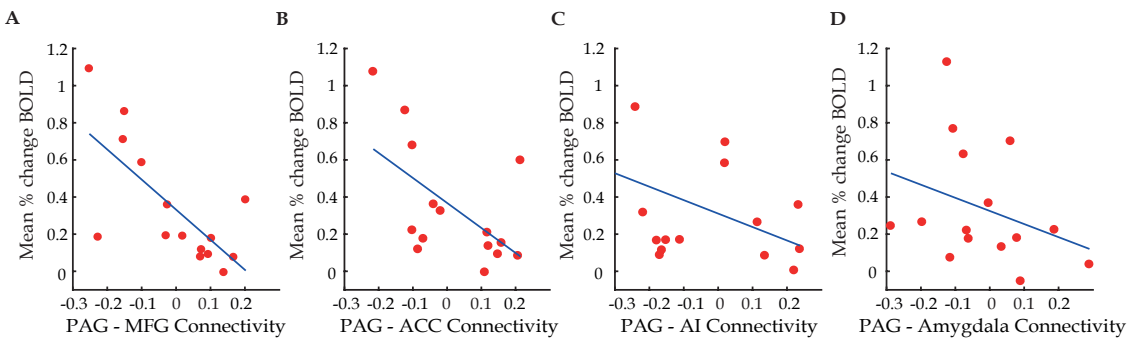

Figure 3.12: Noxious-evoked BOLD activity and pre-stimulus connectivity of the PAG. Mean pre-stimulus functional connectivity of the (**A**) PAG and MFG (r^2= 0.56), (**B**) PAG and ACC (r^2= 0.44), (**C**) PAG and AI (r^2= 0.30), and (**D**) PAG and amygdala (r^2= 0.22) is negatively correlated with noxious-evoked BOLD activity.

3.4.3.3 Noxious-evoked brain activity is inversely correlated with resting state connectivity of the PAG and middle frontal gyrus

Given the significant inverse correlation demonstrated between pre-stimulus connectivity of the PAG and MFG, resting state data acquired in the same infants was used to investigate whether this relationship results from stable connectivity within individuals or reflects a specific pre-stimulus brain state. The resting state functional connectivity of the PAG and MFG was also significantly inversely correlated with the mean percentage change in BOLD activity evoked by noxious stimulation (ß=-1.9, p=0.023; Fig 3.13), accounting for gestational age and postnatal age at study. Thus, consistent with pre-stimulus functional connectivity, infants with greater functional connectivity between the PAG and middle frontal gyri at rest displayed less noxious-evoked brain activity.

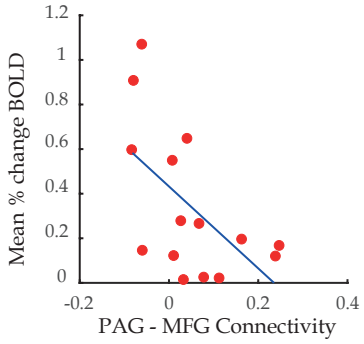

Figure 3.13. Noxious-evoked BOLD activity and resting-state connectivity of PAG - MFG. Resting state connectivity between the PAG and MFG is negatively correlated with the mean noxious-evoked BOLD activity (r^2=. 0.24).

3.4.4 Summary of results

- Experimental noxious stimulation evoked significant BOLD activity in pain-related brain regions in infants.
- Noxious-evoked brain activity was inversely correlated with pre-stimulus functional connectivity of the PAG and MFG, and PAG and ACC.
- Resting state functional connectivity of the PAG and MFG was also inversely correlated with noxious-evoked brain activity.

3.5 Discussion

In Study 1 of this chapter, electrophysiological measures were used to characterise the development of noxious-evoked brain and spinal reflex activity in the preterm period, very preterm (28-32 weeks' gestation) infants through to full-term human infants. Consistent with a previous electrophysiological study (183), nociceptive-specific brain activity was more likely to occur with increasing gestational age and was not observed in very premature infants (183,198). The magnitude of nociceptive-specific brain activity significantly increased with corrected gestational age and oppositely, the amplitude, duration and peak latency of noxious-evoked spinal reflex activity decreased significantly with gestational age. The maturation of brain and reflex activity was assessed by examining the relative proportion of the two signals across the gestational age range and this study demonstrated that the development of these noxious-evoked responses not only occurs concurrently but that the relative proportion of the responses is strongly correlated with gestational age, suggesting a developmental relationship between spinal and supraspinal maturation. This may reflect a change in the balance of descending pain modulation. Study 2 further investigated the emergence of endogenous pain modulation using fMRI data acquired in healthy infants in response to low-intensity noxious stimulation and at rest. Functional connectivity of the PAG to the middle frontal gyrus in the immediate pre-stimulus period was negatively correlated with the mean BOLD activity evoked by noxious stimulation. This relationship was not restricted to pre-stimulus connectivity but was also confirmed in resting state data acquired in the same infants. This could suggest that the developing connectivity of the PAG may play a role in modulating supraspinal noxious inputs in early postnatal life.

3.5.1 Nociceptive-specific brain activity increases with gestational age

In this chapter, noxious stimulation was more likely to evoke nociceptive-specific brain activity than non-specific activity in infants aged 34-35 weeks' gestation or more. This is consistent with previous work by Fabrizi et al., that suggested a critical period at approximately 35 weeks' gestation, whereby sufficient maturation of somatosensory circuitry is likely to have occurred to enable the generation of predominantly specific potentials in response to noxious and touch stimulation (183). Fundamental structural changes occur between 31- and 34-weeks' gestation, including the differentiation of the cortical plate into a six-layered cortex. Developing thalamic connections to layer IV may represent the anatomical substrate necessary for sensory-specific cortical activations (67). Expansion and strengthening of this maturing thalamo-cortical connectivity may underlie not only the emergence of nociceptive-specific brain activity but the increase in magnitude of this potential during the third trimester. Projection of the nociceptive-specific template onto background EEG data collected at rest in the same infants did not show a general increase in amplitude of baseline EEG with gestational age, suggesting that the developmental pattern observed is specific to noxious-evoked activity. An increase in magnitude is unlikely related to a maturational increase in head circumference either, as a larger head circumference in older infants could increase the distance between the source of activity and the recording scalp electrode, more likely resulting in a reduced amplitude of the evoked response (373).

The noxious-evoked potential was on average greatest at the Cz and CPz vertex electrodes. This supports recent work by Verriotis et al., which demonstrated that from 36 weeks' gestation peak amplitude of noxious-evoked activity is detected at the vertex in more than 80% of infants, and in premature infants the percentage of infants with a

peak amplitude at the vertex was greater than 50% (196). In adult studies of noxious-evoked electrophysiological activity, the classical potential evoked by laser, electrical or pinprick stimulation consists of a similar negative-positive deflection that is also maximal at the vertex (194,195,232). This electrophysiological potential is thought to be mediated by a combination of activity from somatosensory, insular and anterior cingulate cortices (374). The negative component is likely generated by the bilateral operculoinsular and contralateral somatosensory cortices (375–377) whilst activity of the cingulate gyrus is thought to contribute to the positive component (377,378). Combined EEG and functional MRI has now been pioneered to investigate the brain regions involved in generating spontaneous bursts of activity in premature infants (379) and this challenging multimodal approach could be applied in future to more thoroughly investigate the brain regions underlying the noxious-evoked electrophysiological potential recorded in this study. The application of denser electrode arrays in future studies would also facilitate source localisation and allow further investigation of the distribution of noxious-evoked activity across the preterm period.

In 39% of infants aged 35 weeks' or more, heel lancing did not evoke a significant nociceptive-specific response above threshold. This is consistent with a previous study of term infants in which heel lancing failed to evoke a significant change in brain activity in 36% of infants (5). Inter-individual variability in the magnitude of the noxious-evoked responses may reflect nociceptive sensitivity, which could be due to genetics, prior experience, environmental influences, or potentially endogenous pain modulation. Slater et al., previously demonstrated that ex-premature infants studied at term-equivalent gestation have greater amplitude noxious-evoked responses than their term-born counterparts (199). A study has also suggested that sepsis affects the magnitude of these

responses (197). Postnatal age, cumulative pain exposure and history of sepsis were therefore accounted for in the models. However, many other factors likely influence noxious-evoked activity and could be accounted for in a larger sample. A longitudinal investigation of noxious-evoked activity in infants repeatedly from extreme prematurity to term-equivalent gestation is currently underway and will provide a better understanding of the developmental trajectory of these responses. This is a challenging study in terms of recruitment, as parents have to commit to infants being studied on multiple test occasions from an early stage of hospitalisation, and there is inevitably a degree of loss of follow-up as a result of deaths or discharges to other centres.

3.5.2 Noxious-evoked reflex activity is refined with gestational age

In study 1, the flexion withdrawal reflex was characterised in response to noxious heel lancing using surface EMG of the ipsilateral biceps femoris and term infants had significantly smaller, shorter and earlier reflexes than premature infants. Reductions in amplitude, duration and latency were significantly correlated with gestational age and these developmental trajectories are consistent with both animal and human studies of reflex physiology. In rat pups, reflex duration reduces during the first few postnatal weeks (121,358). A pinch stimulus evokes motor reflex activity lasting up to 20 seconds in newborn pups and by the third postnatal week this response persists less than one second (121). In this study, reflex duration to heel lancing ranged from >4 seconds to <1 second. A decline in reflex amplitude over the preterm period in human infants is consistent with a decline in amplitude observed in rat pups. It also substantiates the results of a study by Cornellisen and colleagues (148). They reported a developmental reduction in fold increase from baseline specifically between 1000-1250ms after heel lancing in infants

aged between 32-43 weeks' gestation. The authors also suggested this may reflect the absence of inhibitory control in cutaneous circuits (148).

The reduction in latency of reflexes observed in Study 1 over the preterm period is consistent with a decline in latency of cutaneous upper limb reflexes also observed in infants between 33 and 40 weeks' (380), and reductions in lower limb reflexes observed in infants between term birth and 10 months of age (381). This developmental trajectory could be driven by a variety of factors. Nerve conduction velocities significantly increase during the preterm period; tibial nerve conduction dramatically improves from ~6m/s in extremely premature infants to 26m/s in term infants (382). Developmental changes also occur in postsynaptic receptors affecting synaptic current decay (383) and the immaturity of the neuromuscular junction may contribute to the differences in speed and synchronisation of depolarisation (384). In addition to the observed decline in amplitude, latency and duration of reflexes, there was also a marked reduction in the variability of these reflex characteristics in term infants compared with premature infants. The refinement of diffuse and exaggerated reflex behaviour observed in the preterm period may result from maturation of synaptic transmission in the developing neonatal spinal cord and the emergence of descending inhibitory tone.

35% of infants exhibited a reflex response to the control stimulus, consistent with previous studies (148), suggesting poor specificity of this response in early life. In adults, the flexor reflex is a protective nociceptive-specific response, which can be experimentally induced by laser or nerve stimulation and is linearly correlated with pain report (385,386). In neonatal rats, reflexes are also sensitive to tactile stimulation due to a predominance of low-threshold Aß afferent input to the dorsal horn and the gradual

development of nociceptive C-fibre input during the first few postnatal weeks (387,388), accompanied by a functional switch at glycinergic synapses from facilitation to inhibition by postnatal day 14 (127). Exuberant Aß dorsal horn input and weak glycinergic inhibition may also contribute to the lack of nociceptive specificity of the withdrawal reflex in human infants. Hypersensitivity and non-specificity of reflex activity in the preterm period may play a fundamental role in the development and refinement of somatosensory circuitry through experience-dependent mechanisms during the third trimester of human gestation (127,389).

3.5.3 Concomitant maturation of noxious-evoked brain and reflex activity

Both EMG and EEG data were acquired in infants in this study in order to investigate the developmental relationship between noxious-evoked supraspinal and spinal nociceptive activity. The relative proportion of nociceptive-specific brain activity to reflex activity within individual infants significantly increased with gestational age, irrespective of postnatal age, cumulative pain exposure, or history of postnatal infection. Large nociceptive-specific potentials in older infants were therefore concomitant with smaller more acute reflexive responses to heel lancing. The change in balance of nociceptive-specific brain activity and spinally-mediated reflex activity could suggest that maturing nociceptive cortical networks may begin to exert an inhibitory influence on spinal nociceptive processing in infants approaching term gestation.

In rats, the maturation of limb reflex behaviour also coincides with maturation of descending inhibitory pathways of the dorsolateral funiculus in the first three postnatal weeks (122,359) and spinalisation of neonatal pups results in abnormal distribution of reflex receptive fields in the adult animal (359). This suggests that supraspinal input may

play a critical role in the tuning of spinal nociceptive processing. Spinalisation of rodents after the fourth postnatal week results in hyper-excitability of the dorsal horn following a period of spinal shock, reflecting a loss of supraspinal inhibitory tone (390). Human adults similarly develop increased latency and duration of lower limb reflexes following a more prolonged period of spinal shock (391,392). Unlike neonatal rats, human infants at term also exhibit hyperreflexia following a period of spinal shock in response to perinatal spinal cord injury (393). The mechanisms underlying recovery from spinal cord injury are complex but this may also suggest that spinal and supraspinal circuitry in term infants are more developed than suggested by other studies, which have previously equated the entire preterm-term period in humans to the first postnatal week in rats (148). Concomitant maturation of nociceptive brain and reflex activity in human infants could simply result from synchronisation of developmental trajectories and not necessarily a direct causal relationship. Nevertheless, the results are suggestive of a rational link between supraspinal and spinal nociceptive circuit development in human infants, which merits further investigation.

3.5.4 Noxious stimulation evoked BOLD activity in pain-related brain regions

In Study 2, noxious stimulation evoked significant clusters of BOLD activity in areas of the brain that are frequently active during experimental pain studies in adults (80). These included the somatosensory cortices, thalami, anterior cingulate cortex and insular cortices. The clusters of activity were more clearly localised within grey matter than previous studies of noxious-evoked BOLD activity in infants (4,8) and this was likely due to improvements in acquisition parameters (including optimal TE and increased spatial resolution) and use of a neonatal-specific pre-processing analysis pipeline, which we have recently shown improves data quality, and increases sensitivity to signal and spatial

specificity (see Chapter 2). Due to concerns over inflation of family-wise error rates (394), a more stringent cluster-defining threshold of z = 3.1 was adopted, which evidently reduced the number of brain regions with significant activity. Consistent with our previous study in an independent dataset (4), no significant activity was observed in the amygdala or orbitofrontal cortex, regions associated with the affective component of pain and fear (395–399). This could potentially be attributed to the low-intensity of the stimulus applied. Although the 128mN stimulus has been shown to elicit an electrophysiological potential similar in morphology to the potential evoked by heel lancing, it is of much smaller magnitude, and it is not associated with a significant change in facial expression or overt distress of the infant, unlike clinical heel lancing (151). Significant activity in these emotional brain regions may perhaps occur during more salient painful stimuli that infants are commonly exposed to in clinical environments. However, it is not possible to test this hypothesis as the application of a more salient experimental stimulus that causes distress would be unethical and likely result in significant noise corruption due to movement of the participants. Performing a clinically-required painful procedure is not presently possible in the scanner due to spatial and safety constraints, and multiple repeats to improve signal to noise could unlikely be justified. Alternatively, it is possible that the amygdala is simply not active in response to noxious stimulation in early postnatal life, consistent with rodent studies that have demonstrated a lack of noxious-evoked activity in this region in rat pups until postnatal day 10 (400,401).

3.5.5 Noxious-evoked BOLD activity and functional connectivity of the PAG

Based on the results of Study 1, descending inhibitory modulation of spinal nociceptive circuitry may begin to emerge towards term gestation in human infants. This was supported by our recent fMRI study in an independent dataset, which suggested greater pre-stimulus functional network connectivity across the pain modulatory network is significantly associated with lower noxious-evoked brain activity. In Study 2, fMRI data was acquired in infants aged between 35- and 42- weeks' gestation to investigate the connectivity of the PAG to cortical regions involved in descending pain modulation and its relation to noxious-evoked BOLD activity. Pre-stimulus connectivity of the PAG and MFG was significantly negatively associated with noxious-evoked BOLD activity. In adults, correlation between PAG and dorsolateral prefrontal cortex activity has been reported prior to noxious stimulation in the context of placebo analgesia, and prefrontal cortex activity was correlated with placebo-induced pain relief and a reduction in pain-related brain activity (402). Wager et al., suggested that anticipation during placebo may result in prefrontal activity triggering endogenous opioid release within the brainstem resulting in modulation of pain perception (402). It is unlikely however that the effect reported in Study 2 resulted from anticipation because stimulation was applied at a variable interval of at least 25 seconds, to ensure that the infant's foot was stationary and, in a position, allowing accurate perpendicular application of the stimulus. Care was also taken to ensure that the monitor alerting the researcher to apply the stimulus did not change dramatically in brightness, as this could have alerted the infant prior to each application. Significant covariation of the cingulo-frontal cortex and PAG has also been associated with pain modulation by distraction (235).The prefrontal cortex is known to be involved in attention to pain (403–406) and it is possible that infants that attended more during the study had greater pre-stimulus functional connectivity between the PAG

and MFG. Measures of alertness or sleep state were unfortunately not recorded during the study to further explore this theory. However, the relationship between connectivity of these regions and noxious-evoked activity was reproduced using resting state data acquired in the same infants, which suggests that the relationship described between pre-stimulus functional connectivity of these brain regions and noxious-evoked activity was unlikely state-dependent.

In adults, the PAG is functionally connected to the vmPFC/rACC, insula and amygdala at rest (407). Changes in resting-state connectivity of the PAG have furthermore been implicated in several chronic pain conditions suggesting a link between connectivity of this region and vulnerability to pain. Migraine sufferers who develop allodynia have been shown to exhibit significantly reduced connectivity between the PAG and prefrontal regions, anterior cingulate and anterior insular cortices (368). Adults with chronic back pain also exhibit increased functional connectivity of the PAG and ventral medial prefrontal cortex (vmPFC)/rostral anterior cingulate cortex (rACC) compared to matched controls, and this connectivity is negatively correlated with pain ratings (408). Animal studies also provide evidence for the role of frontal-midbrain connectivity in modulating pain-related behaviour. In rats, electrical stimulation of the ventrolateral orbital cortex, an equivalent frontal region, results in antinociception evidenced by suppression of the heat-evoked tail-flick reflex (363) and this effect is abolished by lesioning (409) or GABA microinjections of the PAG (362).

Pre-stimulus functional connectivity between the PAG and ACC was also inversely related to noxious-evoked brain activity, which is consistent with results of our recent study in an independent dataset (243). Stronger co-variation between these two brain

regions in adults has been shown to relate to increased efficacy of endogenous placebo analgesia (410). Although correlation cannot be used to determine causation, it is reasonable to suggest that developing functional connectivity of the PAG may begin to exert a weak inhibitory tone at this stage of development in some infants, modulating ascending noxious input to the brain. A similar modulation of reflex activity could be expected and in future studies application of MRI-compatible EMG to the biceps femoris of the stimulated limb during acquisition of functional MRI could provide evidence to further support or refute this hypothesis. Combined spinal and brain MRI has also been acquired in adults, demonstrating a positive correlation between signal from the spinal dorsal horn and the PAG during noxious thermal stimulation, and the degree of this coupling was found to be predictive of pain report (411). The authors suggested that this system may provide a tonic influence to continuously balance and regulate noxious inputs (411). Future studies could endeavour to adapt combined spinal-brain acquisition protocols for use in infants to further explore the role of the PAG and its connectivity with the spinal dorsal horn during early postnatal development.

3.5.6 Results in the context of the animal literature

The PAG is a phylogenetically conserved midbrain region with descending projections to the RVM and spinal cord, which plays a fundamental role in endogenous pain modulation in humans, primates (412,413), rats (414,415), cats (416,417), and rabbits (418). The results of this chapter suggest the early emergence of descending inhibitory tone involving the PAG in infants around term gestation, which is consistent with our previous study of the network as a whole in an independent set, but is potentially contradictory to studies of rodent pups, depending on the theoretical definition of age equivalence of this system across species. Descending projections from the brainstem to

the dorsal horn are anatomically present but not functionally mature from birth in rats (419). In human infants, the timing of the development of structural neuroanatomical connectivity between cortical and brainstem pain modulatory brain regions has not yet been determined. Advances in non-invasive imaging of white matter connectivity using diffusion tensor imaging (for review see (420)) have been applied to adults to investigate the structural connectivity of the PAG (421) and these could be translated to infants.

Electrophysiological activity has been suggested as a measure through which to compare the functional maturity of the cortex between species. Sleep-related patterns of brain activity in 35-37 week infants are similar to those of rat pups between postnatal days 10-13, which could suggest that the first two weeks of life in rats are roughly equivalent to the preterm period in humans (422). In rats, electrical stimulation of the PAG does not produce inhibition of the tail-flick reflex to noxious heat stimulation until postnatal day 21 (423) and stimulation of the RVM (which receives descending input from the PAG) elicits descending facilitation until postnatal day 28 (150). Extrapolation of these results to humans could suggest that term infants are perhaps unlikely capable of descending inhibitory modulation of noxious input. Invasive non-physiological stimulation in rats may however not necessarily inform us directly on the tonic activity of this developing system in human infants. Furthermore, rodent studies have also demonstrated that the PAG preferentially modulates C-fibre inputs in adult rats (424,425). The noxious stimulus in Study 2 predominantly activates A-delta fibres, according to adult studies (231). However, it is not known at what stage of development this selective modulation arises and whether this is paralleled in human development.

Despite the commonality of many biochemical processes, complex neurobiological sequences and developmental milestones may occur on different timescales across species (426) and linear translation may not always be possible. Structural differences clearly exist across species. Ezra et al., used high-resolution brainstem optimised diffusion sequences and probabilistic tractography to investigate the structural connectivity of the adult human PAG and although they identified similarities in midbrain and hindbrain connectivity to non-humans, forebrain connectivity differed significantly (372). The authors suggested that functional studies of the PAG in non-humans may therefore not be directly translatable to humans. Until now, our understanding of the development of nociceptive circuitry in early life has predominantly been based upon extrapolation from invaluable rodent studies, in which surgical lesions, pharmacological manipulations, and electrical stimulation have allowed the exploration of complex developmental processes. Translational studies are challenging but essential in order to verify or refute our hypotheses based upon these developmental models. The increasing use of MRI in both human infants and animals such as rats, will undoubtedly facilitate more direct translational studies to further investigate the fundamental processes that underlie the development of pain processing in early life.

3.5.7 Study Limitations

Noxious stimulation was delivered by clinical heel lancing in Study 1. The depth of penetration of the blade can vary despite using the same brand of lancet in all infants and unlike the experimental noxious stimulus used in Study 2, the device cannot be calibrated. Calibrated experimental noxious stimuli of graded intensities could be used in a future study of infants across the gestational age range in order to further investigate the development of this system. Noxious-evoked delta brush activity was automatically

identified in premature infants in Study 1 using a burst detection method that identifies the co-occurrence of slow waves (0.5-2 Hz) and higher frequency activity (8-22 Hz) (427). Due to the small number of very premature infants, it was not possible to explore changes in amplitude, frequency composition or distribution of this evoked activity across gestation. New methods of analysing noxious-evoked brain activity are being applied to neonatal electrophysiological data including time-frequency decomposition. Heel lancing has been shown to evoke gamma oscillations in term infants, and, unlike adults, a long latency noxious-evoked energy increase in the fast delta band (2–4 Hz) (428). Future studies could also investigate the development and distribution of these changes across the preterm period and their relationship to other noxious-evoked behaviours such as facial expressions, heart rate variability and reflex activity. Unfortunately, the impact of sleep state or alertness on evoked responses could not be accounted for in these studies and would also be interesting to examine in future work. Accurate electrophysiological classification of sleep state would require application of a wider array of electrodes and longer recordings of activity prior to application of the stimuli.

