

Imaging nociceptive brain activity in the newborn infant



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Abstract

In this thesis electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) are used to investigate the temporal and spatial patterns of noxious-evoked brain activity in newborn infants. EEG was used to investigate responses to graded intensities of experimental noxious stimulation, and evoked brain activity was compared with behavioural and spinal cord activity constituting common surrogate measures of pain in infants. Nociceptive-specific brain activity was elicited in response to all forces of experimental noxious stimulation (applied forces: 32 - 128 mN). In addition, the magnitude of the noxious-evoked response was positively correlated with the magnitude of reflex leg withdrawal, and this relationship was observed in the absence of changes in facial expression.

As fMRI had not previously been used to investigate nociceptive processing in infants at 3 Tesla, initial experiments were conducted to optimise the acquisition parameters. The results from optimisation showed that an echo time of approximately 50 ms should be used in future fMRI studies in infants. Experiments conducted alongside this optimisation used fMRI to investigate the cortical and subcortical structures activated by experimental noxious stimulation (applied forces: 32 - 128 mN) in newborn infants (0 - 11 days old). This was compared with noxious-evoked brain activity in adults (applied forces: 32 - 512 mN). Experimental noxious stimulation evoked a widespread pattern of brain activity in newborn infants that overlapped with the network of brain regions activated by nociceptive processing in adults.

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Abbreviations

ACC - Anterior cingulate cortex

BET - Brain Extraction Tool

BOLD - Blood oxygen level dependent

CSF - Cerebrospinal fluid

EEG - Electroencephalography

EMG - Electromyography

EPI - Echo Planar Imaging

FEAT - FMRIB's Expert Analysis Tool

fMRI - functional Magnetic Resonance Imaging

FMRIB - (The Oxford Centre for) Functional MRI of the Brain

FSL - FMRIB's Software Library

GA - Gestational age

HRF - haemodynamic response function

ISI - Inter-stimulus-interval

MCFLIRT - Motion correction with FMRIB's linear image registration tool

MELODIC - Model-free fMRI analysis using probabilistic independent components

MRI - Magnetic Resonance Imaging

OFC - Orbitofrontal cortex

PACE - Prospective Acquisition Correction

PCA - Post conceptual age

PIPP - Premature Infant Pain Profile

ROI - Region of interest

RVM - Rostral-ventral medulla

STT - Spinothalamic tract

T - Tesla

TE - Echo time

TR - Repetition time

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Chapter 1

Introduction

1.1. Overview of the Introduction

How the human brain develops such that we can experience pain is fundamental to our understanding of human development and paediatric care. In this thesis, electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) are used to investigate noxious-evoked brain activity in newborn infants following non-skin-breaking experimental noxious stimulation. A parallel fMRI study was also conducted in adult participants in order to compare the pattern of noxious-evoked brain activity elicited in adults with that observed in infants.

The overall aim of these investigations was to characterise the temporal and spatial patterns of brain activity evoked by nociceptive stimuli in newborn infants. Reproducible noxious-evoked brain activity has previously been recorded in premature and newborn infants using near-infrared spectroscopy (NIRS) and EEG in response to clinically-required acute noxious procedures [1-3]. However, the reliance on clinical procedures has meant that noxious-evoked brain activity had not been recorded and characterised in newborn infants who did not require invasive clinical procedures, such as clinical heel lancing or cannulation. In this project non-skin-breaking acute mechanical stimulators (trade name: PinPrick, MRC systems) have been used to apply an experimental nociceptive stimulus to the heel of infant participants. These stimulators are commonly used to assess nociceptive sensitivity in chronic pain patients [4].

As the distinction between pain and nociception is integral to the discussion herein, this Introduction begins with a brief description of the two terms. This is followed by an overview of the complex components that underlie the neurobiology of nociceptive

processing in adults, providing an important basis for the comparison between the developed and developing nociceptive systems that are carefully compared throughout this thesis.

Pain is an experience that has developed to protect organisms from actual or potential harm. Pain is also an extremely common health problem and as such is a frequent occurrence during clinical care. The assessment and treatment of pain is complicated further by factors that influence pain perception, such as prior experience or individual sensitivity to pain. The current gold standard for assessing pain is verbal report, allowing the subjective pain experience to be considered in the assessment or treatment of pain. However this presents a problem in patients who are unable to report their pain.

Historically, pain has been undertreated in infants due to a prevailing view that infants could not feel pain. Approximately 30 years ago, a newborn infant would have undergone surgery using muscle relaxants without the provision of adequate analgesia [5]. At this time, this was a relatively common practice in many hospitals across the world, and was due, in part, to the belief that the immaturity of an infant's nervous system meant they were unable to experience pain. Although infants are now routinely provided with pain relief both during and after surgery, analgesia for acute painful procedures is still seldom provided.

Exposure to clinically essential painful procedures is a common occurrence for infants receiving neonatal intensive care, with reports that infants receive an average of 11 painful procedures per day [6]. In addition it is often the youngest and sickest infants that will be exposed to the greatest number of invasive painful procedures and numerous reports have indicated that analgesia is seldom provided [6-9]. The lack of provision of analgesia is coupled with a lack of understanding about how pain is processed in the infant's nervous system, and limitations with regards to the sensitivity of currently available tools for measuring pain in this population. The fourth section of this Introduction therefore comprises a brief overview of the history that led to the lack of

appreciation of pain in infancy and an outline of current practice with regards to assessment and treatment of pain in this population. Next, an overview of current knowledge underlying the development of early human pain processing is provided. While most of the infants studied were born at term (i.e. on or after 37 weeks gestational age), six premature born infants aged between 35 and 37 weeks gestation also had an fMRI scan of their brain activity. Hence the section on development of early pain processing predominantly focuses on changes that occur during the last trimester of pregnancy.

To conclude, detailed descriptions are provided of how brain imaging has been utilised thus far to investigate pain processing in adults and infants. The focus of this final section is primarily on EEG and fMRI, providing general background information about the methodologies as well as detailed information on what each technique can help uncover about nociceptive processing in newborn infants.

1.2. An introduction to pain and nociception

The International Association for the study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [10]. This carefully worded statement “avoids tying pain to the stimulus” and crucially encompasses the multiple dimensions involved in the complex experience of pain. Pain can often be experienced following acute tissue damage, but can also be experienced in the absence of tissue damage. The IASP definition of pain specifies that “if they (a person) regard their experience as pain, and if they report it in the same way as pain caused by tissue damage, it should be accepted as pain.” One prominent feature of this definition is the influence that verbal report can have on the identification of pain in individuals. While verbal report is considered a “gold-standard” and is the most commonly adopted pain assessment method in clinical settings, in certain individuals for whom verbal report may be difficult or impossible (e.g. non-verbal infants, aphasic and aphonic patients), it becomes necessary to adopt alternative surrogate

measures to assess and treat pain. IASP acknowledged this challenge of pain assessment in non-verbal populations when they issued an update to their definition of pain in 2011:

“The inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment.” [11]

The experience of pain requires cognitive processing which occurs within the brain, and more often than not, is evoked by events that occur in the peripheral skin, viscera, bone or muscles. Nociceptors (receptors for detecting noxious stimuli) are present on primary afferents, which are the neurons responsible for detecting actual or potentially damaging stimuli. Therefore nociception is “the neural process of encoding noxious stimuli” [10]. This definition, once again provided by IASP, includes a note as follows:

“Consequences of encoding may be autonomic (e.g. elevated blood pressure) or behavioural (motor withdrawal reflex or more complex nocifensive behaviour). Pain sensation is not necessarily implied.” [11]

It is possible to have nociception in the absence of pain, and similarly pain may be experienced in the absence of a noxious stimulus. Therefore even when the origin of the nociceptive input may no longer exist or be immediately apparent, it is still possible for strong experiences of pain to be reported. Pain can be influenced by a plethora of factors ranging from the current state of the stimulated area (i.e. whether tissue injury has previously occurred), to the psychological state of the individual or higher-order cognitive processing such as mood or attention. Therefore it is the combination of these factors that contributes to the complexities of the pain experience. A dynamic underlying neurobiology of nociceptive pathways has been described, which affords the flexibility required to combine information from so many sources. Overall, pain is an essential component for individual survival and provides protection from harmful, damaging inputs.

1.3. An overview of peripheral and central nociceptive pathways

1.3.1. Primary afferent nociceptors

There are various types of primary sensory afferent neurons present within the skin. Different afferent neurons respond to varying qualities of sensory inputs, for example, noxious or non-noxious inputs. Large diameter, myelinated sensory afferents, called A α - and A β -fibres, are responsible for conducting non-noxious mechanical inputs and are activated by light touch and proprioceptive signals. In contrast A δ - and C-fibres respond to noxious stimuli. Most A δ -fibres (and some C-fibres) are mechanical nociceptors while the majority (approximately 70 %) of C-fibre nociceptors (and some A δ) are polymodal [12]. Polymodal nociceptors are capable of responding to mechanical, thermal and chemical inputs in the noxious range. C-fibre nociceptors are small diameter unmyelinated neurons, with free nerve endings that terminate within the upper epidermal layers. These fibres have thermal thresholds of 43°C and are predominantly activated by laser heat stimuli (a highly localised thermal stimulus which only activates superficial nociceptors in the epidermis [13]). A δ -fibres are small diameter myelinated neurons of which two types have been identified. Type-I A δ -fibres are sensitive to noxious mechanical stimuli and can be found in hairy or glabrous skin [14]. Type-II A δ -fibres have a high threshold to mechanical stimuli but exhibit lower thresholds to thermal and electrical stimulation compared to Type-I afferents [14].

A δ -fibres are myelinated and have diameters of between 2.0 - 6.0 μm [14]. A δ -fibres conduct action potentials at a speed of 12 - 30 m/s, and are the first of the nociceptors to signal a threatening stimulus [14]. This fast detection is accompanied by a precise localisation of the stimulus and may often result in a reflexive response, aiding the quick and effective withdrawal from harmful stimuli. Activation of A δ -fibres is associated with sharp or pricking sensations [14]. C-fibre afferents are unmyelinated with a diameter between 0.4 - 1.2 μm and conduction velocity between 0.5 - 2.0 m/s [14]. These slower afferents also have slower adaptation to changes in the levels of noxious inputs and are

responsible for more sustained signalling of pain following an injury. C-fibres trigger autonomic responses and have been associated with the dull, throbbing pain that follows an insult and promotes protective behaviours during healing [14].

1.3.2. Organisation of nociceptive pathways in the spinal cord

The dorsal horn of the spinal cord is a key point for modulation of sensory inputs into the central nervous system. Primary afferent fibres predominantly terminate within the superficial laminae (lamina I and II) of the dorsal horn [14]. Primary afferents connect with different second order neurons including (i) projection neurons that decussate before ascending the spinal cord *via* the spinothalamic tract, (ii) interneurons responsible for modulation of motor neuron activity and (iii) descending projections from the brainstem and cortex. Interneurons can be excitatory or inhibitory and the balance between excitation and inhibition determines responses to noxious and non-noxious stimuli. These interactions all contribute to the complexity of nociception and the experience of pain within individuals [15,16], and it is thought that alterations to this dynamic central modulation may underlie some chronic pain states [17].

The neurochemical characteristics of primary C-fibre afferents appear to determine the location of termination within the dorsal horn of the spinal cord [18]. C-fibres can be classified as peptidergic or non-peptidergic. Peptidergic fibres express the peptide neurotransmitter named substance P and terminate almost exclusively within lamina I of the dorsal horn [19,20]. On the other hand most non-peptidergic C-fibres terminate in inner lamina II while myelinated A δ -fibres terminate in laminae I (and a subset in lamina V) in the dorsal horn [20].

1.3.3. Ascending cortical pain pathways

Distinct parallel projections have been described which originate in the dorsal horn and transmit messages about nociception to the brain [21]. Lamina I contains a small percentage of ascending projection neurons (approximately 5 %) which form a part of the neo-spinothalamic pathways [20,22]. These neurons quickly transmit nociceptive signals *via* the lateral spinothalamic tract (within the ventral horn), and project to areas of the thalamus and thence the cortex. Alternatively projection neurons that originate within lamina V ascend *via* “phylogenetically old pathways”, including the spinoreticular, and spinomedullary pathways [14,21]. Such pathways have been shown to connect with neurons projecting to the hypothalamus or amygdala [21]. It has therefore been suggested that this pathway encodes affective pain processing, while the neo-spinothalamic tract is responsible for the earlier sensory part of the cortical response to nociceptive inputs [23]. The existence of parallel pathways responsible for the different qualities of pain processing is an interesting observation that has been cited amongst the literature since the late 1980s [24].

1.3.4. Descending cortical pain pathways

An early study by Reynolds in 1969 showed that activation of the periaqueductal grey (PAG) by electrical stimulation induced profound, natural analgesia [25]. This response was so strong that Reynolds was able to conduct a highly invasive laparotomy surgery in three rats without having to provide any anaesthesia [25]. It is now known that numerous brainstem nuclei including the PAG, rostroventral medulla (RVM) and nucleus cuneiformis (NCF) release inhibitory endorphins and enkephalins, which are the body’s natural opioids [14]. These brainstem nuclei have projections down to the spinal cord that are responsible for providing the balance between inhibitory and excitatory inputs that mediate peripheral nociceptive drive. The PAG is a region of grey matter that surrounds the fourth ventricle in the midbrain. This nucleus has projections to the RVM, which in turn has three types of neuronal projections (i) ON (ii) OFF and (iii) Neutral cells [26]. All three

neuronal types project to the dorsal lamina (I, II and V) of the spinal cord where they can modulate nociceptive inputs [26]. OFF cells tonically fire and inhibit ON cell activity *via* inhibitory interneuronal connections [27]. They also cease firing in response to noxious stimuli [26]. ON cells do not fire until they detect nociceptive inputs and therefore typically facilitate spinal nociception [26]. Neutral cells do not fire in response to pain [26]. These distinct neuronal types are under constant modulation, maintaining the delicate balance between excitation and inhibition that allows our central nervous system to rapidly respond to incoming nociceptive stimuli.

1.3.5. Supraspinal nociceptive processing

A complex network of brain regions has been identified, which respond to nociceptive stimulation. This includes numerous cortical and subcortical brain areas [28,29]. Brain areas involved in nociceptive processing include the somatosensory brain regions (S1, S2), insula, frontal cortex, thalamus, putamen and brainstem (see figure 1.1). As mentioned already, the multiple nociceptive tracts within the spinal cord can broadly be segregated into two main pathways, thought to be preferentially involved in the sensory and affective or cognitive aspects of nociceptive processing. This segregation complements the definition of pain as a “sensory and emotional experience”. Such partitioning is also apparent within regions associated with supraspinal pain processing. For example brain areas such as the thalamus, somatosensory cortices (including the primary and secondary regions) and posterior insular (PI) cortices have all been strongly associated with the sensory aspects of pain perception [30-37]. Brain areas associated with affective and cognitive/evaluative dimensions of pain include the amygdala, orbitofrontal cortex (OFC), anterior cingulate cortex (ACC), anterior insular (AI) cortices and the prefrontal cortex [31,38-45].

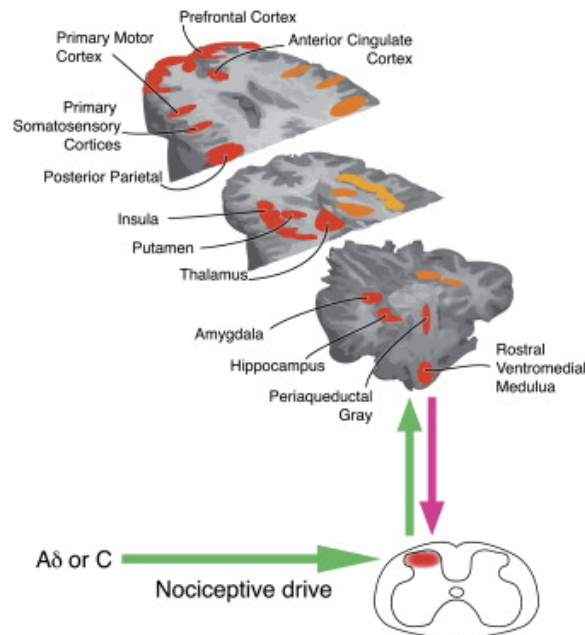


Figure 1.1: Schematic representing numerous brain regions involved in pain processing in adults. Reprinted from Neuron, [29], with permission from Elsevier.

Historically the thalamus was considered a potential contender for a “pain centre” in the brain, however surgeries to lesion the thalamus in patients with chronic pain often resulted in increased pain sensitivity rather than abolishment of pain [46]. While the thalamus may not constitute the pain centre, it is known to be a prominent relay centre for the detection and processing of numerous sensory stimuli (i.e. visual, auditory and tactile inputs) [47,48]. The advent of neuroimaging techniques has introduced the possibility of studying pain in humans non-invasively. Since the introduction of such technology, our understanding of pain has considerably advanced. The use of neuroimaging techniques for studying pain processing in humans will be discussed further in section 1.6.

1.4. Infant pain

In the preceding paragraphs of this Introduction, the complex and dynamic neurobiological framework that underlies nociception and the experience of pain was described. However, at what age does the human nervous system become developed enough to transduce, propagate and process nociceptive information in a similar way to

the mature adult? This question was originally discussed in a clinical context, when investigators wanted to understand whether human infants were capable of feeling pain. This was coupled with an important change to the way that pain was considered. The late 19th century was the era in which the importance and diagnostic value of pain began to be recognised [49]. Prior to the introduction of anaesthesia during surgical procedures in 1846, pain was not considered to be an experience that required treatment [50]. On the contrary, pain was often considered to be an unavoidable and necessary experience in order to promote healing [51]. However, in a landmark lecture series, delivered by Professor J. Hilton between 1860 and 1862, it is clear that attitudes towards pain were changing [49]. Pain was no longer considered a beneficent, unavoidable and necessary experience, but was instead discussed as an unpleasant experience that could and should be treated [49]. Once the treatment of pain became incorporated into clinical care, the difficult question of how to treat pain in non-verbal populations was consequently projected to the forefront and became an important clinical concern.

This section begins by providing a brief historical overview of the understanding of pain in infants, followed by a description of how inquisition into the biological development of infant pain led to the emergence of different techniques for assessing pain in this non-verbal population. To conclude, an outline of the current clinical scenario is provided, including a description of the high level of pain exposure that premature and sick infants are faced with during their time in intensive care.

1.4.1. Historical overview of infant pain

The last 40 years has seen a rapid increase in the number of clinical and research studies investigating the development of nociceptive pathways and pain in infants. This increase in awareness and motivation to understand infant pain has helped refute the long-standing denial surrounding pain in infants. Denial about pain in infants did not begin as an independent phenomenon; in the early 20th century the emergence of the need to treat

pain and suffering had infiltrated clinical practice. Prior to this it was thought that pain was beneficial and even led to debate regarding the value of anaesthesia [51].

A recent review article by Rodkey and Riddell investigated the available infant pain literature with an aim to provide some understanding of the possible views and motivations that ultimately led to, what they describe as, “infant pain denial” [51]. In their paper the authors propose that a combination of severe caution over interpretation of experimental observations and a lack of substantial evidence to refute the common misconceptions regarding infant pain ultimately “contributed to the denial of infant pain” [51]. Early attitudes towards infants included the idea that their limited mental capacity meant they were perceived to be “lower beings” and as such observed responses to sensory stimulation were considered to be purely reflexive [51]. In fact this viewpoint was shared by so many that even when clear pain responses were elicited when infants were pricked in various regions of their bodies with sharp needles, researchers were innately biased towards concluding that infants did not demonstrate an ability to feel pain [52].

During the early 20th century, experimentation of “the reflexive nature” of infant pain continued and extreme caution over the interpretation of experimental findings also persisted. There were however few cases where pain responses were described; one example includes research by Blanton in 1917, where she reports clear facial expression responses and reflexive leg withdrawal of both legs following lancing for blood collection or needle pricks. However, it is striking that despite describing such responses, Blanton then demonstrates reluctance to challenge the well-ingrained consensus that babies do not feel pain. In her report, she concluded that these responses were merely evidence of complex and advanced pain behaviours (i.e. reflexes) and were therefore not an indication that infants feel pain (Blanton’s work is reported in [52]). During this time, a book entitled *Paediatric Anaesthesia* was published which focussed on the use of anaesthesia in children. This was a landmark event as it demonstrated that pain in children had become a serious consideration during clinical practice [52].

In the later part of the 20th century, the real debate over infant pain began to emerge amongst clinicians and scientists. In 1926, Pieper, a German paediatrician published a paper in which he conclusively reported that infants were responding to painful stimulation [51]. This was in direct contrast to work by McGraw who concluded that such observed responses were not convincing evidence to suggest that infants were not decorticate [51,52]. Therefore the belief that infants did not have the capacity to experience pain had yet to be overturned and had by this point also guided anaesthetic protocol. Initially, it was considered appropriate to conduct surgery as soon after birth as possible since infants demonstrate the least reactivity to pain during this period [52]. Since there had been no conclusive demonstration that infants could feel pain, concerns over the side effects associated with anaesthetics resulted in increasing numbers of infants undergoing surgery under the sole administration of muscle relaxants, which were used to stop the infants moving during the surgical procedures.

It is fascinating to discover that much of this was occurring unbeknownst to the public and even to the parents of those infants requiring surgery. 1985 was the year in which the case of one such infant, Jeffrey Lawson, made it into the mainstream media and marked the beginning of a fundamental change in clinical practice. At less than 26 weeks gestational age, Jeffrey Lawson was an extremely young premature infant who required a heart operation shortly after birth. In accordance with clinical practice at the time, Jeffrey's surgery was conducted after he had been administered with a paralytic agent called Pavulon [53]. While Jeffrey survived his surgery, he passed away five weeks later and it wasn't until his mother enquired about the surgery that she realised that he had not been provided with any pain relief. Soon after her son's death, Lawson wrote a letter to the journal, *Birth*, in which she details the numerous organisations that she had contacted in an attempt to increase awareness about this issue and within her letter, she describes the overwhelming rejection she was met with [53]. In the same journal issue, a letter from Harrison also describes the great anguish felt by her and others who were now aware that much of the intensive care their children received did not involve any form of pain relief [53]. These letters convey the unquestioning belief held by physicians that

infants were not capable of feeling pain. The belief was so strong that Harrison recounts the case of one parent who refused to sign a surgical consent form once she found out her child would not be given anaesthetic and was subsequently “reported to local authorities as an abusive parent” [53].

While all these events were occurring in the United States of America, in the United Kingdom, at Oxford University, Anand and colleagues systematically measured changes in cortisol and other metabolites such as glucose, insulin and lactate in infants just before and up to 24 hours after they underwent surgery [54]. From these investigations the group demonstrated that high levels of adrenaline were being released during surgery when limited pain relief was provided [54]. This also correlated with higher concentrations of glucose which remained for over 6 hours following surgery and were coupled with reduced insulin concentrations [54]. Anand and colleagues stated that such prolonged periods of hormonal imbalance could have “detrimental effects on the renal cortex and the cerebral substance” and they also noted that this could be more of a problem in infants born prematurely as these infants showed less capability of increasing their insulin levels compared with term-born infants [54]. In this paper the authors suggest that better provision of anaesthesia could help reduce the stress responses observed in infants and, in 1987, they went on to publish their results from a randomised controlled clinical trial investigating this very premise [5]. This seminal paper demonstrated that infants receiving fentanyl for analgesia during surgery, not only demonstrated significantly reduced hormonal responses following surgery but they also had better outcomes and less post-operative complications, with 50 % of infants in the non-fentanyl group requiring increased ventilator support and exhibiting frequent attacks of bradycardia compared with approximately 13 % in the fentanyl group [5].

The events of Jeffrey Lawson together with this new clinical study ultimately culminated in a statement published by the American Society of Anaesthesiologists and the American Academy of Pediatrics Committee in 1987, in which they wrote:

“There is an increasing body of evidence that neonates, including those born preterm, demonstrate physiological responses to surgical procedures that are similar to those demonstrated by adults and that these responses can be lessened with anaesthetic agents” [55].

These investigations led to an era in which scientific enquiry on infant pain was reignited and since then many studies have investigated the epidemiology and neurobiology of pain in infants requiring prolonged clinical care (described in section 1.4.3). Therefore, while it is now accepted that infants are capable of detecting noxious inputs, the complexity of pain and the difficulty of assessing pain in non-verbal populations means that the assessment and identification of pain is still a challenging clinical problem in the infant population.

1.4.2. Infant pain assessment tools

Observations of infants undergoing clinically painful procedures have been conducted with the aim of identifying a reliable method of measuring pain in this population. The investigation of behavioural responses evoked by nociceptive stimuli includes changes in facial expressions, body movements and cry frequencies. Physiological responses such as changes in heart rate and oxygen saturations have also been investigated [52,56-59].

Grunau and Craig were the first investigators to systematically observe facial expression change following noxious and non-noxious procedures and to compare the responses in order to ascertain specificity for pain [60]. In order to assess facial expression in a systematic way, they adapted the facial action coding system (developed to assess emotional responses in adults [61]) for use in infants. By adapting this method, they presented the first tool for assessing responses to pain in infants, which identified facial actions that were reliably elicited following a painful procedure, and are to this day commonly used to assess infant pain in clinical practice. Dubbed the neonatal facial action coding system (NFCS), this measure incorporated facial actions included brow bulge, eye squeeze and nasolabial furrow, which were three of the most frequently observed actions (occurrence: 96 - 97 %) following a heel lance [60]. In this study and

subsequent studies, Grunau and Craig demonstrate that facial expression shows specificity for noxious inputs by showing that all three facial actions described are elicited by clinically required heel lance, or vitamin K injections shortly after birth, while they are not elicited by non-painful tactile procedures such as swabbing of the heel or thigh [60,62,63].

As well as systematically assessing facial expression, Grunau and Craig also aimed to assess two factors, sleep/awake state (i.e. quiet sleep, active sleep, quiet awake or active awake) and sex, and their relation to pain responses [60]. The authors hypothesised that these factors may affect facial expression response as well as the cycle, latency and duration of cry [60]. While behavioural responses to non-noxious stimuli were modulated by the infant's state, the three facial actions described were equally elicited following a heel lance, regardless of the infant's sleep state [60]. Other facial actions, namely taut tongue and vertical mouth stretch, were also greatest in response to heel lance when infants were in the "quiet awake" state [60].

Sex was not reported to have any significant effect on facial expression responses [60]. However boys were reported as crying more quickly and for longer cycles compared with girls [60]. Other researchers have since investigated the usefulness of cry as a tool to assess pain in infants. While a shorter latency and longer duration of cry have been associated with noxious stimuli, the variability of the presence of cry has limited the utility of this measure. For example, some infants cry to non-noxious as well as noxious stimuli while other infants do not cry at all in response to noxious stimulation. Furthermore, clinical interventions such as being ventilated or intubated, can lead to circumstances that limit the ability of an infant to cry [62,64]. In addition, Grunau and Craig appear to infer that cry will inevitably be associated with environmental changes perhaps implying that facial expression makes a more useful outcome measure [60,62].

Physiological indicators of pain including changes in heart rate, oxygen saturations and respiratory rate have also been investigated as objective quantitative measures in

premature and term infants [59,63,65,66]. Overall, heart rate and oxygen saturation responses are reliably measured following noxious stimuli but they do not demonstrate specificity for pain [63,65,66]. This observation has been confirmed in both preterm and term infants, and is not surprising since substantial variability in autonomic responses has previously been reported across age [63]. When noxious-evoked facial expression responses were compared across age in infants, it was demonstrated that the magnitude of facial expression is positively correlated with gestational age of the infant. Importantly, infants as young as 25 weeks gestation show observable facial expression responses to noxious stimuli [65], and this accordingly increases with age.

The factors and behavioural measures described, including sleep state, gestation of infant, and changes in heart rate, oxygen saturations and facial expression, have since been validated as part of a commonly used tool for assessing acute procedural pain in infants, called the Premature Infant Pain Profile (PIPP) (see table for how to score PIPP in table 1.1) [59]. PIPP allows calculation of pain scores that are additionally weighted for the infant's sleep state as well as gestational age at the time of the study. PIPP is one of a large number of scoring tools developed for assessing acute procedural, prolonged and postoperative pain in infants [67,68]. PIPP is commonly used because it is relatively simple to implement, and is one of the only multidimensional tools that includes facial expression and has been developed and validated for use in both preterm and term infants [52]. Other scoring tools such as Neonatal Infant Pain Scale (NIPS) and Behavioural Indicators of Infant Pain (BIIP) also incorporate facial expression [69,70]. NIPS has a high inter-rater reliability of 0.92 - 0.97, which is 0.07 greater than PIPP, however all of its scoring is done on a binary scale, severely impacting the sensitivity of the tool [52,69]. While BIPS is more descriptive, it is newer and therefore less well utilised and less validated compared with PIPP. However as it focuses more on facial expression and body movements without scores for the more variable physiological parameters, it may present a more sensitive tool for use in clinical settings in future [70].

Indicators	0	1	2	3
GA in weeks	≥ 36 weeks	32 to 35 weeks and 6 days	28 to 31 weeks and 6 days	< 28 weeks
Observe the NB for 15sec				
Alertness	Active Awake Opened eyes Facial movements present	Quiet Awake Opened eyes No facial movements	Active Sleep Closed eyes Facial movements present	Quiet Sleeping Closed eyes No facial movements
Record HR and SpO ₂				
Maximal HR	↑ 0 to 4 bpm	↑ 5 to 14 bpm	↑ 15 to 24 bpm	↑ ≥ 25 bpm
Minimal Saturation	↓ 0 to 2.4%	↓ 2.5 to 4.9%	↓ 5 to 7.4%	↓ ≥ 7.5%
Observe NB for 30 sec				
Frowned forehead	Absent	Minimal	Moderate	Maximal
Eyes squeezed	Absent	Minimal	Moderate	Maximal
Nasolabial furrow	Absent	Minimal	Moderate	Maximal

Absent is defined as 0 to 9% of the observation time; minimal, 10% to 39% of the time; moderate, 40% to 69% of the time; and maximal as 70% or more of the observation time. In this scale, scores vary from zero to 21 points. Scores equal or lower than 6 indicate absence of pain or minimal pain; scores above 12 indicate the presence of moderate to severe pain.
GA – Gestational Age. NB – Newborn.

Table 1.1: Description of the use of the PIPP pain assessment tool. Table describes the scoring methods for (PIPP) [59]. Reprinted from The Clinical Journal of Pain, [59], with permission from Wolters Kluwer Health, Inc.

Increasing emphasis is being placed on the use of multidimensional measures for assessing infant pain [65,71,72]. The availability of newer brain imaging methodology has also allowed the observation of noxious-evoked brain activity in infants (discussed further in section 1.6). One study using such methods reported that 30 % of infants who demonstrated increased cortical activity in response to nociceptive stimulation showed no concomitant change in facial expression [73]. This example demonstrates how assessment of pain using behavioural measures alone may lack sensitivity in a large percentage of infants. Therefore, the investigation of surrogate measures of pain that correlate with noxious-evoked brain activity could lead to a more sensitive way of assessing pain for use in a clinical setting [72,74].

1.4.3. Epidemiology of infant pain

In 2010, the World Health Organisation (WHO) published their first global report on premature birth [75]. They report that an estimated 12.9 million births in 2005 were preterm. This value rose to 15 million of all global births by 2007 [76]. According to the Office for National Statistics (the UK's largest independent producer of official statistics),

7.3 % of infants born in England and Wales in 2012 were premature, and 7 % of those infants were born at less than 28 weeks gestation [77]. Infants born too soon will be admitted to neonatal care units where they will likely stay for the remaining duration of a normal pregnancy. Numerous epidemiological studies conducted over the last 20 years have recorded the number of invasive painful and stressful procedures infants are exposed to during their initial two weeks in intensive care [6-9,78-80].

The report by Barker and Rutter, conducted in the UK, was designed to investigate the occurrence of certain procedures [7]. Barker and Rutter recorded information on 3283 invasive procedures conducted in 54 infants admitted to their neonatal intensive care unit (NICU) during the study period [7]. 56 % of the recorded procedures were heel pricks for blood sampling and the youngest infant in the group, born at 23 weeks gestation, underwent 488 procedures in total, where 212 were endotracheal suctioning and 218 were heel pricks [7]. This total number was approximately 100 more than the infant who received the second highest number of invasive interventions [7].

Unfortunately such high exposure to pain was not out of the ordinary. Johnston and colleagues conducted the first multicentre trial investigating exposure to pain in 14 NICUs in Canada *via* a survey [78]. Of the 239 patients included in the study the average number of procedures per day equalled 2. However, only 21.3 % of patients were recorded to have received analgesia during the study period and of these, medication was provided for an invasive procedure only 17 times [78]. 10 years on, in another large multicentre trial conducted in France, Carbajal and colleagues reported that infants were receiving an average of 10 painful procedures per day with some form of analgesia (either pharmacological or non-pharmacological) provided only 20.8 % of the time [9]. Similarly to Barker and Rutter, Carbajal and colleagues report that the youngest and sickest infants were those exposed to the greatest number of invasive, painful procedures during their time in hospital [7,9]. Given that the study by Carbajal *et al.* was restricted to 14 days per unit, it is shocking that some infants required over 300 painful procedures in that short period of time.

These studies demonstrate that while attitudes towards infant pain have changed since 1987 and infants are no longer subjected to surgery without pain relieving drugs, procedural pain continues to be undertreated in newborn infants during intensive care. While it has become recommended practice to provide analgesia to infants during and after surgery, it is still common in neonatal units in the UK to perform lumbar punctures without any analgesia. In 2008 it was reported that 65 % of NICUs did not have any pain management guidelines [81]. Together, the lack of pain management protocols and the limited availability of appropriate analgesia means that many acutely painful procedures continue to be performed on infants without any pharmacological analgesia. Many analgesics used in neonates and infants have never been licensed for use in this population [82]. Surveys of pain management in one NICU in the Netherlands reported that the use of analgesia for acute painful procedures went from 60.3 % to 36.6 % between 2001 and 2009, which they attributed to studies reporting that morphine was not efficacious, and had detrimental long-term consequences on subsequent development, in newborn infants [6].

The evidence demonstrates that infants undergoing intensive care are still exposed to a high number of invasive painful procedures each day, and analgesia is currently infrequently provided. This, coupled with limited available approved analgesic treatment and further limitations of pain assessment tools, means that clinicians caring for sick infants still have limited tools to adequately detect pain, and to manage and treat it safely and effectively.

1.5. Development of peripheral and central nociceptive pathways

At the beginning of the previous section, the following question was proposed: at what age does the human nervous system become developed enough to transduce, propagate and process nociceptive information in a similar way to the mature, adult system? In the

subsequent section research from the last three decades is presented, in which this question has been investigated in both animal models and human infants.

As many of the studies investigating development of nociceptive pathways have been conducted in rat pups, it is important to conceptualise how the developmental trajectories of rats and humans compare. In 1991, Romijn *et al.* reviewed available experimental literature in order to answer this query. Overall, the authors reasoned, “the pattern of electrocortical activity best reflects the stage of functional maturation of the neocortex” [83]. In this instance, electrocortical activity specifically referred to patterns of brain activity measured during quiet and active sleep using EEG. Hence, they subsequently compared the age at which such patterns emerged in the two species. The study found that these patterns appear at about 35 - 37 weeks, while the same patterns are recorded in rat pups between 10 - 13 postnatal days, coinciding with a marked maturation in the recorded EEG activity [83]. Therefore, this indirect evidence suggests that rat pups of 12 - 13 days or older may best approximate an infant of 37 weeks gestational age [83]. This evidence also highlights that the first two weeks of a rat pup’s life are comparable to preterm and not term infants.

1.5.1. Development of nociceptive afferents

Primary afferent neurons with functioning nociceptors are required for transduction and propagation of nociceptive inputs to the central nervous system. In a series of experiments investigating the properties of primary cutaneous afferents in rat pups *in vivo*, Fitzgerald and colleagues showed that primary afferents are present and functional in the periphery as well as in the dorsal horn of the spinal cord from postnatal day 0 (P0) [84-86]. Primary afferents show responses to both pinching and electrical stimuli, while responses to a highly irritant chemical stimulus (mustard oil), which activates TRPV1 receptors on C-fibres, [14], produced no reflex withdrawal response until P10-11 [84]. By the end of the first two postnatal weeks, (approximated to be a comparable developmental age to a term infant [83]) the firing amplitudes of A and C-fibre afferents are similar to

that recorded from adult rats (reported common amplitudes of 100 μ V - 1 mV) but with longer duration (0.4 - 0.6 ms in rat pups compared with 0.2 - 0.3 ms in adult rats) [85].

Although firing amplitudes of primary nociceptive afferents cannot be recorded in newborn infants due to the invasive nature of such recordings, non-invasive studies of nerve conduction velocity can be used to obtain information about development [87,88]. Nerve conduction studies in infants and children have been performed using electrical stimulation in order to obtain conduction velocities in both sensory and motor nerves in the hand and foot [88-90]. Garcia *et al.* demonstrated that conduction velocities in both sensory and motor fibres in neonates were approximately half that observed in adults (sensory conduction velocity values in the medial nerve in neonates = 24 m/s and in 4 - 6 year olds = 51 m/s, which is comparable to adult values) [90]. Moreover an interesting study by Eyre *et al.* used non-invasive electrical stimulation of the motor cortex, and subsequently of the cervical motor roots at the level of the spinal cord, to evoke muscle activity in the contralateral arm [89]. The group demonstrated that conduction delays present at the synaptic terminal in the spinal cord were high in infants compared with adults, falling to less than one third of the value by 2 years of age, and reaching adult-like values by 5 years of age [89]. Whilst these studies have not investigated nociceptive fibres, they suggest that nociceptive conduction velocity in infants will be reduced compared with adults.

Observations and measurement of reflex withdrawal muscle activity provide evidence suggesting the development of nocifensive behaviour is comparable between rat pups and human infants. For example, primary afferents in neonatal rat pups exhibit extensive prolonged activity, recorded using extracellular neurophysiological recordings, that follows both single or multiple stimuli [85]. In addition to these observed “after-discharges”, electromyography (EMG) also showed exaggerated, longer lasting reflex responses [84]. Fitzgerald and Gibson recorded EMG responses in rat pups while applying different modalities of noxious stimuli (including mechanical, thermal, chemical and electrical modes) to the hind paw [84]. A striking result from this paper was that the

duration of reflex withdrawal responses evoked by a single pinch were 10 - 15 seconds longer during the first week of life compared with responses in 18 day old and adult rats. A subsequent investigation of nociceptive withdrawal reflexes in preterm and term infants similarly demonstrated that the duration of the response to mechanical noxious stimuli decreased with increasing age of the infant [91].

A study comparing reflex withdrawal responses in human infants with rat pups further demonstrated that not only do the observed responses have longer durations, but they are accompanied by generalised movements of other parts of the body in very young infants [92]. In the youngest population of infants studied (< 27 weeks postconceptional which is approximately equal to < 29 weeks gestational age [93]), stimuli that evoked withdrawal reflex of the ipsilateral limb were accompanied by generalised movements of the whole body and the leg contralateral to the stimulation [92]. The incidence of such exaggerated responses decreased with increasing age in infants [92], with responses to mechanical stimuli gradually resulting in the greatest evoked withdrawal within the stimulated limb in term infants, with the ipsilateral limb demonstrating a lower overall response [91,94]. Waldenström *et al.* also showed that noxious laser stimuli applied to the tail of rat pups elicited erroneous reflexive tail flicks towards the stimulus in 64 % of pups at postnatal day 10 [95]. Eventually by P21 the error rate reduces to adult levels of < 10 % [95].

Overall this evidence demonstrates a refinement of reflex withdrawal responses accompanied by a maturation of nociceptive afferents in rat pups. If extrapolated to human infants, this suggests that infants are likely to possess intact nociceptive connections and functioning nociceptive afferents at birth which when activated, should elicit nociceptive-evoked reflex withdrawal activity.

It is notable that investigations of reflex withdrawal thresholds in preterm and term-born infants have shown that the force of mechanical stimulation required to evoke reflex withdrawal activity gradually increases with age [91,92,96]. Investigations of mechanical

reflex withdrawal thresholds in rat pups also show the same increase in threshold with age over the first three postnatal weeks, and the threshold at P26 remains significantly lower than that observed in adult rats [92]. This phenomenon again reflects that observed in term infants, where the median mechanical force required for evoking a withdrawal reflex was 2.1 g [91], which is approximately three times lower than the average mechanical pain threshold in adults [4].

Given that nociceptive afferents exhibit similar firing amplitudes and thresholds to mature nociceptors from birth [85,86], the observed lower thresholds for reflex withdrawal cannot be due to greater sensitivity of primary afferents. Indeed it has been demonstrated that A β -fibre terminals can be observed in laminae I and II in the rat dorsal horn and retract to deeper lamina over the first 3 postnatal weeks [97]. Low intensity stimulation of A β -fibre afferents also elicits C-fos activation within lamina I and II [98]. Beggs *et al.* show that blocking NMDA-dependent activity in the superficial lamina throughout a critical period during the first three postnatal weeks in a rat pup prevents reorganisation and retraction of A β -fibres from the superficial lamina [99]. In addition, low cutaneous reflex withdrawal thresholds are maintained in the same rat pups [99]. Therefore, it appears that an NMDA-dependent process is responsible for retraction of cutaneous sensory fibres from the superficial lamina. Long term potentiation and depression (LTP and LTD) mechanisms rely on NMDA receptors (for review see [100]), and activity-dependent correlation has been shown to help refine inputs in other sensory systems such as the visual cortex [101]. This same mechanism would also explain the importance of tactile sensory input for development of tail flick response specificity in rat pups [95].

Once infants reach term age, their reflex withdrawal thresholds to nociceptive stimuli are higher and repeated stimulation no longer elicits generalised bodily movements [92]. Instead many studies report that habituation to repeated mechanical noxious stimuli is observed by the time infants reach term age [92,96,102]. Habituation to noxious stimuli can be observed in infants as a decrease in the number of reflex responses elicited

following repeated application of a noxious stimulus at the reflex withdrawal threshold. A switch to habituation following repeated stimuli is seen after 32 weeks PCA [92]. This suggests that descending inhibitory nociceptive mechanisms may be contributing to reducing nociceptive inputs by term age.

1.5.2. Development of central ascending and descending pain pathways

Using retrograde labelling, STT projection neurons were shown to be present by embryonic day 18 (E18) in the rat pup spinal cord [103]. Although the diameter of the projections increased across age, the total number of neurons observed did not change when E18 and adult rats were compared [103]. Interestingly a subsequent investigation of functional activity of the STT projection neurons using c-fos labelling showed that no activation could be observed until P3 in rat pups [104]. At P3, the ventral lateral medulla showed marked c-fos expression following formalin injection of the forepaw or hindpaw [104]. This brain region has been shown to be involved in autonomic cardiovascular control, for example Ross *et al.* showed that direct electrical and chemical stimulation led to increases in arterial blood pressure and heart rate in rats [105]. Adjacent regions are also important for inhibition of nocifensive behaviours, for example, stimulation of the lateral reticular nucleus within the rostral ventromedial medulla (RVM) has been shown to suppress the nociceptive tail flick response in adult rats [106]. Other brain regions including the thalamus, hypothalamus and PAG only expressed c-fos activity following formalin injection at P14, and again the PAG has strong associations with descending inhibitory control of the spinal cord [104].

Fitzgerald and Koltzenberg observed function of descending inhibitory pathways by stimulating the dorsolateral funiculus (DLF) in rat pups between P9 and P26 days [107]. The projections through this tract are likely to originate from the RVM and the authors showed that descending projections *via* the DLF to the dorsal horn are present at birth [107]. Despite their presence, descending inhibitory control in response to cutaneous brush and pinch stimuli was not observed until P10, when very weak inhibition was seen,

and adult-like function was present by P22 [107]. Van Praag and Frenk also demonstrated that direct stimulation of the PAG does not produce inhibition of nocifensive tail flick responses until P21 in rats, after which the response increases, demonstrating greater inhibitory drive in the adult rat [108].

Hathway *et al.* also investigated descending inhibitory control within the dorsal horn across age in rat pups. The group showed that GABA had excitatory influence on dorsal horn neurons at P3 but exhibited inhibitory influence by P21. Spinalisation in the P3 pups prior to investigation of reflexes completely abolished the excitatory influence of GABA, demonstrating that supraspinal mediation was essential to observe this reversal [109]. The authors later demonstrated that direct stimulation of the RVM in rat pups was exclusively excitatory, producing increased nociceptive withdrawal reflex until at least P21 [110]. After this age, a biphasic response is observed, with low intensity electrical stimulation (5 - 20 μ A) facilitating reflex withdrawal while greater intensity stimulation (50 - 200 μ A) induced inhibition of reflex withdrawal responses with significant reductions in reflex withdrawal seen by adult age [110]. Together this evidence demonstrates that there is a developmentally regulated change in the descending signals from the RVM, going from solely excitatory to a combination of excitatory and inhibitory in the adult. This coincides with the development of descending inhibition from PAG in rats, which releases inhibitory opioids. Hathway *et al.* showed that blocking opioid action between P21 and P28 prevented the change in activity from the RVM, confirming that opioids are essential for the switch to occur [111]. Whether this relationship is also observed in infants and the developmental trajectory of descending inhibition remains unknown.

It has been demonstrated that both excitatory and inhibitory receptors undergo developmental regulation over the postnatal period in rats (for reviews see [112,113]). An example would be that GABA appears to have an excitatory influence on reflex withdrawal thresholds in rat pups, which is opposite to the inhibitory effect observed in adult rats [109,112]. The mechanism behind this early excitatory response has been linked to less

negative resting state potentials caused by excess intracellular chloride ions present due to a lack of an important potassium-chloride co-transporter within the neural membrane [114]. Koch *et al.* also demonstrated that glycine receptor mediated inhibition is not functional in rat pups until after P14 [115], another example of the delayed maturation of inhibitory receptor function. These differences in modulation, and in particular the observed increase in excitatory drive, in rat pups could render the spinal cord sensitive to altered peripheral inputs. Beggs *et al.* demonstrated that surgical incision of the hindpaw in neonatal rat pups resulted in persistent allodynia and hyperalgesia of reflex withdrawal responses following subsequent injury in adulthood [116]. This was also accompanied by increased microglial activation within the dorsal horn, which, when inhibited, reduced the observed sensitisation [116]. Therefore, it appears that central pathways in rat pups are vulnerable to excessive external noxious inputs during early life. Again, if extrapolated to infants, this could indicate that descending inhibitory drive may be refined over the third trimester of pregnancy. However, such comparisons are highly speculative and further investigation of these mechanisms is required in human infants.

1.5.3. Development of supraspinal central pain processing

Histological studies of post-mortem human foetal brain tissue have been used to investigate the growth of thalamocortical connections *via* the subplate, a transient layer of the cortex just below the cortical plate (see figure 1.2 b) [117]. The cortical plate is a neuronal rich developmental layer that goes on to form the cortex. The growth of these pathways has been identified as the main connectivity event during the period encompassing the third trimester of pregnancy [117]. Moreover, the functional maturity of the thalamocortical pathways will be essential for cortical pain processing.

Histological investigations by Kostovic and colleagues have shown that the subplate forms early in gestation and contains “waiting” thalamocortical afferents from approximately 20 weeks gestational age [118]. 23 - 24 weeks gestation coincides with the age after which a foetus could be considered viable and kept alive with intensive care [119]. The “waiting”

thalamocortical axons then migrate and penetrate the cortical plate [117,120]. The period between 26 and 34 weeks gestation is characterised by synaptogenesis and the increase in thickness of the subplate zone [120]. Thalamocortical axons have also begun to form synaptic connections with the deeper layers of the cortical plate (layers III-VI), (see figure 1.2 g-i) [117,120]. The dissolution of the transient subplate layer begins shortly after it reaches its maximal thickness at 34 weeks gestation [117,118,120], and this is followed by lamination of the cortex, which occurs with inner layers forming before outer layers [120].

The later stage of preterm development, between 35 - 37 weeks gestational age (GA), is signified by growth of the cortical plate to overtake the subplate as the most prominent layer of the developing cortex. In addition, by 35 weeks significant growth of inter- and intra-hemispheric connections become apparent. This can also be detected using DTI imaging, as the prominent diffusion direction changes from radial orientation at 26 weeks to equal radial and axial diffusion by 35 weeks [121]. This elaboration of connections in the cortex coincides with initial reports of functional connectivity observed in thalamocortical pathways [120]. Accumulation of thalamic afferents have been observed in somatosensory, visual, frontal and temporal regions and while all regions show considerable cortical lamination by term age, the frontal region is the last to mature, also showing the slowest dissolution of the subplate region [120,122]. This coincides with changes in EEG brain activity including a reduction in spontaneous or sensory-evoked delta brush activity and an increase in synchrony in activity between the two hemispheres [123-125].

Little is known about the functional development of the network of brain areas required for pain processing. Much of our knowledge in humans comes from brain imaging experiments of the living CNS, which will be described in detail the next section of this introduction.