The results of study 2 are limited by the small sample size and age range of the infants studied. A modified MRI-compatible incubator would have been required to study infants younger than 35 weeks' as current model designs do not permit adequate access to the infant during scanning to allow delivery of a noxious stimulus by a researcher. A device for automated application of a low-intensity noxious stimulus would be necessary to allow characterisation of noxious-evoked BOLD activity in younger infants. Imaging the neonatal brain is particularly challenging due to the small size of the structures and contamination of the signal by head motion and physiological noise caused by cardiac and respiratory rhythms (334). The PAG time-courses were slightly noisier than other

regions of interest (Fig 3.10), which was likely due to the size of the region and its sensitivity to cardiac and respiratory signals due to its location. Components representing noise were meticulously identified and regressed out using independent component analysis (348). The PAG is a small poorly differentiated midbrain region and therefore care was taken to outline the mask for this structure using an adult PAG mask as a guide, in discussion with experienced adult brainstem researchers, and the localisation of the translated mask was visually inspected in each infant. The data was also acquired with greater spatial resolution ($2mm^3$) than our previous study (243). Although PAG connectivity has been related to noxious-evoked activity in this study, PAG activity in response to noxious stimulation has never been demonstrated in infants. Future studies using a brainstem-focussed acquisition protocol could further explore the role of this structure in early development. The PAG is structurally and functionally connected to the RVM in adults and this connectivity is integral to the function of the endogenous pain modulatory system. Study 2 unfortunately did not investigate connectivity between these structures as the RVM was not captured within the field of view in many subjects. The RVM is particularly vulnerable to contamination by physiological noise due to its anatomical location. Simultaneous recordings of cardiac and respiratory rates have now been implemented and will be used to improve the quality of signal obtained from this small brainstem region in subsequent studies.

3.5.8 Clinical Implications

The results of this study have implications for the assessment and management of pain in neonatal care. Firstly, if the electrophysiological measures in Study 1 are to be used in the multimodal assessment of pain or analgesic efficacy, an understanding of the normal developmental trajectory of these responses is essential. Having identified a clear

maturational increase in the magnitude of noxious-evoked activity and decrease in noxious-evoked reflex activity, future studies employing these outcome measures should account for the effect of gestational age. This study re-confirms the suggested timing of the emergence of noxious-specific brain activity and demonstrates that the template of noxious-evoked brain activity is not useful as an outcome measure in infants below 34 weeks' gestation. From 34 weeks' gestation, infants generally have the structural and functional maturity to exhibit electrophysiological evidence of supraspinal noxious processing similar to adults. Therefore, a lack of specific brain activity or a reduction in magnitude of such activity in this infant population could be used to infer a reduction in pain or the analgesic efficacy of an intervention, such as in Chapter 5. The assessment of pain in very premature infants however remains a challenge and the interpretation of delta brush activity has significant ethical implications. It would be dangerous to assume that infants that are too immature to generate noxious-specific brain activity were incapable of pain perception. Whether their unique developmental pattern of brain activity is capable of generating a conscious perception of pain similar to our subjective experiences may ultimately never be known. Nevertheless, noxious stimuli clearly result in considerable widespread and exaggerated supraspinal and spinal activity in these infants, and developmentally-unexpected noxious stimulation of a plastic developing nervous system may have neurodevelopmental consequences (see review (429)). In the absence of conclusive evidence, the cautious assumption that these infants experience pain safeguards against potential suffering and future morbidity.

The endogenous pain modulatory system represents a major target for analgesics, therefore understanding how this system develops in the preterm period is of both fundamental neuroscientific and clinical interest. The PAG is a central site of action for opioids, NSAIDs, and cannabinoids (102,105,107). Electrical stimulation of this region

in rats can produce such potent analgesia that a laparotomy can be performed on a conscious animal without evidence of distress (92). In young rats, electrical stimulation of the PAG however only provides analgesia from postnatal day 21 (423). Systemic administration of opioids during the first two weeks of life does however cause suppression of reflexes (423,430–432). This anti-noceptive effect may arise from purely spinal mechanisms, unlike in adult animals (433), as severing descending pathways in these rat pups does not affect morphine-mediated analgesia, and the antinociceptive activity of morphine appears to increase with the proliferation of opiate receptors until day 15 (430). Understanding the functional development of this system in humans will be essential to understanding the actions of these drugs in developing infants. Opioids are frequently administered intravenously to ventilated premature neonates in intensive care. This may have important consequences for the development of endogenous pain modulation considering that in rodents chronic morphine exposure during a critical period covering the second postnatal week accelerates the development of inhibitory control (128). Furthermore, there is growing evidence that tissue injury and nociceptive input in infancy may affect the balance of descending pain control in later life (244–247,434).

3.6 Conclusion

In summary, these studies suggest that although the descending pain modulatory system is immature in human infants, endogenous inhibitory control of noxious inputs may begin around term gestation, following the development of noxious-specific brain activity. Developing connectivity of the PAG to cortical regions of the endogenous pain modulatory network may influence the magnitude of noxious-evoked brain activity. The results of this chapter provide novel insights into the development of supraspinal and spinal nociceptive processing in human infants and represent an important step towards translation of animal studies of the endogenous pain modulatory system.

Chapter 4

4 Sex differences in nociceptive processing in human infants

4.1 Introduction

Differences in pain exist between the sexes. Women report pain more frequently, with greater severity, at more anatomical sites and are more commonly afflicted by post-procedural and chronic pain including musculoskeletal pain and osteoarthritis, migraine, neuropathic pain, oral pain, and widespread pain (203,435–438). More than 100 experimental studies of sex differences have been conducted across a variety of types of pain, including mechanical, thermal, electrical and chemical noxious stimulation (for an extensive review see (439)). Despite the considerable heterogeneity of these studies in terms of assessment methodology and reported effect sizes, overall the results of meta-analysis strongly suggest that women have greater pain sensitivity and reduced tolerance, with few studies observing a difference in the opposite direction (436). Whether these sex differences reported by both epidemiological and experimental studies arise from fundamental neurobiological differences or psychosocial influences remains a subject of considerable debate.

In adults, electrophysiological studies have demonstrated differences in the magnitude of noxious-evoked brain activity between the sexes (440–445), with females exhibiting greater responses. Neuroimaging studies have more inconsistently revealed sex differences in the brain regions significantly activated by noxious stimulation or pain conditions (446–456). fMRI has been used to study sex differences in functional activity evoked by heat (452,457), visceral (449), distension (448,458,459), pressure (460) and electrical (453) noxious stimuli. Structural, functional and diffusion imaging studies have

also been conducted in patients with chronic pain conditions, including IBS and migraine, and have demonstrated sex differences in the structure (461,462), functional activity (456), functional connectivity (463–465) and structural connectivity (466–468) of brain regions particularly involved in salience, emotion and sensorimotor processing (reviewed by Gupta et al., (469).

Sex differences in nociception and antinociception have been reported in adult rodents, including differences in the context of thermal nociception (470–472), mechanical allodynia (473,474), opioid analgesia (470–472,475–477), and stress-induced analgesia (478), which also depend upon the genetic strain of the animal. Dimorphic responses have also been demonstrated during early postnatal development. In the first week of life, female mice display longer latency nociceptive tail-flick reflexes and a weaker analgesic response to morphine (479). Furthermore, the sex of a rodent appears to modulate the effect of early life pain exposure (480,481) and other stressors (482) on pain responses in adulthood.

It is unclear whether sex differences in nociceptive responses are present in human infants. Several studies have investigated differences in pain-related behavioural responses in infants and yielded conflicting results. In the 1950s and 1960s, several studies employing experimental stimuli reported greater behavioural reactions in female infants (483,484). Grunau and Craig subsequently studied infants undergoing clinical heel lancing and reported a shorter latency time to cry and facial response in male infants (131). However, Guinsburg and colleagues conversely reported that female infants expressed more facial features of the NFCS than males in response to this procedure (485). A study of infants undergoing vaccination however reported lower pain scores in

female infants using the Modified Behavioural Pain Scale (MBPS) (486). Several subsequent studies have reported no significant sex differences in facial expression using the NFCS (487) or the BIIP score (153). Although, these studies were notably in premature infants and facial expressions are less robustly evoked at this stage of development (141,198).

Behavioural responses such as facial expressions are assessed subjectively, can be strongly elicited by non-noxious stimuli (140), and do not always correlate with underlying brain activity (162). Measures of noxious-evoked brain activity can offer a more direct insight into physiological differences in nociceptive processing. Using near-infrared spectroscopy to record evoked changes in the concentration of oxygenated haemoglobin (HbO2), Bartocci et al., observed that male premature infants (aged between 28- and 36-weeks' gestation) had a greater increase in HbO_2 over the ipsilateral and contralateral hemispheres than female infants in response to venepuncture (161). More recently, Verriotis et al., have specifically investigated sex differences in electrophysiological brain activity evoked by heel lancing in infants aged between 29- and 42-weeks' gestation and reported a more widespread distribution of the nociceptive event-related potential in female infants, and no difference in response amplitude (373). These studies included a broad range of gestational ages and neither reported or accounted for demographic features such as pain exposure, illness severity, or sepsis, which may impact pain responses particularly in premature infants.

Studying noxious-evoked responses in newborn infants facilitates the investigation of biological differences in nociceptive processing with minimal influence of psychological and sociocultural factors, which are challenging to untangle in adults. A detailed history

of each infant's antenatal and postnatal life can be examined to facilitate balancing of demographic variables that could potentially confound analyses of sex differences. In the previous chapter it was demonstrated that nociceptive-specific electrophysiological brain activity is more frequently elicited in infants aged 35 weeks' gestation or more and experimental noxious stimulation can be used to evoke BOLD activity in pain-related brain regions in such infants. The aim of this chapter was to investigate whether sex differences in nociceptive processing exist in healthy infants aged between 36- and 42-weeks' gestation, with the prediction that female infants would be more sensitive to noxious stimuli, consistent with the adult literature, and therefore display greater noxious-evoked activity. In Study 1, electrophysiological measures were used to compare noxious-evoked brain activity and reflex activity between the sexes, and in Study 2 functional MRI was used to compare noxious-evoked changes in BOLD activity.

4.2 Study 1

4.2.1 Methods

4.2.1.1 Participants

Healthy infants on the postnatal ward aged between 36- and 42- weeks' gestation at birth who required a heel lance for clinical purposes within the first postnatal week were included in this study. Infants were excluded if they required invasive resuscitation at birth, had intra-uterine growth restriction (IUGR), were small for gestational age, or if they had required admission to the Newborn Care Unit or a lumbar puncture for the investigation of sepsis. 38 infants (19 male and 19 female) met the inclusion criteria. The two groups were comparable across the demographic characteristics listed (Table 4.1). None of the infants had congenital abnormalities.

		Male (n=19)	Female (n=19)
Gestational age at birth (weeks)		40.3 (37.4 – 40.9)	39.1 (37.0 - 40.7)
Gestational age at study (weeks)		41.9 (37.6 – 41.6)	39.9 (37.9 – 40.9)
Postnatal age (days)		2 (1-5)	3 (1-5)
Birthweight (g)		3678 (614)	3555 (709)
Multiple pregnancy		0	1
Mode of delivery			
	SVD (%)	6 (32)	9 (47)
	Assisted (%)	8 (42)	6 (32)
	Caesarian (%)	5 (26)	4 (21)
APGAR at 5 min		10 (0.6)	10 (0.7)
Skin-breaking procedures		4 (1-5)	4 (2-7)

Table 4.1: Study 1 participant demographics. Data are presented as median (IQR), mean (SD) or number (%). *SVD = spontaneous vaginal delivery.*

4.2.1.2 Study Procedure

EEG electrodes were sited on the scalp and EMG electrodes were sited on the ipsilateral and contralateral biceps femoris of each infant, and data were recorded using the equipment and parameters previously described in Chapter 2. Electrophysiological data

were recorded during a control heel lance and subsequent clinical heel lance, following which blood was collected for tests requested by the clinical team.

4.2.2 Analysis

4.2.2.1 EEG analysis

The template of noxious-evoked brain activity was projected onto the epoched woody-filtered EEG data at each electrode as previously described (see Chapter 2) in order to calculate the magnitude of brain activity evoked by the control heel lance and clinical heel lance respectively. EEG data contaminated by artefact or with poor or negative fitting of the template were excluded. 31 infants (16 male and 15 female) were therefore included in the final analyses with artefact-free data recorded from electrodes Cz, CPz, C3 and C4 during heel lancing. Data from the FCz electrode were excluded for 5 infants (3 female, 2 male) due to artefact or technical error (accidental detachment of electrode).

The difference in magnitude of noxious-evoked brain activity in response to the control lance and heel lance at the Cz vertex electrode was compared between the sexes using a linear mixed effects model with the magnitude as the response variable, individual infant as a random effect, and sex and procedure (control, lance) as fixed effects. In order to analyse sex differences in response to heel lancing across the pericentral electrodes (Cz, CPz, C3, C4, and FCz), a linear mixed effects model was also used with individual infant as a random effect, and electrode and sex as fixed effects, and an interaction was included between these two variables. A post-hoc Holm test was used to account for multiple testing.

4.2.2.2 EMG analysis

The EMG data collected from the ipsilateral and contralateral limb were pre-processed as described in Chapter 2. Data from two infants of each sex were not included in group analyses due to noise artefact or as they were significant outliers (see Fig 4.1). The mean RMS of the reflex activity in the first 1000ms post-stimulus, the duration of reflex activity, and the overall amplitude of the reflex between its start and end (defined using methods described in Chapter 3) were compared between the sexes. Linear mixed effect models were run for each measure of reflex activity. In each model, I included subject as a random intercept term, and sex, gestational age and side of reflex as fixed effects, with an interaction between sex and reflex side. A square root transformation was applied to the magnitude data to correct for right-skew. For each analysis, models were analysed in R statistical software and model fits were verified using Q-Q plots, checking the distribution of residuals and homogeneity of variance.

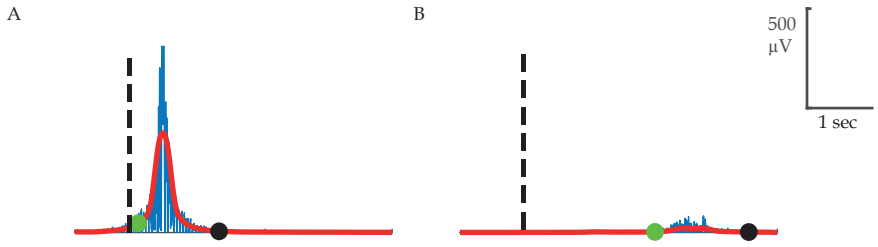

Figure 4.1: EMG examples. Examples of EMG traces excluded from analysis with an unusually high peak activity >1000µV (**A**) or an extremely delayed reflex onset (**B**). The dashed line indicates the point of heel lancing.

4.2.3 **Results**

38 infants underwent heel lancing for clinically-requested blood tests during which electrophysiological data were acquired for the analysis of noxious-evoked brain and reflex activity. There were no adverse events.

4.2.3.1 Noxious-evoked reflex activity was not different between the sexes

Artefact-free EMG data was acquired in 17 participants of each sex. Overall, infants exhibited ipsilateral reflex activity to heel lancing with median magnitude 27.1 ± 4.6μV and median duration 2.0 ± 0.2 seconds, and contralateral reflex activity of smaller magnitude (median magnitude 12.1 ± 5.0 μV) and similar duration (median duration 1.8 ± 0.2 seconds). None of the infants exhibited a significant ipsilateral or contralateral reflex in response to the control heel lance. There was no significant effect of sex, gestation at study or reflex side on the mean RMS of reflex activity to heel lancing (linear mixed effects model: interaction sex and reflex side: χ^2=0.37 p=0.54; sex: χ^2= 0.29, p=0.59; gestation at study χ^2= 2.01, p=0.16; reflex side: χ^2=1.99, p=0.16; Fig 4.2). There was no significant effect of sex or reflex side on the duration of reflex activity to heel lancing (linear mixed effects model - interaction sex and reflex side: χ^2=1.02, p=0.31; reflex side χ^2=0.09, p=0.76; sex: χ^2=1.18, p=0.28; Fig 4.2). However, duration of the reflex significantly decreased with gestational age (gestational age at study: χ^2=5.85, p=0.016). Lastly, there were no significant effects of sex or reflex side on the overall amplitude of activity to heel lancing (linear mixed effects model - interaction reflex side and sex: χ^2=2.47, p=0.12; reflex side χ^2=0.07, p=0.79; sex: χ^2=0.42, p=0.52; Fig 4.2). However, the magnitude significantly decreased with gestational age at study (gestational age at study: χ^2=6.53, p=0.011).

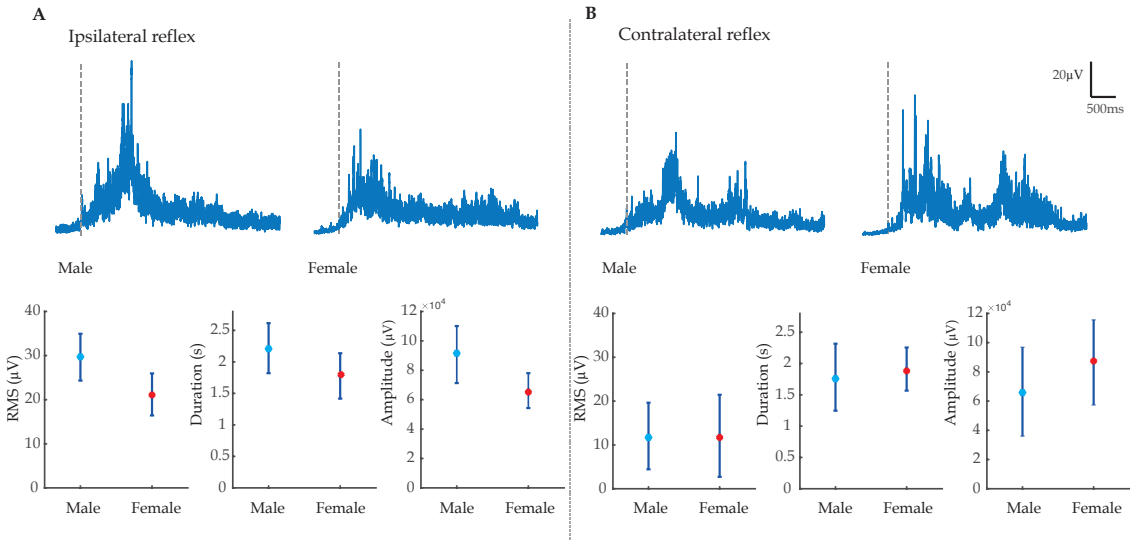

Figure 4.2: Noxious-evoked reflex activity in male and female infants. A. Average ipsilateral reflex activity in male (n=17) and female (n=17) infants. Median RMS, duration and amplitude for each sex are plotted below. **B.** Average contralateral reflex activity in male (n=17) and female (n=17) infants. Median RMS, duration and amplitude of the reflex for each sex are plotted below. Error bars indicate standard error. *The dashed line on the EMG indicates the point of heel lancing.*

4.2.3.2 Greater magnitude of noxious-evoked brain activity in female infants

A noxious-specific potential was identified at the vertex (Cz electrode) in all infants in response to heel lancing with a mean magnitude of 1.03 ± 0.64 (mean ±SD; Fig 4.3). Heel lancing evoked a significantly greater magnitude of brain activity than the control lance at the vertex (linear mixed effects model: χ^2= 19.56, p<0.001) and there was a significant interaction between sex and procedure (control vs lance) (linear mixed effects model: χ^2= 6.95, p=0.008). The magnitude of noxious-evoked brain activity to heel lancing was greater in female infants compared to male infants, accounting for gestational age at study (post-hoc Holm test: t= 2.71, p=0.039). However, there was no significant difference in the magnitude of the potential evoked by the control lance between the sexes, accounting for gestational age (post-hoc Holm test: t= 0.06, p=0.95). Gestational age at study was

positively associated with the magnitude of both the noxious-evoked potential to the control and lance (linear mixed effects model: χ^2= 6.95, p=0.008).

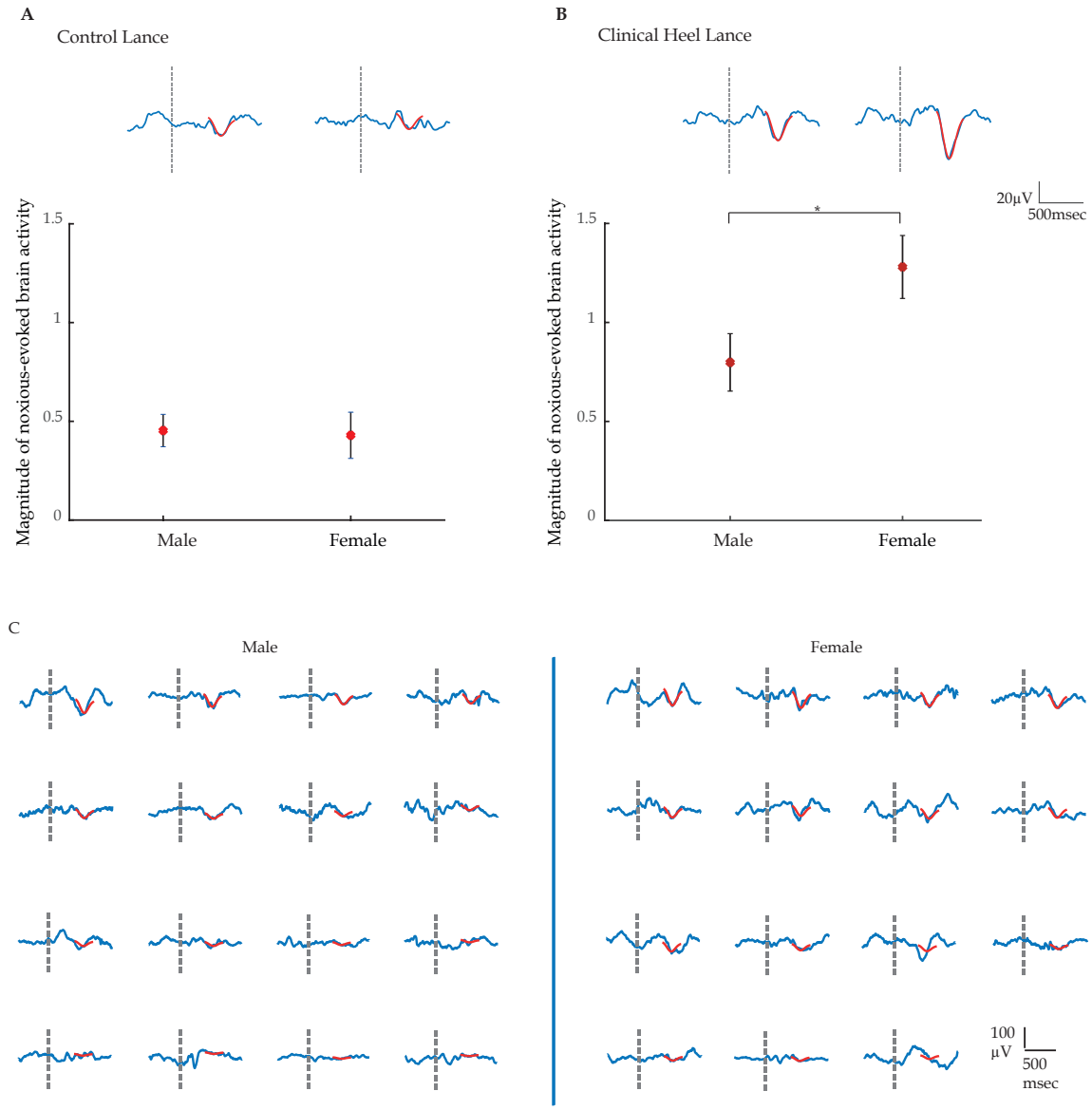

Figure 4.3: Sex differences in noxious-evoked brain activity.

A. Mean magnitude of brain activity evoked by the control lance at the Cz electrode in male and female infants. **B**. Mean magnitude of brain activity evoked by heel lancing at the vertex electrode Cz in male and female infants. **C**. Brain activity evoked by heel lancing for each individual infant, divided by sex and ordered by magnitude of noxious-evoked brain activity.

4.2.3.3 Distribution of noxious-evoked brain activity across pericentral electrodes

The template of noxious-evoked brain activity was used to investigate activity across the peri-central electrodes in response to heel lancing (Fig 4.4). In male infants, there was no significant difference in the magnitude of activity at Cz compared to the peri-central electrodes, accounting for gestation at study (linear mixed effects – electrode: χ^2=4.73, p=0.32, gestation at study: χ^2=0.05, p=0.84). In female infants, activity at Cz was significantly greater than each of the peri-central electrodes, accounting for gestational age at study (overall effect: χ^2=31.17, p<0.001; post-hoc Holm test: Cz – CPz: t=3.57, p=0.005; Cz – C contra: t=5.05, p<0.001; Cz - C ipsi t=4.99, p<0.001; Cz - FCz t= 4.93, p<0.001).