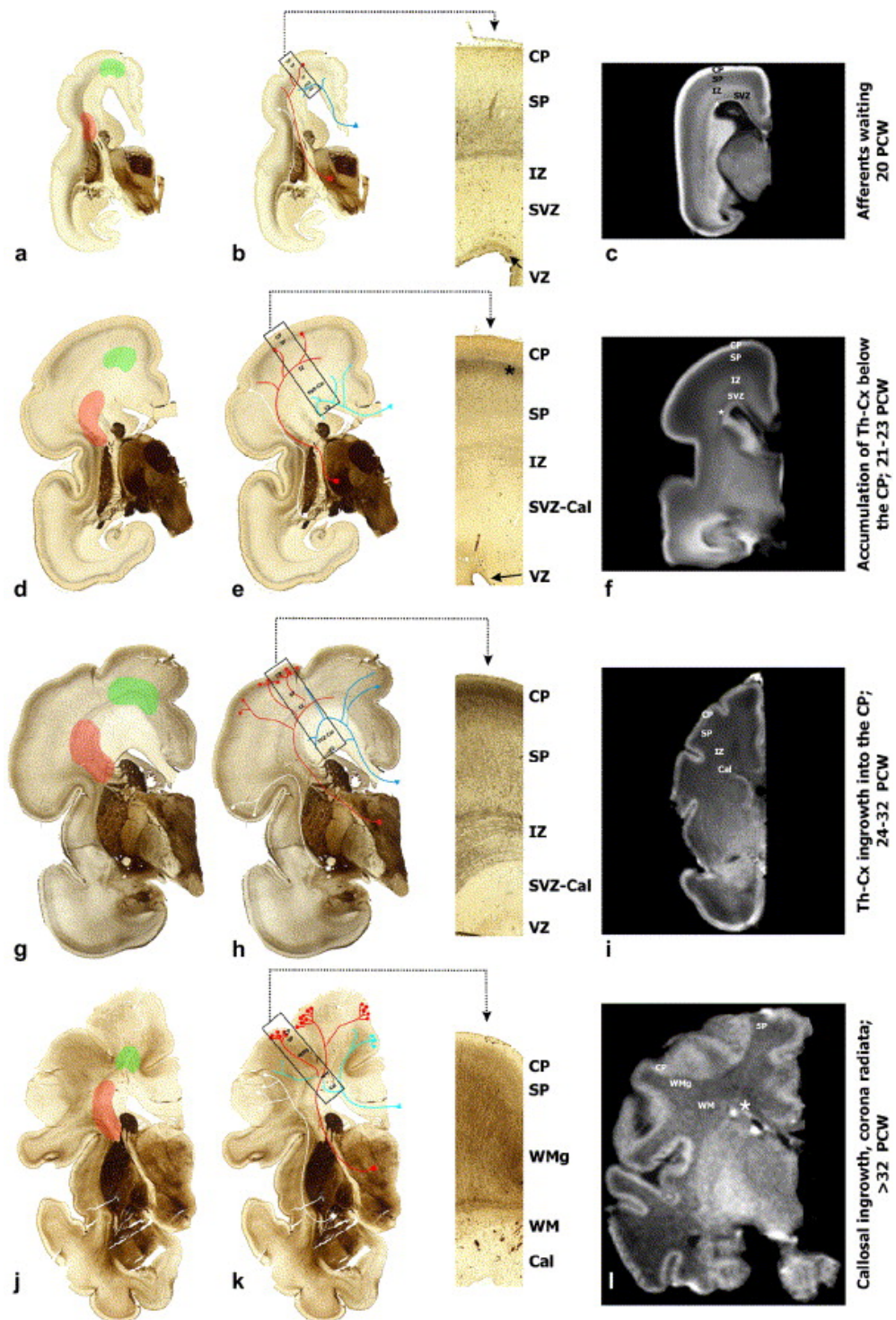


Figure 1.2: Growth of thalamocortical connections during different stages of development. Figures shows developmental changes occurring in the 20th post-conception week (PCW) (a, b, c), fetal phase (d, e, f), early preterm phase (g, h, i) and late preterm phase (j, k, l). Gestational age is approximately two weeks greater than PCW. Reprinted from *Seminars in Fetal and Neonatal Medicine*, [120], with permission from Elsevier.

1.6. Imaging nociceptive brain activity

1.6.1. Emergence of the investigation of functional brain activity in infants

Animal studies and histological studies of human brain tissue initiated the enquiry into the development of nociceptive pathways in the peripheral and central nervous system. However, although investigations of the thalamocortical pathways in human studies were thorough, they were lacking insight into the functional integrity of the observed collection of neurons, a crucial indication of their maturity for processing nociceptive stimuli.

The emergence of non-invasive brain imaging techniques such as near-infrared spectroscopy (NIRS), electroencephalography (EEG), magneto encephalography (MEG) and functional magnetic resonance imaging (fMRI) have revolutionised both clinical and research investigation into the function of the living human central nervous system (CNS). All four techniques have been instrumental in providing a window into the functional activity occurring in the newborn infant's brain. The use of NIRS to investigate cortical responses in newborn infants was very popular when the technique was first employed and, to begin with, was more commonly used in infants than adults [124]. NIRS optodes emit infrared light and use a detector to measure the absorbance of light by haemoglobin. NIRS thereby enables detection of changes in the concentration of oxygenated and deoxygenated haemoglobin in the cortex. The penetration of the optical technique was shown to be approximately 6.3 - 8.5 mm below the surface of the scalp where the optode was positioned in neonates [126]. NIRS optodes can be placed on a newborn infant's head at the bedside (i.e. within a clinical environment), and, although the technique is limited to recording cortical haemodynamic changes (as the infrared light does not penetrate to deeper cortical regions), NIRS provides a high temporal resolution, with an accuracy of 50 ms [28]. Hence, by recording over time it becomes possible to observe increases and decreases in the concentration of oxy- and deoxy-haemoglobin in response to external stimulation [124,127]. Researchers first reported use of NIRS to investigate cortical haemodynamic changes in newborn infants in 1985 [128] and later in 2006 it was

reported as being utilised to observe functional brain activity in response to nociceptive stimuli in infants [1,2].

In 2006, NIRS was used to record changes in haemodynamic concentrations following venepuncture and heel lance in newborn premature and term born infants [1,2]. These studies demonstrated that noxious stimulation evoked increases in oxygenated haemoglobin concentration detected at optodes placed above the somatosensory cortices [1,2]. In addition, Bartocci *et al.* reported that responses to innocuous stimuli were significantly smaller than responses to noxious stimuli, and Slater *et al.* reported that responses contralateral to the site of the stimulus were greater than changes recorded in the ipsilateral somatosensory cortex [1,2]. NIRS and MEG have otherwise mainly been used to investigate innocuous sensory-evoked responses such as tactile [129-133], auditory [134,135], and visual responses [136,137] in preterm and term infants.

1.6.2. EEG and the measurement of noxious-evoked brain activity

EEG is a non-invasive technique that measures the synchronised electrical currents generated by groups of neurons in the brain [138]. Small electrodes placed on the scalp detect changes in electrical activity, mainly generated by the movement of positive sodium and potassium and negative chloride ions in and out of the extracellular neuronal space, which generate electronic field potentials [138]. Activity can either be excitatory or inhibitory and electrodes detect the overall increase or decrease in electron concentration that results [138]. Since an overall change in electrical activity is being detected, the presence of equal and opposing positive and negative charge would result in a neutral signal detected by the scalp electrodes; therefore EEG is only sensitive to large unipolar changes in signal [138]. The changes described can be detected with extremely high temporal resolution (1 ms [28]), with typical sampling rates of 2,000 Hertz achieved during EEG acquisition (Curry 7, Compumedics Neuroscan).

EEG is an extremely useful tool, but is not without its limitations. The spatial accuracy of EEG is dependent on the number of electrodes placed on the scalp. Electrode placements range from a minimum of 8, recommended for use in premature and full term infants [125], to as many as 512 electrodes, now available for use in adults (Curry 7, Compumedics Neuroscan). The feasibility of use of each montage depends on the size of the participant or patient's head. The montage chosen also depends on the type of signals being investigated, for example a larger number of electrodes may be required for an investigation of EEG during sleep compared with an investigation of auditory evoked potentials. In addition, with fewer electrodes, the ability to determine the source of the electrical activity is reduced. This is because a very large synchronous response originating from a more distal cortical location may resemble a smaller amplitude response originating nearer the surface of the cortex, closer to a particular electrode site [138]. Finally, the distance from the source of neural activity to the EEG electrodes determines the polarisation of the recorded signal. Therefore inferences regarding the excitatory or inhibitory function of the population of neurons from which the signal originates cannot be made based on recording of EEG activity. For example, an excitatory post-synaptic potential (EPSP) generated at a location near to an extracellular recording electrode (such an EEG scalp electrode), caused by an influx of positive ions into the neuron, will result in a depolarisation across the neuronal membrane. This activity causes a negative deflection in the field potential recorded at the scalp electrode. However, a negative deflection will also be recorded following an inhibitory post-synaptic potential (IPSP) generated at a location distant to the scalp electrode. Similarly, a locally generated IPSP and an EPSP generated further away or deeper in the cortical layers will both result in a positive deflection in the EEG signal. These examples therefore demonstrate the necessary caution with which EEG signals should be interpreted (for further information see *Electroencephalography: Basic Principles, Clinical Applications and Related Fields*, 5th Edition [138]).

Despite some of the limitations described, EEG is nonetheless an invaluable technique for investigating normal and abnormal brain function for both clinical and research applications. The fact that EEG can be recorded non-invasively and by the bedside means it can be relatively simply employed in infants, children and adults. Neurophysiological recordings are most commonly used clinically in the assessment of patient epilepsy and are integral in correct diagnosis and management of epileptic syndromes or patients with coma or encephalopathy [124,138]. Recording of brain activity during seizures, between 24 and 48 hours following a seizure or for example during sleep, allows the investigation and identification of abnormal excitability, depressed or disorganised brain activity [124,138].

EEG recordings are also used to investigate event related potentials (ERPs) or evoked brain activity [139]. The most common approach is to provide sensory stimuli such as auditory or photic (visual) stimulation and compare the morphology of the evoked brain activity to that which has been characterised in normal, healthy individuals [139]. Observation of evoked brain activity to external stimulation is useful because it allows inferences to be made about brain function. Stimuli can also be precisely timed to an EEG recording [140,141] and repeated numerous times, allowing averaging of the evoked response across trials [139]. This facilitates removal of unrelated recorded background activity and additionally improves sensitivity to the evoked response to allow detection of even small amplitude changes [139].

More recently, EEG has been used to investigate noxious-evoked brain activity in adults [28,142]. The most commonly used noxious stimulus in adults is a laser heat stimulus which applies a direct and concentrated pulse of heat energy to the upper epidermal layers of the skin, activating both C-fibre nerve endings and A δ -fibre dendrites [143,144]. The evoked response observed following application of a laser stimulus consists of a waveform with two negative peaks. The first negative-positive component occurs 140 - 240 ms after the stimulus and corresponds to propagation *via* A δ -fibres, which are thought to signal the initial sensory aspect of the pain response [144-146]. Using EEG, this

response has been localised to the primary and secondary somatosensory cortices [147-149]. Subsequently, a second negative-positive component with a latency greater than 1000 ms is also elicited by laser stimulation [150]. This later component has been linked with slow-conducting C-fibres [150,151] and localised to the ACC [147-149]. Beydoun *et al.* showed that when participant's paid less attention to a noxious stimulus, the peak amplitude of the later response and perceived intensity of the stimulus were reduced, thereby suggesting an association with affective pain processing [152]. Lee *et al.* reproduced this result using a paradigm that applied laser stimuli to the dorsum of the hand in pairs at varying distances [153]. By altering the distance, the participant's ability to detect distinct stimuli was altered and this correlated with the magnitude of the later component, while the initial sensory component crucially remained unchanged [153]. Thus the authors suggested that the later evoked response was representative of later processing of the nociceptive input, likely associated with affective aspects of pain. In addition to these characteristic components of the laser-evoked response, Treede *et al.* also noted that a maximal amplitude response is observed at the vertex, at electrode Cz which is positioned at the midline, above the somatosensory cortices [154].

In 2010, Slater and colleagues reported using EEG to investigate noxious-evoked brain activity in newborn infants [3]. By recording brain activity in response to a clinically-required heel lance, Slater *et al.* characterised a nociceptive-specific evoked potential in infants aged between 35 and 39 weeks gestation [3]. Using principal component analysis, they showed that the evoked response was maximal at both Cz and CPz electrodes and constituted a negative-positive wave that had its average negative and positive peaks at 420 and 560 ms respectively [3]. This response was preceded by an earlier potential that was recorded in response to both the noxious heel lance and a control stimulus (which constituted the heel lance rotated by 90 degrees so that its spring-loaded blade released into the air and did not cut the infant's skin). The earlier negative-positive complex appears on average at 150 and 260 ms [3]. The authors therefore suggest that the earlier response represents a sensory component generated by the non-noxious aspects of the

stimulation [3]. The presence of both a sensory response, followed by a later evoked potential is much like the responses observed to laser-evoked stimuli in adults [153].

In 2001 Magerl and colleagues demonstrated that PinPrick stimuli predominantly activate A δ -fibres [155] and the stimuli have been widely used to assess mechanical sensitivity in patients. Researchers had also utilised the stimulators during fMRI experiments, but until recently the stimulators had never been used to investigate noxious-evoked potentials in adults using EEG [141]. Iannetti and colleagues used a novel method of time-locking a 128 mN force PinPrick to EEG recordings and demonstrated that a reproducible negative-positive complex was evoked on average 111 ms after the onset of the stimulus [141]. The authors state that, although this response is quicker than the range for A δ -fibre activation from laser-evoked stimulation, type I-AMH nociceptors have been shown to exhibit faster conduction velocities compared with the more heat sensitive type II-AMH fibres, therefore accounting for the slight reduction in response latency [141]. Investigation of the topography of the response revealed that both the negative and positive components of the response were maximal at the midline, and the application of capsaicin to the stimulus site caused a 92 % increase in the amplitude of the N-wave (after correction for habituation) [141]. Finally this study also reported that the PinPrick-evoked potential was reduced by approximately 50 % in an individual with a known lesion to their spinothalamic pathways rendering one side of their body with impaired thermal and nociceptive sensitivity [141]. Quantitative sensory testing revealed that the participant only responded to 56 % of nociceptive stimuli applied to extremities on the impaired side of the body compared with 100 % of the noxious stimuli applied to the contralateral side [141], thus providing important evidence that PinPrick-evoked stimuli are propagated *via* the spinothalamic tract.

Using a novel method of time-locking PinPricks to EEG recordings with a high-speed camera, Hartley, Poorun and colleagues used PinPrick stimuli to investigate noxious-evoked changes in brain activity in anaesthetised children [156]. Importantly, the authors did not observe the evoked potentials that would be expected in awake individuals.

However, a significant increase in power in the delta frequency band (0 - 3 Hz) was identified following applications of the 512 mN force of experimental PinPrick stimuli to the dorsum of the hand, compared with delta power in the pre-stimulus baseline periods [156]. Prior to these investigations, PinPrick-evoked brain activity had not been recorded in children using EEG and is, to date, yet to be recorded in newborn infants.

1.6.3. fMRI and the measurement of noxious-evoked brain activity

In this section I will be describing the emergence and utility of magnetic resonance imaging (MRI). MRI has become an increasingly common diagnostic and clinical research tool and one of its greatest strengths is that it allows a non-invasive window into the entire human body. MRI can be used to obtain structural images of the body, as well as investigating the function of the nervous system. MRI allows observation of functional brain activity in cortical and subcortical structures and, as it does not involve any radioactive chemicals, it is an appealing method for use in infants and children.

MRI enables the production of high quality structural images of the brain with isotropic millimetre voxel resolution [157]. This includes the segregation of different brain tissues, which is possible due to the different properties that each tissue exhibits within a magnetic field. Properties such as the relaxation rates within different brain regions and tissue types (for a detailed description see Chapter 3) enable contrast to be observed between tissues, which are used to capture detailed images of the underlying structure.

In addition to providing invaluable insights into the structure of the living brain, MRI can also be used to investigate brain function. Functional MRI is primarily used to observe changes in blood flow occurring in the brain when a participant is at rest, performing a task or experiencing sensory stimuli. When groups of neurons within a brain area increase their neural activity, metabolic demand is increased, and consequently the blood flow to that area also increases. The increase in blood flow is delayed compared with the change in neural activity, taking approximately 6 seconds to peak in an adult [158] and

consequently results in an increase in the volume of oxygenated haemoglobin flowing to the active region. Haemoglobin present within the blood is diamagnetic (possesses only paired electrons) when oxygenated and paramagnetic (possesses an unpaired electron) when deoxygenated. This change in the electronic property of haemoglobin results in a change in the magnetic field detected by the MRI scanner. This change is called the blood oxygen level dependent (BOLD) response and can be measured and used to investigate the functioning of the brain during fMRI studies. The spatial resolution of fMRI varies between 1 and 5 mm depending on the size of the region being scanned [157]. This is because the voxel size directly impacts the acquisition time of the scan, therefore studies investigating the whole brain tend to use larger voxel sizes of 3 - 5 mm [157]. While this resolution is inferior compared to NIRS, fMRI affords the important advantage of being able to investigate changes in blood flow across the whole brain, including both cortical and subcortical brain areas.

The observation of BOLD changes within the brain was first made *in vivo* by Ogawa *et al.*, who imaged an anaesthetised rodent's brain at 4 and 7 T [159,160]. The detection of changes in blood flow in the brain was suggested to be correlated with neuronal activity [160], the relationship between which has since been found to be considerably more complex [161]. In 1996, a study reported investigating the changes in cerebral blood flow in a cat's occipital cortex in response to visual stimulation [162]. Malonek and Grinvald were able to demonstrate that a local and specific increase in deoxygenated haemoglobin within a brain region, (peaking 2 seconds after the stimulus) was followed by the gross general perfusion of the region and its surrounding areas with oxygenated haemoglobin which peaked 5-6 seconds after the stimulus [162]. It was not until the early 21st century that evidence of this was also demonstrated using simultaneous electrophysiological and fMRI recordings in anaesthetised and awake monkeys [163,164]. A small number of studies have also been able to record neuronal activity from human patients with implanted electrodes and compare the observed activity to BOLD responses in separate participants performing the same task within an fMRI scanner [165,166]. While this evidence provided validity to the suggestion that BOLD activity was closely correlated with

neuronal firing, the conclusion remained a controversial one since Logothetis and colleagues had also demonstrated that local field potentials (LFP) (mainly generated by the summation of post-synaptic potentials), were also well correlated with BOLD response [163]. Further scientific investigation has suggested that BOLD is in fact more specifically correlated with LFPs, which better represent the input to, as well as the intrinsic processing of, the underlying groups of neurons [161,167].

Importantly, for the purpose of this thesis, fMRI studies have enabled the investigation of specific brain regions and their contribution to pain processing in humans [29]. The numerous brain regions activated in response to pain and nociception are highlighted in figure 1.1 [29]. The somatosensory (S1, S2), thalamic, and posterior insular cortices provide examples of brain regions that process discriminative sensory nociception [30-37]. The anterior insula (AI), posterior parietal cortex, ACC and OFC are examples of brain regions associated with cognitive and emotional processing of painful inputs [31,38-45]. Neuroimaging investigations of pain in adults have afforded neuroimaging scientists the ability to study specific brain regions, as well as being able to investigate brain activity in pain patients without having to subject them to invasive imaging methods. A clear example of the investigation of small brain regions includes numerous studies investigating the role of the periaqueductal grey (PAG), a region of grey matter within the midbrain in the brainstem, in the modulation of descending pain processing [168,169]. These studies have shown that both distraction and placebo result in greater activation in the PAG, which is in turn correlated with lower subjective pain ratings reported by participants [168,169].

A common technique used in pain studies is to calibrate stimuli to a subject's individual pain threshold (defined as the intensity of stimulation where $50 \pm 10\%$ of the stimuli are described as painful [170]). Ploner and colleagues investigated participant's brain activity in response to noxious thermal laser stimuli applied at the individual participant's near-pain threshold and, using this method, demonstrated that greater functional connectivity between the anterior insula (AI) and the PAG during the pre-stimulus period was

predictive of whether participants rated the resulting stimulus as painful [170]. This study is therefore a useful example of the fact that the pre-stimulus or background level of activity within brain areas involved in pain processing contributes to resulting responses to noxious inputs [170,171].

Chronic pain patients experience constantly fluctuating levels of pain. Baliki *et al.* used fMRI to investigate whether the fluctuating levels of pain experienced by chronic back pain patients correlated with BOLD responses [172]. In a very detailed study, the group demonstrated that the medial prefrontal cortex (including the rostral ACC) was the sole brain region significantly activated during high levels of spontaneous pain [172]. The authors suggest that this could likely be related to individual's negative emotional state [172]. Therefore it is *via* a combination of pain imaging in both healthy subjects and those with chronic or neuropathic pain that we have developed a more thorough understanding of specific functions associated with the numerous brain regions commonly activated during pain processing [28,29].

Functional MRI studies investigating nociceptive processing have been conducted using a wide range of nociceptive stimuli including thermal, mechanical and chemical stimulation (see [28]). Thermal stimulation may involve the application of cold pressers, participants placing hands in to ice-cold water or application of thermal heat stimuli; heated optodes or laser stimuli are most commonly used. In addition, application of chemicals such as capsaicin to the skin allows the generation of central sensitisation in otherwise healthy participants, resulting in a useful human model of chronic or neuropathic pain. Mechanical experimental noxious PinPrick stimuli have also been used in investigations of nociceptive processing in adults (examples include [173-175]). The stimulators have also been incorporated into quantitative sensory testing (QST) methods for assessing nociceptive sensitivity and pain thresholds [4]. Iannetti *et al.* have also suggested that the investigation of evoked responses following noxious mechanical stimuli provides greater clinical utility since alterations of mechanical sensitivity are more often reported in neuropathic and chronic pain patients [141].

Within the last 20 years, MRI research has been extended to investigations of the infant brain. MRI was first and foremost successfully used to examine structural development [176-179]. During gestation and the early postnatal period, an infant's brain undergoes considerable developmental changes. One study showed that between 29 and 41 weeks an infant's brain volume is increasing by 22 ml/week [178]. This dramatic increase in volume is accompanied by a marked increase in the gyration of the brain (see figure 1.3 [180]). The central sulcus can be reliably identified in infants from 26 - 28 weeks gestation [181,182]. The brain also develops from having a predominantly smooth cortical surface at 25 - 26 weeks gestation to undergo a rapid increase in gyri and sulci between 30 - 37 weeks gestation [178,180,182].

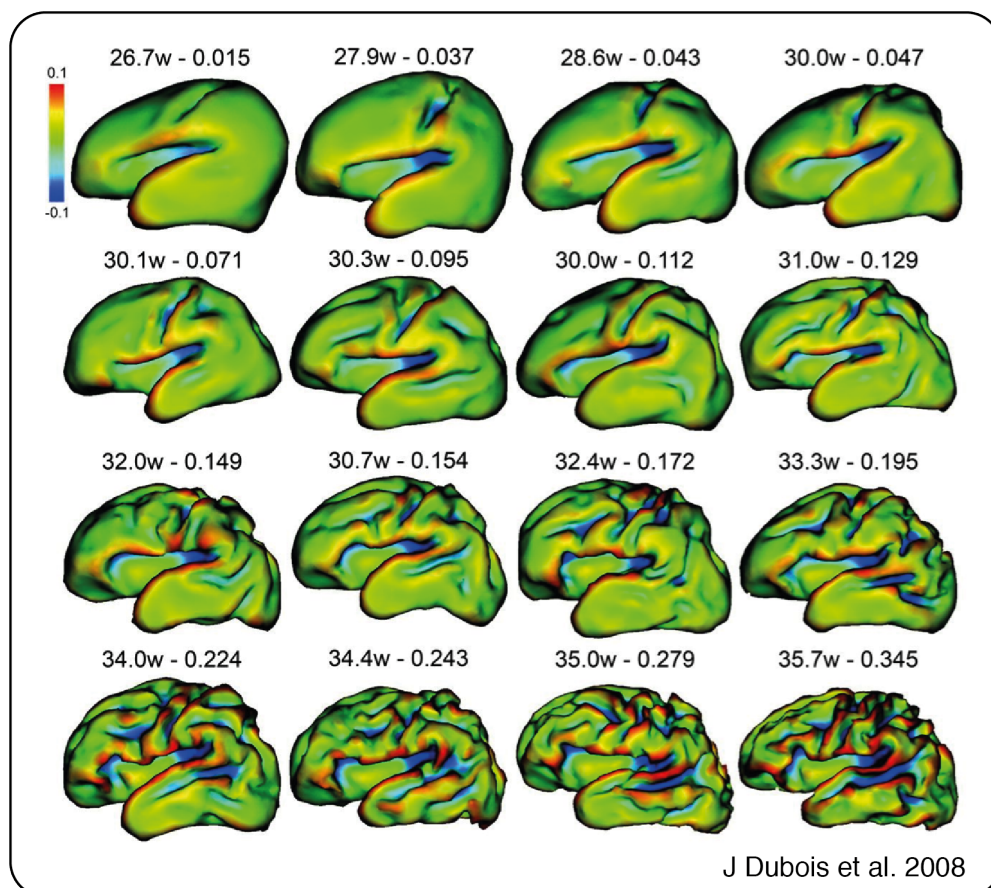


Figure 1.3: Images showing early cortical folding between 30 and 35 weeks gestational age. Figure reprinted from *Cerebral Cortex*, [180], with permission from Oxford University Press.

In addition to rapid structural changes, it has also been shown that infant brains are lower in lipid content due to a lack of myelinated nerve fibres [183] and contain high levels of water compared with the adult brain, which falls as lipid concentration increases [184]. Overall, it is apparent that the infant brain differs from the adult brain in more ways than just the size and structure. Therefore, it is recommended that structural MRI images should be collected using T2 contrast [185]. This is because the contrast observed between grey and white matter in infants is greater in T2-weighted images compared to the T1-weighted images normally collected in adults [185]. The authors suggest that this difference is likely to be driven by the differences between tissue water content [185]. Differences between structural imaging parameters provide one example of the considerations that are required when imaging infant's brains. The differences in underlying microstructure as well as the physiology of the brain make it likely that any parameters used while scanning in adults will not necessarily apply for use in infants.

Functional MRI has enabled a long-awaited insight into the developing infant brain. By adding non-essential research scans onto the end of clinically required MRI investigations, researchers began investigating brain activity in response to different sensory stimuli across early development. Scans tacked on to the end of research scans in this way therefore often involved premature infants and included investigation of visual [186-188], auditory [189-192], olfactory [193] and somatosensory stimuli [194,195]. Early studies often produced conflicting results since both negative and positive BOLD responses were at times being observed within the same study paradigm [187]. The uncertainty regarding the underlying physiology of the BOLD response in infants appeared to suggest that fMRI could not reliably be used to assess sensory development. However, a recent computational analysis has shown that these conflicting results were likely due to the application of inappropriate haemodynamic response functions (HRFs) to the infant fMRI data [196]. A study by Arichi and colleagues investigated evoked BOLD activity in term and preterm infants following the application of a brief somatosensory stimulus and showed that positive BOLD responses were reliably evoked [194]. The authors were able to determine this through the flexible application of a HRF, calculated from the data itself,

and made possible through advancement in the methods available for statistical analysis of fMRI data [197]. Furthermore, in a subsequent study, Arichi and colleagues also demonstrated that the presentation of an odorant liquid to an infant produced reliable positive BOLD activity in the piriform cortex, suggesting that evoked positive BOLD is not restricted to somatosensory responses [193].

In addition to the investigation of sensory-evoked BOLD responses in infants, studies have also been performed to investigate resting state networks (RSNs), as well as DTI and functional connectivity. RSNs allow investigation of the natural fluctuations in BOLD response across the brain while a participant is lying in the scanner, not performing a task. Doria and colleagues recently investigated RSNs in infants and showed that visual, auditory, somatosensory, motor, default mode, frontoparietal, and executive control networks are all observable in a full term infant [198]. Allievi *et al.* provided a demonstration of DTI in newborn infants, showing that the application of passive motor stimulation to the infant's hand produced activation along corticospinal tracts known to process motor functions [195]. Finally, functional connectivity has recently been used to show that by term equivalent age the topographical organisation of thalamocortical connections have already developed to resemble the organisation seen in adults [199], including connections to primary sensory, motor and visual cortices. The functional connectivity and resting state data in particular are both supportive of the hypothesis that evoked sensory stimuli could be expected to produce reliable BOLD responses in all sensory networks.

Although much of the newborn infant MRI research thus far has used field strengths of 1.5 - 2.4 T, researchers have begun to use 3 T MRI to investigate brain activity responses to different sensory stimuli, including sensory mechanical and visual stimuli, in newborn infants [188,194]. To my knowledge, only one feasibility pilot study has investigated noxious-evoked brain activity using fMRI. This study was conducted in both term and premature infants at 1.5 T [200]. Cutaneous mechanical von-Frey hair stimuli were used in 11 infants, and, of these 11, one infant had experimental noxious PinPrick stimuli at a

force of 64 mN applied to their foot [200]. The investigators successfully demonstrate the feasibility of using experimental noxious stimuli during fMRI studies in infants and report significant activity in the somatosensory cortices as well as the precuneus, frontal area and SMA following PinPrick stimulation [200]. It should, however, be noted that these results were only reported from two epochs in a single subject, hence reproduction in larger groups of infants is required to corroborate the observed result [200].

As described in this section, MRI provides clinicians and researchers with millimetre-precise spatial resolution that provides the most detailed imaging available of the entire living human CNS. In addition, the spatial resolution afforded by functional MRI, although not as precise as the structural resolution, is still offset by the advantage of the technique being non-invasive and capable of imaging the entire brain including deep brain regions. While NIRS is the only other method that can provide improved spatial resolution of functional brain activity, the technique is limited to the measurement of cortical haemodynamic changes. This makes fMRI the ideal tool, particularly for the investigation of nociceptive processing, because of the important involvement of deeper brain structures such as the thalamus, basal ganglia and brainstem. As the BOLD response, measured during fMRI experiments, is an indirect measurement of the coupling between neural activation and an increase in blood flow to the activated region, the temporal resolution of fMRI is intrinsically limited by the underlying physiology of this output parameter. Therefore, while fMRI provides an unparalleled source of spatial resolution, techniques that measure direct neural activity such as EEG are also essential in investigating the temporal dynamics of evoked brain activity. The two methods have been adopted separately in this thesis in order to investigate nociceptive processing in the infant brain. By using the same stimulus and making the experimental protocol as similar as possible, the aim was to provide a description of both the temporal and spatial characteristics of noxious-evoked brain activity in infants.

1.7. Thesis overview

In this thesis, electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) techniques are used to investigate the temporal and spatial patterns of noxious-evoked brain activity in newborn infants.

Noxious-evoked brain activity has previously been recorded in premature and newborn infants using near-infrared spectroscopy (NIRS) and EEG in response to clinically required acute painful procedures. However, the reliance on clinical procedures has meant that noxious-evoked brain activity had not been recorded and characterised in newborn infants who did not require invasive clinical procedures. In this project non-invasive, acute mechanical PinPrick stimulators, commonly used to assess nociceptive sensitivity in chronic pain patients, were used to stimulate the heel of the foot in newborn infants. The same stimulators were used during recordings of brain activity using either EEG or fMRI in newborn infants within the first 11 days since birth. EEG was used to investigate evoked brain activity following graded intensities of experimental noxious stimulation and responses were compared with behavioural and spinal-cord activity constituting common surrogate measures of pain in infants. In addition, fMRI's high spatial resolution was utilised to investigate the cortical and subcortical structures activated by experimental noxious stimulation in infants and this was compared to noxious-evoked activity in adults following the same type of stimulation. As fMRI had not previously been used to investigate nociceptive processing in infants at 3 T, initial experiments were conducted in order to optimise the acquisition parameters for use during future studies.

The initial optimisation experiments reported in this thesis demonstrate that a TE of 50 ms should be recommended for use in future fMRI studies in infants. This is longer than the TE of 30 ms that is typically used in adult studies. Additionally, it is demonstrated that nociceptive-specific brain activity is elicited in response to all forces of the experimental noxious stimulators used (32 - 128 mN). The magnitude of the noxious-evoked response is positively correlated with the magnitude of reflex leg withdrawal

response, and this relationship is observed even in the absence of a change in facial expression. This evidence suggests that reflex withdrawal muscle activity could represent a sensitive correlate of nociceptive processing in infants.

Finally, experimental noxious stimulation evoked a widespread pattern of brain activity in newborn infants that closely resembled that seen adults. The evoked activity observed in both adults and infants overlapped with the network of brain regions reported in the literature to be activated by nociceptive processing. The results demonstrate that noxious-evoked activity involved brain regions associated with both sensory and affective aspects of pain processing. Thus, given that pain is described as a “sensory and emotional experience” and the adult participants described the noxious stimulation as painful, these results provide evidence that newborn infants may have the capacity to experience pain in a similar way to adults. Given the high exposure to acute painful procedures recorded during neonatal intensive care, these results highlight the importance of developing new and effective strategies for assessment and management of pain in infants. In addition, it is also suggested that the brain imaging tools and techniques adopted in this thesis could be used in future assessment of pharmacological pain relief in infants, opening up a future avenue for testing the safety and efficacy of analgesics in this population.

Chapter 2

General Methods

This chapter describes the General Methods that are used in all three Results chapters. It includes the acquisition and processing methods used in this work for data collected using EEG, EMG, infant clinical pain scoring and MRI techniques.

2.1 fMRI methods used to investigate nociceptive processing in newborn infants

MRI research in newborn infants is an innovative and expanding research area. Part of this DPhil project involved initiating the set-up for scanning the first infants at the Centre for Functional Magnetic Resonance Imaging of the Brain (FMRIB) at the University of Oxford. In addition this work represents the first studies where functional magnetic resonance imaging (fMRI) has been used to record cortical and subcortical brain activity in response to an experimental noxious stimulus in newborn infants at 3 T.

Before the research commenced ethical approval was obtained, which was followed by the coordination of the institutional approvals required to enable safe MRI scanning of infants. Some of the important considerations involved during this initial set-up period have been detailed here, including safety and recruitment strategy. Safety was of paramount importance and included separate considerations (i) during transport of the infant to FMRIB (ii) during the scanning session and (iii) following the study. See figure 2.1 for a general outline of all infant MRI study protocols.

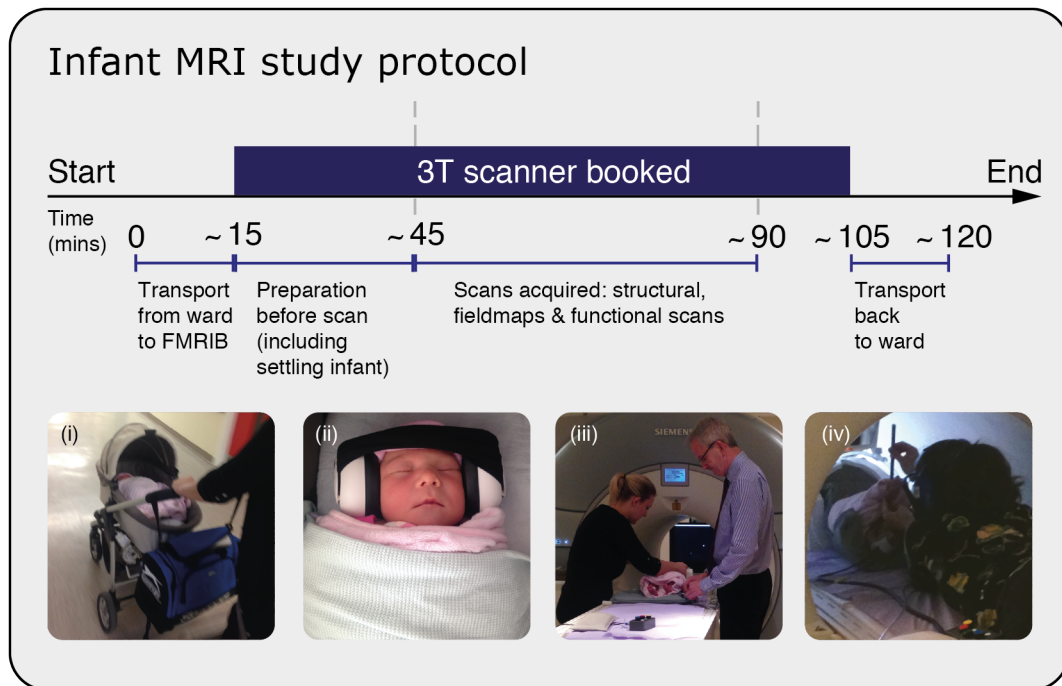


Figure 2.1: Outline of infant MRI study protocol. Picture inserts show (i) an infant being transported to FMRIB for a research scan, (ii) an infant wearing ear protection, ready to be scanned, (iii) an infant being prepped before going into the bore of the magnet (including placement of padded cushions around the head and pulse-oximeter monitoring prior to being swaddled with the left foot exposed for stimulation), and (iv) an infant having their left foot stimulated with a PinPrick stimulator.

2.1.1. Safety considerations during transport to and from FMRIB

Although the recruitment criteria specifically stated that all infants taking part in the study would be well and clinically stable (as judged by a clinical member of staff), it was not a requirement for the infants to have been discharged from hospital prior to taking part in the study. Therefore, in order to ensure all infant's safety during transportation to and from the wards, and during the study, a clinical member of staff (trained in neonatal life support) was present throughout the study period. Infants were transported in a pram and clinical staff carried medical equipment in case of an emergency. The equipment included suction devices in case the infant vomited and/or aspirated and intubation equipment, including bag and mask and an oxygen cylinder.

2.1.2. Safety considerations during the study session

Since most infant participants were inpatients on the neonatal or maternity unit at the John Radcliffe Hospital, all MRI studies were conducted with a minimum of one research member, one clinical member of staff and one radiographer present. In addition to this, during all scanning, the infants would have their vital signs (oxygen saturation and heart rate) continuously monitored using an appropriate 3 T compatible pulse-oximeter (Nonin Fibre Optic Pulse Oximeter; Nonin Medical, Minnesota, or an Expression monitor, Invivo Corporation, Orlando). Resuscitation equipment was available in case of emergencies and the OMRI unit (which is less than 10 meters from the FMRIB Centre) was the first point of call in an emergency. OMRI is a clinical MRI unit set up for both adult and paediatric scanning and therefore have an emergency trolley of medical essentials appropriate for use in infants. Finally, a specific emergency call-out was set up with the paediatric crash team at the John Radcliffe Hospital. This ensured that the team were aware of FMRIB's location so as to facilitate the quickest possible response time.

2.1.3. Safety considerations following the study

The standard operating procedures were also written in the event that an incidental finding was seen on an infant's MRI scan. FMRIB's incidental finding protocol, which details the procedure to be followed for adult participants, was edited so that it was suitable for the neonatal population. This protocol was adapted so that a paediatric neuro-radiologist and neonatal consultant would be notified in the event that an incidental finding was observed.

It was particularly important that bespoke SOPs were generated for the infant studies as a recent study using MR imaging to investigate the prevalence of intracranial haemorrhage reported that 26 % of newborn babies scanned between 1 and 5 weeks had evidence of a minor brain bleed following a natural delivery [201]. Such small bleeding will resolve without treatment, however, this is an example of a potential incidental finding that could

frequently occur in research studies and therefore warranted careful consideration and planning.

2.1.4. Recruitment strategy

A specific recruitment strategy was adopted for MRI studies conducted throughout my DPhil project. In order to ensure availability of all required research personnel, scanning sessions were booked in advance. Infants were then recruited to take part in the study at pre-allocated times. This recruitment strategy presented some challenges. As most infants were healthy term-born participants, it is common for these children to leave hospital within 24 hours after birth. Consequently, this meant that much of the recruitment was done the day before the study session was booked. Unfortunately, the difficulties in recruiting this population meant it was not possible to book more than one scanning session per week. Overall 34 infants were included in this DPhil project (see section 2.2.4), resulting in a recruitment success rate of approximately 1.8 studies per month (studies commenced in July 2013 and continued until January 2015 when the 3 T scanner was decommissioned prior to being upgraded).

2.2 Ethics and recruitment

2.2.1. Ethical approval

Studies were approved by the Oxford Research Ethics Committee (REC) of the National Research Ethics Service (NRES). All research conformed to the standards set by the Declaration of Helsinki and Good Clinical Practice guidelines.

MRI and EEG studies in newborn infants were conducted as part of the ethics application entitled “*Investigating pain in the developing human brain*” (REC reference 12/SC/0447), on which I am a named investigator.

Adult MRI and psychophysical studies were conducted under the following ethical approvals, “*Human pain perception*” (REC reference 11/SC/0249) and “*Adult Electroencephalography (EEG) and Electromyography (EMG) responses to noxious punctate and tactile stimuli.*” (CUREC Ref: MSD/IDREC/C1/2011/143).

2.2.2. EEG and EMG recruitment

12 infants took part in a study involving simultaneous recording of EEG and EMG in response to experimental noxious stimulation. An additional 4 infants had EEG and EMG recorded during a clinically required non-noxious control and noxious heel lance. The study was conducted on the maternity or neonatal units at The John Radcliffe Hospital, Oxford. Prior to obtaining consent for the studies involving experimental noxious stimuli, parents were shown the highest force of stimulator that was going to be used during the study (applied force: 128 mN, trade name: PinPrick Stimulators, MRC Systems). Parents gave informed, written consent for their infant prior to taking part in the study.

2.2.3. Clinical pain scoring recruitment

For the infant clinical pain scoring studies, 35 parents were approached and asked whether their infant’s facial expression and oxygen saturation and heart rate could be monitored during presentation of experimental noxious stimulation. Of those parents, 10 gave informed, written consent.

2.2.4. MRI recruitment

Over the course of my DPhil, more than 274 parents were asked if they would attend an MRI scanning session at FMRIB with their newborn infant. From all parents approached, 34 parents gave informed, written consent and 31 took part in an MRI study.

All parents provided informed, written consent before the study commenced. Prior to obtaining consent for infants, parents were shown the highest force of experimental stimulator that was going to be used during the study (applied force: 128 mN, trade name: PinPrick Stimulators, MRC Systems). Three sets of parents withdrew from the study after giving consent; two of the three were discharged from hospital prior to the study session. During the study parents were able to accompany their baby and, in nearly all cases, one or both parents chose to do so. From the 31 infants that attended a scanning session, one infant was not scanned due to the baby being overly restless and crying when placed on the MRI bed. Additionally, a technical failure during two studies rendered the data from those studies unusable.

10 adults were approached about taking part in an MRI study at FMRIB. All adults were recruited from the John Radcliffe Hospital and were either members of staff or students. All of the adults approached about taking part gave consent and attended one 2-hour scanning session. At a later stage all adults were asked whether they would also attend a separate session in which a psychophysical experiment on pain ratings was conducted. All of the adults that took part in the scanning session also gave consent and attended the psychophysics session.

2.3 Experimental stimuli

2.3.1. Experimental noxious stimulation

Acute mechanical noxious (non-skin-breaking) stimulation was applied using graded mechanical nociceptive stimulators (PinPrick Stimulators, MRC Systems). Different PinPrick stimulators can be used to apply different forces of mechanical noxious stimulation ranging between 8 and 512 mN, and the stimulators used in all of the experiments were MRI compatible. The manufacturer, MRC Systems, provided an original Testing Certificate with the PinPricks demonstrating that the stimulators were calibrated

to apply the expected forces (see Appendix 1). The force applied by the stimulators was rechecked to confirm the accuracy of the applied force (see Appendix 2).

In adults, five intensities of stimulation were applied to the dorsum of the left foot (applied force: 32, 64, 128, 256 and 512 mN). In infants, three intensities of stimulation were applied to the sole of the left foot (applied force: 32, 64, 128 mN). Greater force was not applied in infants to avoid risk of tissue damage. In all cases, stimuli were delivered by the experimenter in one smooth motion and lasted approximately 1 second.

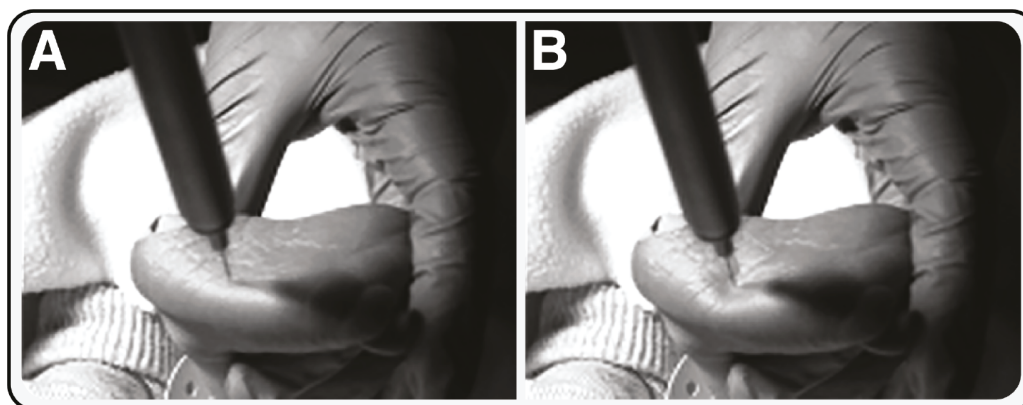


Figure 2.2: Stimulation of an infant's heel with a 128 mN PinPrick. Images are from a study using EEG to record noxious-evoked brain activity. A high-speed camera was used to record the exact time at which the 128 mN PinPrick stimulus is applied to the sole of an infant's left foot.

2.3.2. Heel lance and non-noxious control lance

To verify previously published results [3,91,140,202-204], noxious-evoked brain activity was recorded in response to a clinically required heel lance in a small group of four infants. All heel lances were performed by a trained member of clinical staff, as part of the infant's routine care. All heel lance recordings were preceded by a background period, and non-noxious control lance (where the lancet was rotated by 90 degrees and held against the infant's foot so that the blade did not contact the infant's heel when released).

2.4 Infant study protocols

2.4.1. EEG and EMG recording techniques

EEG electrodes were placed on the scalp in order to record evoked, electrophysiological brain activity. EEG was recorded at eight scalp electrodes (Ambu Neuroline disposable Ag/AgCl cup electrodes) in positions Cz, CPz, C3, C4, FCz, Oz, T3 and T4 according to the modified international 10-20 system. The reference electrode was positioned at Fz and the ground was placed on the forehead. EEG conductive paste (Elefix EEG paste, Nihon Kohden) was used to optimise contact with the scalp. Impedances were reduced by gently rubbing the skin with EEG preparation gel (NuPrep gel, D.O. Weaver and Co.) prior to electrode placement. EMG was used to record reflex muscle activity in response to noxious stimulation. Bipolar EMG electrodes were placed on the biceps femoris of the left (stimulated) leg (Ambu Neuroline 700 solid gel surface electrodes) (see figure 2.3). Infants were then tightly swaddled with their left foot exposed for stimulation.

Electrophysiological activity was acquired with the SynAmps RT 64-channel headbox and amplifiers (Compumedics Neuroscan), with a bandwidth from DC - 400 Hz and a sampling rate of 2 kHz. CURRYscan7 Neuroimaging Suite (Compumedics Neuroscan) was used to record the activity. All equipment conformed to the electrical safety standard for medical devices, IEC 60601-1. The lance and control lance were time-locked to the EEG and EMG recordings using an event-detection interface and accelerometer [140]. Experimental noxious stimuli were time-locked to the electrophysiological recording using a high-speed camera (Firefly MV, Point Grey Research Inc.) that was directly linked to the recordings [156]. The point of stimulation was then manually event-marked after the study, by identifying the time in the video recording where the barrel of the experimental stimulator was first depressed. Video recordings were captured at 220 frames per second, with a precision of 9 ms and accuracy of 4.5 ms. EEG data was filtered between 0.5 and 70 Hz with a notch filter at 50 Hz. Epochs of EEG brain activity were extracted with 500

milliseconds before and 1000 ms after the onset of the stimulus (determined by the event-mark).

EMG signals were filtered between 10 - 500 Hz with a notch filter at 50 Hz (and harmonics). Epochs were extracted during a window from 500 ms before the stimulus to 1000 ms after the stimulus, and baseline corrected to the pre-stimulus mean, and rectified. The data was divided into 250 ms bins and in each bin the root mean square signal was calculated. The post stimulus mean was calculated as the mean across the four bins following the stimulus (0 - 1000 ms) and the pre-stimulus mean was calculated across the two bins prior to the stimulus.

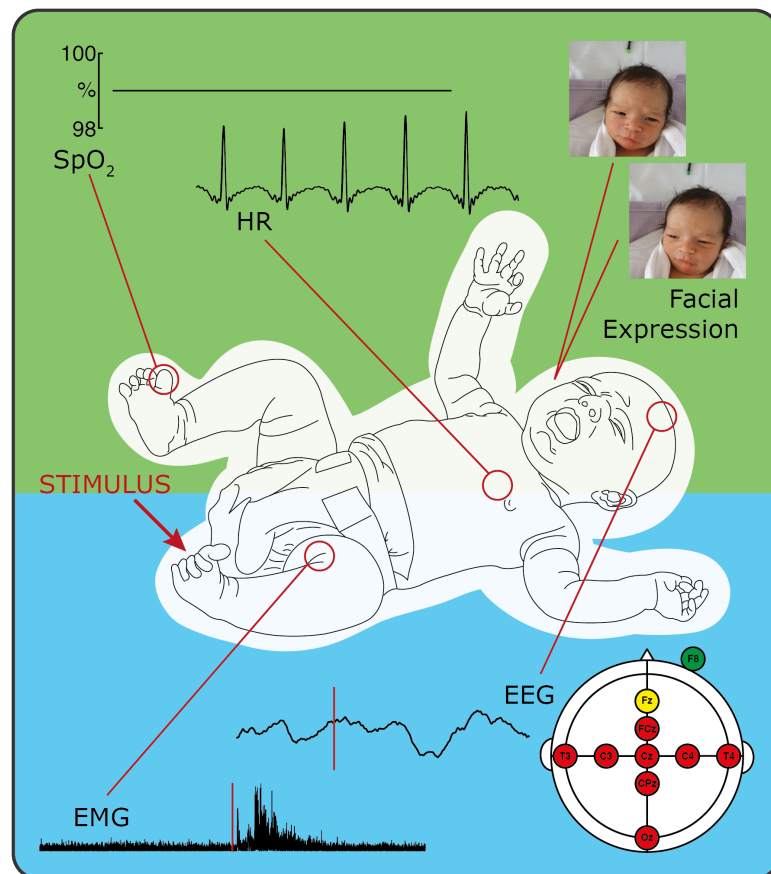


Figure 2.3: Schematic representing the techniques used during the EEG, EMG and clinical pain scoring studies. Techniques used during the EEG and EMG study are shown in the blue section, and techniques used during the clinical pain scoring studies are shown in the green section. Stimuli were always applied to the left foot, therefore oxygen saturations were monitored *via* a probe placed on the right foot. The red circles indicate the placement of equipment for monitoring the different responses. The diagram in the bottom right shows the electrode placement scheme used in all infants during EEG studies.