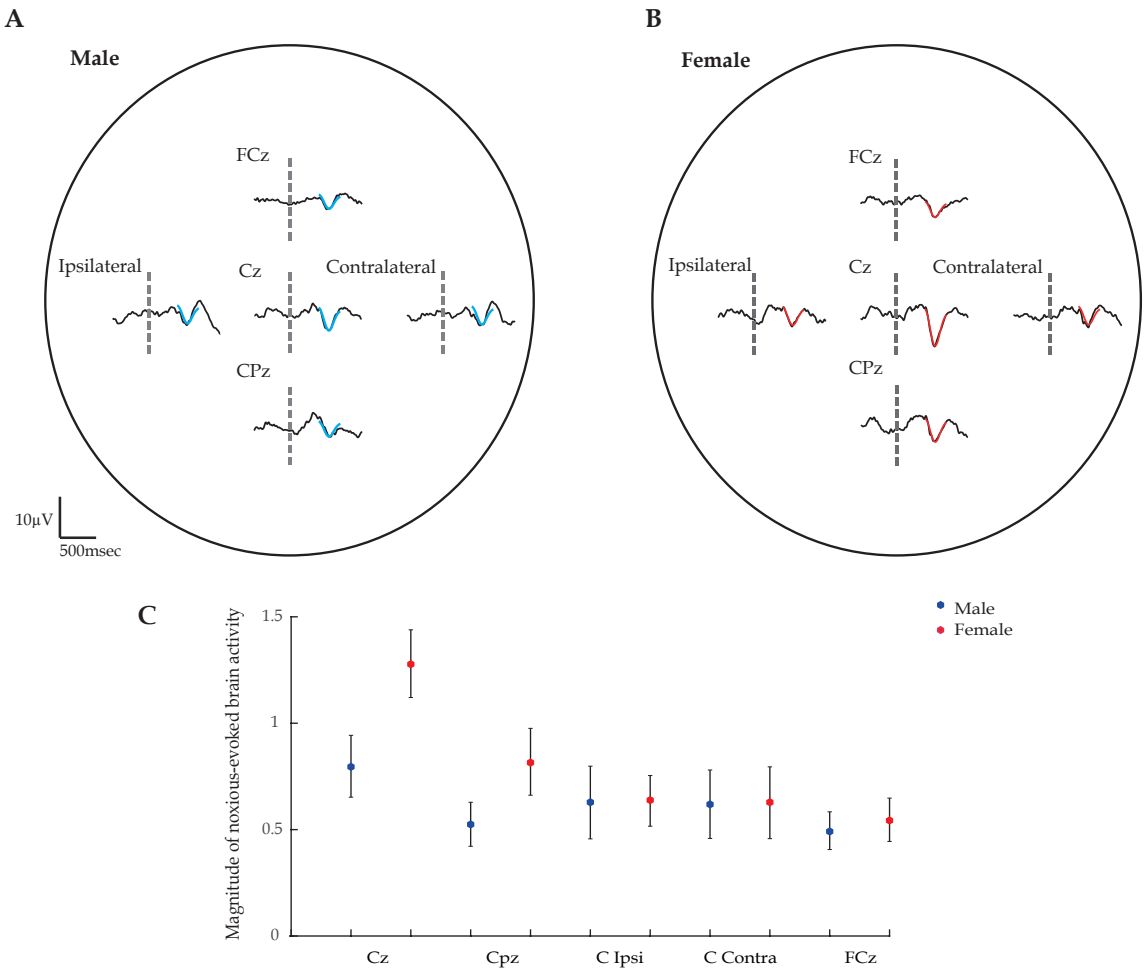

Figure 4.4. Sex differences in noxious-evoked brain activity across the brain. Mean noxious-evoked activity at central electrodes across the brain in male infants **(A)** and female infants (**B**). The template of noxious evoked activity is overlaid over each mean woody-filtered EEG trace. **C.** The mean magnitude of noxious-evoked activity at each electrode for male (blue) and female (red) infants. Error bars indicate standard error of the mean.

4.3 Study 2

4.3.1 Methods

4.3.1.1 Participants

As for Study 1, only healthy infants aged between 36- and 42- weeks' gestation at birth within the first ten postnatal days were included. Infants were excluded if they required invasive resuscitation at birth, had congenital abnormalities, or if they had required admission to the neonatal unit or a lumbar puncture for the investigation of sepsis. 10 male and 10 female infants were included in the final analysis, ensuring the two groups were comparable across the demographic characteristics listed (Table 4.2).

	Male (n=10)	Female (n=10)
Gestational age at birth (weeks)	39.1 (37.9 – 40.6)	38.8 (37.3 – 39.1)
Gestational age at study (weeks)	39.4 (38.0 – 40.7)	39.1 (38.0 – 39.6)
Postnatal age (days)	2.0 (1.0 – 2.0)	3.9 (2.0 – 5.0)
Birthweight (g)	3484 (728)	3377 (549)
Multiple pregnancy	2	1
Intra-uterine growth restriction	1	1
Mode of delivery		
SVD	4	2
Assisted	2	3
CS	4	5
APGAR at 5 min	9.9 (range: 8 -10)	9.8 (range: 9 -10)
Infants with history of suspected sepsis	2	3
Skin-breaking procedures	3 (0 – 10)	2.5 (0 – 5)

Table 4.2: Study 2 participant demographics. Data are presented as median (IQR), mean (SD) or number.

4.3.1.2 Study Procedure

Infants were transported from the postnatal ward to the Wellcome Centre for Integrative Neuroimaging, and following examination and application of ear protection were settled into a cradle in the Siemens Prisma 3T scanner as described in Chapter 2.

Following the acquisition of structural data, a 128mN Pinprick stimulator was applied ten times to the left foot of each infant with a minimum inter-stimulus interval of 25 seconds during acquisition of functional MRI data.

4.3.1.3 Analysis

Individual MRI data were pre-processed as described in Chapter 2. The mean frame-wise displacement was calculated using the function *fsl_motion_outliers*, providing an estimate of participant motion. Each infant's brain volume was also estimated using a common mask of the cerebrum, defined from the 40-week gestation standard, which was warped to the individual's structural space. Brain volume was estimated from the number of voxels and voxel volume.

For higher-level group analysis, a non-parametric permutation technique was applied to reduce the impact of between-subject variance and noise. The average stimulus-evoked positive response across male and female infants was estimated from a combined 4D data set of the positive COPE from first-level subject analyses using FSL's Randomise (370) to generate 5000 permutations. In order to avoid arbitrary setting of a cluster threshold, threshold-free cluster-enhancement was implemented that captures the amount of cluster-like local spatial support for an activation (488–490). A non-parametric one sample t-test was used to identify significant clusters, with 3mm of variance smoothing. TFCE images were thresholded at a p-value threshold of $p<0.05$ and used to mask the tstat image. The function *Cluster* was used to extract clusters of significant activity and their local maxima, and their location was anatomically defined using the M-CRIB atlas. The average positive evoked activity for each sex was also obtained. The average group stimulus-evoked

negative response was again estimated using the same method using the negative COPEs from first-level subject analyses.

In order to identify areas in which there were sex differences in brain activity in response to noxious stimulation, contrasts of female>male activity and male>female activity were performed using permutation testing (Randomise with 5000 permutations, TFCE, 5% FWER) with gestational age at study and estimated subject motion as nuisance covariates. The function *Cluster* was used to identify clusters of voxels in which activity was significantly different and their local maxima. Regions of significantly different activity were defined using peak local maxima and the M-CRIB atlas. In order to investigate the activity underlying the significant differences identified, activity masks for each region with a sex difference were created using masks from the M-CRIB atlas. FSL's *Featquery* was then used to calculate the percentage signal change in BOLD in these areas in male and female infants.

4.3.2 **Results**

fMRI data was acquired during repeated application of the 128mN stimulus in 10 male and 10 female infants to investigate differences in noxious-evoked BOLD activity. There was no significant difference in estimated brain volume between the sexes (mean volume ± SD male: 284,684 ± 45,959; female: 306,457 ± 44,752; t-test: p=0.30) or estimated head motion during scanning between the sexes (mean FD ±SD male: 0.69mm ± 0.44; female: 0.64mm ± 0.45; t-test: p=0.78). In response to noxious stimulation of the left foot, clusters of positive BOLD activity were identified in the bilateral postcentral gyri, insular cortices, and thalami, as well as the contralateral precentral cortex and secondary somatosensory cortex on average across male and female infants (Fig 4.5; Table 4.3). Peaks of activation were also identified in the superior parietal, superior temporal and supramarginal cortices. There was however no significant negative BOLD activity on average across male and female infants.

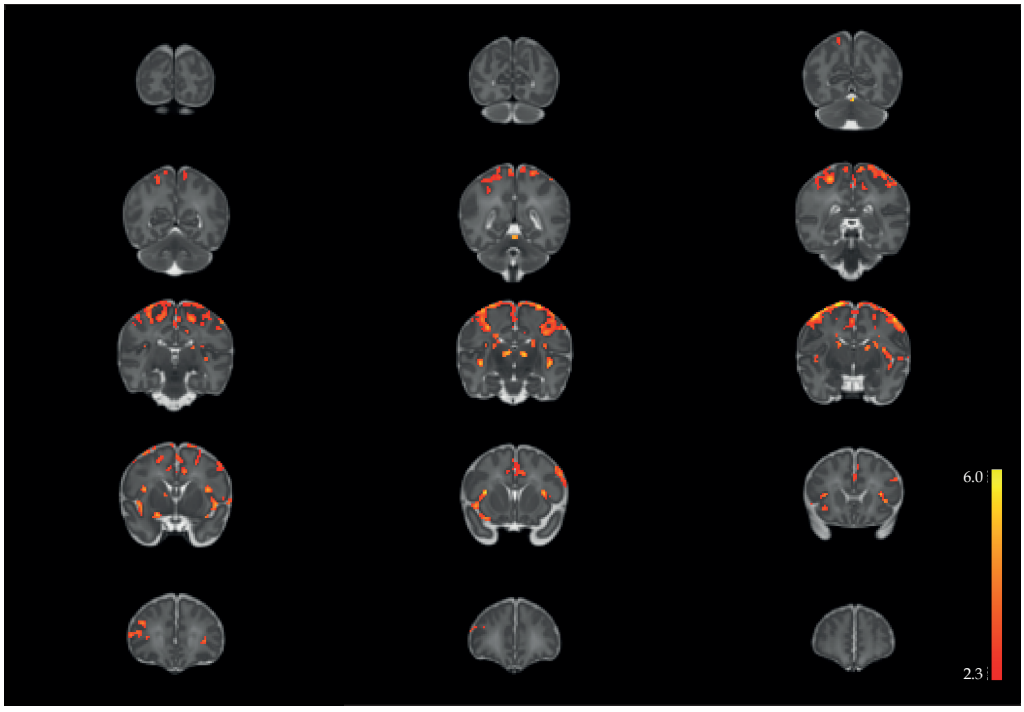

Figure 4.5: Noxious-evoked BOLD activity. Mean significant positive BOLD in response to the 128mN noxious experimental stimulus across both male and female infants (n=20).

CLUSTER	SIZE	REGION	HEMISPHERE	PEAK	COORDINATES		
1	3946	Precentral gyrus	Contralateral	5.14	31.0	-11.0	45.0
		Postcentral gyrus	Contralateral	4.81	17.5	-15.5	54.0
		Superior parietal	Contralateral	4.54	19.0	-29.0	45.0
		Postcentral gyrus	Ipsilateral	4.36	-5.0	-30.5	52.5
2	609	Parsopercularis (S2)	Contralateral	5.82	-23.5	-2.5	16.5
		Superior temporal	Contralateral	4.67	28.0	-17.0	9.0
		Insula	Contralateral	4.26	23.5	8.5	13.5
3	503	Insula	Ipsilateral	4.82	-20.0	2.5	16.5
		Parsorbitalis	Ipsilateral	3.9	-20.0	-11.5	10.5
4	117	Subcortical matter	Contralateral	4.78	11.5	-12.5	24.0
5	78	Thalamus	Ipsilateral	4.39	-8.0	-9.5	21.0
6	73	Thalamus	Contralateral	4.51	7.0	-15.5	18.0
5	71	Supramarginal	Contralateral	3.80	31.0	-26.0	27.0
		Superior temporal	Contralateral	3.38	28.0	-24.5	19.5
6	7	Cerebellar hemisphere	Ipsilateral	4.87	1.0	-47.0	-1.5

Table 4.3: Localisation of noxious-evoked BOLD activity. Brain regions with local maxima of significant noxious-evoked BOLD activity on average in male and female infants.

4.3.2.1 Noxious stimulation evoked greater BOLD activity in female infants

Analysis of average group activity in female infants revealed significant activity across core brain regions involved in nociceptive processing (Fig 4.6), whereas no significant average group activity was detected in male infants. Examination of this group's uncorrected tstat data suggested that activity was evoked in core regions, such as the somatosensory cortices, insula and thalamus, which failed to survive multiple comparisons correction (Fig 4.6).

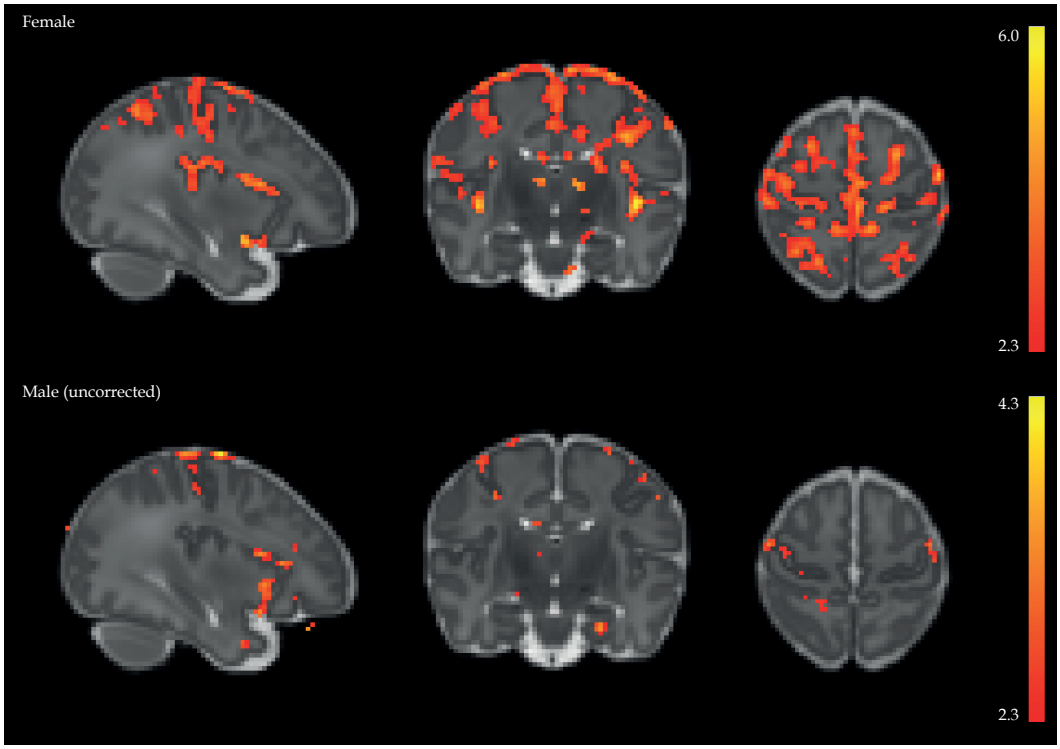

Figure 4.6: Noxious-evoked brain activity in female and male infants. *Top panel*: Mean positive BOLD activity evoked by noxious stimulation in female infants (n=10, 5000 permutations, TFCE, 5% FWER) overlaid on a 40-week neonatal brain template. *Bottom panel*: Uncorrected raw t-stat image of mean positive BOLD activity evoked by noxious stimulation in male infants (n=10) overlaid on a 40-week neonatal brain template. The same sagittal, coronal and axial views are used to facilitate visual comparison.

A contrast between the sexes accounting for gestational age and average subject motion, revealed clusters of voxels in several brain regions in which female infants had significantly greater activity in response to noxious stimulation compared to male infants (Fig 4.7). Peaks of significantly greater activation were identified bilaterally in the paracentral lobules, precuneus, and inferior parietal cortices (Table 4.4). Clusters of greater activity were also identified in the contralateral insula and superior frontal cortex as well as the ipsilateral superior frontal cortex. There were however no clusters of significantly greater activity in male infants compared to female infants.

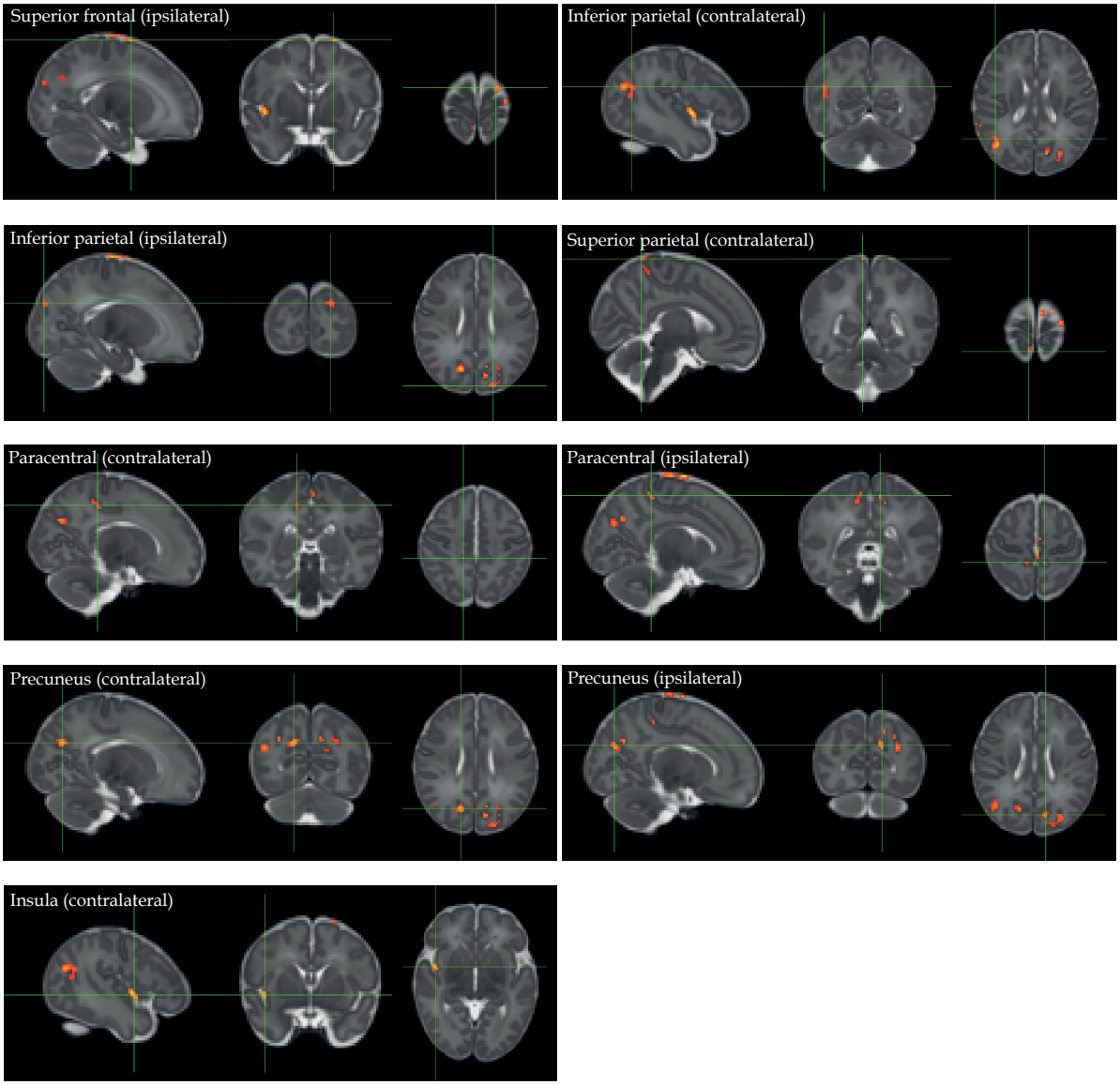

Figure 4.7: Brain regions with clusters of greater activity in female infants. The peak voxel for each cluster is identified at the intersection of the green lines in the coronal, axial and sagittal views of each structure.

CLUSTER	SIZE	REGION	HEMISPHERE	PEAK	COORDINATES		
1	79	Superior frontal	Ipsilateral	4.38	-12.5	-6.5	52.5
2	68	Inferior parietal	Contralateral	3.74	29.5	-42.5	24.0
3	46	Inferior parietal	Ipsilateral	3.75	-11.0	-60.5	27.0
4	37	Superior parietal	Contralateral	3.57	5.5	-36.5	54.0
		Paracentral	Contralateral	3.47	10.0	-27.5	37.5
5	29	Precuneus	Contralateral	4.56	11.5	-48.5	27.0
6	26	Insula	Contralateral	4.71	29.5	-5.0	7.5
7	26	Precuneus	Ipsilateral	4.23	-6.5	-53.0	25.5
8	8	Paracentral	Ipsilateral	3.71	-5.0	-30.5	43.5

Table 4.4: Localisation of greater BOLD activity in female infants. Brain regions with local maxima of significantly greater noxious-evoked BOLD activity in female infants.

Analysis of the percentage signal change in BOLD in these areas of significant difference revealed that female infants had positive activity in these clusters, whereas male infants had weak positive or negative changes in BOLD activity on average within these areas (Fig 4.8).

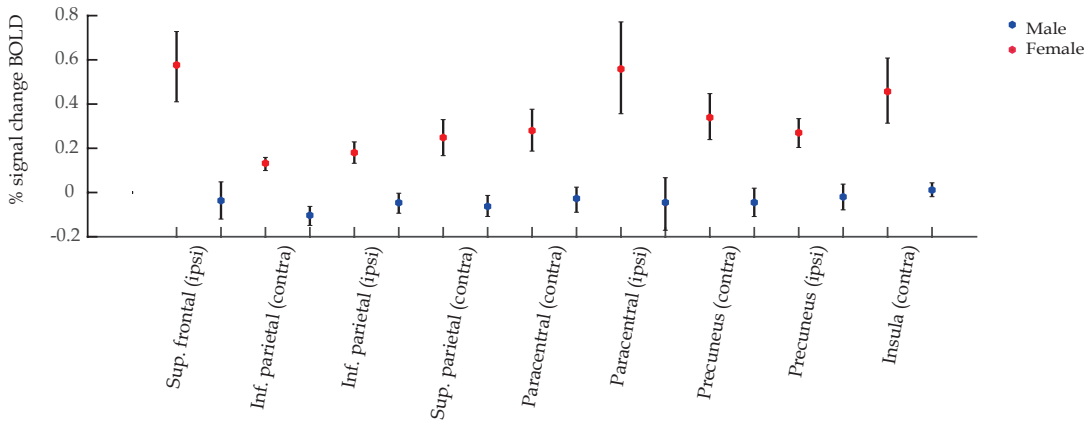

Figure 4.8: BOLD activity in brain regions with sex differences. Mean (SEM) percentage signal change in BOLD within clusters with greater activity in female than male infants.

In order to investigate whether these sex differences in functional activity could be due differences in brain volume, the contrast analysis of female>male activity was repeated with estimated brain volume as a nuisance covariate in place of gestational age (as both could not be included due to multicollinearity). Clusters of greater activity in female infants were identified in most of the same brain regions as the previous analysis, including the bilateral paracentral lobules, precuneus, inferior parietal lobules, and contralateral insula. However, in this analysis clusters of greater activity in female infants were also identified in the ipsilateral isthmus cingulate and posterior cingulate, and in the contralateral superior temporal gyrus.

4.4 Discussion

The electrophysiological and fMRI studies in this chapter provide evidence of sex differences in brain activity evoked by noxious stimulation in infants. Study 1 revealed significant differences in brain activity evoked at the vertex by heel lancing, with females demonstrating greater magnitude of the nociceptive-specific potential. Consistent with this, fMRI analysis of a separate cohort of infants revealed significant sex differences in clusters of activity evoked by low-intensity experimental noxious stimulation of the foot, with female infants exhibiting greater activity bilaterally in the inferior parietal lobules, precuneus, and paracentral lobules, and in the contralateral superior parietal lobule and insula. Despite these differences in brain activity, there was no significant difference in the magnitude or duration of reflex activity of the ipsilateral or contralateral limb between the sexes.

Many theories have been proposed to explain how and why sex differences in pain occur (see review (436)). Differences have been attributed to genetic effects from sex chromosomes (491), psychological traits such as anxiety and catastrophising (492,493), the differential expression of a variety of pain-related neurochemicals and receptors (494,495), and the sociocultural effects of gender (496). Furthermore, there are competing theories relating to the effects of sex steroids, one attributing differences to an 'activational' process during adulthood, and the other attributing them to 'organisational' effects of these hormones during brain development (497,498). The results of this chapter suggest sex differences in noxious-evoked brain activity can be observed in the first postnatal weeks and therefore lend support to genetic, neurochemical and organisational effect theories.