2.4.2. Clinical pain scoring recording techniques

Clinical pain scores, based on behavioural and physiological observations were calculated using the Premature Infant Pain Profile (PIPP) [59]. Facial expression change was recorded using a handheld camera and oxygen saturations were calculated using a pulse oximeter probe placed on the infant's right foot (OxiMax N-600 pulse oximetry monitor, Nellcor). The point of stimulation was identified on the camera by an LED light, which was coordinated to flash simultaneously at the time of the stimulus by pressing on a foot pedal. Heart rate was calculated from the ECG recording, acquired using the SynAmps RT 64-channel headbox and amplifiers, using a standard three lead placement on the chest.

A trained, clinical neurophysiologist supporting the study calculated all the PIPP scores. The facial expression component of the PIPP was analysed by viewing video footage recorded during the experiment. The videos were cut into 45 s epochs, which included a 15 s baseline and 30 s after the stimulus. The presence of each facial expression (nasolabial furrow, eye squeeze, and brow bulge) was assessed individually. The observer was blinded to the stimulus type when analysing the videos and did not know whether the video footage was from the baseline period or following stimulation. The physiological indices in the PIPP score were calculated by obtaining the mean heart rate and oxygen saturations in the 15 s before the heel lance, and the maximum heart rate and minimum oxygen saturations from the 30 s after the heel lance. Custom-made data analysis software was used to automatically calculate the mean baseline heart rate and oxygen saturation. The behavioural state score was calculated by observing the infant's sleep state and facial movements on the video footage in the 15 s before the heel lance.

2.4.3. MR Image acquisition

As described in section 2.1.1, all infants were accompanied and transported to FMRIB by a member of clinical staff from the Neonatal Unit trained in neonatal life support. The clinical member of staff remained with each infant throughout the study to ensure the

infant's safety and wellbeing. Before leaving the Maternity Unit, infants were screened for loose metal items (including metal poppers on clothing that were in direct contact with the skin) and the first level of ear protection was fitted (neonatal ear putty and ear muffs (Minimuffs, Natus Medical Inc.)), which provided sound attenuation levels of 23 and 7 dB respectively. Parents were given the option to accompany their infant to FMRIB and to sit inside the MRI scan room throughout the scans. Parents accompanying infants were also screened for MRI safety and provided with adequate ear protection (MRI safe ear defenders, JSP Big Blue, attenuation 30 dB). Infants were fed before being placed on a vacuum-positioning mattress on the MRI bed. Next a pulse-oximeter probe was placed on the right foot (i.e. the foot that was not to be stimulated). Pulse and oxygen saturations were monitored throughout scanning using a 3 T MRI compatible monitor (NONIN pulse-oximeter or Expression monitor, Invivo Corporation). The infant was then swaddled, ensuring that the left foot remained outside of the blanket in order to allow stimulation during the scan. Finally, the vacuum-positioning mattress, which contained MR-safe beads, was gathered around the infant so that it provided additional support, before the air was removed from the interior, leaving the mattress in a rigid shape. This method provided an additional layer of support to the infant, which much like swaddling restricted motion during scanning and encouraged the infant to settle.

All MR imaging was acquired on a Siemens Magnetom Verio 3 T scanning system using a Siemens 32-channel adult head coil, the same as that used for the adult scans. Once the infant's head was positioned inside the head coil, ear-defenders (Em's 4 Bubs Baby Earmuffs, Em's 4 Kids, sound attenuation 23 dB) were placed over the first level of ear protection. Finally, extra soft foam padding was placed around the ear-defenders to restrict head movement.

Once infants were settled, they were positioned in the centre of the bore of the scanner. During the first 10 minutes of scanning a full brain structural image, fieldmaps and in some infants short echo planar imaging (EPI) sequences were acquired for calculation of $T2^*$ (see Chapter 3). These sequences did not require disturbing the infants and allowed

additional time for infants to settle inside the scanner before experimental stimulation was applied. Scanning sessions lasted between 60 and 90 minutes (see figure 2.1). The length of the session was primarily dependent on how settled the infant was.

T2-weighted imaging sequences are recommended to obtain good contrast in structural images in newborn infants (unlike T1-weighted images, which are typically used for adults) [179,185]. Full brain T2-weighted structural images were obtained for all infants (repetition time (TR) = 13871 ms; echo time (TE) = 89 ms; flip angle 150°; resolution 1x1x1 mm; slices = 80). All structural scans were visually checked at the time of acquisition for motion artefact and if necessary a repeat scan was obtained during the session.

Fieldmap images were obtained for post-acquisition correction of gradient field effects. Echo time (TE) during EPI sequences was varied during initial scanning sessions in order to investigate the optimal TE for use in newborn infants (TE range: 30 - 70 ms). In addition, in a subset of infants, short EPI scans were collected at various TEs (n = 7, TE range: 30 - 80ms; n = 1, TE range: 5.33 - 29.09 ms). The protocol and analyses following acquisition of these images are described in Chapter 3.

Prospective Acquisition Correction for head motion (*PACE*) was applied during all EPI scans. *PACE* is a motion correction technique that tracks the position of the head during scan acquisition and applies a real-time correction for large head movements [205]. All stimuli were time-locked to the fMRI data by the experimenter pressing a button at the same time as the stimuli were applied. This button press was registered and recorded using Neurobehavioural System's *Presentation*[™] Software.

Finally, once all scanning was completed, the infant and parents were transported back to their ward by the research team and clinical member of staff, as described in section 2.1.1.

2.5 Adult study protocol

2.5.1. MR Image acquisition

fMRI of the brain was performed on all participants. Adult participants arrived at FMRIB where they were asked to remove items of loose metal and were screened for MRI safety by a Radiographer. Following successful screening, ear protection was provided (foam ear plugs, 3M, attenuation 36 dB) and adults were made comfortable while lying on the MRI scanner bed.

Full brain T1-weighted structural images were obtained for adults (MPRAGE; TR = 2040 ms; TE = 4.7 ms; flip angle 8°; resolution 1x1x1 mm; axial slices = 192). This was followed by acquisition of field map images for correction of gradient field effects. Next, five T2-weighted EPI sequences were acquired including application of *PACE* (in order to keep the acquisition as close to the infant protocol as possible), but with the typical TE used for adult studies (TR = 3280 ms; TE = 30 ms; flip angle = 90°; FOV = 192mm; imaging matrix 64x64; resolution 3x3x3 mm; slices = 50; average total volumes = 96). During the EPI sequences experimental noxious stimuli were applied as described in section 2.3.1.

2.6 MRI data processing

2.6.1. Adult data preparation and first level analysis

All data processing were carried out using the FMRIB's Software Library (*FSL*) [206-208]. *FSL version 5.0* was used for adult data. All adult T1-structural images and gradient fieldmap images had the skull surface and non-brain regions removed from the image using FMRIB's brain extraction tool (*BET*) [209]. Fieldmap images were collected and used for correcting field inhomogeneity within the static magnetic field (caused by the participant lying in the scanner).

All first level analyses were run using FMRIB's Expert Analysis Tool (*FEAT*, version 6.0). Functional data were spatially smoothed (full width half maximum = 5 mm) and temporal filtering (high pass cut off = 90 s) was also applied. Motion artefacts were minimised using Motion Correction with *MCFLIRT* [210] and by the addition of motion-derived explanatory variables (EV) in the models. Motion-derived EVs were generated using *FSL* motion outliers. The function uses the plots generated by *MCFLIRT* to identify individual volumes within the functional EPI, during which a large deviation in head position occurred, A volume is identified as an outlier, and therefore removed from further analysis using the following threshold: $Q3 + 1.5 * IQR$, where $Q3$ is the 75th percentile of the data and IQR is the interquartile range. A binary text file is then generated identifying the volumes with high motion parameters (i.e. outliers). This text file consists of a binary 2D matrix (size = the number of outlier volumes x the total number of volumes in the functional EPI), and can be added to form an additional confound EV in the general linear model (GLM) when running stats for the first level analysis. Each EV effectively removing the signals associated with the identified time-point from the analysis.

The timing of each applied stimulus (accurate to 0.1 s) was extracted from a file generated during the study using *Presentation*[™]. The timing of the stimulus was then used to generate a custom timing file consisting of triplets of numbers. The triplets describe; the onset of the stimulus (accurate to 0.1 s), the duration of the stimulus (i.e. 1 s) and the value of the input during that period (i.e. 1 as all stimuli were of equal strength). *FEAT* used this information to create a model of the expected BOLD response given the stimulus timings. Time-series analyses were performed using a general linear model (GLM) by convolving the experimental model with a standard adult HRF (mean peak = 6 s (sd = 3 s)). This approach was used to identify voxels in the brain that had significant increases or decreases in BOLD activity (threshold at $z = 2.3$ with a corrected cluster significance level of $p < 0.05$, contrast EVs used: +1 or -1).

All functional data were registered, via each individual's structural image, to a standard template brain (described fully in section 2.6.3).

2.6.2. Infant data preparation and first level analysis

All data processing were carried out using *FSL version 4.9.1*. [206-208]. *FSL version 4.9.1* was used for infant data as it incorporates *FEAT version 5.98*, which does *not* use boundary-based registration (*BBR*). This was necessary in order to achieve successful registrations of infant images (for further details see section 2.6.3). *BET* was used to remove non-brain structures from the infant T2-weighted structural images [209]. Parameters within *BET* were adjusted to produce the optimal brain extraction per image. In general the option to improve *BET* for a small field of view in Z produced a good brain extracted structural. In the infant field maps, brain extraction was achieved using a mask of the infant's brain-extracted structural image to guide the field map preparation.

All first level analyses were run using *FEAT* (*version 5.98* was used for registration and pre-stats and *version 6.0* for further statistical analysis). Wherever possible, infant data processing were done in the same way as the adult analyses described above. Functional data were spatially smoothed and temporal filtering was applied as in adults since the voxel size of the acquired EPI data was the same in both groups. Motion artefacts were minimised using *MCFLIRT* [210] and by the addition of motion-derived explanatory variables (EV) in the models.

There were two key differences between the adult and infant analyses. Firstly, the registration used for the infant analyses was different to adults (the steps taken are described in detail in section 2.6.3). Secondly, as well as the use of *MCFLIRT* and motion EVs in both groups, independent component analysis (ICA) was applied using *MELODIC* (model-free fMRI analysis using probabilistic independent component analysis) and components representing movement were removed from the data (this process is described in detail in section 2.6.4).

First-level statistical and post-statistical analyses of each infant's functional images were conducted using *FEAT* (*version 6.0*). Stimulus timing files were prepared and input into

the analyses in the same way as described in section 2.6.1. *FEAT* used this information to create a model of the expected BOLD response given the stimulus timings. Time-series analyses were performed using a general linear model (GLM) by convolving the experimental model with an infant HRF (mean peak = 7 s (sd = 3.5 s)), described by Arichi *et al.* [211]. The resulting model corresponds to the expected BOLD response within any given voxel, and is included as an explanatory variable in the GLM. The analysis then generates a parameter estimate per explanatory estimate included in the model, which can be tested, in order to determine whether the observed responses within a voxel match the expected responses. This approach was used to identify voxels in the brain that had significant increases or decreases in BOLD activity (threshold at $z = 2.3$ with a corrected cluster significance level of $p < 0.05$, contrast EVs used: +1 or -1).

2.6.3. Registrations

Infant fMRI data were collected across an age range of 35-43 weeks gestation. During this period of development the volume of the brain is rapidly increasing and the patterns of gyri and sulci are becoming more defined from week to week [180,212] (figures 1.4 and 2.4). Analysis of newborn infant data therefore requires an adapted approach when compared to the most commonly adopted method for analysing adult data.

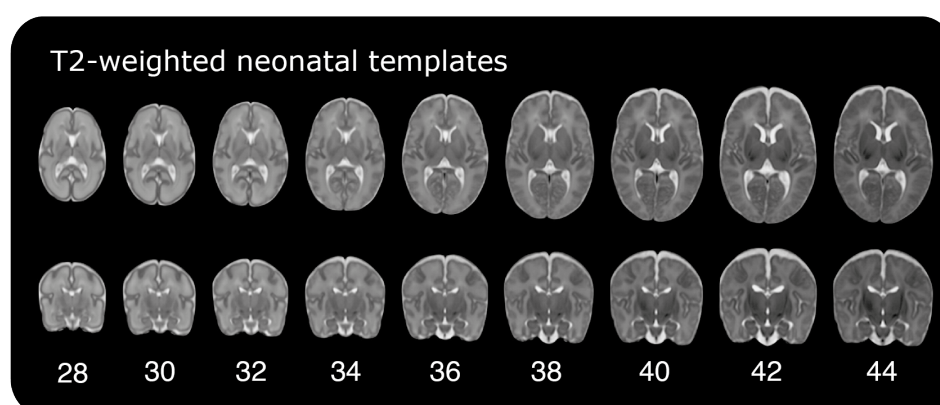


Figure 2.4: T2-weighted neonatal weekly gestational age template images [212]. Images of template brains (generated for each gestational week) were constructed from structural scans of 204 premature neonates. Reprinted from NeuroImage, [212], with permission from Elsevier.

A three-step registration approach is most commonly used for registration of adult data and requires three images and three transformations to be performed (see figure 2.5). For each adult, three steps are taken as follows; (i) the EPI image was registered to the subject's T1-weighted structural image, with a rigid body transformation, six DOF and BBR, using FMRIBs Linear Image Registration Tool (*FLIRT*) [210,213,214]; (ii) the T1-weighted structural image was registered to a standard structural template brain called the MNI template (<http://www.bic.mni.mcgill.ca/ServicesAtlases/ICBM152Nlin6>) using FMRIBs Non-linear Registration Tool (*FNIRT*) with a non-linear transformation and 12 DOF; and (iii) the functional image is registered to the final standard image using the individual's high-resolution structural as a guide.

This registration of low resolution functional images to high resolution structural images is extremely important as it allows for each voxel within the data to be most accurately located onto an image of the individual subject's brain [210,213]. This optimised three-step method was used to register all of the adult fMRI data however, for infants, an additional step was utilised in order to optimise registration within this subject group.

For infants a 4-step registration approach was used (figure 2.5): (i) the EPI image was registered to the subjects T2-weighted structural images using *FLIRT*, with a rigid body transformation with six DOF and no BBR [210,213]; (ii) the T2-weighted structural images were registered to a neonatal specific template image, which corresponded to the gestational age of the infant at the time of the study [212]; (iii) the template images were then registered to a standardised infant template, corresponding to a 40-week gestation infant [212]; and (iv) the functional image was registered to the standard 40-week template using the individual structural and weekly templates as guides. The second and third stages of the infant registration were carried out using a non-linear transformation (*FNIRT*) and 12 DOF. All weekly template brain images were available from www.brain-development.org. The final registration step allowed all resulting analyses to be viewed in a common space with one common background image. The 40-week gestation template was chosen because it most closely matched the median age of the infants. This was

beneficial because it reduced the difference between the infants at the highest and lowest ages, therefore reducing the magnitude of the registration required between the weekly templates.

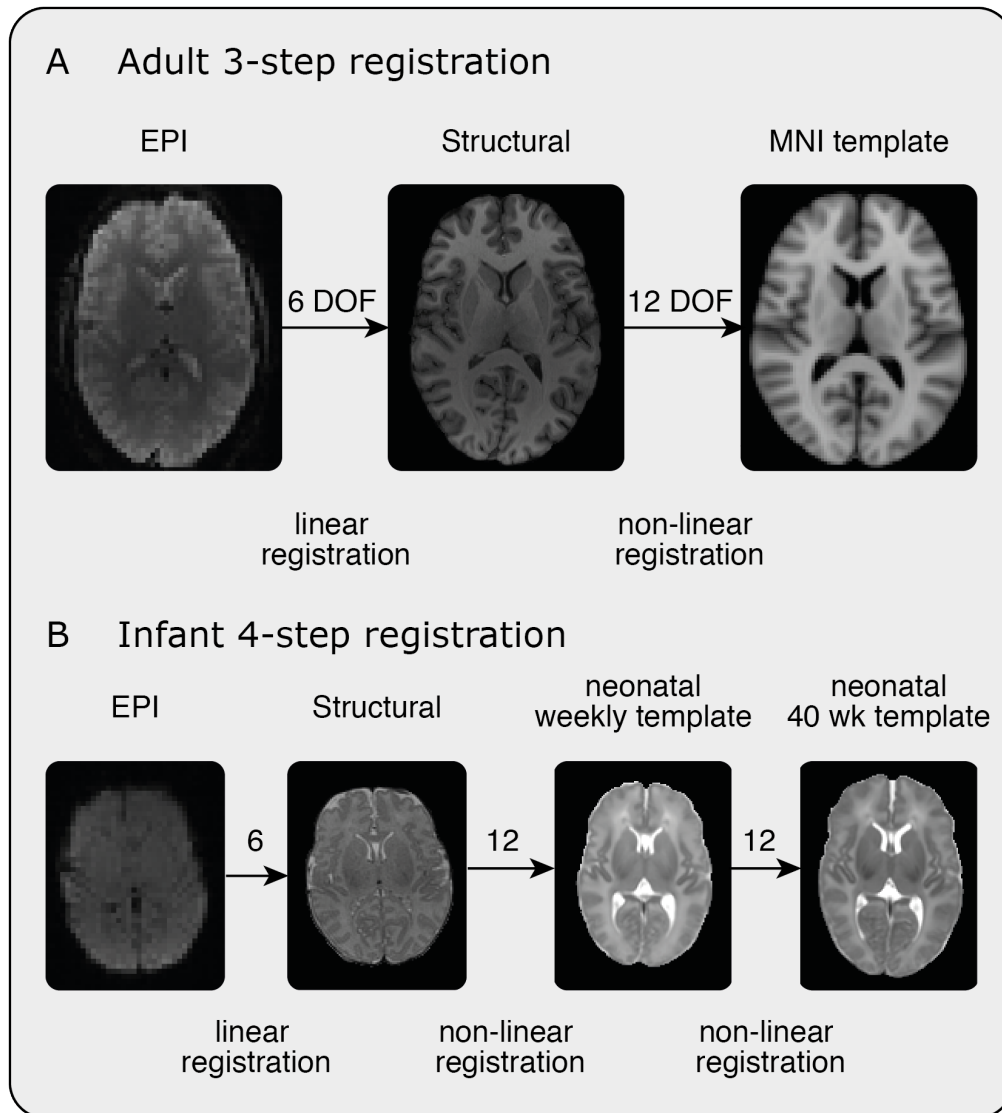


Figure 2.5: Steps involved in registration of adult and infant images. Schematic represents (A) adult 3-step and (B) infant 4-step registration methods. Examples of EPI and structural images were taken from the data used in this thesis. Numerical values above the arrows in section (B) represent the degrees of freedom of the registration method used. An MNI 2mm template brain is used as the standard template brain for adults while a 40 week template image generated by Serag *et al.* is used for infants [212].

In infants, it was also necessary to use an earlier version of *FEAT* (version 4.9.1.) when running registrations. This was because *FEAT* was not previously automated to use

boundary-based registration (*BBR*). *BBR* is a method by which segmentation of the structural is used to separate white matter boundaries (since they are usually more clearly defined). These boundaries can then be used to guide better registration of the EPI to structural [214]. Below is an example of an EPI to structural registration obtained when *BBR* is adopted in the infant studies (figure 2.6 A). Excessive distortion and stretching of the edges of the image can be observed. This is likely due to the fact that the infant EPI images have very little contrast between their tissue types therefore *BBR* struggles to discern tissue boundaries. Instead a 6 DOF registration is adopted, utilising landmarks within the EPI and structural images such as ventricle boundaries to successfully register the images (figure 2.6 B). Finally the structural to standard template registration (steps two and three of the 4-step infant registration) also had to be altered so that the template space was that of the infant sized standard images and not the adult MNI image (figures 2.6 C-D). Together these alterations allowed the production of successful registrations of all infant structural and functional images.

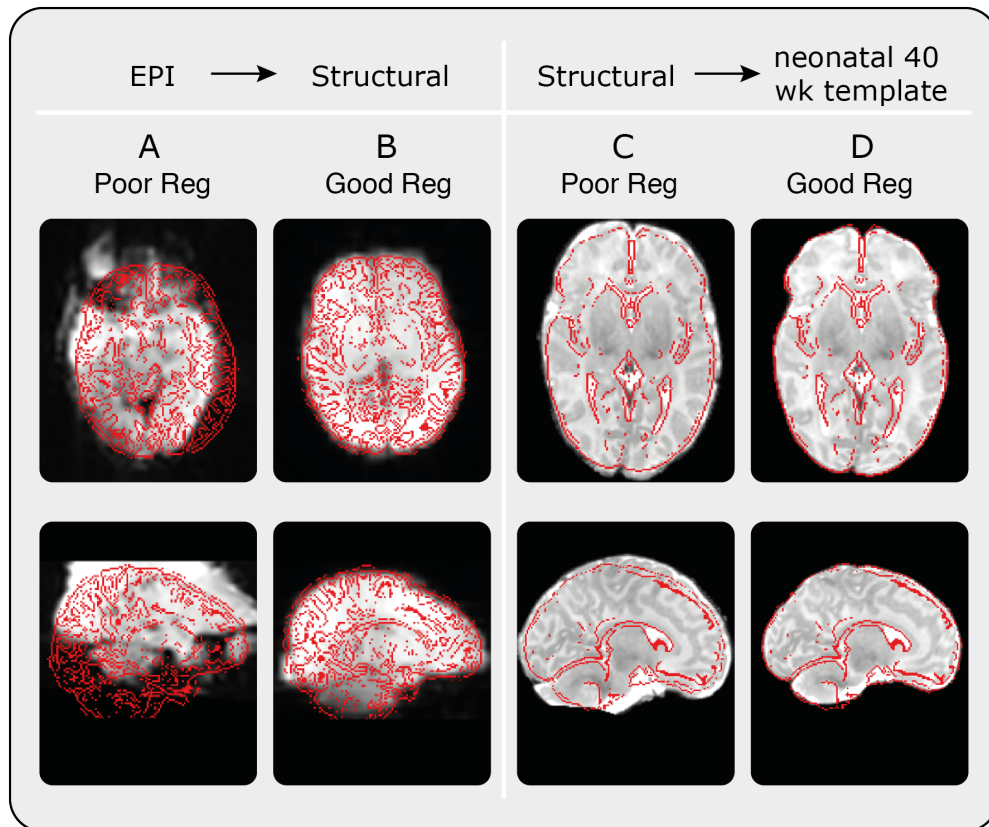


Figure 2.6: Representations of poor and significantly improved registrations from infant data analysis. Examples are of EPI to structural data (A, B) and structural to standard 40 week template image (C, D).

2.6.4. Motion correction

Excessive motion can result in unusable functional MRI data. The main methods for reducing motion during adult scans are to (i) place cushioned pads around the head to reduce the available room for head rotation and (ii) ask participants to remain as still as possible during scanning. In infants, it is possible to adopt the first method however, unless the infants are asleep or very settled, there is a high chance that they will move during the scanning sequence. Therefore, in this following section, three methods are described that have been adopted to reduce motion effects during acquisition (prospective) and data processing (retrospective): (i) *PACE* (ii) *MCFLIRT* and explanatory variables (EVs) for large head displacement, and (iii) removal of independent components of brain activity resembling movement.

2.6.4.1. *PACE* for head motion

Prospective acquisition correction (*PACE*) is a technique developed by Thesen *et al.* that uses real time image-based tracking to assess images as they are collected and consequently adjust the acquisition frame of reference if the subject has moved [205]. This can very simply be described as the application of a rigid body transformation in between consecutive volumes. Due to the motion expected in the infant scanning, *PACE* was used to optimise data collection. In order to be as consistent as possible when collecting data across the two groups, *PACE* was applied to EPI scans collected in both adults and infants.