4.4.1 **Noxious stimulation evoked greater EEG activity in female infants**

The magnitude of noxious-evoked activity at the vertex Cz electrode was greater in female infants than male infants. There was however no significant difference in the magnitude of activity evoked by the control lance procedure at this site. This suggests that the sex difference observed was not due to a general hyper-responsiveness of these female infants to stimulation. Greater activity at the vertex in female infants is consistent with electrophysiological data of experimental pain in adults. Several large-scale studies characterising evoked potentials at the vertex (Cz) in response to a contact heat stimulus have reported greater amplitude of evoked responses in women compared to men (440,441,443). These potentials are proposed to arise primarily from Aδ fibre stimulation and are also maximally recorded at the vertex. Greater amplitude of mean averaged responses from Fz, Cz and Pz have also been observed in women compared to men in response to noxious CO_2 laser stimulation (442).

The magnitude of noxious-evoked brain activity at the Cz electrode was significantly greater than activity at the pericentral electrodes in female infants. Mean activity at the pericentral electrodes was however similar in magnitude in male and female infants. This suggests that the significant difference at the vertex was not simply due to a general increase in reactivity across the female brain. Another study has also investigated sex differences in noxious-evoked brain activity in neonates but the authors did not observe a significant difference in the magnitude of evoked potentials between the sexes (373). It is important to note that there were considerable population and methodological differences between the studies. Verriotis and colleagues included a broad range of gestational ages, with very premature infants as young as 29 weeks' gestation, whereas Study 1 included only healthy term infants from the postnatal ward who had not required

admission to neonatal care. Prior pain exposure, sepsis and illness were not reported or accounted for and could have impacted the magnitude of noxious-evoked brain activity in the study by Verriotis and reduced the power of the study to detect sex-related differences in the magnitude of the response. In animals, cortical brain regions only express high differential concentrations of androgren and oestrogen receptors during critical periods of late foetal and early postnatal development, which may underlie the development of sexual dimorphism of certain brain structures and functional responses (499). Therefore, it is also possible that differences in the magnitude of responses between the sexes may not manifest until the peri-term period and thus would not be observed across the broader age range encompassing very premature infants. There are also methodological differences between the studies. Verriotis et al., used peak detection based on manual inspection to determine the presence and amplitude of a noxious-evoked potential, which is subjective, unlike the automated application of the template of noxious-evoked brain activity used in Study 1. The authors nevertheless concluded that female infants are more likely to have a widespread response to noxious stimulation than males. This significant difference may have been driven particularly by differences in activity at electrodes beyond the pericentral region examined in Study 1. It is possible that inclusion of a wider array of electrodes could also have yielded differences in the distribution of activity between the sexes in this chapter.

Gestational age was found to have a significant effect on the magnitude of noxious-evoked activity, consistent with the results of Chapter 3, and despite the much narrower age range of this study. Also consistent with Chapter 3, the magnitude of noxious-evoked activity was on average greatest at the vertex regardless of sex. This is consistent with the results of the study by Verriotis et al., in which the maximum amplitude of the potential

was detected at the vertex in greater than 80% of infants aged 36 weeks' or more (373). Increased inter-hemispheric synchronisation from 35 weeks' gestation due to exuberant development of callosal cortico-cortical connectivity may underlie this observation (66). Electrophysiological potentials at the vertex can arise from bilateral activation of underlying cortical regions (373,500) and therefore the increased magnitude of responses at this site in female infants could potentially result from greater bilateral activation of underlying brain regions and/or increased inter-hemispheric functional connectivity. Greater overall structural connectivity (501) and greater inter-hemispheric white matter connectivity have been reported in adult women (502) and therefore could potentially arise during early development.

4.4.2 **Noxious stimulation evoked greater BOLD activity in female infants**

As in Chapter 3, positive BOLD activity was evoked by noxious stimulation in brain regions commonly reported in studies of pain in adults including the somatosensory cortices, thalami, and insular cortices. On comparison of noxious-evoked activity between the sexes, female infants had clusters of significantly greater activity in several brain regions, which is consistent with Study 1, in which increased magnitude of noxious-evoked activity was detected at the vertex in female infants. Clusters of significantly greater activity in female infants were notably observed in the precuneus, inferior parietal lobules and paracentral lobules bilaterally, which could theoretically contribute to an increase in the summative electrophysiological activity recorded at the vertex. In future, a combined EEG-fMRI study could be used to investigate this hypothesis.

The precuneus and inferior parietal lobules are core brain regions of the default mode network, a dispersed group of structures that exhibit correlated low-frequency activity and high metabolic activity at rest (503), which have been associated in adults with self-

oriented attention, mind-wandering (504–508) and background monitoring of the external environment (509). Interestingly, many studies in adults have reported greater connectivity of this canonical network in women compared to men (510–516). These brain regions are however commonly deactivated by goal-directed tasks and pain (457). Some studies have reported stronger deactivation with increasing pain (517,518), whereas others have reported the opposite (457). Activation rather than deactivation of the precuneus was observed in infants in this chapter and is consistent with our previous study in a separate cohort of infants (4), and a subsequent analysis of that data using more stringent thresholding (243). This brain region is not commonly associated with the representation of pain and the significance of activation versus deactivation is currently unclear. Interestingly, a PET study identified impaired deactivation of default-mode network brain regions and impaired functional connectivity of these regions in adults in a minimally-conscious state compared to healthy controls (519). The authors suggested this may imply "preserved but different-from-normal perception of pain" in these patients (519). In adults, a study investigating somatosensory evoked potentials with source localisation analysis has also suggested that pain-evoked activity in the precuneus particularly is associated with pain sensitivity, as patients with greater activity of this brain region required smaller increments in sural nerve stimulation to evoke pain (444). The authors proposed a role of the precuneus in 'promoting salient self-referential information to consciousness' (444). The precuneus has also been implicated in sensitivity to pain in the context of allodynia. Witting and colleagues observed that pain reported in response to a non-noxious stimulus was associated with increased activity of the contralateral precuneus (520). If noxious-evoked activity in the precuneus relates to pain sensitivity, the result of Study 2 of this chapter could potentially be interpreted to suggest greater pain sensitivity in female infants, consistent with much of the adult

literature. There is also evidence of sex differences in this brain region in adults with chronic pain. Women that suffer from migraines have been found to have a thicker precuneus, with greater activity in response to noxious thermal stimulation, and exhibit greater functional connectivity of this structure to the somatosensory cortex and amygdala than men with migraines (456).

It is interesting to note that the precuneus is functionally connected to several brain regions in which clusters of significantly greater activity were also identified in female infants in this study. Its central portion is functionally connected to the inferior parietal lobule and superior temporal sulcus, and its anterior portion to the paracentral lobules and insula (521). The anterior insula plays a pivotal role in emotional processing, interoception, and attention (522–524) and a study recently demonstrated that activity in this region relates to the perceived intensity of painful and non-painful stimuli (525). Greater noxious-evoked insula activity in women has previously been reported using PET in the context of thermal pain, and this activity was associated with greater perceived pain intensity (447). The authors did however suggest that these differences could relate to psychophysical differences and proposed that future studies should endeavour to equalise perceived pain intensity of stimulation between the sexes to allow comparison of activation patterns. Increased BOLD activity of the anterior insula has since been reported in women exposed to visceral pain induced by oesophageal distension (amongst other regions), despite no significant difference in perceived pain intensity (448). Unfortunately, in the absence of verbal report it is not possible to balance perceived pain intensity in the infant population. Although, PIPP scoring could potentially be used as a proxy and sex differences in brain activity of infants with similar PIPP scores could be investigated.

4.4.3 **No evidence of sexual dimorphism in reflex activity**

Consistent with the results of Chapter 3, gestational age at study had a significant effect on reflex duration and amplitude, with older infants exhibiting earlier and smaller reflexes. Despite sex differences in brain activity, there were however no differences in the RMS in the first second, duration or overall amplitude of reflexes between male and female infants. These results are consistent with a study in adults, in which supra-threshold reflex responses to noxious stimulation did not differ significantly (526). Differences in spinal nociceptive sensitivity have however been demonstrated in adults using graded electrocutaneous stimulation of the sural nerve to determine reflex thresholds. Several studies have reported that a lower intensity of stimulation is required to elicit a nociceptive flexion reflex in females compared to males (526–530). Application of graded intensities of experimental noxious stimulation rather than heel lancing could allow investigation of potential sex differences in reflex threshold in early postnatal life. Repeated stimulation would also allow investigation of temporal summation, whereby repeated noxious stimulation leads to increased excitability of spinal cord neurons, as sex differences have been reported in experiments employing noxious heat, electrical and mechanical stimulation in adults (531–535).

4.4.4 **Study Limitations**

Certain methodological choices and challenges of interpretation merit discussion. In both studies of this chapter, the sample sizes were relatively small. This is in part due to the inherent challenges of recruiting healthy newborn infants (imminently due to be discharged home) to studies concerning pain, and efforts to optimally balance demographic characteristics between the groups to maximise the study's power to detect differences related to sex. Nevertheless, studies of sex differences in adults have also been

published with between 8-15 subjects per group (435,446,447,449–453) and adult studies are inevitably less able to balance and control for potential confounding factors over a much longer lifespan.

In study 1, only one intensity of noxious stimulation was applied, which could not be calibrated or repeated to increase signal to noise as it was a clinically-required tissue-damaging stimulus. Application of a repeated non-distressing experimental stimulus (such as the stimulus used in the fMRI study) could allow further exploration of differences in nociceptive thresholds and stimulus encoding. As mentioned in the discussion of electrophysiological results, only data from pericentral electrodes were recorded, which limits conclusions regarding the distribution of noxious-evoked brain activity. Furthermore, only differences in activity within the narrow time-window of the noxious-evoked template were examined and future studies could investigate sexual dimorphism of later responses, employing time-frequency decomposition of the data as discussed in Chapter 3. Lastly, increased head circumference could theoretically result in decreased magnitude of evoked potentials in male infants. However, head circumference is correlated with gestational age and therefore this variable had to be excluded from statistical models due to multicollinearity. Head circumference is not generally included as a covariate in adult studies of electrophysiological sex differences and considering the balanced and narrow range of gestational ages in this study, it is unlikely that this variable influenced the results.

In study 2, efforts were also made to balance potential confounding factors such as age, birthweight, pain exposure, and movement during scanning. Nevertheless, as discussed in the previous chapter, attentional and sleep states could also potentially influence

noxious-evoked BOLD activity but could not be accounted for. Overall estimated brain volume was not significantly different between the two sexes. There is however considerable evidence from both in-vivo and post-mortem studies of sexual dimorphism of the human brain that despite having a smaller intracranial volume on average women have greater grey matter volumes of certain structures relative to their cerebral size. These include areas involved in nociceptive processing such the thalamic nuclei (536), anterior cingulate gyrus (537), dorsolateral prefrontal cortex (538) and superior temporal cortex (538–540). Another study has also reported significantly larger volumes of the precuneus and superior frontal sulcus in women, which are brain regions in which clusters of greater activity were identified in Study 2 (514). Furthermore, there is evidence that sexual dimorphism of the brain's structure is present from birth as full-term male infants have on average 9% greater intra-cranial volumes than females, which is similar to differences observed in children (541,542) and adults (543,544). It is possible that sex differences in volumetric and functional connectivity may also exist for specific brain regions in early development, although these may not necessarily correspond directly to differences identified in adults. Voxel-based morphometry could be used in future studies to investigate whether volumetric differences underlie some of the results of this chapter, and this would require further optimisation of the T2 scan parameters used to acquire structural data in these infants.

A whole brain analysis was employed as there was no clear consensus from adult studies of sex differences in noxious-evoked brain activity from which to develop a clear hypothesis for a region-constrained analysis in infants. Furthermore, fMRI studies of sex differences in adults have included experimental heat (452,455,457), electrical (453), or visceral pain stimuli (448,449), and this may account for some of the heterogeneity in results of regions exhibiting significant differences. Threshold-free cluster enhancement

was used to identify significant BOLD activity, to avoid arbitrary thresholding, and maximise sensitivity to signal (545). However, one of the consequences of this methodological choice is reduced clustering or 'bleeding' of activity, making interpretation of localisation more challenging. Nevertheless, clear clusters of significant differences in activity were identified on comparison between the sexes.

Assigning a function to brain regions based on clusters of activity is challenging and even more so in the developing brain. Cautious application of reverse inference is necessary, projecting the functional roles of structures defined in a fully mature nervous system onto those of a developing system. It is unknown whether the areas of increased activity identified are fundamental to the percept of pain in infants, or are involved in sensitivity or salience detection, or perhaps represent a developmental pattern of brain activity which later facilitates the perception of pain as we understand it. Statistical thresholding allows us to assign importance to a region based on a cluster of voxels within an anatomically-defined structure. It is unknown however whether it is more biologically important to have a highly significant small cluster of activity within a region or an overall mean increase in activity of a region. Despite these limitations in interpretation, it is clear that both EEG and fMRI have consistently demonstrated greater activity in female compared to male infants in response to noxious stimulation, which is consistent with much of the adult literature and could potentially indicate greater pain sensitivity in female infants.

4.4.5 **Clinical significance**

If quantitative and qualitative neurobiological sex differences in pain processing exist in early life, they are clinically-relevant as they could alter the long-term consequences of early pain exposure and the efficacy of analgesics. The degree of cumulative pain exposure in neonatal care has been associated with reduced thalamic development and

overall brain size at 18-months particularly in female ex-premature infants (546). In children born prematurely, sex-dependent differences in cortisol levels have also been reported (547). Furthermore, in adults born prematurely, sex-dependent differences in pain sensitivity have been observed, with males displaying reduced baseline sensitivity and females exhibiting increased sensitivity to noxious cold (548). Rodent models may provide further mechanistic insights into sex-specific effects on nociceptive processing and guide the development of sex-dependent interventions to mitigate the long-term consequences of procedural pain in neonatal care (429). Historically, there has been a significant male bias in preclinical pain studies (549), which is only now being addressed to ensure sex-specific mechanisms are recognised and sex-specific findings are not inappropriately generalised, impeding the development of analgesic interventions (436).

4.5 Conclusions

Overall, the studies of this chapter provide evidence of significant differences in noxious-evoked brain activity between the sexes. Noxious stimulation evoked greater magnitude of electrophysiological activity at the vertex and significantly greater BOLD activity across multiple cortical brain regions in female compared to male infants. The extent to which these differences are biologically relevant in early life deserves further investigation. Sex differences in supraspinal nociceptive processing and potentially in pain sensitivity may exist from birth. Sexual dimorphism of noxious-evoked responses should therefore be considered and accounted for in future studies of analgesic and pain-relieving strategies in infants.

Chapter 5

5 The analgesic efficacy and safety of oral morphine for procedural pain in non-ventilated infants

5.1 Introduction

Despite accruing evidence that repetitive pain in early life has significant short-term effects and long-term repercussions for infants, pain in neonatal care remains inadequately managed due to a lack of effective evidence-based analgesics (550). Eye examinations are an example of a painful procedure that is repeatedly conducted in prematurely-born infants during their hospitalisation and post-discharge in outpatient clinics. The procedure allows early detection and assessment of retinopathy of prematurity (ROP), a developmental retinal vascular disorder (Fig 5.1). Normal neuronal and vascular development of the retina is arrested by premature birth and aberrant vascularisation can occur due to compensatory mechanisms (551). Fortunately, the introduction of ROP screening programmes has significantly reduced the risk of significant visual impairment in prematurely-born infants through earlier identification and treatment of this pathological process (552). The examination is a skilled procedure involving pharmacological pupillary dilatation, insertion of a metal speculum, thorough inspection with a binocular indirect ophthalmoscope, and manipulation of the globe with scleral depression. Unfortunately, this critical procedure is painful (33,553), stressful and destabilising (554), and is repeatedly performed until the retina is fully vascularised or continues to be performed if ROP is identified and requires monitoring or treatment (555). ROP examinations cause tachycardia (556,557), hypertension (556), desaturations (556,557), raised salivary cortisol (558), high behavioural pain scores (for review (317)), and increase apnoea rates in the subsequent 48 hours (554). Use of a speculum and

indenter allows optimal visualisation of the peripheral retina but is known to cause pain in children and adults and therefore is not used without local anaesthetic in these patient groups (555). The UK *Guideline for the screening and treatment of retinopathy of prematurity* recommends that topical anaesthetic eye drops are instilled prior to ROP examinations involving speculum insertion (555). However, systematic review of the two randomised controlled trials (559,560) of topical anaesthesia has revealed that the degree of analgesia (reduction in PIPP scores) provided by this intervention remains inadequate (561). Comfort care techniques including sucrose (562–571), swaddling (571), and non-nutritive sucking (562,565,569,571) are also suggested but are not endorsed as essential standard care due to a lack of robust evidence. Although some reductions in pain scores have been reported, none of these pain-relieving strategies, alone or in combination, have been shown to adequately reduce the pain of the procedure or reduce the subsequent physiological instability(317,572). Considering PIPP scores are frequently greater than 12 in infants undergoing ROP examinations (317), indicating moderate to severe pain, treatment with a pharmacological intervention should be considered.

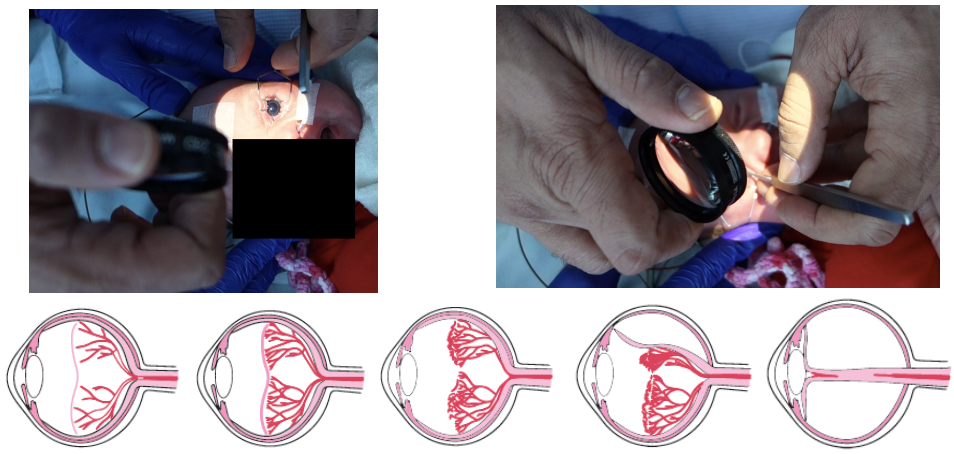

Figure 5.1: Examination for identification and staging of retinopathy of prematurity.
Above: Examination for retinopathy of prematurity with an eyelid speculum and indenter. *Below:* Illustration of the stages of retinopathy of prematurity from abnormal vessel growth to complete detachment of the retina.

Morphine is a potent analgesic in children and adults. In neonatal care, it is frequently administered intravenously for sedation and comfort during mechanical ventilation and additional boluses are often given in practice to treat elevated pain scores or prior to painful procedures (38,573). As reviewed in the introduction to this thesis, there is however contradictory evidence of the analgesic efficacy of morphine in this patient population. Although formularies such as the British National Formulary for Children (290) and guidelines (574,575) provide recommended oral doses of morphine for pain relief in neonates, there is no context given as to when this treatment should be administered. A single pilot study has previously investigated the analgesic efficacy of oral morphine for ROP screening, but the trial was stopped early due to changes in the Medicines and Healthcare products Regulatory Agency (MHRA) regulations (291). Six infants received a large dose of oral morphine (200micrograms/kg) prior to ROP screening and there were no reports of respiratory depression (confirmed by personal communication). The oral route of administration is advantageous as many infants undergoing ROP screening in the latter part of their hospitalisation will no longer have intravenous access. No RCTs have been completed to date investigating the safety and efficacy of oral morphine for acutely painful procedures in healthy non-ventilated premature infants.

Neurophysiological measures of brain and reflex activity have previously been used to assess the analgesic efficacy of sucrose (253) and topical anaesthetic (5) in infants but these measures have unfortunately not yet been characterised in response to retinopathy of prematurity examinations. Fortunately, in our local Newborn Care Unit, routine blood tests are frequently scheduled on the same morning as ROP screening examinations. Therefore, in the Poppi trial, we were able to opportunistically study the effects of oral

morphine in these infants who required a heel lance on their scheduled date of ROP screening, providing the opportunity to assess multiple pain-related measures, maximising our understanding of the effects of morphine across the developing central nervous system (CNS).

The overall aim of the trial was to test whether a 100 μg/kg dose of morphine sulphate administered orally to non-ventilated infants prior to heel lancing and ROP screening could safely reduce procedure-related pain (assessed using clinical pain scores and noxious-evoked brain and reflex activity) and reduce clinical instability (assessed by continuous monitoring of physiological parameters 24 hours before and after the clinical procedure).

5.2 Methods

5.2.1 Study design

A double-blinded, placebo-controlled randomised controlled trial was conducted on the Newborn Care Unit at the John Radcliffe Hospital, Oxford between September 2016 and November 2017. The trial was registered with the Lancet, and the full trial protocol was published (576).

5.2.2 Participants

The participants were premature infants born at <32 weeks' gestation or with a birthweight <1501g (fulfilling National criteria for ROP screening), aged between 34- and 42-weeks' gestation, in order to allow use of a template of noxious-evoked brain activity validated in this age range. Inclusion and exclusion criteria are listed in Fig 5.2. Eligibility was re-assessed at randomisation, study commencement and prior to the drug administration.

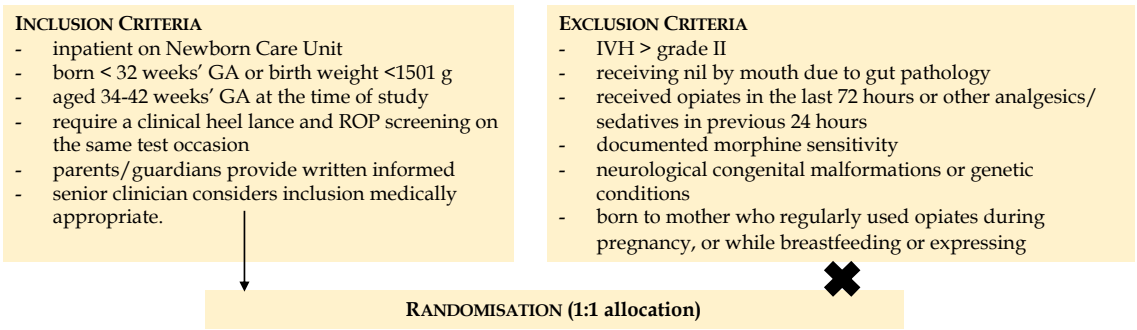

Figure 5.2: Poppi trial inclusion and exclusion criteria.

5.2.3 Intervention

A dose of 100 μg/kg was chosen based on recommendations in the British National Formulary for children (290) and local experience with this dose for ROP laser surgery. Pharmacokinetic modelling of morphine in neonates suggests an oral bioavailability of approximately 46% (577) due to maturing metabolism and clearance in infants, making this dose approximately equivalent to 46 μg/kg intravenously. Morphine sulphate (200μg/ml) and placebo (an inactive carrier solution) were manufactured by Stockport Pharmaceuticals Production Unit and delivered to the John Radcliffe Hospital Pharmacy in 10ml amber bottles labelled with a pack ID. The solutions were indistinguishable by colour, consistency and odour.

5.2.4 Randomisation

Using a website hosted by the NPEU CTU, we randomised enrolled participants to a pack ID. A minimisation algorithm was used to balance important demographics between the two groups (see Table 5.2). All members of the research and clinical teams, and parents were blinded to treatment allocation. An envelope containing a single-use access code to the randomisation website was stored on the unit that could be used by the clinical team to unmask treatment allocation in the event of an emergency. Any infants who became clinically unstable during the pre-intervention monitoring period did not receive the drug or placebo and were instead studied at the next appropriate occasion without re-randomisation.

5.2.5 Study Procedure

Heel lances and ROP examinations were performed on the same test occasion as part of the infant's scheduled clinical care. No unnecessary or additional blood tests or deviations from standard examination procedure were made for the study. A timeline of the trial procedures is provided in Fig 5.3. In order to establish a baseline of physiological stability for each infant, heart rate, respiratory rate, oxygen saturations, oxygen requirement and 6-hourly blood pressures were monitored for 24 hours prior to the planned clinical procedures. 60 minutes prior to the heel lance, 100µg/kg of morphine sulphate or placebo (of equivalent volume) was administered orally or via the nasogastric tube if in situ. As per local and national guidelines, mydriatic eye drops (Tropicamide 1% and Phenylephrine 2.5%) were administered for pupillary dilatation. Electroencephalography (EEG) and electromyography (EMG) electrodes were then sited on the infant's scalp as previously described in Chapter 2. 60 minutes after drug administration, video monitoring commenced, and a heel lance control and heel lance were performed. Infants were settled after blood collection and displayed no signs of distress prior to commencement of ROP screening. A senior ophthalmologist instilled topical local anaesthetic (proxymetacaine 0.5%) eye drops prior to insertion of the speculum and performed binocular ophthalmoscopic fundus examination of each eye using a Flynn-style indenter. EEG and EMG leads were subsequently removed, and physiological monitoring and documentation was continued for 24 hours to establish post-procedure physiological stability. A rotation of clinically-trained researchers (myself, another doctor, and a skilled neonatal nurse) remained with each infant for at least six hours post-drug administration.

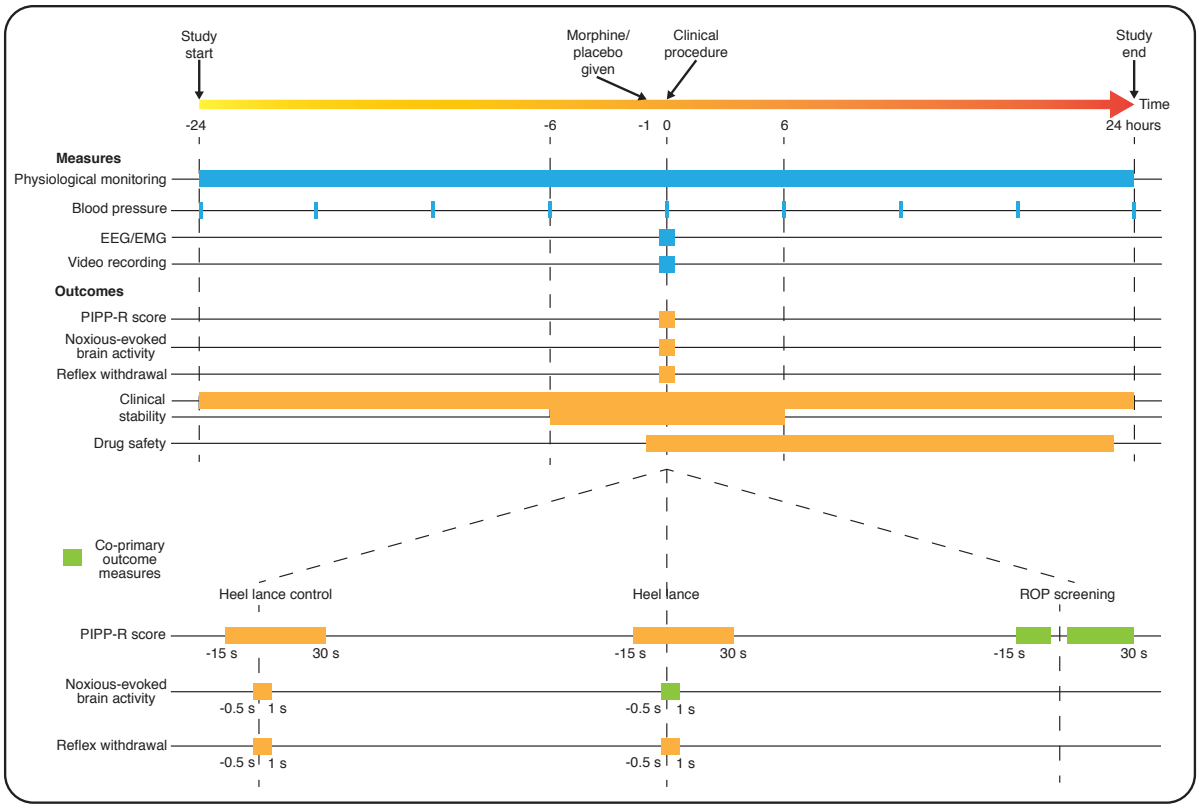

Figure 5.3: Trial outline. Timeline of the procedures and outcome measures over the 48-hour study period of each infant.

5.2.6 Experimental Recording techniques

An IntelliVue MX800 Phillips monitor was used to continuously record heart rate from ECG (sampling rate 250Hz), respiratory rate from an impedance pneumograph (sampling rate 62.5Hz), and oxygen saturations from a pulse oximeter, and ixTrend software (Excellence software) was used to continuously download the data. Procedural and clinical events were electronically annotated at the cotside during the 48-hour study period. Blood pressures were non-invasively measured 6-hourly using a neonatal cuff of appropriate size.

A clear view of facial expressions was recorded for 15 seconds before and 30 seconds after the heel lance control, the heel lance and the completed ROP screen (after removal of speculum from the second eye) respectively. The duration of ROP screening, from insertion of speculum into the first eye to removal of speculum from the second eye, was determined by retrospective review of video recordings.

EEG and EMG data were acquired using equipment and parameters previously described in Chapters 2.

5.2.7 Outcomes

The co-primary outcomes of the trial were a clinical pain score, the Premature Infant Pain Profile- Revised (PIPP-R), described in Chapter 2, in response to ROP screening and noxious-evoked brain activity evoked by heel lancing, identified using a published EEG-based template (5), validated in infants from 34 weeks' gestation. A magnitude of noxious-evoked brain activity at Cz was obtained as previously described in Chapter 2. The template was also projected onto data from other electrodes in a post-hoc analysis of noxious-evoked activity across the brain.

Secondary outcome measures were the PIPP-R score and reflex withdrawal activity in response to heel lancing. EMG data was processed as described in Chapter 2 and the magnitude of reflex withdrawal was defined using the mean RMS activity in the 1000ms post-stimulus.

Further secondary outcomes measured clinical stability in terms of episodes of oxygen desaturation, bradycardia, tachycardia, apnoea and significant increases in respiratory

support (as defined in Table 5.1) during the 6- and 24-hour periods after the clinical procedure. Increased respiratory support was defined as a significant increase in oxygen requirement or an increase in respiratory support modality (Table 5.1).

Parameter	Definition
Desaturations	O_2 saturations < 80% for ≥ 10 seconds
Bradycardias	heart rate < 100 for ≥15 seconds
Tachycardias	heart rate > 200 for ≥15 seconds.
Apnoeas	bradycardia with cessation of breathing > 20 seconds or apnoea recorded by clinical team
Respiratory support	Grade 1: self-ventilating in air Grade 2: low flow O_2 (0.01–0.35 litres/minute; 100% oxygen) Grade 3: high flow O_2(1–8 litres/minute) or CPAP or duoPAP (21–100% oxygen) Grade 4: mechanical ventilation (21–100% oxygen)
Significant increase in O_2 requirement	Increase > 10% O_2 Increase in flow rate >0.04 litres/minute (if low flow O_2) Increase in flow rate >1 litre/minute (if high flow O_2)

Table 5.1: Definitions of clinical stability. Ranges of physiological parameters used in the assessment of clinical stability.

5.2.8 Adverse events

Clinical investigators identified adverse events in the 24 hours after trial drug administration from review of clinical documentation and determined the attribution and expectedness of events with reference to the summary of Product Characteristics. Expected adverse events were also recorded, which included suspected sepsis, anaemia, minor changes in oxygen requirement (less than changes regarded as significant in the trial – see Table 5.1), and electrolyte imbalances on blood results. Expected serious adverse events included necrotising enterocolitis, intracranial abnormalities (haemorrhage, infarction or white matter damage), late-onset invasive infection, retinopathy of prematurity, patent ductus arteriosus, and congenital abnormalities.

The trial stopping boundary was defined prior to trial commencement using a group sequential method for apnoeic episodes requiring intervention with non-invasive positive pressure ventilation, with a one-sided Gamma spending function, (gamma= 4.5, type I error rate= 0.2, estimated power= 0.79). This boundary was selected by the DMC after consideration of hypothetical scenarios and was based on a placebo group event rate of 7%, and a difference between the group rates of 12%. The DMC reviewed all trial data and detailed summaries of adverse events after 31 infants had been studied.

5.2.9 Statistical Analysis

A power calculation was used to determine that a sample size of 132 infants was required to detect a 2 point-reduction in PIPP-R scores from a post-ROP PIPP score of 8.3 ± 3.5 in a previous study, with 90% power (two-sided significance level $p<0.05$). In an adult study, a 40% reduction in EEG response equated to a significant reduction in verbally reported pain scores (297), and 132 infants was also calculated as sufficient to achieve this reduction with a power of 90% (two-sided significance level $p<0.05$). The sample size was inflated to 156 infants (78 per trial arm) to compensate for multiple births and 10% loss to follow-up.

According to the skew of the data, mean (SD) or median (IQR) values are reported. Significant differences with 95% confidence intervals were tested using t-tests, in the case of PIPP-R scores, and using the Wilcoxon ran-sum test with the Hodges-Lehmann estimator for median differences with 95% CI for noxious-evoked brain and reflex activity. Inter-rater and intra-rater reliability were assessed using intra-class correlation from a random effects model with 95% confidence intervals. These were 0.98 (95% CI

0.97–0.99) and 0.98 (0.95–0.99) respectively for heel lance PIPP-R scores, and 0.97 (0.94–0.99) and 0·89 (0.79–0.95) respectively for retinopathy of prematurity screening. For each physiological outcome (bradycardia, tachycardias and desaturation), a standardised difference was calculated for the 6-hours before and after the clinical intervention, and another for the 24-hours before and after. The standardised difference was calculated for each infant as the difference in the number of events in the time-period before and after the clinical intervention, as a proportion of the total events in the time period. A negligible constant (0.01) was added to each event counted to prevent analysis issues related to zeros. Standardised differences were compared between the treatment groups using a Wilcoxon rank-sum test. Risk ratios were used to compare infants with new-onset or increased apnoeas in the 6- and 24-hour periods post-intervention and a risk difference was used to compare the number of infants requiring increased respiratory support in the 6 and 24-hour periods.

In an exploratory analysis, the effect of treatment on noxious-evoked brain activity was analysed across the pericentral electrodes (Cz, CPz, C3, C4, and FCz) using a linear mixed effects model with magnitude of noxious-evoked brain activity as the response variable, and treatment, electrode and gestational age as fixed effects, and an interaction between treatment and electrode. Individual infant ID was also included as a random effect to account for the multiple electrode readings for each infant. After finding a significant effect of electrode, direct comparisons between brain activity at different electrodes were made using estimated marginal means with a post-hoc Holm's correction for multiple testing.

In an additional exploratory analysis, time courses were calculated for each physiological variable (heart rate, respiratory rate and oxygen saturation) by averaging the values for each infant in moving one-hour intervals, overlapping by half an hour, and plotted hourly. The average time courses for each treatment group were compared using non-parametric cluster analysis (578), in which t-statistics were calculated at each time point. Time-points with a t-statistic greater than the 97.5^{th} percentile of the t-distribution were identified as significant and significant clusters were identified using random permutation analysis (10,000 permutations).

Data were analysed using MATLAB (version R2017a) and R statistical software.

5.3 Results

The main results presented in this chapter have been published in the Lancet (579).

Thirty-one infants were recruited prior to early trial cessation. One infant was fully consented but was withdrawn from the study by their parents prior to drug administration due to imminent discharge (Fig 5.4). 15 infants received 100 μg/kg of oral morphine and 15 received a placebo solution. The infant demographics and clinical characteristics are reported in Table 5.2.

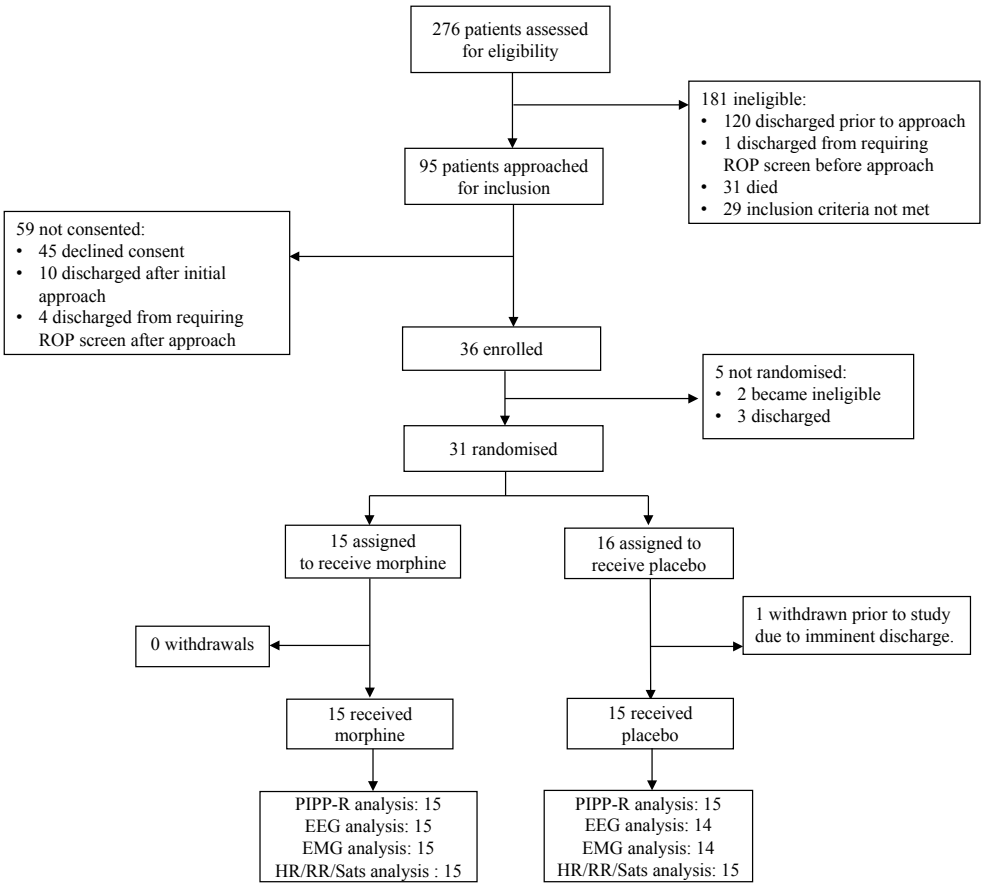

Figure 5.4: Trial recruitment. Electrophysiological data from one infant in the placebo group was excluded from analyses due to artefacts.

Characteristics at birth	Morphine (n=15)	Placebo (n=15)
Gestational age (weeks) *	28.1 (26.3-30.1)	28.6 (27.9-29.7)
Birthweight (g)	1107.0 (328.7)	1173.4 (349.5)
Birthweight z-score	-0.4 (0.9)	-0.2 (1.0)
Intra-uterine growth restriction	2 (13.3%)	3 (20.0%)
Apgar score at 10 minutes	10.0 (9.0-10.0)	10.0 (8.0-10.0)
Mode of delivery		
Spontaneous vaginal delivery	8 (53.3%)	5 (33.3%)
Caesarean section	7 (46.7%)	10 (66.7%)
Gender (male)	12 (80.0%)	8 (53.3%)
Multiple pregnancy	4 (26.7%)	4 (26.7%)
Characteristics at time of randomisation		
Gestational age (weeks) *	34.7 (34.1-35.1)	34.7 (34.1 – 35.1)
Days ventilated *	0.0 (0.0-7.0)	2.0 (1.0-17.0)
Days since morphine given *	46.5 (33.5-49.0)	19.0 (15.0-39.0)
Infants with IVH (I or II)	3 (20.0%)	2 (13.3%)
History of Surgery *	0 (0.0%)	1 (6.7%)
Characteristics at time of clinical procedures		
Gestational age (weeks)	35.0 (34.3-35.4)	34.9 (34.3-36.3)
Postnatal age (weeks)	50 (28-58)	49 (43-59)
Weight (g)	2048.5 (425.9)	2127.5 (330.9)
Presence of gastric tube *	14 (100.0%)	15 (100.0%)
Duration ROP screening (sec)	97 (82-108)	91 (83-110)
Diagnosis of ROP	2 (13.3%)	2 (13.3%)
Level of care		
ITU	1 (6.7%)	1 (6.7%)
HDU	5 (33.3%)	9 (60.0%)
LDU	9 (60.0%)	5 (33.3%)
Time between drug and heel lance (minutes)	61 (57-66)	63 (58 -70)

Table 5.2: Trial participant demographics. *Data are median (IQR), mean (SD) or number (%). *denotes criteria used in minimisation algorithm at randomisation.*

The trial was stopped following a planned interim review by independent members of the Data Monitoring Committee (DMC) and Trial Steering Committee (TSC). They determined that the predefined stopping boundary had been crossed and concluded that there was evidence to suggest that oral morphine at this dose has the potential to cause harm without analgesic benefit.

5.3.1 Validation of pain-related outcome measures

In order to ensure the sensitivity of the measures of analgesic efficacy used in the trial, we compared the magnitude of responses to the heel lance and ROP screening (regardless of treatment allocation) to responses to the control and background activity. The magnitude of each outcome measure was significantly greater following the noxious interventions. The average PIPP-R score for all 30 infants was 4.6 ± 3.2 (mean ± SD) following the non-noxious control stimulus; 8.2 ± 3.6 following the heel lance; and 10.8 ± 3.3 after retinopathy of prematurity screening (Figure 5.5A). Considering a PIPP-R score of less than 7 indicates little or no pain, it is reassuring that the control lance response was within this range. Overall, 60% of infants (18/30) had PIPP-R ≥7 following heel lancing, and 90% of infants (27/30) had scores in this range following ROP screening. 30% of all infants scored more than 12 following ROP screening, indicating moderate to severe pain (135).

The magnitudes of the noxious-evoked brain activity and reflex withdrawal activity were also significantly greater following heel lancing compared with the non-noxious control stimulus and background activity (noxious-evoked brain activity: background: 0.16 ± 0.19, control: 0.18 ± 0.20, heel lance: 0.95 ± 0.69; reflex withdrawal: background: 3.15 ± 2.46, control: 6.08 ± 6.07, heel lance: 26.76 ± 21.88; Fig 5.5).

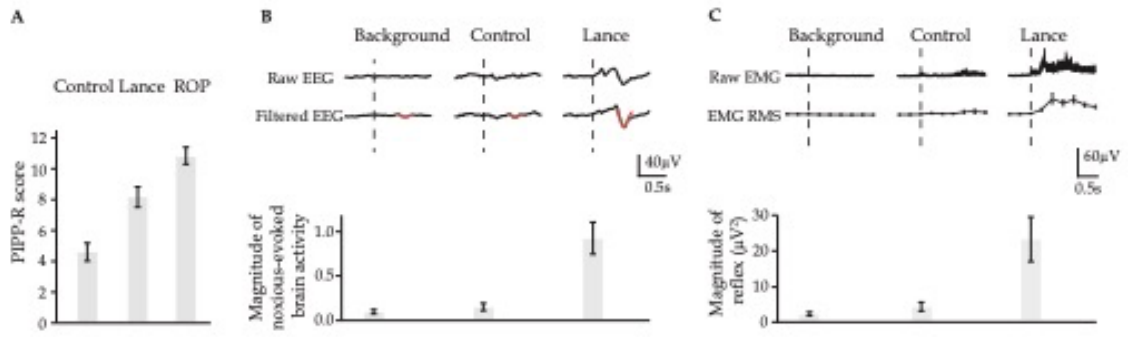

Figure 5.5: Validation of pain-related outcome measures. A. Mean (SE) PIPP-R score of all infants (regardless of treatment arm) following the control heel lance, heel lance, and ROP screening. **B**. Average EEG traces at rest (background) and following the control heel lance and heel lance. The Woody-filtered EEG is overlaid with the noxious-evoked brain activity template (red) and the median (SE) magnitude is shown below for all 3 conditions. **C**. Reflex activity at rest (background) and following the control heel lance and heel lance in all infants. The RMS of the mean reflex activity for each event is shown with the median ± SE below.

5.3.2 Oral morphine did not reduce measures of noxious-evoked activity

The PIPP-R score following ROP screening was not significantly different between morphine-treated infants and infants who received placebo (morphine: PIPP-R = 11.1 ± 3.2; placebo PIPP-R = 10.5 ± 3.4; p=0.66, t-test, 95% CI: -1.9 – 3.02; Fig 5.6A). 13/15 infants had a PIPP score >7 in both the morphine group and in the placebo group. Similarly, the magnitude of noxious-evoked brain activity following heel lancing did not significantly differ between infants in the two treatment arms (morphine: 1.13±0.83; placebo 0.74 ± 0.46; p=0.13, t-test, 95% CI: -0.12 – 0.12; Fig 5.6B). 14/15 infants who received morphine had noxious-evoked brain activity greater than the mean background threshold compared with 13/14 infants who received placebo (Fig 5.7).

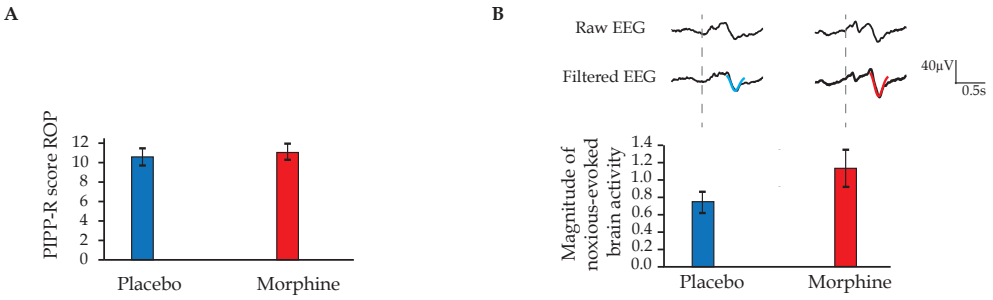

Figure 5.6: Co-primary outcome measures. A. Mean (SE) PIPP-R scores after ROP screening for infants who received placebo and morphine. **B.** Median (SE) magnitude of noxious-evoked brain activity after heel lance for infants who received placebo and morphine. The group average Woody-filtered EEG is overlaid with the template of noxious-evoked brain activity (blue= placebo; red=morphine).

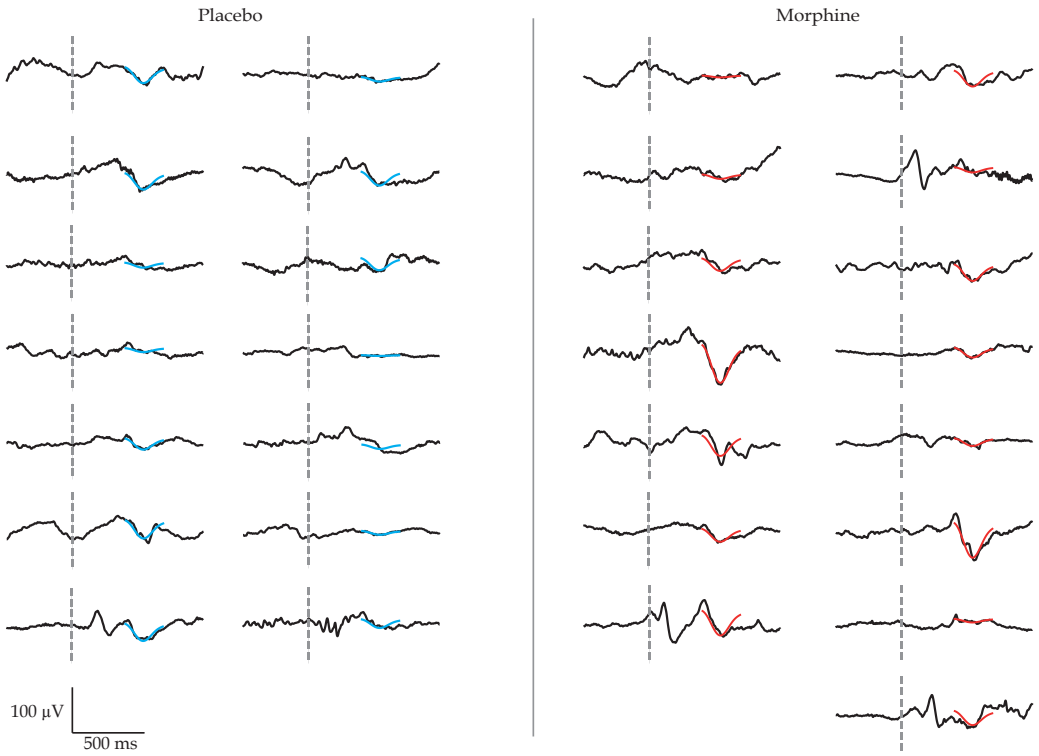

Figure 5.7: Noxious-evoked brain activity of placebo and morphine infants.
For each infant, the Woody-filtered EEG is overlaid with the template of noxious-evoked brain activity (blue= placebo; red=morphine).