2.6.4.2. *MCFLIRT* and explanatory variables (EVs) for large head displacement

Motion correction with FMRIB's linear image registration tool (*MCFLIRT*) was applied during all first level analyses. *MCFLIRT* applies motion correction to a 4 D functional data set following acquisition. A rigid body (linear) transformation is used to register each volume, using a rigid body transformation, to a reference volume (the middle volume in the functional image is used as the reference). *MCFLIRT* also generates plots depicting the level of correction that was performed across the functional image. Below are some examples of *MCFLIRT* plots generated from data from infant participants (figure 2.7).

Using *MCFLIRT* also enabled the assessment of functional images for the presence of stimulus-correlated movement. Movement that is present at the same time or immediately following repeated stimuli can bias the data, potentially resulting in activation that is related to movement and not to the experimental noxious stimulus in question. Therefore all acquired data were visually assessed for such correlates. An example of EPI data with high stimulus-correlated movement is presented in figure 2.7 A.

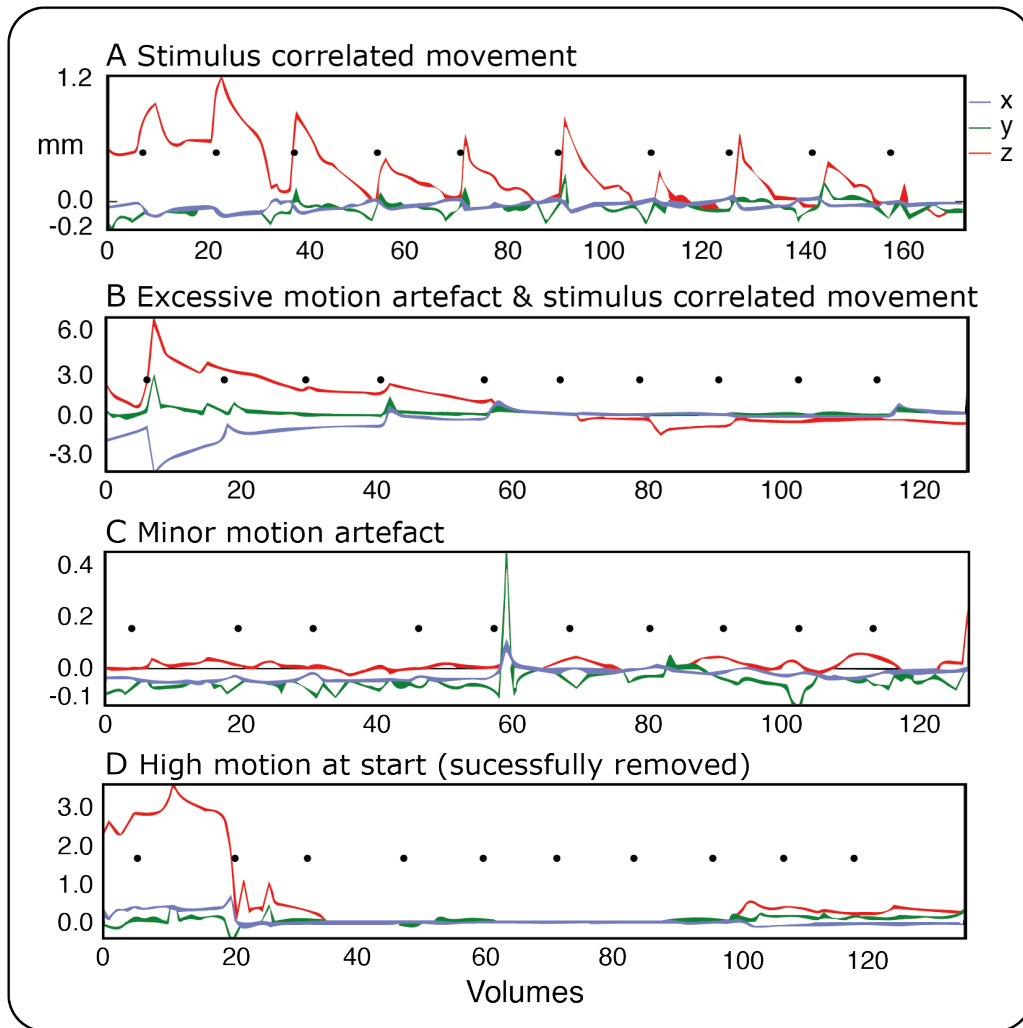


Figure 2.7: Examples of *MCFLIRT* plots used to accept and reject infant data. Motion plots generated from (A, B) rejected and (C, D) accepted functional images from infant data. Coloured lines represent both rotations and translations in mm in different directions (x: left/right, y: anterior/posterior, z: inferior/superior). Black circles represent the volume in which a 128 mN noxious PinPick stimulus was applied to the infant's left foot.

Despite the general consensus that the identification and removal of excessive motion is important in fMRI analyses, many analyses reported in the literature fail to accurately describe how subject's were screened for excessive motion and inclusion of detailed exclusion criteria is sparse [215]. Wilke *et al.* describe a method of combining total rotation and translation of individual voxels to give a value of total displacement in mm for an individual subject's scan, and suggest this methodology for individually assessing subject motion during fMRI scans [216]. This was the same methodology adopted by the function in *FSL* called *fsl_motion_outliers*. Therefore, as well as using *MCFLIRT*,

fsl_motion_outliers was used with a framewise-displacement-root-mean-square (fdrms) option to calculate the displacement (by combining rotations and translations) between each volume and the reference volume. *Fsl_motion_outliers* then uses the framewise displacement parameters to determine motion outliers per volume across the image (further details of this method was provided in section 2.6.1.). The outlier volumes then effectively have their signals removed from further analysis. These methods were adopted identically during both adult and infant analyses.

As well as using *MCFLIRT* to identify stimulus correlated motion, images with excessive motion artefact such as that shown in figure 2.7 B were also identified. These images were later reassessed following motion artefact removal using EVs and the application of independent component analysis (described in section 2.6.4.3). Images where the filtered functional data still exhibited visible motion artefacts were deemed to have failed motion artefact removal and removed from further analysis.

2.6.4.3. Independent Component removal

All of the analyses described thus far adopt rigid body correction of motion to reduce movement artefacts within functional data. However, it has been demonstrated that combining these methods with independent component artefact removal is effective at removing any remaining residual motion [217]. Independent component analysis can be used to split functional MR data into temporally and spatially distinct components of activity. *MELODIC* (model-free fMRI analysis using probabilistic independent components analysis) can be used during fMRI analysis in *FSL* to generate spatial maps and corresponding time series components from a single EPI data input. The independent components (IC) describe patterns of activity in the data that are independent to subsequent components. It has been demonstrated that this method can be used to identify and remove components from the data that resemble movement artefact [218,219]. Examples of ICs resembling movement artefact and true data respectively are shown in figure 2.8 A - B.

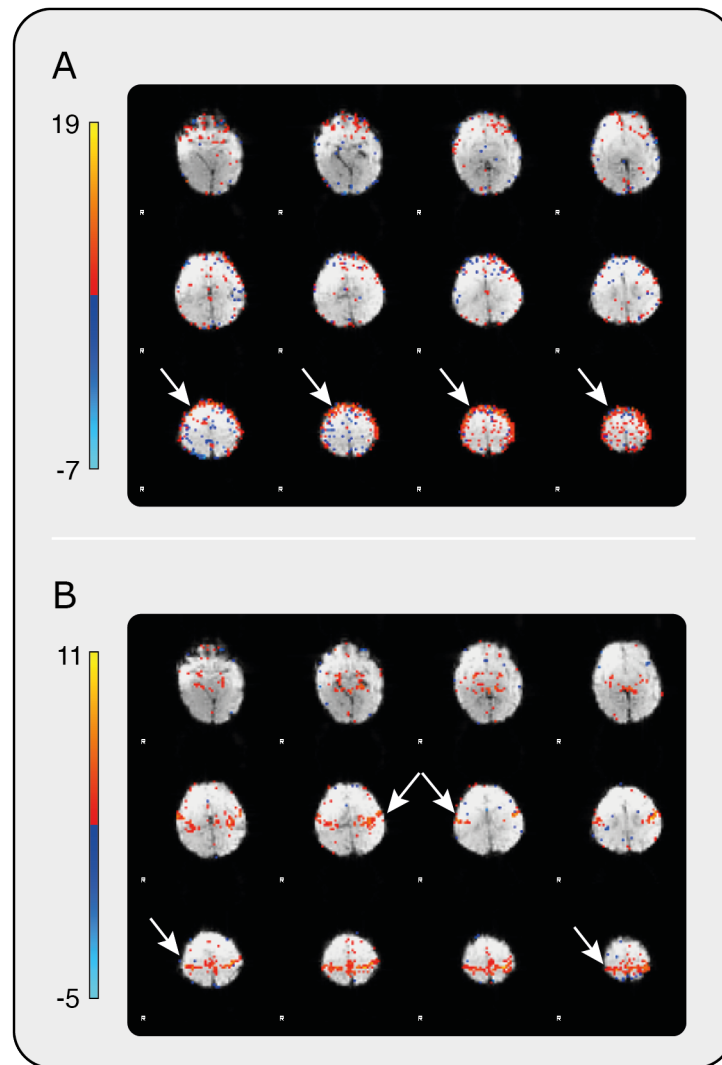


Figure 2.8: Examples of independent components generated from data from a single infant. Arrows identify (A) a rejected component with ringing around the brain, which suggests that the component is representing motion, and (B) an accepted component showing activation across the sensorimotor cortex.

2.6.5. Comparable atlases for infants and adults

A voxel-based conjunction analysis was not performed between adult and infant participants because of extreme differences between infant and adult brain anatomy, which would make such an analysis unreliable. Instead, anatomical brain regions were classified using the adult Harvard-Oxford cortical and subcortical atlases [220] and a neonatal-specific atlas, which uses similar anatomical nomenclature as the Harvard-Oxford atlases [221] (see table 2.1). Using the full list of regions from the two atlases, a

comparable list consisting of 25 brain regions was created (see table 2.1). As the cerebellum was not identified in either the adult or neonatal atlas, and the brainstem not identified in the neonatal atlas, masks that were available as part of the standard templates in each population were used to identify these regions. As is apparent from the list of brain regions in table 2.1, comparisons between the infant and adult groups were considered on a gross anatomical scale. For example, the temporal gyrus, visual cortex, and brainstem were each considered as single structures. Figure 2.9 shows the adult and infant atlases in their original segregated forms before being combined to form the 25 comparable brain regions.

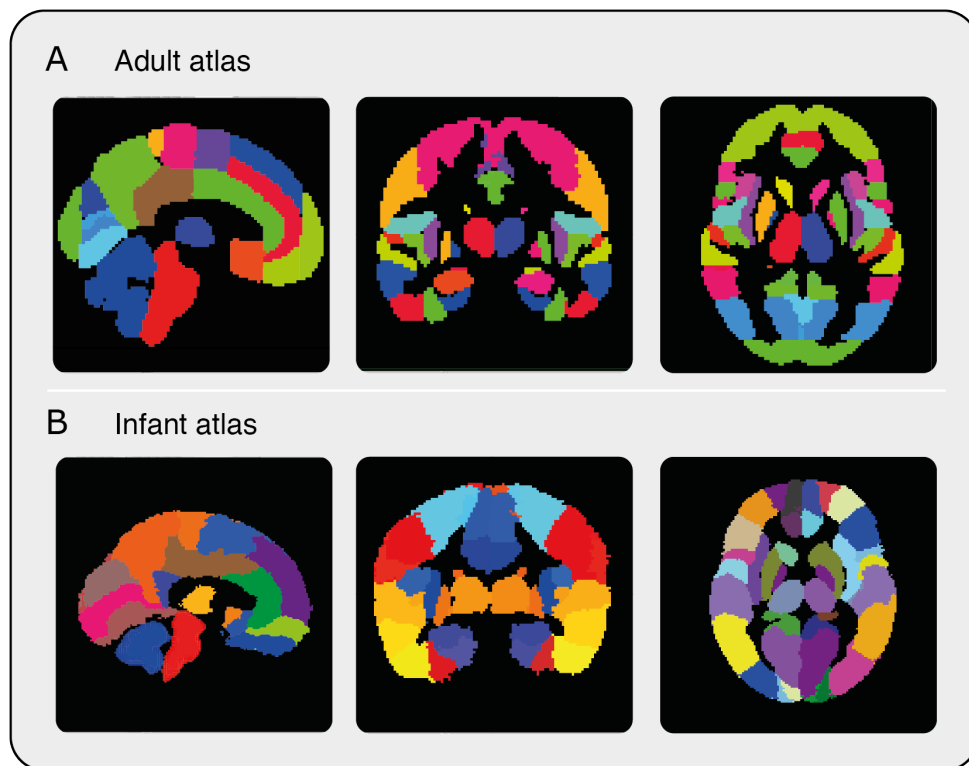


Figure 2.9: Original adult and infant atlases. Coloured images depicting (A) the original adult Harvard-Oxford atlas [220] (with MNI Cerebellum mask included) and (B) the original infant atlas generated by Shi *et al.* 2011 [221] (including a cerebellum and brainstem mask taken from the 40 week standard template image generated by Serag *et al.* [212]). Each colour represents a separate brain region as it is described in the original atlas lists (given in Table 2.1).

Region no. & name used in analysis		Adult Harvard-Oxford atlas regions combined	Infant neonatal atlas regions combined
1	Precentral gyrus	1. Precentral gyrus	1. Precentral gyrus
2	Frontal gyrus	1. Middle frontal gyrus, 2-3. Inferior frontal gyrus (pars opercularis & pars triangularis), 4. Subcallosal cortex, 5. Superior frontal gyrus, 6. Paracingulate gyrus ^(a) .	1. Middle frontal gyrus, 2-3. Inferior frontal gyrus (opercular & triangular), 4. Olfactory, 5. Rectus gyrus, 6-7. Superior frontal gyrus (dorsal & medial).
3	OFC	1. Frontal orbital cortex, 2. Frontal medial cortex, 3. Frontal pole.	1-4. OFC (inferior, medial, middle & superior).
4	Insula	1. Insular cortex	1. Insula
5	ACC	1. Anterior cingulate gyrus	1. Anterior cingulate gyrus
6	Hippocampus	1. Hippocampus	1. Hippocampus
7	Amygdala	1. Amygdala	1. Amygdala
8	Visual Cortex	1. Lingual gyrus, 2. Cuneal cortex, 3. Supracalcerine cortex, 4. Intracalcerine cortex, 5-6. Lateral occipital cortex (inferior & superior divisions), 7. Occipital Pole.	1. Lingual gyrus, 2. Cuneus, 3. Calcerine cortex, 4-6. Occipital gyrus (superior, middle & inferior).
9	Postcentral gyrus	1. Postcentral gyrus	1. Postcentral gyrus
10	Angular gyrus	1. Angular gyrus	1. Angular gyrus
11	Precuneous	1. Precuneous cortex	1. Precuneous
12	Caudate	1. Caudate, 2. Accumbens	1. Caudate ^(b)
13	Putamen	1. Putamen	1. Putamen
14	Pallidum	1. Pallidum	1. Pallidum
15	Thalamus	1. Thalamus	1. Thalamus
16	Auditory Cortex	1. Heschl's gyrus (H1 & H2)	1. Heschl gyrus
17	Cerebellum ^(c)	1. Cerebellum	1. Cerebellum
18	Brainstem	1. Brainstem	1. Brainstem ^(d)
19	Supplementary Motor Cortex	1. Juxtapositional lobule cortex (formerly Supplementary Motor Cortex)	1. Supplementary motor area
20	Parahippocampus	1-2. Parahippocampal gyrus (anterior & posterior divisions)	1. Parahippocampal gyrus
21	PCC	1. Posterior cingulate gyrus	1. Middle cingulate gyrus, 2. Posterior cingulate gyrus.

Table continued on the next page.

Region no. & name used in analysis		Adult Harvard-Oxford atlas regions combined	Infant neonatal atlas regions combined
22	SMG	1-2. Supramarginal gyrus (anterior & posterior divisions)	1. Supramarginal gyrus
23	Temporal Gyrus (15 full adult regions and 6 infant)	1-2. Superior temporal gyrus (anterior & posterior divisions), 3-5. Middle temporal gyrus (anterior, posterior and temporo-occipital divisions), 6-8. Inferior temporal gyrus (anterior, posterior and temporooccipital divisions), 9. Temporal pole, 10. Fusiform cortex (occipital division), 11-13. Fusiform cortex (temporal (anterior, posterior and occipital divisions)), 14. Planum polare, 15. Planum temporale.	1. Superior temporal gyrus, 2. Middle temporal gyrus, 3. Inferior temporal gyrus, 4-5. Temporal pole (middle and superior), 6. Fusiform gyrus
24	Parietal lobule	1. Superior parietal lobule	1. Superior parietal gyrus, 2. Inferior parietal lobule, 3. Paracentral lobule.
25	Opercular Cortex	1-3. Central, frontal and parietal opercular cortices.	1. Rolandic operculum
TOTAL		57 adult regions	47 infant regions

Table 2.1: Details of how adult and infant brain regions from the original atlases were compared between the two populations. A final comparable list of 25 brain areas was created. (a) The Paracingulate gyrus was not described as a separate structure in infants and appeared combined with the anterior Cingulate gyrus (ACC). (b) In the infants the Caudate included both the Caudate and Accumbens as described in the adult atlas. (c) The Cerebellum did not feature as an indexed structure in either atlas and therefore masks for the Cerebellum were taken from the adult MNI and infant template images respectively (<http://www.bic.mni.mcgill.ca/ServicesAtlases/ICBM152Nlin6>: Accessed on September 10th 2016) [212]. (d) The Brainstem was not included as a structure in the infant atlas, therefore a mask of this region was used from the infant template images [212].

Using this methodology, brain activity could be investigated within the gross anatomical brain regions described. In order to do this, the ROI was isolated from the appropriate atlas and registrations from the atlas space to either standard template space or the original subject's functional space was used to investigate noxious-evoked brain activity within the ROI. An example of the registration of a particular ROI from high-resolution standard space to an individual subject's functional EPI space is given in figure 2.10.

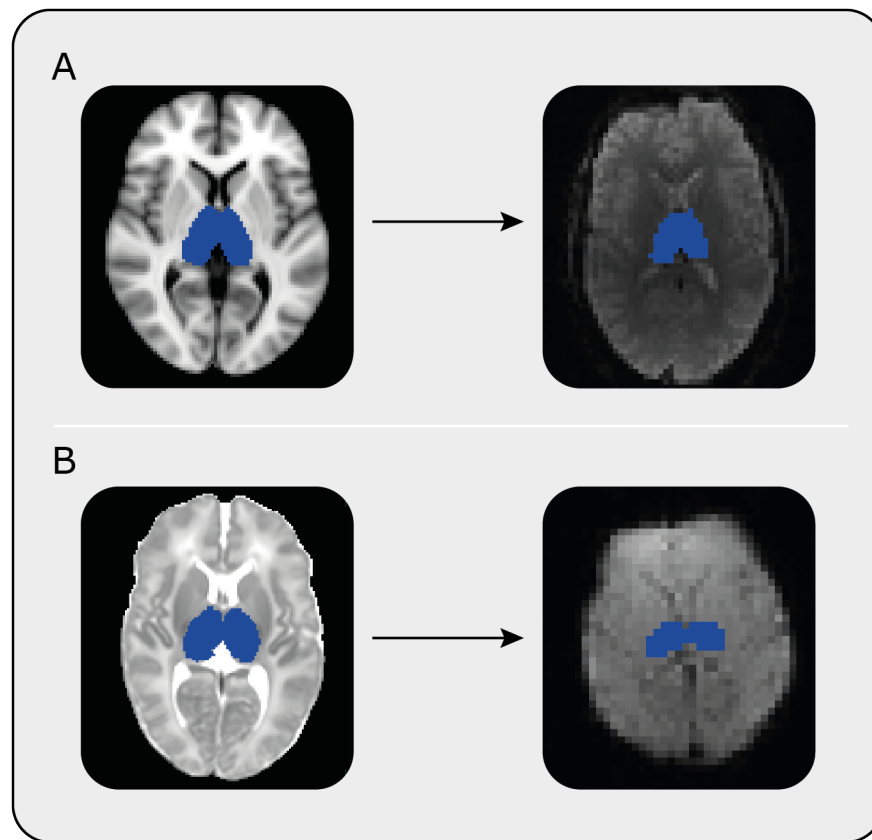


Figure 2.10: Example of the registration of an ROI mask from standard space to an individual subject's functional space. Registration of a mask of the thalamus (blue) from standard to functional space in (A) one adult and (B) one infant.

Chapter 3

Investigating optimal MR Imaging parameters for scanning newborn infants

3.1. Introduction

MR imaging is based on the phenomenon of nuclear magnetic resonance (NMR). MRI is the specific investigation of the proton, which is present in abundance within water molecules in the human body. Single protons have dipoles within them and therefore, when protons within the body, are placed within a strong magnetic field, bulk magnetisation occurs (i.e. the vast majority of protons align in the direction of the magnetic field - known as the longitudinal or B_0 plane) [157]. This direction of alignment then becomes the low energy state for the protons. During an MR experiment, a resonant field (RF) pulse is applied through an RF coil, which aligns the protons orthogonally with the B_0 plane; this new plane is known as the transverse plane [157]. Immediately after the protons have been excited, they are in a high-energy state and from this point they begin to relax back into the low-energy state of the B_0 plane. This transition from the transverse to the longitudinal plane can be detected as a free induction decay (FID) throughout the brain during MR experiments [157].

Three variables describe the different types of relaxation observed following excitation by an RF pulse. Relaxation from the transverse back to the longitudinal B_0 plane occurs in the order of seconds and involves the protons (which have a special property called “spin” and are referred to in this chapter as “spinning protons”) releasing energy *via* spin to other molecules in the surrounding tissue (referred to as the lattice) [157]. This process is therefore often termed “spin-lattice relaxation” or longitudinal relaxation and has the variable T1. T1 is mathematically represented as an exponential recovery of magnetisation (see figure 3.1); the value for T1 is given as the time taken for recovery of the signal by 63 % [222]. In addition to losing energy to the lattice, the spinning protons will also interact

with neighbouring spinning protons. This type of relaxation occurs in the order of milliseconds, is referred to as the “spin-spin relaxation” or transverse relaxation and has the variable T2 [157]. T2 relaxation is a constant that describes the exponential decay of transverse magnetization (see figure 3.1). The value for T2 is given as the time taken for decay of the signal by 37 %, (or $1/e$) [223]. T2* relaxation is similar to T2, however T2* describes the exponential decay of transverse magnetization caused by a combination of “spin-spin relaxation” and magnetic field inhomogeneity effects [157]. Inhomogeneity present within the magnetic field means protons within a voxel may not experience the same magnetic field and this results in a more rapid decay of transverse magnetisation compared to T2 (see figure 3.1).

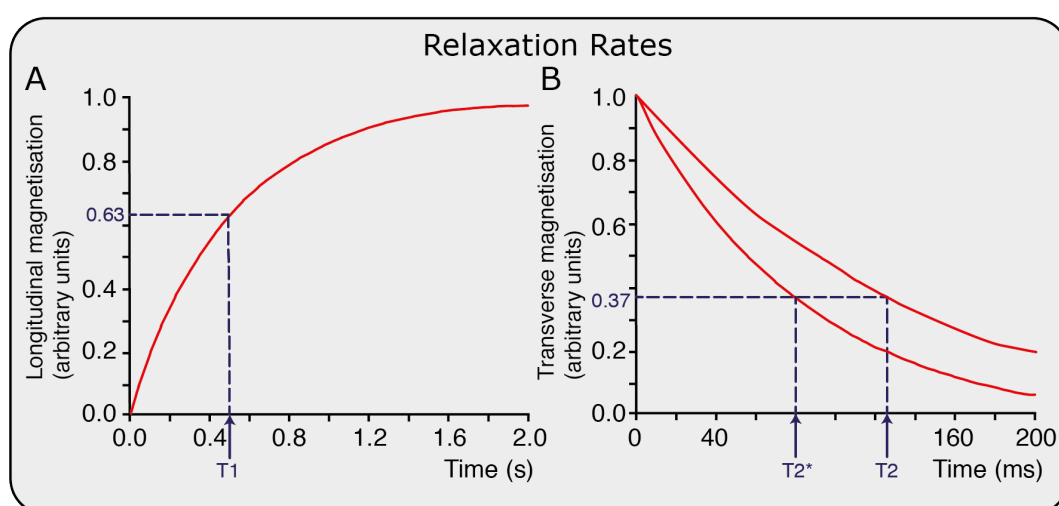


Figure 3.1: Mathematical representation of relaxation rates. (A) T1 longitudinal relaxation and (B) T2 and T2* transverse relaxation curves are shown. The relationship between T2 and T2* decay curves are also depicted in (B).