Consistent with these results, the PIPP-R score and magnitude of reflex activity evoked by lancing did not significantly differ between the groups (PIPP-R: morphine: 7.9 ± 3.4,

placebo: 8.5 ± 3.9, p=0.66, t-test, 95% CI: -3.3 – 2.1; reflex withdrawal: morphine: 28.11 ± 17.80, placebo: 25.31 ± 26.19, p=0.74, t-test, 95% CI: -14.15 – 19.76; Fig 5.8).

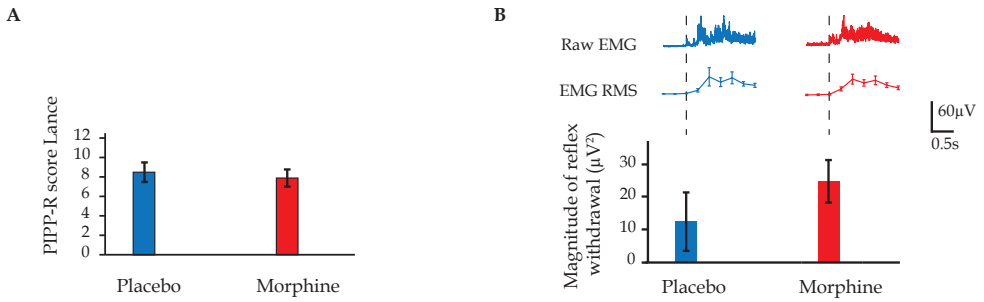

Figure 5.8. Secondary outcome measures. A. Mean (SE) of the clinical PIPP-R score in response to heel lancing. **B.** Median (SE) magnitude of reflex activity of the ipsilateral limb in response to heel lancing.

5.3.3 Effect of morphine on noxious-evoked brain activity across the brain

In a post-hoc exploratory analysis comparing the magnitude of responses at other electrodes, the greatest response was consistently identified at the Cz electrode in both infants that received placebo and infants that received morphine (Fig 5.9). The magnitude of noxious-evoked brain activity was not significantly reduced in infants that received morphine at any individual electrode. The median magnitude at every electrode was instead greater in infants that received morphine compared to infants that received placebo, although not significantly. The difference in magnitude of noxious-evoked brain activity between infants who received morphine and infants who received placebo did not differ significantly between electrodes (linear mixed effect model: treatment x electrode: χ^2=6.10, p=0.19). In addition, there was no overall effect of treatment ((linear mixed effect model: treatment: χ^2=2.60, p=0.11) or gestational age (linear mixed effect model: GA: χ^2=0.01, p=0.92). However, the magnitude of noxious-evoked brain activity did significantly differ between electrodes (linear mixed effect model: electrode: χ^2=34.25, p<0.001), with activity being greater at Cz and CPz compared with FCz (estimated

marginal means comparison: CPz-FCz: t=2.95, p=0.024; Cz-FCz: t=4.29, p<0.001; CPz-Cz: t=1.39, p=0.50)

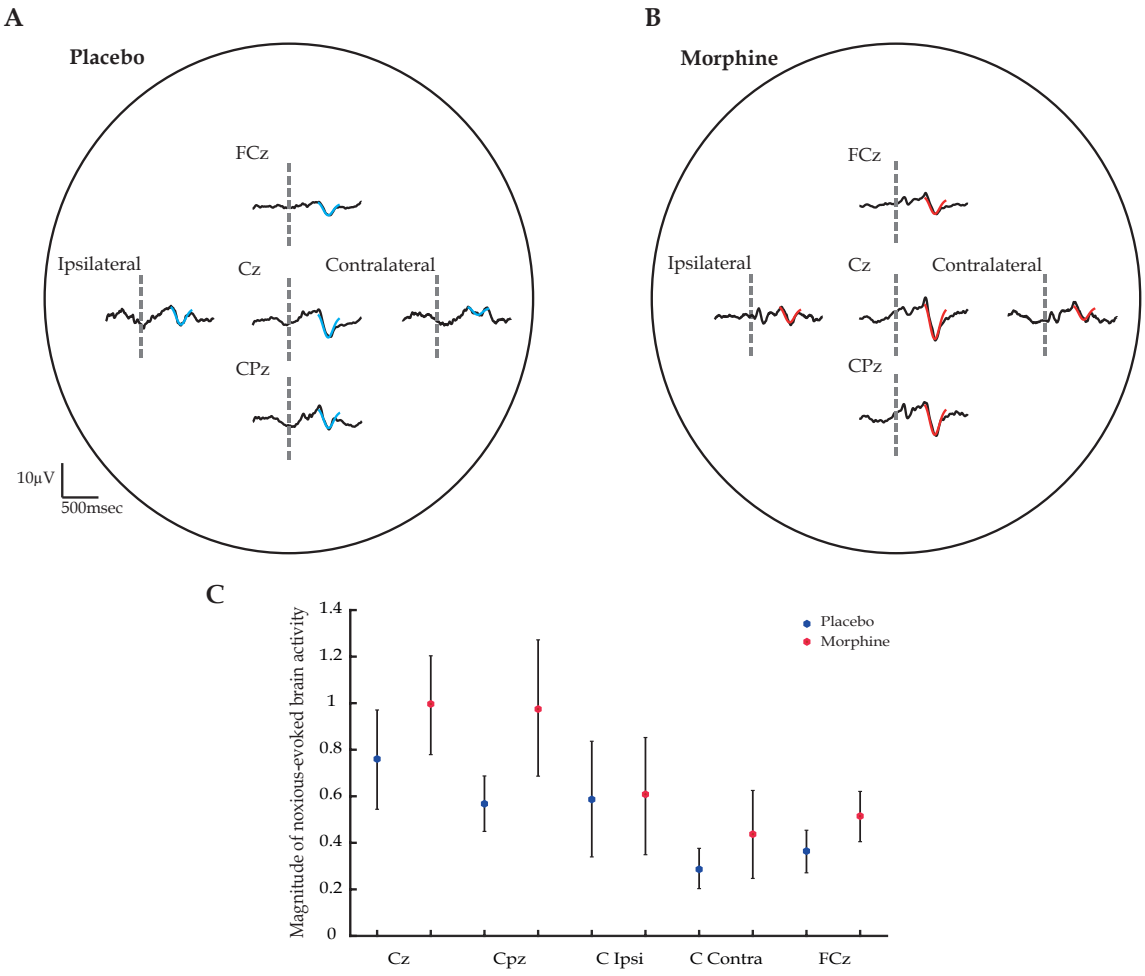

Figure 5.9: Magnitude of noxious-evoked brain activity across central electrodes. For each electrode, the Woody-filtered EEG is overlaid with the template of noxious-evoked brain activity in infants that received placebo (**A**) and infants that received morphine (**B**). Median (SE) magnitude of noxious-evoked brain activity after heel lance at each of the central electrodes in infants who received placebo and morphine **(C)**. C ipsi = central electrode over the hemisphere ipsilateral to the site of heel lancing. C contra = central electrode over the hemisphere contralateral to the site of heel lancing.

5.3.4 Oral morphine disrupts physiological stability in non-ventilated infants

Infants who received morphine experienced significantly more episodes of oxygen desaturation in the 6-hour and 24-hour periods following the clinical intervention (standardised difference in 6 hours: morphine: 0.57 (0.84) median (IQR), placebo: -0.06 (0.57), Wilcoxon rank-sum test, $p = 0.0008$, 95% CI: 0.33 – 1.00; standardised difference in 24 hours: morphine: 0.22 (0.90), placebo: 0.00 (0.29), $p = 0.020$, 95% CI: 0.039 – 0.72; Fig 5.10), and significantly more episodes of bradycardia in the 24-hour period following the clinical intervention compared with the placebo group (standardised change: morphine: 0.43 (0.99), placebo: 0 (0.9), $p = 0.020$, 95% CI: $1x10^{-5}$ – 0.99; Fig 5.10).

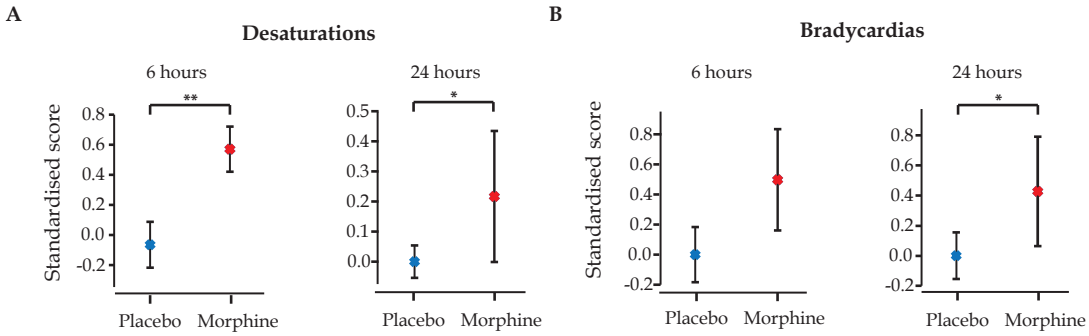

Figure 5.10: Standardised measures of physiological stability. A. Median (SE) of the standardised difference in number of episodes of desaturation in the 6 hours post-procedure compared with the 6 hours pre-procedure. **B**. Median (SE) of the standardised difference in number of episodes of desaturation in the 24 hours post-procedure compared with the 24 hours pre-procedure. **C**. Median (SE) of the standardised difference in number of episodes of bradycardias in the 6 hours post-procedure compared with the 6 hours pre-procedure. **D**. Median (SE) of the standardised difference in number of episodes of bradycardias in the 24 hours post-procedure compared with the 24 hours pre-procedure.

Seven of the 15 infants who received morphine experienced more apnoeas in the 6-hour period following the clinical intervention compared with 3 of the 15 infants who received placebo (morphine: 7/15 infants, placebo: 3/15 infants, risk ratio = 2.33, $p = 0.15$, 95%

CI: 0.74 – 7.35). Eight of the 15 infants (53 %) who received morphine experienced more apnoeas in the overall 24-hour period following the clinical intervention, compared with 3 out of 15 infants (20%) who received placebo (risk ratio = 2.7, p = 0.085, 95% CI: 0.9 – 8.2). Four of the 15 infants who received morphine also required a significant increase in respiratory support in the 6 hours post-procedure and 5 of the 15 infants required an increase in the overall 24 hours post-procedure. This was significantly more than the infants who received placebo, none of which required a significant increase in respiratory support (6 hours: risk difference = 0.27, p = 0.020, 95% CI: 0.04 – 0.49; 24 hours: risk difference = 0.33, p = 0.006, 95% CI: 0.09 – 0.57).

There was no significant difference in the number of episodes of tachycardia in the 6-hour or 24-hour period following the clinical intervention, nor in the number of episodes of bradycardia in the 6-hour period following the clinical intervention (standardised change tachycardias 6 hours: morphine: 0 (0), placebo 0 (0), p = 0.33, tachycardias 24 hours: morphine: 0 (1.45), placebo: 0 (0), p = 0.58, bradycardias 6 hours: morphine: 0.50 (0.99), placebo: 0 (0.82), p = 0.07).

5.3.5 Oral morphine causes concern of potential harm in non-ventilated infants

The pre-defined safety stopping boundary was passed due to the occurrence of apnoeas requiring significant intervention in the morphine group. 3 of the 15 (20%) infants who received morphine required resuscitation with NIPPV in the 24-hours after the clinical intervention, compared to none in the placebo group. None of the infants in the trial developed hypotension, although blood pressure was only measured 6-hourly non-invasively (Fig 5.11).

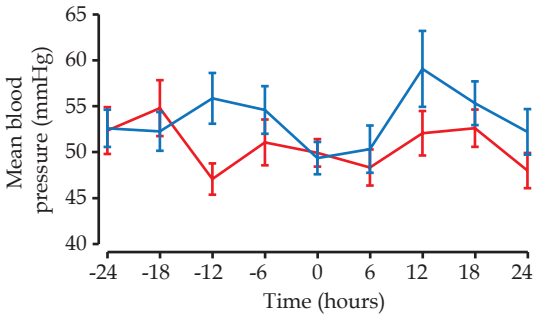

Figure 5.11. Mean blood pressure. The group mean ± SE of the mean blood pressure measured every 6 hours across the 48-hour trial period for infants who received placebo (blue) and morphine (red). 0 hours indicates the time of the clinical procedure.

5.3.6 Adverse Events

In the 24 hours after drug administration, eight of the 15 infants who received morphine (53%) experienced respiratory adverse events, which we blindly reported as 'possibly' or 'probably' related to morphine administration (Table 5.3). Two of these infants required transfer from low dependency to high dependency care. The degree of respiratory compromise of one infant caused the clinical team to unmask their treatment allocation and subsequently administer 100 µg/kg naloxone intramuscularly and a further 10µg/kg intravenously, approximately 8 and 9 hours after the administration of morphine respectively. In comparison, only one of the infants who received placebo had a mild respiratory adverse event, which was blindly assessed as unrelated to treatment.

ADVERSE EVENTS	Onset post-drug		Treatment	Grade	Attribution	Allocation
	hrs	min				
Nasal congestion	11	56	Saline drops	Mild	Not related	Placebo
Rash	4	4	Cream	Mild	Not related	Placebo
Profound desaturations	17	59	Facial oxygen	Mild	Not related	Placebo
Recurrent desaturations	8	9	Stimulations	Mild	Possibly	Morphine
Recurrent desaturations	1	58	Facial O_2	Mild	Possibly	Morphine
Apnoea	2	13	NIPPV, ↑ high flow O_2	Moderate	Possibly	Morphine
Recurrent apnoeic episodes	2	39	Stimulations, ↑low flow O_2	Moderate	Possibly	Morphine
Recurrent apnoeas	1	28	Stimulations, NIPPV x3	Moderate	Possibly	Morphine
Recurrent desaturations/ bradycardias/apnoea	2	3	High flow O_2 ↓Feed volume	Moderate	Possibly	Morphine
SERIOUS ADVERSE EVENTS						
Persistent hypoventilation/ desaturations	6	0	HDU Transfer High flow O_2	Moderate	Possibly	Morphine
Recurrent apnoeas	6	24	NIPPV HDU Transfer High flow O_2 Naloxone x2	Moderate	Probably	Morphine

Table 5.3: Adverse Events. Onset, treatment, severity and attribution of adverse events during the 24 hours post- administration of morphine or placebo. HDU= high dependency unit.

5.3.7 Cardiorespiratory effects of bolus oral morphine

We conducted additional post-hoc analyses on the continuous physiological measures to further investigate the cardiorespiratory effects of morphine administration. Morphine caused significant prolonged reductions in both heart rate and respiratory rate. The mean heart rate of infants who received morphine was significantly lower than the mean heart rate of infants who received placebo from 30 minutes to 6 hours post-drug administration (cluster-corrected nonparametric analysis, $p = 0.0001$; Fig 5.12). The mean respiratory

rate of infants who received morphine was also significantly lower than the mean respiratory rate of infants who received placebo from 1 hour to 8.5 hours post-drug administration (cluster-corrected nonparametric analysis, p = 0.003; Fig 5.12). The maximum effect of morphine on these physiological parameters occurred at approximately 2.5 hours; the mean heart rate was on average reduced by 13.9 beats per minute and the mean respiratory rate was reduced by 8 breaths per minute (Fig 5.12). The mean oxygen saturation of infants who received morphine did not drop significantly below the mean saturations of infants who received placebo. However, 13-15 hours after the start of the clinical procedure, the mean oxygen saturation of infants in the placebo group was significantly lower than infants who received morphine (cluster-corrected nonparametric analysis, p=0·022).

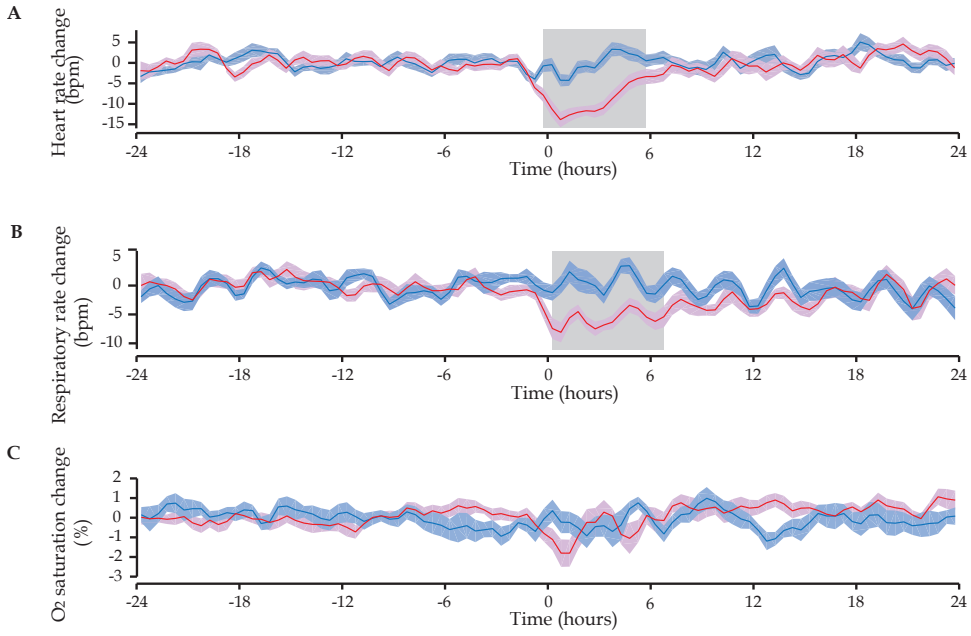

Figure 5.12: Trends in clinical stability. Mean (SE) heart rate (**A**), respiratory rate (**B**), and oxygen saturation (**C**) change over the 48-hour trial period for infants that received placebo (blue) and morphine (red). Periods during which the treatment groups differed significantly are shaded in grey. Zero indicates the time of the clinical procedure.

5.4 Discussion

The trial demonstrated that the administration of oral morphine at a dose of 100μg/kg to infants born prematurely prior to a screening examination for retinopathy of prematurity has a significant negative impact on cardiorespiratory stability, without suggestion of analgesic benefit.

5.4.1 Oral morphine did not reduce pain-related outcome measures

The magnitude of the clinical pain score, noxious-evoked brain activity and reflex withdrawal activity were not significantly different between treatment arms. Unfortunately, due to early cessation of the trial for safety concerns, the study was underpowered, and it was not possible to conclusively determine the analgesic efficacy of oral morphine using the primary outcome measures. However, there was a trend across the electrophysiological measures (noxious-evoked brain activity at every electrode and reflex activity following heel lancing) towards greater responses in the morphine-treated infants. This should be interpreted with caution. However, it is interesting to consider that if this trial had been conducted as a pilot on which to base a larger multicentre trial, it would not have been possible to power a further study to detect an analgesic effect of morphine.

Significant reductions in noxious-evoked brain activity by pain-relieving interventions have previously been demonstrated in both adults and infants using small sample sizes. The study in adults on which the trial was powered involved only 6 individuals, and it clearly demonstrated a significant attenuation of laser-evoked potentials following the administration of morphine. The advantage of this study was the within-subject control. Similarly, the template of noxious-evoked brain activity used in the Poppi trial has

previously been used to demonstrate a significant effect of local anaesthetic in infants (5). In this case an experimental noxious stimulus was applied to treated and untreated skin to allow within subject comparison. Considerable inter-individual variation in pain responses was evident in the control group of the Poppi trial. Testing the effect of morphine within-subject would have significantly increased the power of the study by automatically accounting for the many factors that may influence inter-individual variability in pain responses which include, but are not limited to, gestational age, sex, pain experience, and attention. In future studies investigating pain-relieving strategies for ROP examinations, administration of an analgesic drug prior to the examination of one eye could be considered to allow comparison with examination of the other eye, reducing the number of infants exposed to the experimental treatment under investigation.

5.4.2 Oral morphine caused significant cardiorespiratory instability

This trial has provided evidence that oral morphine at a dose of 100 μg/kg has the potential to cause harm in non-ventilated prematurely-born infants. The predefined safety stopping boundary was passed, because three of the 15 infants assigned morphine (20%) had apnoeic episodes requiring resuscitation with NIPPV in the 24 hours after drug administration, compared with no infants in the placebo group. Another infant was started on high flow oxygen after developing signs of respiratory insufficiency, which may have masked or prevented them from developing severe apnoeic episodes requiring NIPPV. This infant arguably could also have been included in the safety boundary as commencement of high flow oxygen therapy due to morphine-induced hypoventilation represented a considerable escalation of therapy (from self-ventilation in air), was an upsetting setback for parents, increased the level of care the infant required, and may have prolonged their hospitalisation. The Data Monitoring Committee played an invaluable

role in reviewing interim data to ensure patient safety. Importantly, in order to recommend cessation of a trial, they are not required to prove harm at a significance level (580). Their recommendation was not solely based upon the predefined stopping boundary but a holistic view of efficacy data as well as detailed accounts of all adverse events, which were experienced by more than half of the infants that received morphine.

Infants who received morphine experienced significantly more episodes of bradycardia and desaturation over the 24-hour period following the clinical procedure and a third of them required a significant increase in respiratory support. Cardiorespiratory effects are certainly not an unexpected side effect of morphine. However, the degree and frequency of compromise was surprising considering the dose of morphine in the trial was within the range provided in the British National Formulary (290), within the wider range recommended in neonatal guidelines, and was half the dose used in a previous (incomplete) trial (291). Opioids are known to produce dose-dependent respiratory depression with decreased responsiveness to CO_2 and alter the periodicity of breathing resulting in pauses, periodic breathing and apnoeas (581). Respiratory compromise likely results from activation of μ-opioid receptors expressed in the brainstem's respiratory centres, such as the pre-Bötzinger complex, that generate respiratory patterns (582). The cardiorespiratory effects of morphine reported in the Poppi trial are more pronounced than effects observed in previous studies of intravenous morphine. A Canadian survey of opiate use revealed that intravenous morphine was 'sometimes' given for pain relief prior to central line placement in non-ventilated infants (583). In a retrospective study of the safety of morphine in this context, Taddio et al., reported that three-quarters of infants received ~50 μg/kg, approximately equivalent to the oral dose administered in the Poppi trial, and a quarter received ~100 μg/kg intravenously (305). Five of the 43 infants in the

study experienced respiratory depression requiring interventions including bag/mask ventilation, increased ventilatory support, and naloxone reversal. However, there were other factors likely contributing to respiratory insufficiency; one infant had been extubated the previous day, two infants had experienced desaturations in the period prior to morphine administration, and two infants had inadvertently received morphine overdoses. Apnoeic episodes occur commonly in premature infants due to immature respiratory control and decrease in frequency with increasing gestational age (581). In the Poppi trial, a baseline of cardiorespiratory stability was established over the 24-hour period prior to drug administration for each infant to allow more accurate characterisation of the effects of morphine on the variable and fragile cardiorespiratory physiology of premature infants.

The safety of intravenous boluses of morphine have also been investigated in infants receiving continuous positive pressure ventilation (303). A significant increase in apnoeas was reported in a subgroup of patients that received more than 30 μg/kg. These infants were however considerably more premature than infants in the Poppi trial and were therefore likely to have poorer clearance rates of morphine and have been at greater risk of respiratory compromise. This study did highlight the occurrence of delayed apnoeas 3-4 hours post-morphine administration, substantiating the results of the Poppi trial, in which apnoeas were observed even 6 hours post-administration of the oral preparation of the drug. This is potentially relevant in the context of infants receiving morphine for intubation or during mechanical ventilation as these delayed effects may unnecessarily prolong short periods of ventilation due to apparent poor respiratory drive or precipitate re-intubation if these late-onset events are not expected. Pain is commonly assumed to antagonise the side effects of opiates. In the Poppi trial, it is likely that the pain of the

clinical procedures was not sustained enough to antagonise the cardiorespiratory effects of morphine, resulting in the delayed adverse events observed. Shorter acting synthetic opioid derivatives would potentially allow a better balance and timing of procedural pain to drug effects, theoretically reducing the likelihood of adverse cardiorespiratory events. Fentanyl may therefore merit investigation as an analgesic for procedural pain in this patient population.

Although hypoxic events are common and manageable in an intensive or high dependency care environment, their potential impact should not be underestimated, and particularly so when they are iatrogenic and occur in stable infants in a low-dependency environment. Frequent hypoxic events may affect long-term morbidity in these infants by perpetuating a pro-inflammatory/pro-oxidant cascade, contributing to the development of retinopathy of prematurity, poor growth, and adverse neurodevelopmental outcomes (584). The acceptability of side effects of any treatment is context-dependent and relies upon demonstration of a positive balance of benefits outweighing potential adverse consequences. In the Poppi trial, the occurrence of cardiorespiratory adverse events, the increased monitoring requirements and the need for increased respiratory support in infants who received morphine could not be justified by any demonstrable benefit.