The rates of different relaxation variables are dependent on the tissue from which the signal is obtained. In general, if protons are in close contact with other protons and macromolecules then this enables more rapid transfer of energy so relaxation occurs more quickly. Consequently relaxation within brain tissue occurs fastest in white matter, followed by grey matter and then cerebrospinal fluid (CSF) [157]. Examples of relaxation times in the adult brain at 3 T are given in tables 3.1 and 3.2. These properties of brain

tissues can be utilised together with the different relaxation variables described to enable T1 and T2 tissue contrast, used to produce images of the brain during MR experiments [157].

Average T1 and T2 for grey and white matter (n = 19)		
	T1 (ms $\pm$ S.E.)	T2 (ms $\pm$ S.E.)
White matter	832 $\pm$ 10	79.6 $\pm$ 0.6
Grey matter	1331 $\pm$ 13	110 $\pm$ 2

Table 3.1: Example of T1 and T2 relaxation times measured in adults at 3 T [224]. Three regions of interest (ROI) were investigated to generate the average for white matter (WM) (WM regions: frontal, parietal and occipital) and six ROIs for the grey matter (GM) average (GM regions: frontal, parasagittal frontal, insular, parietal, parasagittal occipital and occipital). The data in this Table has been reprinted from the Journal of Magnetic Resonance Imaging, [224] with permission from John Wiley and Sons.

Average T2* in the brain at 3 T (n = 7, males)		
ROI	T2* (ms $\pm$ S.E.)	
Frontal white matter	44.7 $\pm$ 1.2	
Frontal grey matter	51.8 $\pm$ 3.3	
Occipital white matter	48.4 $\pm$ 4.5	
Occipital grey matter	41.6 $\pm$ 2.0	

Table 3.2: Example of T2* values measured in adults at 3 T [224]. The data in this Table has been reprinted from the Journal of Magnetic Resonance Imaging, [224] with permission from John Wiley and Sons.

As described in the Introduction, functional MRI can be used to record BOLD responses, which are indirect measurements of changes in blood volume, occurring in the brain when a participant is performing a task, experiencing sensory stimuli or lying at rest in the scanner. When groups of neurons activate in synchrony, metabolic demand to that brain region increases. Consequently an increase in the volume of oxygenated blood to the activated brain area can be observed approximately 6 seconds after the onset of increased neural activity [158]. Haemoglobin present within the blood is diamagnetic (possesses only paired electrons) when oxygenated and paramagnetic (possesses an unpaired electron) when deoxygenated, which is induced by oxidation from Fe^{2+} to Fe^{3+} [222]. This change in the electronic property of haemoglobin can be detected as a change in the T2*-

weighted signal collected during an EPI acquisition and is used to identify increases and decreases in oxygenated blood within a region of the brain [157]. More deoxygenated blood present within a brain region results in more paramagnetic interference with the surrounding protons [157]. This thereby causes quicker $T2^*$ relaxation in the presence of deoxygenated haemoglobin and slower $T2^*$ relaxation in the presence of oxygenated haemoglobin [225]. This change is called the blood oxygen level dependent (BOLD) response and can be measured and used to investigate the functioning of the brain during fMRI studies (see section 1.6.3).

The change in BOLD signal in fMRI studies is dependent on the echo time (TE) and the time constant that describes the decay of transverse magnetisation ($T2^*$). The TE is the time interval (expressed in milliseconds) between an RF pulse and the acquisition of signal during an EPI sequence (see figure 3.2). Ogawa *et al.* observed that the amplitude of a visual-evoked BOLD response was greater when the TE was increased during their initial MRI studies investigating the BOLD response [160]. This can be explained by considering the $T2^*$, which decays more slowly in the presence of oxygenated haemoglobin. An increase in oxygenated haemoglobin within an activated brain area will increase the $T2^*$ contrast observed between that region and those adjacent to it. The greater the $T2^*$ contrast the higher the BOLD signal increase. Therefore allowing more time (i.e. a longer TE) before acquiring the $T2^*$ -weighted signal causes an increase in BOLD contrast. These changes are also dependent on the make-up and structure of the underlying brain tissues.

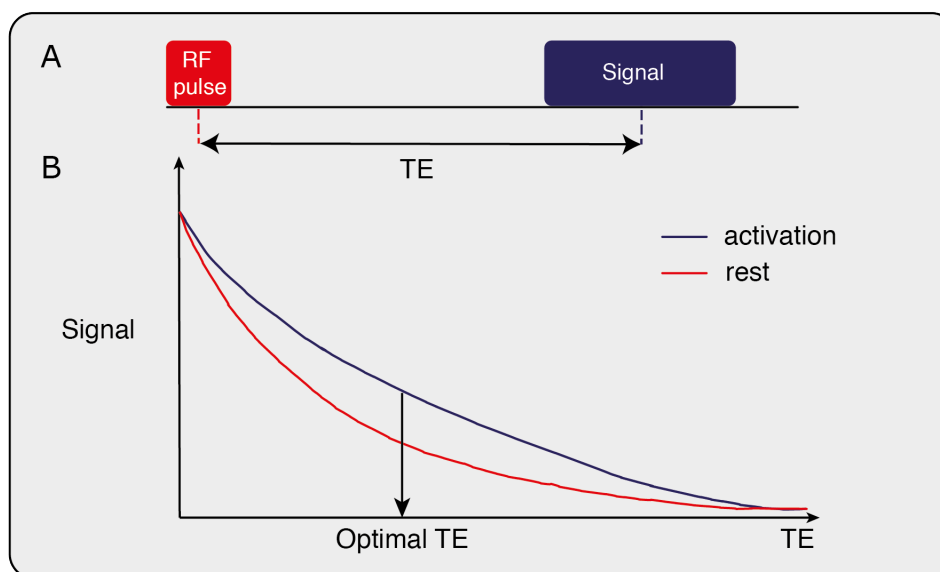


Figure 3.2: TE in fMRI experiments. (A) Schematic representing the TE parameter during a gradient echo pulse sequence. (B) Graphical representation of the optimal TE for use during a gradient EPI sequence. The black arrow represents the point at which optimal BOLD contrast is achieved, which is when the difference is greatest between T_2^* in active and resting brain regions.

In 1994 Van Gelderen *et al.* investigated the T_2^* relaxation rates and optimal TE for use in BOLD fMRI experiments and showed that the optimal TE, which was obtained when the difference between T_2 and T_2^* relaxation was greatest, was approximately equal to the T_2^* [226]. Therefore theoretically, the maximum sensitivity of the change in BOLD signal should be observed when the TE is equal to T_2^* . However, in practice susceptibility of the transverse relaxation to thermal and physiological noise, means this relationship is often not maintained [227], thus a shorter TE detects the highest signal. Fera *et al.* investigated the optimal TE for use in adults and reported that the highest sensitivity to BOLD was detected when a TE of 30 ms was used. Most adult fMRI studies, across a range of stimulus modalities and brain regions, use a TE of 30 ms, despite regional variations in the optimal TE being reported [224,228].

In infants the optimum TE has not been established, however as the infant brain has higher water content and lower lipid concentration compared to adults [179,183] a longer TE of 45 ms has been used in many imaging studies performed at 3 T [194,198]. It is important to use the optimal TE if the maximal contrast in BOLD signal and highest

resolution images are to be recorded. Moreover, as variations across stimulus modality may also have an effect on the optimal response, it was of particular note for this thesis that the optimum TE for investigating nociceptive processing in infants at 3T had not been calculated.

To date few studies have investigated T2 and T2* in infants (for examples see [185,229-232]), and none have reported T2* in infants at 3T. A study by Counsell and colleagues in 2003 investigated T2 values in grey and white matter in preterm-born infants between 25 and 41 weeks post menstrual age at 1 T [230]. The authors demonstrated that a significant reduction in T2 was observed across gestational age in frontal, occipital and central white matter regions ($p < 0.0001$) [230]. Another study by Rivkin *et al.*, conducted using a 1.5 T scanner, demonstrated that longer T2* values were recorded in the infant brain compared to adults [231]. The authors also suggested that the TE should be increased compared with adults in order to maximise the BOLD signal obtained from the infant brain. Subsequent MRI studies, conducted using a 3T scanner, also reported longer T2 values in infants. One study reported average T2 values for term infants as 210 and 123 ms for white and grey matter respectively [185]. This result highlights the substantially increased T2 value for white matter in infants compared to adults, as the average white matter T2 value in adults is reported to be 79.6 ms (see table 3.1). While the T2 value for grey matter in adults is also higher compared with infants (average grey matter T2 in adults = 110 ms, table 3.1), the difference between the adult and infant values was not as great.

In this study a series of fMRI experiments were performed, in which noxious stimulation was applied to the heel of the infant's foot while data was acquired at different TEs. The aim was to establish the TE that resulted in the greatest percentage BOLD signal change across the whole brain and in specific anatomical brain regions. In addition, as this method can be confounded by potential movement artefact, image variation due to hardware instability and variable participant responses to the stimulation, T2* maps were also generated using two approaches. As opposed to the data collected for analysing

optimal TE, both methods of T2* map generation involved data collected at rest. For the first approach, six consecutive volumes of EPI data were acquired at increasing TEs (range: 30 - 80 ms) in seven infants. The successive images were used to generate T2* maps of the whole brain and investigate T2* variation across different brain regions. A second approach utilised a single-shot acquisition technique, developed by Clare *et al.*, and recommended for use in investigating the optimal TE within a particular brain region in adult studies [228]. Using this method, a single-shot gradient image spanning multiple TEs (from a single RF excitation) was acquired in one infant and adult participant, and these images were used to generate T2* map images for comparison with the first approach. Differences in the regional variation in the T2* values between the adults and infants are also reported. The infant T2* values were compared to the TE where the maximum percentage signal change was recorded.

3.2. Methods

The infants recruited to this investigation either took part in one or both studies investigating optimal TE and T2* in the infant brain. As collection of all possible sequences would have required infants to spend longer in the scanner, increasing the risk of significant movement artefact, data collection was staggered for each section of this investigation. To avoid the excessive application of noxious stimulation each infant had a maximum of three EPI sequences collected in any single study investigating TE and a small number of infants also had data acquired for generation of T2* maps (see table 3.3).

3.2.1. Participants

The parents of 14 newborn term infants (4 females, mean GA at study: 40.23 weeks, mean PNA at study: 3.5 days) and 1 adult (female, 29 years) gave informed written consent and took part in this study. Participants were included in 3 separate studies:

Study 1: infants in study 1 took part in the TE investigation

Study 2: infants in study 2 had six single-volume EPI scans collected at a range of TEs for T2* investigation

Study 3: one infant and one adult participant had a single-shot T2* sequence acquired for generation of whole-brain T2* maps for comparison with those in Study 2

Demographics of the infant and adult participants and information on the sequences acquired during each study are provided in table 3.3.

Infant Participants									
Demographics			Study 1					Study 2	Study 3
Session (Participant no.)	IA @ study (weeks +days)	PNA @ study (days)	TE investigation					Number of TE sequences acquired	T2* multiple EPI (M) / single-shot (S)
			TE30	TE40	TE50	TE60	TE70		
01 (05)	37+4	3	x	x	x			3	
02 (06)	37+5	4	x	x	x			3	
03 (09)	41+5	4	x	x			x	3	
04 (14)	39+3	2	x		x		x	3	
05 (15)	40+2	5		x			x	2	
06 (16)	39+6	2		x	x			2	M
07 (17)	41+5	3		x	x			2	M
08 (18)	40+5	4			x			1	M
09 (19)	38+4	2				x		1	M
10 (20)	39+1	5				x		1	
11 (21)	39+5	1				x		1	M
12 (22)	42+5	2				x		1	M
13 (24)	41+5	5							M
14 (27)	42+3	7							S

Adult Participant	
Demographics	Study 3
Session (Participant no.)	T2* multiple EPI (M) / single-shot (S)
Age (years)	
01 (01)	29
	S

Table 3.3: Demographics and information on study groups for participants. M indicates the acquisition of 6 short consecutive EPI scans of one volume each. S indicates the acquisition of a single-shot T2* sequence consisting of multiple TEs (see section 3.2.3).

Summaries of the participants within each study group are given below:

Study 1: 12 term born infants (3 females, mean GA at study: 39.9 weeks, mean PNA at study: 3.1 days) had EPI scans collected at multiple TEs (see table 3.3).

Study 2: 7 term born infants (2 females, mean GA at study: 40.7 weeks, mean PNA at study: 2.7 days) had 6 volumes of EPI acquired in succession.

Study 3: One infant (42 weeks gestation, 7 days PNA) and one adult participant (29 years) had a single-shot T2* scanning sequence acquired [228]. The two images are compared in a separate analysis.

3.2.2. Investigating optimal TE

3.2.2.1. Protocol

During a single scanning session between one and three 6-minute echo planar imaging (EPI) scans were collected per infant using different TEs (see table 3.3).

During each EPI scan a noxious stimulator (PinPrick Stimulators, MRC Systems) calibrated to apply a force of 128 mN was used to stimulate the heel of the infant's foot. The heel was stimulated in trains of 10 stimuli per individual EPI acquisition, with a minimum inter-stimulus interval of 25 seconds. To avoid the excessive application of noxious stimulation to the infants a maximum of 3 trains of noxious stimulation were applied to each infant.

3.2.2.2. Calculating percentage BOLD signal change to find optimal TE

Significant group activations at each TE were investigated using single-subject first level fMRI analysis, which was conducted as described in section 2.6. To calculate the optimal

BOLD response, the mean percentage signal in a chosen region of interest (ROI) was plotted against the TE used to acquire the data. The optimal BOLD response was defined as the TE that provided the greatest increase in signal [227]. A second order negative polynomial was fit to the data as it best represented the change in signal observed at increasing TE. The fitted polynomial was determined by conducting regression analysis, which included subject as a factor. The resulting parameter estimates were used to plot the regression line and determine the TE at which the greatest BOLD response occurred. In adults a negative quadratic has also been used to describe the relationship between increasing TE and increase in BOLD signal at 3 T [227,228]. Mean percentage increase in BOLD per EPI data were averaged at each TE and the mean and standard error of the mean plotted.

Mean percentage increase in BOLD was calculated across the whole brain for each EPI scan. A whole brain mask excluded any voxels of CSF identified from the standard template brain (i.e. within the sulci and ventricles). In addition, four ROIs were selected in order to investigate the regional variation across different brain areas. The four selected ROIs were the bilateral ACC, frontal gyrus, thalamus and postcentral gyrus. *Featquery* was used to obtain a mean percentage BOLD increase in all regions.

3.2.3. Investigating optimal T2*

Two separate experimental protocols were used to investigate T2* values in infants.

3.2.3.1. Protocols

During each infant's scanning session, a gradient EPI sequence was used to acquire whole brain images across a range of TEs. In Study 2, 6 separate single-volume EPI images were acquired in succession and each image had a different TE (image acquisition parameters: list of TEs: 20, 30, 40, 50, 60, 70, and 80 ms, TR = 3.8 s, flip angle = 90°, Voxel size = 3 x 3 x 3, FOV = 64 x 64 x 33). In Study 3, a single gradient EPI sequence was used to collect

images at 12 different TEs (range: 5.33 - 29.09 ms) during the same sequence [228] (Infant sequence; TR = 1.85 s, flip angle = 70°, Voxels size = 1.5 x 1.5 x 2, FOV = 128 x 128 x 44. Adult sequence; TR = 2.36, flip angle = 70°. Voxel size = 1.97 x 1.97 x 2, FOV = 128 x 128 x 64). Full brain coverage was achieved for both participants. The image was then used to calculate the rate of T2* signal decay per voxel across the brain. All data for groups 2 and 3 were acquired at rest and all infants had their scan acquired at the start of the scanning session.

3.2.3.2. Calculating T2* in the infant and adult brain at 3T.

Each volume of data collected captured the amount of T2*-weighted signal per voxel at the specified TE. The signal relating changes in BOLD per voxel of the brain to the specified TE, is described by equation 3.1:

$$S = S_0 \exp(-TE/T2^*)$$

Equation 3.1: Equation describing the relationship between TE and T2*-weighted BOLD signal. S = signal obtained from the EPI. S_0 = Average baseline signal across the volume. TE = echo time (milliseconds). T2* = a time constant describing the exponential decay of the signal.

Regression analysis was conducted across all TEs in order to calculate the T2* constant using equation 3.1. For Study 2 each volume of EPI data (collected at a different TE) was loaded into *MATLAB* where regression analysis was carried out per voxel. A T2* map of the brain was created in *MATLAB* and subsequently used to calculate mean T2* values for the whole brain and then the four brain areas; ACC, frontal gyrus, thalamus and postcentral gyrus. In order to identify brain areas a 4-step registration was carried out (as described in the section 2.6.3). The EPI image with the shortest TE was registered to each participant's individual structural, followed by the appropriate weekly template and then the standard infant template. This allowed ROIs from the infant atlas to be registered to the single-volume EPIs in order to calculate average T2* values across the region. A mask of each ROI in the participant's EPI space was loaded into *MATLAB* and used to select the signal from the ROI and calculate an average T2* value. Outlier values were removed from

the calculation of the mean using the following equations: $outliers \leq Q1 - 1.5*(Q3 - Q1)$ and $outliers \geq Q3 + 1.5*(Q3 - Q1)$, where $Q1$ and $Q3$ refer to the first and third quartiles calculated from the data and $(Q3 - Q1)$ denotes the interquartile range. Finally $T2^*$ values were averaged across participants to produce the overall means.

For Study 3, a custom script (generated and supplied by Dr. S. Hurley) was used. The script performs the regression described and generates a 3D image of the whole brain where each voxel has a value that corresponds to the rate of change in $T2^*$ per voxel. $R2^*$ is a variable used to describe the rate of change in the $T2^*$ relaxation. Following generation of the $R2^*$ map images, each image was brain extracted. Brain extraction was performed manually because the bright signal from areas outside the brain precluded acceptable brain extraction results using *BET*. Following brain extraction, registrations of the individual $R2^*$ map to the participant's structural and then to the weekly and standard templates were conducted. This allowed ROIs to be generated for the whole brain and then the four brain areas listed in the description for Study 2. Once the desired voxels had been selected, *MATLAB* was used to take the reciprocal of each $R2^*$ value to generate $T2^*$ values per voxel. Mean $T2^*$ values per ROI were then generated. Outliers were again excluded in the same way as mentioned for Study 2.

3.3. Results

3.3.1. Study 1: Optimising TE

The use of the optimal TE during fMRI experiments enables the detection of the maximal change in BOLD signal. Significant activity from data collected in individual subjects at different TEs is shown in figure 3.3. Significant positive BOLD responses can be observed at all TEs between 30 and 70 ms. Sensitivity to BOLD responses increase between TEs of 30 and 50 ms as shown by the increase in the number of active voxels across the whole brain (Figure 3.3 A - C). In contrast less overall significant BOLD response is present at TE = 60 ms and there is a marked reduction in significant BOLD at a TE of 70 ms (figure 3.3 D, E). The number of significant voxels at TE = 70 ms is comparable to the number observed at TE = 30 ms, however it is notable that the activity is observed in different brain regions and there is greater distortion of signal in the EPI image at TE of 70 ms compared with a TE of 30 ms (figure 3.3 A, E).

Activity at 40 ms was present across multiple brain regions including the posterior insula and opercular cortex (figure 3.3 B). At TE = 50 ms, the observed activity was more widespread across the brain with stronger activation observed across the bilateral sensorimotor cortices and the thalamus (figure 3.3 C). No significant negative BOLD was observed at any of the TEs investigated.

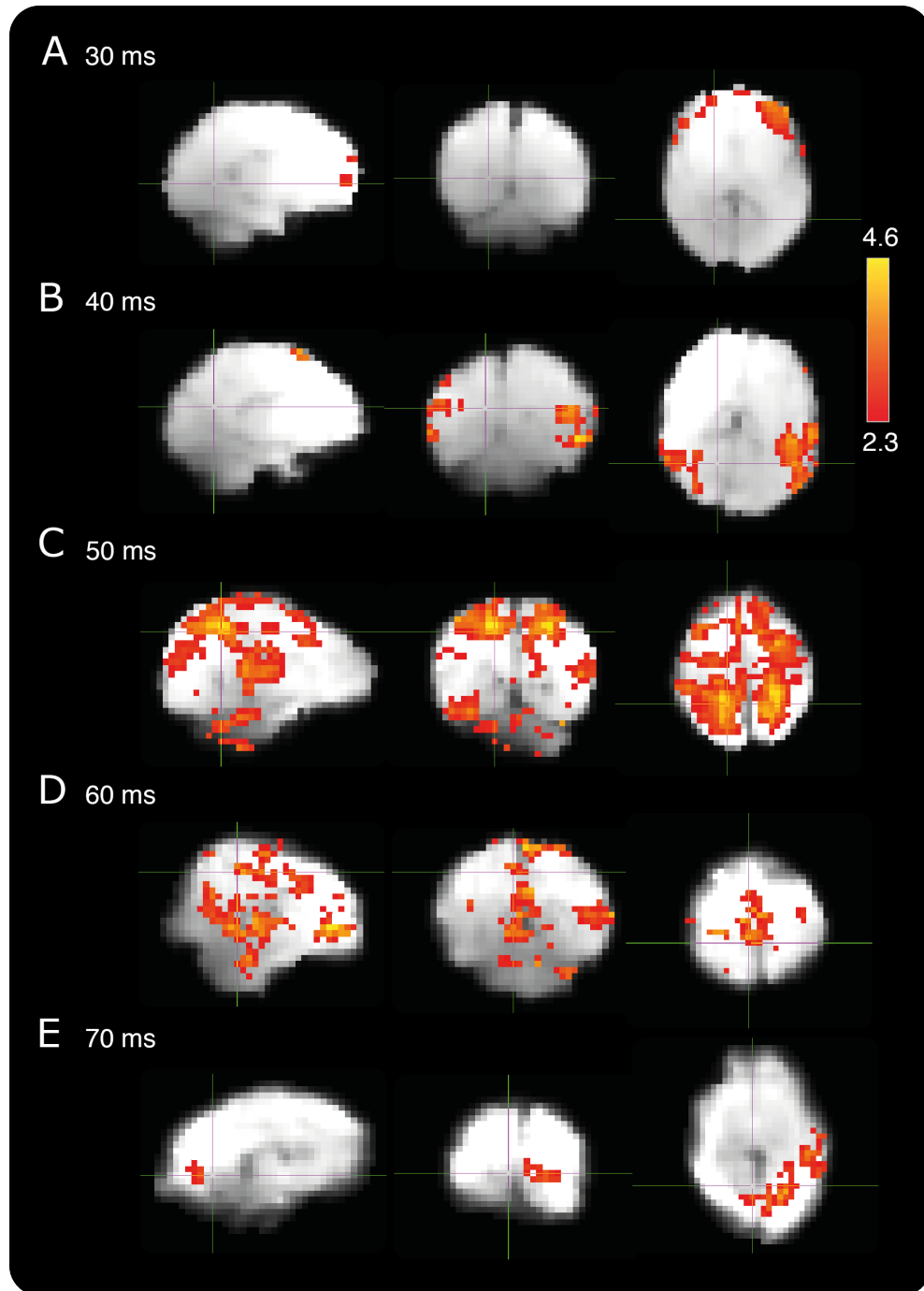


Figure 3.3: Significant brain activity at different TEs. Individual subject's significant brain activity is shown at (A) TE = 30 ms (Subject 2), (B) 40 ms (Subject 1), (C) 50 ms (Subject 6), (D) 60 ms (Subject 9) and (E) 70 ms (Subject 4). A red-yellow colour scale represents z-values for active voxels. Activity is overlaid onto mean functional EPI data shown in the sagittal, coronal and transverse planes.

The mean percentage increase in BOLD (relative to baseline) is reported across the whole brain at 5 TEs between 30 and 70 ms ($n = 23$, figure 3.4). The optimal TE was calculated to be 53 ms. 90 % of the maximal percentage BOLD increase is achieved within the range: 46 -

60 ms. The fitted regression line indicates that the increase in percentage BOLD signal between 30 and 40 ms is 0.21 % and between 40 and 50 ms is 0.09 %.