5.4.3 The ethics of placebos in neonatal clinical trials

In the Poppi trial, infants received standard pain-relieving strategies consistent with local practice in addition to the administration of morphine or an inert placebo. The use of placebos in randomised controlled trials for procedural pain in neonates is a divisive and controversial issue. A paper by Bellieni and Johnston condemned the practice and advocated that infants in control groups should receive “high-level or validated analgesic

treatments"(585). In the Poppi trial, both infants who received placebo and infants who received morphine were swaddled during heel lancing, thus providing both arms of the trial with an evidence-based non-pharmacological intervention for reducing pain from this procedure (586,587), which has previously been used in control arms of analgesic trials (588). Swaddling is also classified as a high-level evidence-based treatment for heel lancing by Bellieni & Johnston in their ethical review paper (585).

Advocates of sucrose/glucose administration for pain relief, would consider failure to administer sucrose prior to heel lancing in the Poppi trial unethical as they believe that equipoise no longer exists for this treatment (589). However, sucrose does not reduce nociceptive-specific brain activity (253), which for many calls into question its effectiveness in truly reducing pain. Despite seventy-four studies with over 7000 subjects being conducted on the use of sucrose for neonatal procedures and a Cochrane review concluding its effectiveness in reducing pain from heel lancing (249), sucrose/glucose is not nationally (in the UK) or universally adopted as standard practice (590). A survey of 175 NICUs across 8 European countries revealed that oral sucrose/glucose use for heel lancing ranged between 5% and 100% of units across countries, and only 12% of UK units employed the practice (590). The UK guidelines for *Good Practice in Postoperative and Procedural Pain Management,* endorsed by the Royal College of Paediatrics and Child Health, furthermore reflect this in their recommendation that "sucrose or other sweet solutions can be used" for blood sampling, making the intervention optional rather than strongly recommended (34).

A review of all pain-related neonatal studies undertaken prior to 2010, revealed that 89% of control groups have received a placebo or no treatment (591). The Declaration of

Helsinki, adopted by the World Medical Association in 1964, provides authoritative ethical guidelines for human research (325) and in its most recent revision in 2013 an amendment was added concerning the use of inert placebos. Patients may receive a placebo or less effective treatment than the best proven one only if a compelling and scientifically sound argument can be made and patients are not subjected to additional risks of 'serious or irreversible harm'. Strong advocates of sucrose may argue that repeated exposure to pain in early life can have long-term consequences and therefore not providing infants with sucrose prior to heel lancing is unethical. However, the infants in this trial have had hundreds of heel lances without sucrose prior to participating in the study, and a single heel lance with sucrose is unlikely to impact their outcomes, in the same way that that undergoing this single minor procedure without sucrose is unlikely to cause them significant harm.

Advocates of sucrose also recommend its use for ROP examinations. A recent meta-analysis of pain-relieving interventions for ROP examination, which included combinations of sweet taste, topical anaesthetic, paracetamol, breastmilk, singing, non-nutritive sucking and nitrous oxide, concluded that there is currently no optimal analgesic strategy for this procedure. None of the treatments were effective in reducing the PIPP scores to minimal or no pain and the authors were unable to demonstrate the significant superiority of any combination of interventions (317). In the Poppi trial, infants who received placebo and were swaddled during ROP screening according to standard local practice had a mean PIPP-R score of 10.5 ±3.4, which should not warrant concern of unethical conduct as it is less than the median PIPP score achieved with topical anaesthetics (PIPP: 15) and with the addition of sweet taste (PIPP: 11) reported in the most recent meta-analysis (317).

5.4.4 Strengths and limitations of the trial

The Poppi trial employed a novel comprehensive multimodal approach in order to thoroughly assess analgesic efficacy with various measures quantifying drug effects at multiple levels of nociceptive processing as well as the effects on cardiorespiratory physiology. Had cessation of the trial not been necessary due to safety issues, this methodical approach would have yielded the most detailed evidence of the effects of morphine in this patient population to date. The trial design was unusual in that it assessed the effect of the drug on more than one commonly performed procedure, opportunistically maximising data acquired on the effects of the drug. The monitoring of these infants was more rigorous than any previous trials of analgesics in this patient population. The infants were at least 34 weeks' gestation at the time of study and therefore, as discussed in previous chapters, were more likely to reliably display robust and specific responses to noxious procedures, in terms of facial expressions, noxious-evoked brain activity and selective reflex withdrawal activity. The trial was conducted to a high standard and there were no deviations from exclusion or inclusion criteria. The infants were not critically ill or clinically unstable and most infants who received morphine were cared for in a low-dependency setting and were self-ventilating in air.

The conclusions that can be drawn from this study are unfortunately limited by the early cessation of the trial and only apply to infants of at least 34 weeks' GA. Having studied only 20% of the intended sample size, conclusions cannot be made on the analgesic efficacy of this treatment. Early cessation also unfortunately resulted in minor demographic imbalances. Half as many infants had been previously ventilated in the morphine group (morphine: 6/15; placebo:12/15) and only four of these 15 infants had previously received morphine. It is possible that first exposure to morphine could perhaps

account for more pronounced adverse effects. However, three of the four infants previously exposed to morphine developed respiratory adverse events in the trial. This would suggest that morphine naivety may not have contributed to the adverse effects observed.

A further limitation of the study design was the timing of the intervention in relation to drug administration. Considering the analgesic efficacy of morphine is controversial, the timing of peak analgesic efficacy has not been established. Studies of intravenous morphine for central line insertion in neonates have previously suggested an analgesic effect when the procedure was performed within 20 minutes of drug administration. Skin blood flow and heart rate response to central line insertion were significantly attenuated by the administration of 50-100 μg/kg of intravenous morphine 10-15 minutes pre-procedure (592). Similarly, Taddio et al., reported that heart rate and brow bulge scores were significantly lower in infants that received 100 μg/kg of morphine on average 17 minutes (SD 14) prior to central line placement (289). Clearly the oral route will have a longer time to maximum concentration (T_{max}) due to absorption. In the Poppi trial, we administered morphine one-hour pre-procedure following guidance in the Paediatric and Neonatal Dosage Handbook (20th Edition, 2013), local practice guidelines for morphine administration prior to neonatal eye surgery, and a previous incomplete trial of oral morphine for ROP screening (291). The timing was also consistent with studies of older patient populations, which have reported T_{max} of 50 minutes after oral morphine administration in adults (593) and 40 minutes in children (594). The physiological timecourses in the Poppi trial suggest a peak cardiorespiratory effect approximately 2 hours after drug administration. However, a significant reduction in heart rate and respiratory rate in morphine-treated infants was evident 30 minutes and 1-hour

post-drug administration respectively, suggesting considerable absorption of the drug by the time the clinical procedures were performed. Furthermore, it cannot be assumed that the minimum effective analgesic concentration of morphine would necessarily coincide with the peak cardiorespiratory effect. Measuring morphine and its metabolites in the trial would have allowed us to more directly address this issue. Nevertheless, studies have previously struggled to determine a relationship between morphine serum concentration and analgesic effect in infants (285,595) and morphine concentrations in children ranging widely between 4 and 65ng/mL have been associated with analgesia (596–598).

Although oral administration negates the requirement of intravenous access, this route has the disadvantage of variable absorption and reduced bioavailability due to first-pass metabolism. Variable absorption of morphine could account for some infants experiencing more adverse effects than others. Having not measured pharmacodynamic parameters, it is not possible to determine whether this was the case. Mydriatic eye drops containing both anticholinergic and adrenergic agents administered prior to ROP screening are also known to delay gastric emptying (599). For this reason, infants in our Unit receive a slower feed following ROP examination. Administration of these drugs at approximately the same time as morphine could have affected drug absorption, furthermore compounded by the action of morphine itself on gastric emptying (600). The timecourse of adverse events and physiological effects observed in the trial however suggest effective absorption of the drug and there were no reported episodes of abdominal distension in infants in the study. Ultimately it is not possible to determine whether significant gastric delay and absorption occurred without having measured gastric residuals and pharmacokinetic parameters.

Despite employing a variety of pain-related outcome measures, neither PIPP-scoring or nociceptive brain activity could be assessed during the ROP examination. Changes in brain activity evoked by ROP examination have not yet been characterised but this is an area of active research. PIPP-R scoring was performed 1-minute post-procedure rather than during the procedure itself. Previous studies have assessed PIPP scoring prior to ROP, at speculum insertion, 1-minute post-examination and 5-minutes post-examination. Accurate assessment of a facial score during the procedure is however not possible, as both eye squeeze and brow bulge are impeded by speculum insertion. Duration of nasolabial fold could perhaps have been used in isolation for PIPP-R scoring, however this would have reduced the power and validity of this accepted outcome measure. In future studies we may consider repeated PIPP-scoring to enable better characterisation of the reaction to and recovery from the procedure. Sedation scoring and measures of prolonged pain were also not included and should be considered in future studies of analgesics for procedural pain.

5.4.5 Pain and distress

ROP examinations entailing forcible eyelid retraction and probing of the eye are undoubtedly distressing. However, it is ultimately impossible to conclude whether neonates undergoing the procedure truly experience pain as we perceive it, other than by using clinical pain scores and inferring their experiences from the verbal reports of children and adults undergoing similar procedures. In these older populations, eye examinations involving scleral depression are reportedly distressing and uncomfortable (601). In verbal patient groups, communication provides the opportunity to disentangle pain from distress. Clinical pain scores used in neonatal practice, such as PIPP scores, that assimilate both behavioural and physiological responses, will be elevated by distress

and discomfort. For example, median PIPP scores of 8.5 (IQR: 6.25-10.75) have been reported in response to nappy changes in infants receiving neonatal intensive care (140). However, despite raised PIPP scores indicative of pain, we do not attempt to treat non-noxious procedures such as nappy changes with analgesic agents as we assume that the procedure is likely distressing rather than painful. Even if an analgesic agent were to be 100% effective in reducing pain caused by an inherently distressing procedure, PIPP scoring would likely fail to demonstrate the analgesic effect, as a result of the persistence of distress unrelated to pain. It is perhaps for this reason that studies of pain-relieving strategies have thus far been unable to demonstrate absolute reductions or consistent effects on behavioural scores in the case of needle-related procedures (602), lumbar punctures (603–605) and ROP examinations (for review (317)). Even in the absence of pain, ROP screening will likely remain distressing. The only way of reducing this distress would be to administer an agent with not only analgesic, but sedative properties. Reducing distress would likely be beneficial, as it is unclear whether the long-term effects of repeated painful procedures in early life result from stress or pain. Morphine has dose-dependent sedative properties that can be safely exploited in ventilated infants. However, in non-ventilated infants the dose required to provide light sedation would likely pose significant unacceptable risks of cardiorespiratory depression. Until we identify a short-acting agent with both analgesic and sedative properties that can safely be administered in non-ventilated infants, the stress of ROP examinations will likely continue to mask the effect of any analgesics.

5.5 Conclusion

In conclusion, the administration of 100 μg/kg oral morphine to non-ventilated prematurely-born infants aged 34–39 weeks' gestation at study prior to ROP examinations produced significant cardiorespiratory effects without any effect on pain-related outcomes. Firm conclusions regarding the analgesic efficacy of this treatment cannot be made due to early cessation of the trial. Continuation of the trial to determine the effectiveness of the drug would have exposed further infants to an unacceptable risk of harm. Considering the cardiorespiratory events were significant and frequent enough to precipitate safety concerns, oral morphine should not be administered to non-ventilated infants for relief of pain associated with retinopathy of prematurity screening using binocular indirect ophthalmoscopy. Multimodal outcome measures and rigorous physiological monitoring employed here should set a precedent for future neonatal clinical trials, facilitating further progress towards identifying safe and efficacious analgesics for this vulnerable patient population.

Chapter 6

6 Discussion

6.1 Thesis overview

In the absence of verbal report, nociceptive brain activity can provide a surrogate measure of pain that can be used to improve our understanding of how infants encode painful experiences. These brain-derived measures can provide objective and sensitive endpoints to help identify effective pain-relieving interventions. However, if measures of nociceptive brain activity are to be useful in assessing analgesic efficacy in infants, we need to understand how these responses mature and are modulated during development, and how they are influenced by key biological and environmental factors. The work in this thesis investigated the effects of age, connectivity of the PAG, sex and lastly the administration of morphine on nociceptive responses in infants.

Electrophysiological measures were first used to characterise both the development of noxious-evoked brain and spinal reflex activity across the preterm period. Nociceptive-specific brain activity was evoked and increased in magnitude from 32 weeks' gestation, providing a clear developmental trajectory for future studies employing this measure in infants. Noxious-evoked spinal reflex activity was however shown to oppositely decrease in amplitude, duration and peak latency with gestational age. The development of these noxious-evoked responses was not only observed to occur concurrently, but their relative proportion strongly correlated with gestational age, suggesting a developmental relationship between the maturation of spinal and supraspinal nociceptive pathways, which could reflect the emergence of descending pain modulation towards term gestation. In order to investigate the potential influence of descending pain modulatory brain regions

on noxious-evoked brain activity in infants, fMRI was then used in peri-term infants to assess connectivity between the PAG, a core region for descending modulation, and key cortical brain regions known to be involved in descending pain modulation in adults. Connectivity between the PAG and ACC and between the PAG and a region of the prefrontal cortex (MFG) in the pre-stimulus period was significantly negatively associated with noxious-evoked BOLD activity. Connectivity between the latter regions during rest was also negatively associated with noxious-evoked activity. Thus, infants with increased connectivity of these regions had reduced noxious-evoked activity. Infants around term gestation may begin to modulate their responses to noxious input via the PAG and developing descending pain pathways. Further investigation of the development of functional and structural connectivity of modulatory brain regions in neonates is important as many pharmacological (104,105,107) and non-pharmacological pain interventions such as massage and stroking (606,607) seek to engage the endogenous pain modulatory system, and animal and human studies suggest this system may be altered by preterm birth and early pain exposure (244–247,608).

The sexual dimorphism of pain is a growing area of neuroscientific and clinical interest. The increased sensitivity of women to experimental pain and their susceptibility to developing chronic pain conditions (435) is well documented in adults. Electrophysiological and neuroimaging studies have provided evidence of sex differences in noxious-evoked brain activity (440–442,446,447,449–453,460). In Chapter 4, both electrophysiological and fMRI measures of noxious-evoked brain activity revealed significantly greater responses in female infants compared to male infants of comparable gestation, weight and postnatal age. Greater noxious-evoked EEG activity was observed at the vertex in female infants. Consistent with this result, clusters of increased noxious-

evoked BOLD activity were identified bilaterally in several brain regions in female infants, including the paracentral lobules, precuneus, and inferior parietal lobules, which could potentially underlie a summative increase in electrophysiological activity recorded at the midline. Increased BOLD activity in female infants was identified in several brain regions that have been associated with pain sensitivity in adults, including the precuneus (444), parietal cortex (520) and anterior insula (447). These results from a small sample of demographically-matched infants suggest that sex differences in nociceptive processing may arise early in human neurodevelopment. Further investigation of quantitative and as well as qualitative sex differences in nociceptive processing in infancy is warranted as they may impact individual pain experience and the consequences of early pain exposure (429).

In the final experimental chapter of this thesis, brain activity was employed as a co-primary outcome measure to investigate the efficacy of oral morphine for procedural pain in prematurely-born infants. Unfortunately, due to early cessation of the trial, the study was underpowered to determine the efficacy of the drug and no significant differences were observed in the magnitude of noxious-evoked brain activity, clinical pain scores or reflex activity between the two groups. However, the study did not solely report efficacy. Rigorous safety monitoring and a pre-determined safety boundary facilitated early identification of potential harm. Despite the dose employed being commensurate with dosing recommendations in the British National Formulary for Children (290) and local practice for neonatal laser eye surgery, dedicated 24-hour physiological monitoring before and after drug administration confirmed the significant cardiorespiratory effects of this drug in non-ventilated infants. Most importantly, an unacceptable rate of adverse events occurred in the absence of evidence of benefit, suggesting there may be no

therapeutic window for oral morphine in this context. This trial sets a new standard for the comprehensive parallel assessment of both efficacy and safety of analgesic agents in neonatal care using multimodal measures of nociceptive activity and physiology.

6.2 Limitations and further work

6.2.1 The challenge of assessing pain in premature infants

Pain assessment presents the greatest challenge in very premature infants and as such has been identified as a key research priority (41). As demonstrated in Chapter 3, in very premature infants aged less than 32 weeks' gestation noxious stimulation generally evokes non-specific delta brush activity instead of nociceptive evoked potentials (183). This non-discriminative brain activity can also be elicited by innocuous sensory stimuli of various modalities (185,609). In a recent study, we have re-confirmed that the developmental switch from non-specific to modality-specific brain activity (described in Chapter 3) occurs at ~33 weeks' gestation and furthermore demonstrated that the maturation of discriminative facial expressions follows the same developmental trajectory (198). Very premature infants therefore display poorly discriminative facial and brain activity responses, which could crudely be interpreted to mean that all stimulation, regardless of modality, is equally aversive or non-aversive at this developmental stage.

Facial expressions are used to communicate with a caregiver and this signalling is very costly for extremely premature or critically ill infants, in terms of energy expenditure and is associated with a risk of increasing intracranial pressure (610). Therefore, the absence of this signal may not necessarily represent the absence of pain. Equally, from an evolutionary perspective it is unlikely that any selective pressure exists on foetuses in

utero to effectively communicated a difference between noxious and non-noxious stimuli in the late second or early third trimester. Considering the lack of a required receiver to observe facial expressions, there is likely no adaptive benefit to developing accurate behavioural signalling in utero (198). The poorly discriminative behavioural responses of sick or premature infants therefore present a significant problem for the assessment of pain and analgesia. For this reason, the effects of sex (in Chapter 4) and oral morphine (in Chapter 5) were only investigated in clinically stable infants aged 34 weeks' gestation or more at study. In order to test the analgesic efficacy of drugs in very premature infants we require the development of novel measures. It is possible that the saliency or modality of sensory stimulation may drive subtle differences in the frequency composition or topology of delta brush activity in very premature infants. This deserves further investigation in order to provide a much-needed outcome measure for this patient population, which often suffers greater exposure to painful clinical procedures to manage the consequences of their extreme prematurity.

6.2.2 **Assessment of prolonged procedural and non-procedural pain**

A notable limitation of the work in this thesis is its focus on the assessment of immediate acute pain responses. Nociceptive brain and reflex activity are measured within milliseconds and seconds of the noxious stimulus, and PIPP-R scoring used in the Poppi trial records changes in behaviour and physiology over a period of 30 seconds. Assessing pain beyond the acute noxious insult remains a major challenge. Most of the clinical pain scores to date have been developed to measure acute procedural pain. However, in practice, there is concern about prolonged, persistent, and chronic pain, in the context of significant tissue injury or inflammation. Additionally, there are concerns about 'discomfort', which is poorly defined but often cited in the context of prolonged

interventions such as mechanical ventilation. These types of pain are much harder to identify and quantify, and concerted attempts to provide consensus definitions have failed (611). Several scoring systems have nevertheless been developed that attempt to address this issue, including EDIN (612), NPASS (613), ComfortNeo (614) ALPS-Neo (615), and CRIES (616), which are broadly based on behavioural and physiological indicators also used in acute pain assessment. However, these measures have not been widely adopted in clinical practice. A European survey across 243 centres revealed that only 10% of neonates are assessed daily for non-procedural continuous pain (617). Without validated measures, decisions regarding pain management are too often based on informal assessment and individual clinical judgement. Objective, sensitive, and specific pain measurement tools are needed in order to appropriately initiate and titrate pain medications and prevent suffering and the potential adverse effects of both under-treated pain and excessive analgesic drug provision.

Clinical pain measures require consideration of context as well as the neurodevelopmental stage of the infant. Acute pain measures have been used to record responses to procedures that would not generally be considered painful. Recently, a study used the Neonatal Infant Pain Scale to score pain during non-invasive echocardiogram assessments, involving application of an ultrasound probe to the chest to image cardiac physiology. The authors concluded that these assessments cause ‘significant pain’ in premature infants (618). Such handling is likely stressful for a premature infant, but it is unlikely that this non-invasive procedure triggers nociceptors and elicits pain requiring analgesia. Although pain is often accompanied by distress, distress can occur in the absence of pain. Biomarkers that fundamentally distinguish pain from non-painful distress are needed in order to guide the provision of analgesia and sedation.

Electrophysiological noxious evoked potentials used throughout this thesis are valuable in the context of a brief noxious stimulus. Heel lancing is one of the most common painful procedures performed in neonatal intensive care and provides the ideal brief acute stimulus for precise time-locking of the EEG. Noxious-evoked potentials provide a useful measure of the nociceptive responsiveness of the infant, valuable in assessing nociceptive development (as in Chapter 3) and in assessing modulation by individual factors such as sex, or external factors such as administration of an analgesic (as in Chapters 4 and 5). However, further study is needed to characterise changes in brain activity beyond the immediate millisecond post-stimulus period, which would be useful in the context of more prolonged or intermittently painful procedures such as ROP screening, chest drain insertion, PICC insertion, tracheal intubation, and endotracheal suctioning. In the Poppi trial, heel lances were routinely performed prior to ROP screening in our centre, thus providing an opportunity to test the efficacy of pre-emptive morphine for both procedures. Coupling procedures of interest to routine heel lancing may however not always be practical or desirable due to concerns relating to cluster care (619). Using time-frequency decomposition and machine learning to study longer periods of electrophysiological data collected from infants both pre-procedure and post-procedure and infants at rest with high clinical pain scores may reveal new electrophysiological markers of pain.

6.2.3 **What is a clinically significant reduction in pain-related measures?**

Clinical trials, such as the Poppi trial in Chapter 5, aim to demonstrate whether an intervention can significantly reduce pain compared to placebo or a standard intervention. However, the demonstration of a statistically significant reduction does not necessarily

equate to clinically significant results for the patient. Meta-analyses of large randomised clinical trials of analgesics in adults have demonstrated that a 30% reduction in pain intensity achieved on a scale or scoring system may represent the threshold at which most patients would begin to describe a meaningful reduction in pain (620–622). One study specifically investigating the minimum clinically important difference in pain scoring using the Visual Analogue Scale (range: 0 - 100mm), identified that a mean reduction of 30mm was associated with report of 'adequate pain control' (623). In the absence of verbal report, assigning clinical significance to a pain score change is even more challenging. In the Poppi trial, a 2-point reduction in PIPP-R score was defined as a clinically-meaningful reduction used to calculate the power and sample size of the trial, as this threshold has previously been used in other neonatal clinical trials of analgesics investigating topical anaesthetics (624) and intravenous morphine (267). However, some randomised clinical trials in infants have investigated a 1-point reduction (625), whilst others recommend a three-point difference (626) or a 20% reduction should be required (627). The ultimate and often unachievable goal of pain management is analgesia, the complete relief of pain. Considering a PIPP score of less than 7 is generally attributed to mild pain or none at all, we should perhaps be aiming to provide interventions that reduce scores into this range.

The Poppi trial was also originally powered for a 40% reduction in magnitude of nociceptive brain activity, the co-primary outcome measure of the study. Equating a 40% reduction in magnitude of the evoked potential to an analgesic effect was based upon limited and indirect evidence from an adult study of morphine (297). The authors recorded laser-evoked potentials in six patients started on sustained release morphine for non-cancer chronic pain. Morphine doses varied significantly between subjects (range:

30 -150mg per day) and their laser-evoked potentials were tested before initiation of treatment and after patients reported sufficient pain relief. A significant reduction in the mean amplitude of the evoked potential was observed from 19.3 ± 4.5 to 12.5 ± 3.3μV, which accompanied a significant reduction in laser pain rating and laser pain threshold. Although this 35% percentage decrease in magnitude of the potential was associated with pain relief, it is possible that lesser reductions could provide a degree of analgesia. In infants, a ~30% reduction in magnitude of the noxious-evoked potential has previously been demonstrated following the application of a topical anaesthetic (5). Also, more recently, we have demonstrated that stroking an infant at a velocity to activate c-tactile fibres prior to heel lancing attenuates noxious-evoked brain activity, and a ~40% reduction in magnitude was observed with this intervention (607). Therefore, a 30-40% reduction was likely a valid threshold for efficacy, particularly for an agent like morphine, which in adults is a classic potent analgesic against which other drugs are tested (628).

6.2.4 **Sex differences in analgesia**

The results of Chapter 4 suggest sex differences in nociceptive brain activity may exist from birth. Sex differences in nociceptive processing could have an impact on responses to analgesic drugs (629). Preclinical studies have provided considerable evidence of differences in the efficacy of opioids (470,630–638) with males displaying increased sensitivity to this analgesic agent. These differences cannot be attributed to sex differences in opioid pharmacokinetics (634,639–641) but may result from reported sex differences in the anatomy and functional organisation of the PAG, the central brain region of opiate-sensitive neural circuits (642–644). Greater expression of opioid receptors in this brain region has been demonstrated in males (643,645). Studies of sex differences in the analgesic efficacy of morphine in human experimental and clinical

studies have however yielded inconsistent results (204). A meta-analysis of the literature only revealed an overall sex-specific effect when restricted to studies of patient-controlled morphine (646). Reduced opioid consumption in women was interpreted as greater efficacy. However, this could be biased by other factors such as drug adverse effects. In the Poppi trial, reported in Chapter 5 of this thesis, 80% of infants who received morphine were male. This significant sex bias was likely due to early cessation of the trial. However, a general sex imbalance exists in the preterm population as males are more likely to be born prematurely than females (647–649). Therefore, this bias could potentially have remained even if the trial had continued to its intended sample size, as sex was not included as a minimisation factor accounted for at randomisation. Considering the effect of sex on the magnitude of nociceptive brain activity reported in this thesis, a sex bias could have potentially affected the results of the completed trial. In future studies of opiates in infants, sex should be included as a minimisation factor at randomisation and sex differences in opioid sensitivity in infants merit investigation.

6.2.5 **Further investigation of the analgesic efficacy morphine**

Despite the conclusions of a Cochrane review, intravenous morphine is routinely administered to ventilated infants in neonatal care in many centres (283). The results of the Poppi trial further call into question the general use of morphine in neonates. If this drug does not significantly attenuate nociceptive input at the doses administered, its use may not be justified considering the potential adverse effects on cardiorespiratory stability, ventilation requirements, growth and dampening of critical non-nociceptive sensory input (650,651). In rodent models, chronic morphine exposure in the first weeks of life has been associated with regional apoptosis (311), and impairment of learning (308) and hippocampal development (310). However, further studies have demonstrated

that appropriate administration of morphine for severe pain or repetitive mild pain may be neuroprotective and mitigate the long-term consequences of early pain (130,312). Morphine should only be administered to treat pain, which antagonises its undesired effects. The timing, context and duration of morphine administration are likely key to preventing the potential long-term adverse neurodevelopmental effects of both repetitive pain exposure and the drug itself. Intravenous morphine is also commonly administered to neonates with hypoxic ischaemic encephalopathy during therapeutic hypothermia; 88% of cooling centres in the UK administer the drug mostly to prevent 'discomfort' (652). Although initial studies suggested opioid administration during hypothermia may have a neuroprotective effect (653), a recent international multi-centre study failed to demonstrate any such benefit and suggested the treatment may result in prolongation of hospitalisation (652). Additionally, there are concerns of neurotoxicity due to drug accumulation during hypothermia (654,655), prolongation of ventilation and recovery (656) and compromise of prognostication (657). In light of these risks, it is important to determine the benefits and adverse effects of morphine in this context. A randomised controlled trial of morphine versus placebo for infants undergoing therapeutic hypothermia is being proposed to address this clinical equipoise. Without definitive evidence, clinicians are understandably reluctant to stop an intervention they believe may protect infants from discomfort. Inclusion of multimodal nociceptive measures in such a trial would further advance our understanding of the analgesic efficacy of morphine in neonates.

6.2.6 **Fentanyl for procedural pain in infants**

At the time of designing the Poppi trial, morphine was the most frequently used pharmacological analgesic in neonatal intensive care (573). Fentanyl use is however now more common, and this drug may present distinct advantages to morphine in terms of timing, route of administration, and adverse effects (39,658). Over 30 years ago, Anand and colleagues first demonstrated that fentanyl improved the stress response and recovery of premature infants undergoing cardiac surgery (30). Despite limited pharmacokinetic, analgesic efficacy and safety data, its use in neonatal care has increased in ventilated infants and for painful procedures (39,659). Fentanyl is a synthetic opioid that is 50-100 times more potent than morphine and has a potentially preferable pharmacological profile (660). It rapidly traverses the blood-brain barrier due to its high lipid solubility and has an earlier onset of action and peak effect. Due to a lack of histamine release, there is also a lower risk of haemodynamic compromise (661). However, as for morphine, studies of the analgesic efficacy of fentanyl in neonates are limited and heterogeneous (662), with only some studies demonstrating reductions of biochemical markers of stress (663,664) and attenuation of validated pain scores (265,665,666). There is also conflicting evidence of its cardiorespiratory effects, with several studies reporting an increased need for ventilatory support (663,665) whilst others have observed no such effect (664). Although there is a likely risk of respiratory depression with fentanyl, it may be less than the risk associated with morphine. An observational study comparing morphine and fentanyl for laser eye surgery reported that apnoeas were three times more likely in infants receiving morphine (667). Chest wall rigidity is however a concern with fentanyl, although the risk of this complication may be mitigated by slower administration (668). Intravenous access is often a barrier to the administration of analgesics in neonates. However, fentanyl can be administered intra-nasally. This route was initially trialled effectively in neonates

receiving palliative care (669). Since then it has been introduced into practice for non-ventilated infants undergoing painful procedures including intravenous cannulation, PICC line insertion and minor surgical procedures in countries such as Canada (670), with minimal evidence of respiratory depression (671). These initial reports are encouraging but large-scale randomised controlled trials including a dose-ranging study and multimodal validated outcome measures are needed to determine the safety, efficacy and optimal dosing of this drug in infants. The Poppi trial provides an ideal paradigm with which to comprehensively test this drug in a subsequent clinical trial for procedural pain relief in infants.

6.2.7 **fMRI to investigate pain in infants**

In Chapters 3 and 4, fMRI was used to investigate brain activity in response to an experimental noxious stimulus. Regions with significant changes in BOLD were similar to previous studies and activity observed in adults (4). However, it is unclear how specific this brain activity is to the experience of pain as a non-noxious control stimulus was not applied. The electrophysiological template employed in EEG studies reported in this thesis has however been validated using a variety of innocuous stimuli (5). Noxious stimuli are inherently salient and brain activity previously identified as 'pain-specific' likely includes activity related to attention and arousal. This issue of specificity has inspired considerable debate in the field of pain neuroimaging (84). Several studies have demonstrated that brain regions most frequently activated by noxious stimulation also respond to salient non-noxious stimuli (85,86,672,673). Furthermore, activity in these brain regions can also be evoked by noxious stimulation in individuals with a loss-of-function sodium channel mutation resulting in congenital pain insensitivity (82). Overall,

the activity described is arguably more ‘pain-selective’ than ‘pain-specific’ (86). However, it is perhaps unsurprising that mechanical touch and mechanical pain would stimulate activity in similar brain regions considering they are on a continuum whereby increasing pressure of mechanical touch can become painful.

Multivariate pattern analysis is a powerful analytical approach that has been applied in adults to generate three-dimensional signature patterns of brain activity reflecting various types or aspects of pain experience (674). This has allowed a shift away from the arbitrarily thresholded individual voxel towards a distributed, dynamic pattern of brain activity evoked by a noxious event. Wager et al., first applied this methodology to derive a signature of thermal pain, termed the Neural Pain Signature (NPS), which could discriminate painful from non-painful salient stimulation (674). The NPS has since been validated in independent datasets (675) and studies have demonstrated its modulation by opiates (676), serotonin-reuptake inhibitors (677), and expectation (678). Other specific neural signatures have since been developed including the Stimulus Intensity Independent Pain Signature (SIIPS1), which correlates with pain report (679), and a Signature of Analgesic Efficacy (SAE) (680). We are now applying validated neural signatures characterised in adults such as SIIPS and NPS to the data acquired in this thesis to further explore the patterns of noxious-evoked brain activity in early life, how they differ from the adult, and how and when they may differ between the sexes.

In recent years, the focus of neuroimaging has expanded from pure localisation of activity to investigation of the integration of activity and the functional connectivity between brain regions underlying complex cognitive processing and perceptions (for review see (681). In Chapter 3, correlation analyses were used to investigate the functional

connectivity between the PAG and regions known to be involved in descending pain modulation. This is a commonly used but simplistic analysis of connectivity. Considering greater correlation of signal between two regions could relate to simultaneous increases in signal, decreases in uncorrelated signal, or complicated changes in amplitude of one or both signals, complex modelling approaches are being devised to more clearly express changes in functional connectivity (682), which could be applied to infant data in the future. In adults, baseline fluctuations in brain activity between brain regions at rest have also been shown to significantly predict brain activity in response to a task (683). Furthermore, Boly and colleagues demonstrated that differences in perception of noxious somatosensory stimuli can be predicted from baseline activity in brain regions involved in generating pain perception (241). Using the combined stimulus-evoked and resting state data set acquired in infants for this thesis, further work will investigate whether activity in brain regions evoked by noxious stimulation relates to functional connectivity of these brain regions at rest.

6.3 Concluding remarks

In the absence of communication, electrophysiology and MRI can provide a mechanistic insight into nociceptive processing in infants and facilitate the investigation of intrinsic and extrinsic factors that modulate their pain responses. This thesis demonstrates that noxious-evoked brain activity in infants is affected by gestational age, developing connectivity of endogenous pain modulatory brain regions, and sex. Pre-emptive oral morphine however failed to attenuate noxious-evoked brain activity, along with other multimodal measures of pain, despite considerable cardiorespiratory effects. Although these results were obtained in a small sample due to early trial cessation for safety, they further call into question the efficacy of morphine and highlight the urgent need for trials of evidence-based analgesics in this patient population. Considering the consequences of early pain exposure and the increasing drive to ventilate premature infants non-invasively, it is essential that safe and effective analgesics are identified based on a thorough understanding of the development of nociceptive processing and pain-related responses, and are tested through rigorous and comprehensive clinical trials, which transparently assess the balance of risks and benefits of drug administration.

Appendix I : Patient Information Leaflet

Oxford University Hospitals NHS
NHS Trust

Neonatal Unit
The John Radcliffe
Headley Way
Headington
Oxford
OX3 9DU

Tel: 01865 221355
Fax: 01865 221366

NRES Committee South Central – Oxford C
REC Ref: 12/SC/0447
Version: **5**
Version Date: **09/06/2015**

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

during these procedures. At the same time we will measure other physiological factors such as muscle activity, heart rate and oxygen saturations.

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 stinuli. 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

during these procedures. At the same time we will measure other physiological factors such as muscle activity, heart rate and oxygen saturations.

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,

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All studies have a dedicated team of clinicians, radiographers and research personnel that will ensure the safety of your baby at all times.

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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.

The principal investigator for this project is Dr Rebeccah Slater.

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 (tel01865 234537, rslater@fmrib.ox.ac.uk) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email heather.house@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.

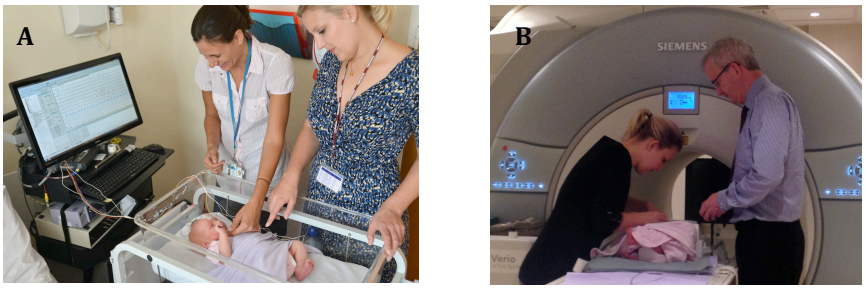
All information regarding their medical records will be treated as strictly confidential.

12. Who has reviewed the study?

All proposals for research involving newborn babies/infants are reviewed by an ethics committee before they can proceed. This study was reviewed by the NRES Committee South Central Oxford C, Room 002, TEDCO Business Centre, Rolling Mill Road, Jarrow, NE32 3DT. Tel: 0191 428 3564

13. Contact for further information.

Dr Rebeccah Slater *Associate Professor of Paediatric Neuroimaging* *University of Oxford*	Telephone: 01865 234537 E-mail: rebeccah.slater@paediatrics.ox.ac.uk
Dr Eleri Adams *Consultant Neonatologist* *Oxford University Hospitals NHS Trust*	Telephone: 01865 221356 E-mail: Eleri.adams@ouh.nhs.uk



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

Thank you for reading this information sheet.

Appendix II : Parent Consent form

Oxford University Hospitals NHS
NHS Trust

Neonatal Unit
The John Radcliffe
Headley Way
Headington
Oxford
OX3 9DU

Tel: 01865 221355
Fax: 01865 221366

NRES Committee South Central – Oxford C

REC Ref: 12/SC/0447

Version: **5**

Date: **09/06/2015**

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 initial box

1 I confirm that I have read and understood the information sheet dated **09/06/2015 (version 5)**, 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 I would be happy for my baby to be studied on one occasion for the NIRS / EEG / FMRI Study. *(please delete as appropriate)* ☐

Oxford University Hospitals NHS
NHS Trust

6 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 / FMRI Study. *(please delete as appropriate)* ☐

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

8 I agree to take part in the above study ☐

CONSENT FORM

Title of project: Investigating pain in the developing human brain

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

Name of parent	Date	Signature

Name of Person taking consent (if different from researcher)	Date	Signature

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 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.

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

PIPP-R Scoring - ROP

Date	Start time for ROP	Duration of ROP	Video number	Study number
D D / M M / Y Y (Day / Month / Year)	h h : m m (24hr)	m m : s s		

Procedural Pain in Premature Infants
poppi

Were retinal photographs taken? Yes ☐ **No** ☐

**NB: For PIPP-R scoring only score gestational age & behavioural state if score generated on any other measure (HR, Sa02, Facial Expression)*

Indicator		0	1	2	3
Gestational age*	CGA at study:	>= 36 weeks'	32 weeks' to 35+6 weeks'	28 weeks' to 31+6 weeks'	< 28 weeks
Behavioural state*	Facial features present in baseline? **Yes** ☐ **No** ☐	Active awake: eyes open, facial movements	Quiet awake: eyes open, no facial movements	Active sleep: eyes closed, facial movements	Quiet sleep: eyes closed, no facial movements
Maximum heart rate	Baseline: / Maximum:	0 to 4 beats per minute increase	5 to 14 beats per minute increase	15 to 24 beats per minute increase	> 24 beats per minute increase
Minimum oxygen saturations	Baseline: / Maximum:	0% to 2% decrease	3% to 5% decrease	6% to 8% decrease	> 8% decrease
Brow bulge	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: > 20 seconds
Eye squeeze	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: > 20 seconds
Nasolabial furrow	Duration:	None: <3 seconds	Minimum: 3 to 10 seconds	Moderate: 11 to 20 seconds	Maximum: > 20 seconds

Latency to any facial expression: ☐☐ **seconds**

Is baby sucking a dummy during facial scoring? Yes ☐ **No** ☐

TOTAL PIPP-R SCORE	

Appendix IV: Poppi trial Parent Information Leaflet

Procedural Pain
in Premature Infants

poppi

Parent Information Leaflet

We would like to tell you about a study this hospital is carrying out for babies who have been born early. Before you decide, it is important for you to understand why the research study is being done and what it will involve. Please read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information.

Study title

Poppi: Procedural pain in premature infants.

Short title: Is morphine an effective analgesic for procedural pain in infants?

What is the purpose of the study?

The overall aim of our research is to find an effective way of treating pain in babies. Babies who are born prematurely are exposed to many painful procedures as part of their essential medical care. As they are unable to tell us when they are in pain, it is difficult to know when they need pain medication or to determine if the pain medication is working. We are looking at whether using a pain relieving medication (morphine) reduces the pain and discomfort caused by routine eye examinations and blood tests. To help us assess this we have developed a way of detecting pain in babies by non-invasively measuring brain activity.

You have been invited to take part in this study because your baby was born prematurely. As part of your baby's routine clinical care your baby will need regular eye tests from 32 weeks gestation to look for retinopathy of prematurity – this is an eye disease related to prematurity. Routinely your baby will be given anaesthetic eye drops for this test as it is considered to be painful and stressful to your baby. They would not be given any additional pain relief (other than standard comfort techniques such as swaddling or use of a dummy), even though the anaesthetic eye drops are not thought to be completely effective. Morphine is a medication we use routinely in the neonatal unit. Your baby will probably have received morphine to make them more comfortable if he or she has been ventilated. We want to find out whether a single dose of morphine given orally can relieve the pain caused by the eye exam and by a routine blood test which will be performed at the same time.

If your baby is due to have an eye exam and blood test we will randomly assign him or her to receive either an oral dose of morphine or a placebo (a harmless inactive solution that does not contain morphine). After your baby receives the medication we will monitor him or her to see if the medication causes any reduction in pain. We will do this by videoing his or her facial expressions, and by gently placing monitoring leads on your baby's body to record oxygen saturations, heart rate, muscle activity and brain activity during the routine blood test and eye exam. Your baby will probably already be monitored for some of these measures as part of their normal care.

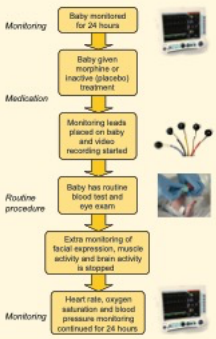
We also want to find out if the pain medication can help make your baby more stable and comfortable for the rest of the day following the examination. In order to determine this, we will record his or her heart rate and oxygen saturations and blood pressure for the 24 hours before and 24 hours after the eye test so that we can identify whether the pain medication has helped make your baby more comfortable.

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 agree to your baby taking part, we will ask you to sign a consent form. If you decide you do not want your baby to take part, we fully understand and assure you that this will have no adverse effect on your baby's normal clinical care.

What is involved in the study?

Babies who are included in the study will have their blood tests and eye tests completed in the routine way. 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.



Medication

When investigating if a treatment works it is necessary to compare it to an inactive (placebo) treatment so we can be sure any differences are due to the treatment. We allocate babies randomly to each group so that the groups are equal in number. This is called a 'blinded' trial which means that neither you, your doctor or the research team will know which treatment group your baby has been allocated to (although if your doctor needs to find out he/she can do so). It is only at the end of the trial when results are being analysed that we will find out which group your baby was in.

Your baby's details will be entered into a computer programme that will randomly select whether your baby will receive morphine or an inactive solution that looks the same (a placebo). This will be given to your baby (in their mouth) one hour before their eye examination. Your baby will have a 50% chance of receiving morphine and a 50% chance of receiving the inactive placebo.

Monitoring for pain

Your baby's reaction to the blood test and eye exam will be recorded on a video camera, and extra monitoring will be used to see if the medication is effectively reducing the discomfort caused by the procedures.

1. We will video your babies face and calculate a pain score based on how much they change their facial expression during and after the procedures. Pain scores are often used on the neonatal unit to measure infant pain.
   Video footage or photographs will not be used for public use and only for study data analysis.
2. We will measure your baby's brain waves using a technique called EEG (electroencephalography). EEG is routinely used on the neonatal unit, on children's wards and in clinics to look at infant brain activity. Monitoring leads (silver discs) will be gently placed on your baby's head to measure the brain waves. This is not painful or harmful to your baby.
3. We will measure how much your baby moves during the eye test and blood test by measuring their muscle activity. We will put two EMG (Electromyography) discs on your baby's thigh muscle to measure their reaction to the tests. EMG is a safe non-invasive technique used to record muscle activity.

4. Your baby will probably already have their heart rate and oxygen saturation monitored for clinical reasons but if these monitors are not in place then a small probe will be wrapped around your baby's wrist to record these measures.

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, EMG and ECG have been used on the neonatal unit, wards and clinics for almost 20 years without adverse effects.

Morphine is a pain-relieving drug that is routinely used in children and adults to treat acute pain. Morphine is routinely used in the neonatal unit to sedate babies when they are ventilated, although to-date few studies have been carried out where morphine has been administered to babies to provide pain relief prior to invasive procedures. The dose of morphine (100 µg/kg) will be administered by mouth and has been approved by the neonatal pharmacist. Although morphine can have effects on breathing rate and blood pressure, a study that used twice this dose did not report any adverse side effects. We do not anticipate that your baby will experience these side effects and your baby will be monitored very closely by the staff on the neonatal unit .

What are the possible benefits of taking part?

We cannot guarantee any direct benefits. At present we don't know whether giving a pain relieving medication (morphine) reduces the pain and discomfort caused by eye exams and blood tests. We are carrying out this study to help doctors make the right decisions about the care of preterm babies in the future.

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 were in the neonatal 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 or were receiving. We require this information as these factors may influence the way a baby copes with pain.

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

What if something goes wrong?

The University of Oxford, as Sponsor, has appropriate insurance in place in the unlikely event that your baby suffers any harm as a direct consequence of your participation in this trial. 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 Eleri Adams on 01865 221356 or email Eleri.adams@ouh.nhs.uk, or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email ctrg@admin.ox.ac.uk.

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 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.

Will taking part in this study be kept confidential?

We will collect personal information about your baby. Due to the nature of neonatal research the National Perinatal Epidemiology Unit (NPEU) policy is to keep personal data for a period of no less than 25 years in order for us to follow-up on health related issues which may become relevant in the future. All relevant medical information and video footage will be kept securely and will only be seen by the research team and people from the sponsor, the Oxford University Hospitals NHS Trust or regulatory authorities who ensure that studies such as these are carried out safely. They may also look at your baby's records to check that the study is being carried out correctly. Unidentifiable data from this study may be shared with other authorised groups who are carrying out similar work.

Will I be paid for taking part in this study?

No payment or expenses will be made for taking part in this study.

Who is organising and funding the research?

The study is sponsored and run by researchers at the University of Oxford and NPEU, Clinical Trials Unit and is funded by the Wellcome Trust and National Institute of Health Research, EME Programme. None of your doctors will be paid for enrolling your baby onto the study.

Withdrawal from the project

You are free to withdraw your baby from the study at any time without having to give a reason. The clinically required procedures will go ahead subject to a review by a suitably qualified clinician. We will ask you whether data already collected may be retained and used for the purposes of the trial.

Who has reviewed the study?

All research that involves NHS patients has been approved by an NHS Research Ethics Committee before it goes ahead. Approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision to take part or not. The Northampton Research Ethics Committee has reviewed and approved this study. This study has also been approved by the Medicines and Healthcare products Regulatory Authority (MHRA) who oversee the safety of new and existing drugs.

What will happen to the results of the research study?

At the end of the study, the results will be analysed and published in a medical journal. We will send you a summary of the final results of the study. A copy of the full journal article can be requested from the NPEU. Your baby will not be identified in any report or publication about the study.

Contact for further information:

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If you would like to contact an independent organisation to discuss the inclusion of babies in research studies generally we suggest that you contact Bliss, a special care baby charity. Bliss contact details are:

Bliss
for babies born too soon, too small, too sick

Chapter House, 18-20 Crucifix Lane, London SE1 3JW

Freephone Family Support Helpline: 0500 618 140

Thank you for reading this information sheet

The POPPI trial is funded by the National Institute for Health's EME Programme and The Wellcome Trust

Appendix V: Poppi trial Consent form

Procedural Pain in Premature Infants
poppi

Consent Form

Please complete in black ballpoint pen

Study Number: ☐☐☐☐☐

Baby's name: _____

Title of study: POPPI Trial (Procedural pain in premature infants)

Short title: Is morphine an effective analgesic for procedural pain in infants?

Chief Investigator: Dr Eleri Adams

Principal Investigator: Dr Rebeccah Slater

		Please initial box
1.	I confirm that I have read and understood the information leaflet (Version 4.0, 12 April 2016), 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 my baby at any time, without giving any reason and without my baby's medical care or legal rights being affected.	☐
3.	I understand that relevant sections of my baby's medical records and data collected during the study, may be looked at by individuals from the Research team, Sponsor, Regulatory Authorities or Oxford University Hospitals NHS Trust, where it is relevant to my baby taking part in this research. I give permission for these individuals to access to my baby's records.	☐
4.	I agree that personal identifying information will be collected and stored at the co-ordinating centre (NPEU CTU). I understand that any information will be treated confidentially.	☐
5.	I consent to my baby being videoed during the study. I understand that recorded images will not be used for public use, only analysis. I understand that only anonymised data will be published or presented at meetings.	☐
6.	I agree for my baby to take part in the POPPI Trial.	☐

Name of parent/guardian	Name of health professional taking consent
Signature	Signature
D D / M M / Y Y	D D / M M / Y Y

The POPPI trial is funded by the National Institute for Health's EME Programme and The Wellcome Trust

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REC Reference 15/EM/0310
Version 4.0 12 April 2016

npeu Clinical Trials Unit

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Original copy to Poppi Co-ordinating Centre, 1 x copy to Parent; 1 x copy to site file; 1 x copy to baby's notes

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