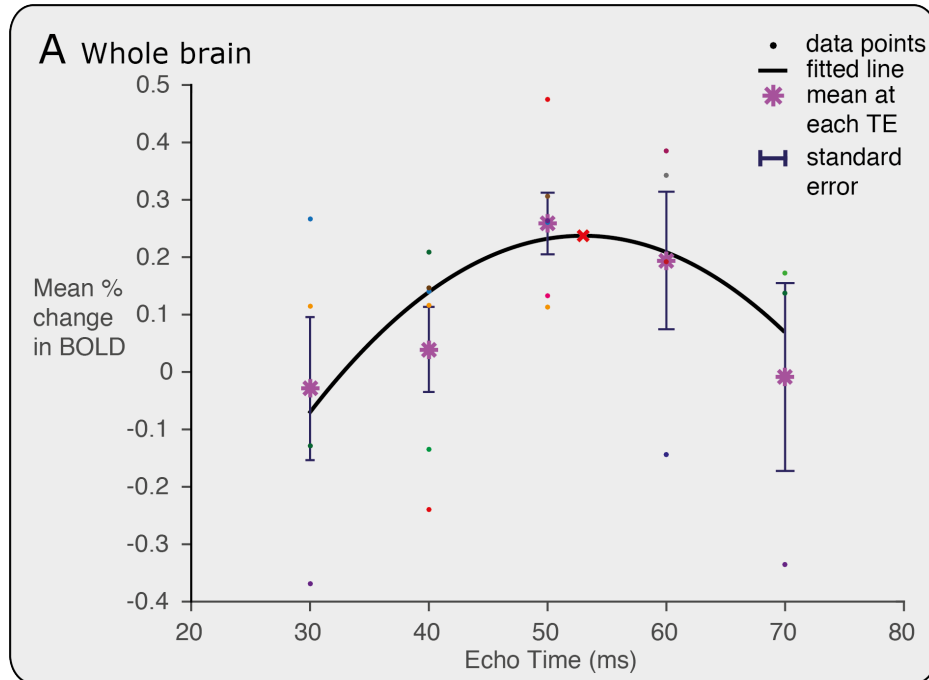


Figure 3.4: Investigation of the optimal TE at which maximal BOLD contrast is detected across the whole brain. Mean percentage change in BOLD was calculated from EPI data collected at different TEs ($n = 23$, TE range: 30 - 70 ms). Red cross indicated the peak of the regression line (TE = 53.1 ms, percentage BOLD increase at peak TE = 0.24).

Regional variation of optimal TE has previously been reported in adults [228], therefore optimal TE was also investigated within 4 ROIs shown in figure 3.5. Data from all ROIs had peak increases in the mean percentage signal between 52 and 54 ms (figure 3.5 and table 3.4). The peak TE together with the percentage BOLD increase at the peak TE is reported in table 3.4. The peak TE across all brain regions fell within the range for the optimal TE across the whole brain. This data suggests that a single optimised TE could be used for acquisition across the whole brain in infants. The percentage increase in BOLD at the peak TE ranged from 0.13 to 0.44 % across the different brain regions and these values fell within the confidence interval limits for the whole brain. The greatest percentage increase in BOLD was observed in the postcentral gyrus, which suggests that noxious stimulation in infants evoked particularly strong activation in this brain region.

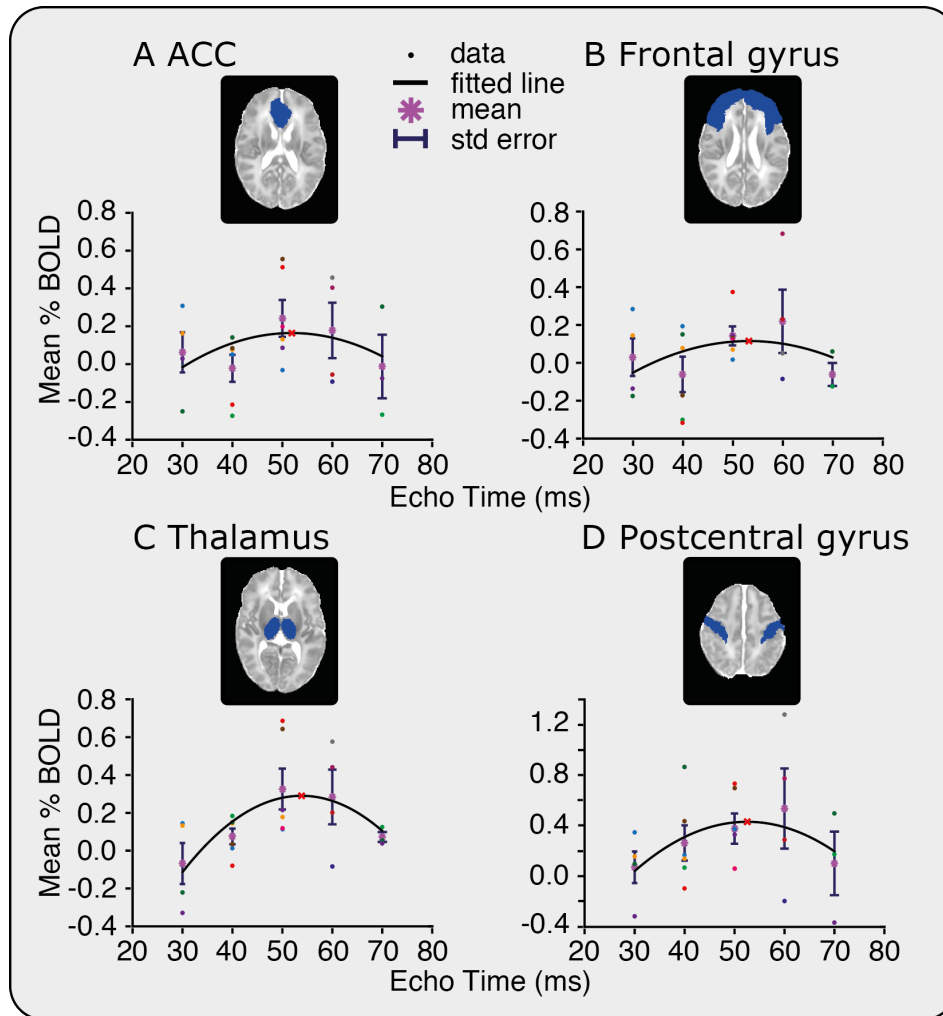


Figure 3.5: Optimal TE investigated within four ROIs. Examples of each ROI mask are depicted on a 40-week standard infant template. The mean percentage increase in BOLD at varying echo times in the bilateral (A) ACC, (B) frontal gyrus, (C) thalamus and (D) postcentral gyrus is shown. Red cross on each graph represents the peak of the regression line. Values for the peak TE and percentage increase in BOLD at the peak TE are given in table 3.4.

Region of interest	Peak TE (range) in ms	% BOLD increase at peak TE (confidence interval)
Whole brain	53.1 (46.7 - 59.5)	0.24 (0.04, 0.44)
ACC	51.9 (44.1 - 57.2)	0.17 (-0.36, 0.69)
Frontal gyrus	53.3 (45.4 - 57.3)	0.13 (-0.47, 0.72)
Thalamus	53.8 (47.1 - 59.8)	0.29 (-0.02, 0.60)
Postcentral gyrus	52.5 (44.8 - 59.1)	0.44 (-0.09, 0.98)

Table 3.4: Peak TE within four ROIs. The peak TE (including the range for which 90 % of the maximal signal is obtained) and percentage increase in BOLD at the peak TE is given for each ROI in figure 3.5.

3.3.2. Study 2: Investigating T2* with six consecutive single-volume EPIs

Examples of a single participant's EPI images (length: one volume) acquired at increasing TEs between 30 - 80 ms are shown in figure 3.6. The images were acquired at rest and demonstrate that greater signal dropout is observed at increasing TEs, with most noticeable dropout observed when the TE was equal to 70 or 80 ms. It is interesting to note that while the EPI image collected at a TE of 30 ms has the greatest signal compared to the other images, this TE would not have been suitable for detecting significant BOLD increase following noxious stimulation (figure 3.3).

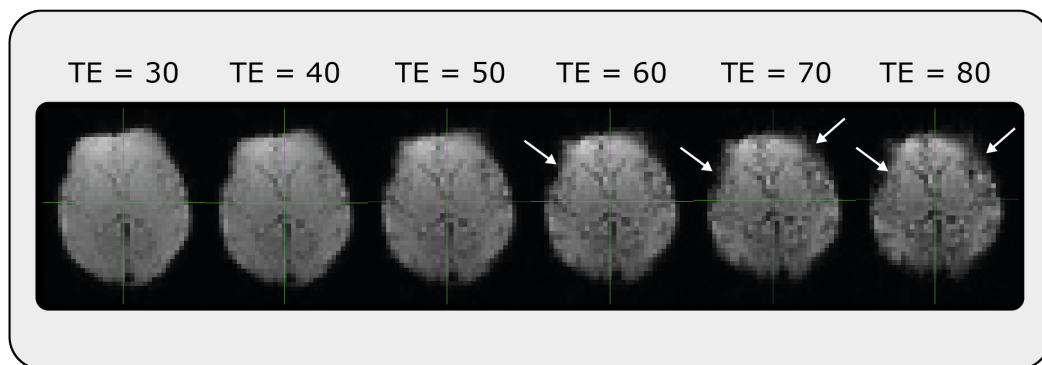


Figure 3.6: EPI volumes at increasing TEs. Traverse slices of EPI volumes from a single participant, collected at increasing TEs (indicated in the figure). Greatest signal dropout is observed at longer TEs, as indicated by the white arrows. MNI coordinates are the same for all images ($x = -3$, $y = -24$, $z = 14$ mm).

The signal obtained from the EPI images can be used to calculate the time constant describing the decay of transverse magnetisation ($T2^*$). Regression across the signal obtained at each TE was used to generate a $T2^*$ map across the whole brain. $T2^*$ values were then investigated across the whole brain and within four ROIs (the same as section 3.3.1.). Figure 3.7 A-E depicts average histograms of the $T2^*$ values within the whole brain and four ROIs. From the histograms it is notable that the $T2^*$ values across the infant brain and within the different ROI have a broad distribution, such that the modal region of the histogram plots does not necessarily represent the mean value (see table 3.5).

The mean T2* within each ROI was calculated (table 3.5) and consistent with previous reports in adults [224,233], the mean T2* values reported in each ROI are longer than the peak TE reported in Study 1.

Region of Interest	Mean T2* $\pm$ sd	Range mean values
Whole brain	86 $\pm$ 5.3	76-91
ACC	112 $\pm$ 10.0	97-125
Frontal gyrus	75 $\pm$ 11.7	61-98
Thalamus	104 $\pm$ 5.7	97-113
Postcentral gyrus	77 $\pm$ 6.9	68-84

Table 3.5: Mean T2* values calculated within different ROIs from 7 infant T2* maps.

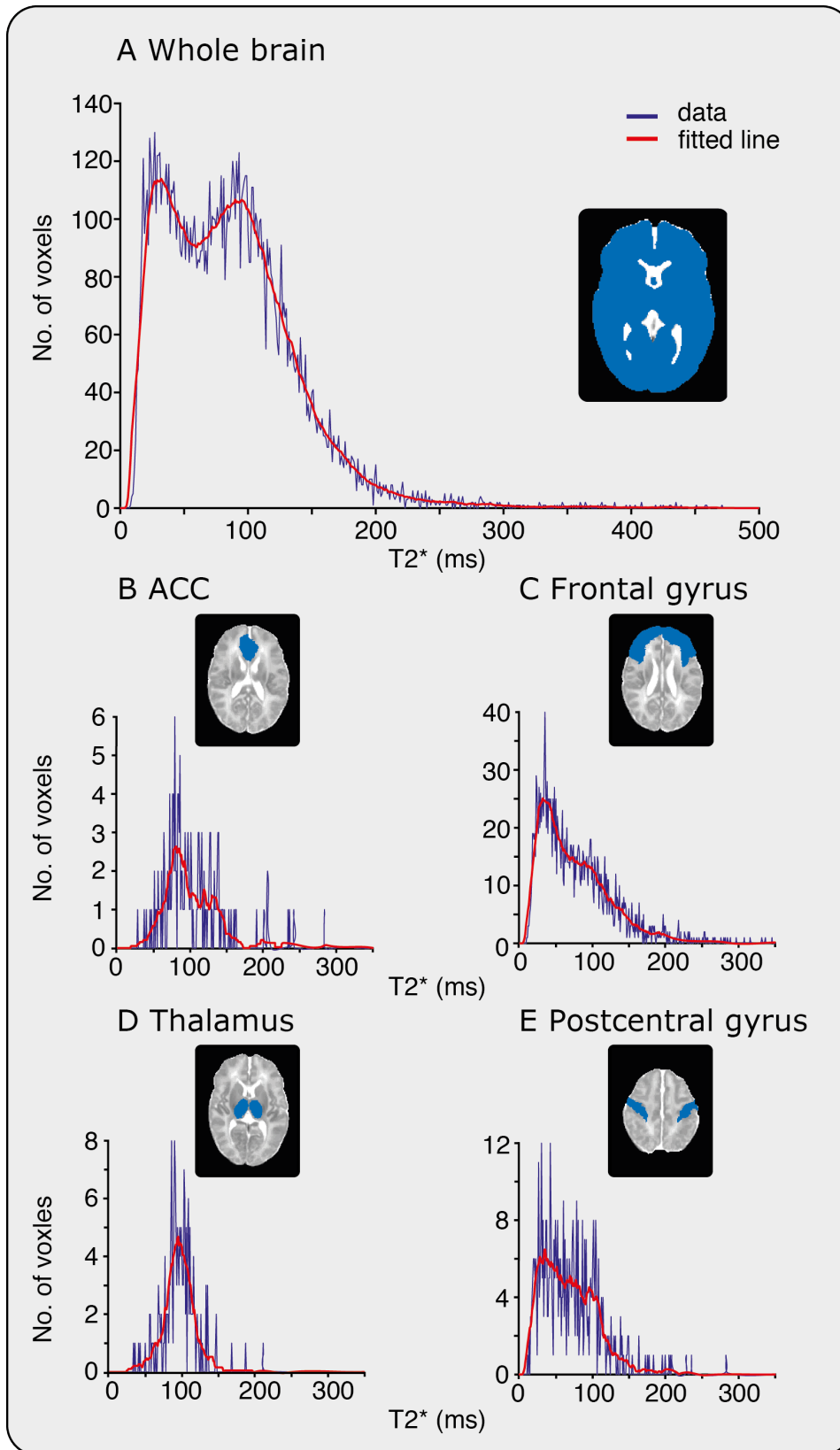


Figure 3.7: Histograms of T2* values in ROIs take from one infant's data. (A) Whole brain and four smaller ROIs (B-E) are shown.

3.3.3. Study 3: Single acquisition T2* maps in adults and infants

Single-shot acquisitions were used to generate whole brain T2* maps in one infant, and for comparison, in one adult participant. Observation of the T2* maps revealed that greater T2* values (brighter voxels) were present across the whole infant brain in comparison to the adult. The brighter regions appear to be present in both grey and white matter and there is additionally less grey/white matter differentiation in the infant brain compared to the adult (figure 3.8). In figure 3.9, the infant T2* map is depicted with a range of 0 to 300 ms in order to observe regional variability. The thalamus appears more uniform and darker (i.e. containing shorter T2* values) compared with cortical brain regions. This observation is supported by data shown figure 3.7.

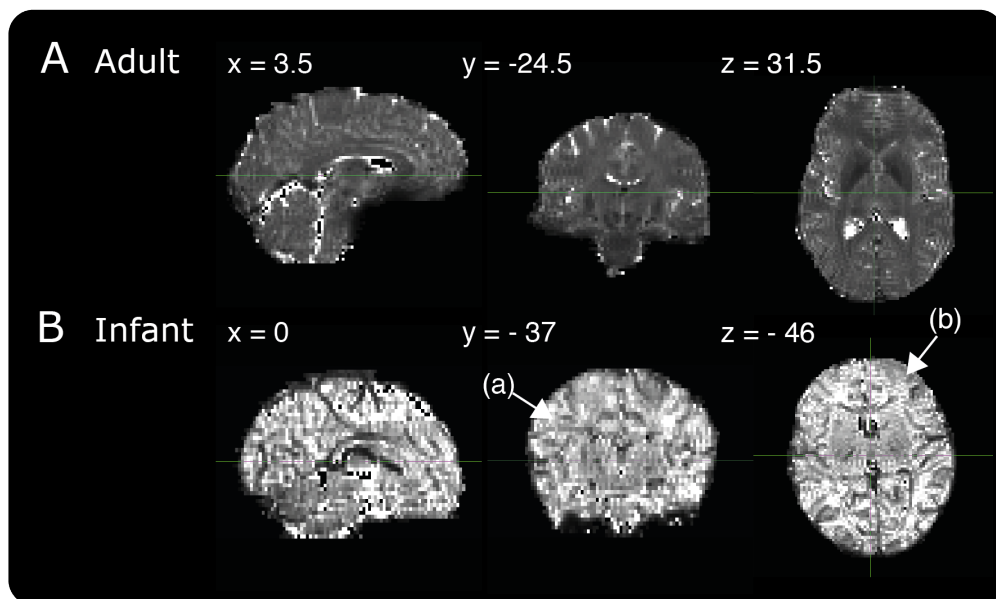


Figure 3.8: Adult and infant T2* maps. Sagittal, coronal and transverse planes of (A) one adult and (B) one infant's T2* maps. MNI coordinates are given for each plane (in mm). Brighter voxels indicate larger T2* values. Both images are scaled the same with a minimum of 0 (black/dark) and maximum of 170 ms (white/bright).

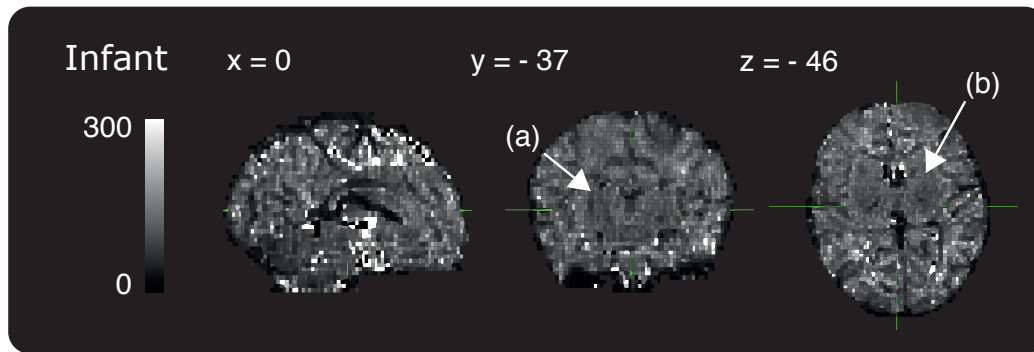


Figure 3.9: Infant T2* map. Sagittal, coronal and transverse planes of one infant's T2* map. MNI coordinates are given for each plane (in mm). No clear distinction is observed between grey and white matter in the cortical brain regions. The thalamus is highlighted using arrows (a) and (b), as a region of particularly uniform T2*.

The T2* maps recorded in the single infant participant in Study 3 were used to obtain average T2* values for the same ROIs reported for the analysis of Study 2. In comparing the T2* values with those obtained from the 7 infants in Study 2, all values, apart from the thalamus, were higher than those reported in Study 2. As the T2* values reported in Study 3 were calculated from data in a single participant, the standard deviations of the mean T2* values are also much greater than those from the group analysis (table 3.5). Adult T2* values are consistently lower than those reported for the infants in all ROIs. This is in agreement with a study by a different group at 1.5 T [231]. Adult T2* values were as expected for 3 T and match the literature [224,233]. The standard deviations for the T2* values are much greater in the single infant participant compared with the adult (table 3.6).

Region of interest	Mean T2* $\pm$ sd in region	
	Adult	Infant
Whole brain	52.1 $\pm$ 11.3	111.0 $\pm$ 44.0
ACC	65.6 $\pm$ 13.6	131.0 $\pm$ 44.5
Frontal gyrus	57.7 $\pm$ 12.6	118.3 $\pm$ 39.5
Thalamus	49.9 $\pm$ 7.5	107.4 $\pm$ 20.4
Postcentral gyrus	56.1 $\pm$ 11.8	108.3 $\pm$ 36.5

Table 3.6: Mean T2* across the whole brain and four ROIs. Mean T2* values (and standard deviations of the mean) are reported. T2* means were calculated for adults and infants from the T2* map shown in figure 3.8.

Theoretically, the maximum sensitivity of the change in BOLD signal will be recorded when the TE is equal to T2*. Overall, in this study, the mean T2* values reported in infants across the brain (Study 2, n = 7, T2* = 86 ms; Study 3, n = 1, T2* = 111.0 ms) are consistently higher than the maximal TE (Study 1, n = 12, optimum TE = 53.1 ms). The typical TE used in adult fMRI experiments is 30 ms and this is also lower than the mean T2* reported from the adult brain in this investigation (Study 3, n = 1, T2* = 52.1 ms).

3.4. Discussion

Overall, this investigation has demonstrated that a longer TE should be used in fMRI investigations with infant participants. Investigation of significant BOLD responses at increasing TEs showed that use of the typical TE used in adult experiments (30 ms) resulted in a marked reduction in significant noxious-evoked brain activity being detected in infants. This highlights the importance of optimising imaging parameters for use in this population. The results show that the optimal TE for use during studies investigating nociceptive processing in newborn infants is 53.1 ms; 23.1 ms longer than the commonly used TE for adult studies. In infants the mean T2* value reported across the whole brain was 86 ± 5.3 ms, which was longer than the optimal TE of 53.1 ms. This is consistent with adult studies where a TE of 30 ms is most commonly used, while longer T2* values are reported [224,227,233].

3.4.1. Maximum BOLD signal in infants is recorded when TE is approximately 50 ms

Longer T2* values were observed in infants at 3 T compared to adults; with an average T2* of 111 ms and 52.1 ms reported across the whole brain in individual subjects respectively. This result is in agreement with Rivkin *et al.* who investigated T2* values in four ROIs within the frontal, occipital and thalamic brain areas in infants and adults and consequently suggested that longer TEs should be used when scanning infants [231]. The newborn infant's brain undergoes considerable postnatal growth and development. This maturation includes increases in synaptic connections and myelination of white matter

tracts in the brain which all increase the proton density and concentration of macromolecules within the brain [124,179]. The infant brain is therefore very different from an adult brain and has also been shown to have higher water content and lower lipid concentrations due to low levels of myelination [183]. These differences are believed to contribute to the longer relaxation times observed in the infant brain [185]. Given that the overall rates of relaxation within the brain tissues in infants is longer, the time taken to reach maximum BOLD signal contrast would also increase. Moreover since BOLD contrast is maximal when the difference between T2 and T2* weighted contrast is greatest, acquiring signal earlier will coincide with a point of lower signal contrast. Therefore, by using the same TE as that used for adults (TE = 30 ms), it is likely that suboptimal signal would be acquired in infants. This would lead to recording a reduced BOLD signal and consequently significant activation (or deactivation) could potentially be missed.

Infant brains are not just small adult brains, they differ in size as well as underlying structure and this was the key reason that analysis of the two populations was completed separately and then compared. For example, the immaturity of the frontal cortex is a good illustration of the substantial difference between the adult and infant brains [234]. The newborn infant's frontal cortex has approximately half the synaptic density, and one third of the volume that it will reach by adulthood [184,235]. The frontal cortex undergoes gradual synaptic pruning that lasts into early adolescence, therefore it is the last region to fully mature in humans [124,236]. In contrast, brain areas including the primary motor, sensory, auditory and visual regions have been shown to mature earlier in development [237-239]. For example the auditory cortices in newborn infants have a pattern of higher synaptic density in cortical layers 2 and 3 that resembles that seen in adults, while the prefrontal cortex exhibits a more immature pattern of approximately equal synaptic density across all the cortical layers [235,240]. Synaptic pruning and lineation of the cortex is accompanied by approximately 40 % postnatal reduction in synaptic density [240,241]. This process has yet to occur in infants, so the brain has increased, less-refined, bilateral connections, and a lack of glial proliferation as this continues to develop during postnatal life [124]. A recent functional connectivity study reported that the structure of

the thalamus in term infants closely resembled the adult thalamus [199], emphasizing the timelines at which different brain regions mature during development. Fluctuation of maturation timelines could result in substantial variation amongst relaxation rates and subsequently regional variation in optimal TE in infants however, the investigation of optimal TE presented in this chapter demonstrated similar peak TEs across all the selected ROIs.

3.4.2. Optimal TE is lower than the mean T2*

Rivkin and colleagues reported longer T2* values in infants compared to adults and used the theoretical relationship that optimal BOLD signal is acquired when TE is equal to T2* to conclude that longer TEs should be used when conducting fMRI studies in newborn infants [231,242]. However, the optimal TE suggested by this experiment is lower than the mean T2*, which is in agreement with evidence suggesting that in practice optimal BOLD signal is acquired at a lower TE compared to the theoretical observation of $TE = T2^*$ [227]. This relationship is influenced by sensitivity of the T2-weighted signal to the inhomogeneity of the magnetic field within the scanner as well as physiological noise such as changes in heart rate, breathing rate [227]. Therefore lower TEs are used to detect the maximal signal contrast before signal decays under the influence of noise. Based on the results of the mean T2* obtained from the infant brain in this study, the optimal TE for use in infants should be approximately 86 ms. However, the results of our TE investigation demonstrate that percentage change in BOLD signal was maximal when the TE was approximately 50 ms. The mean T2* value reported in adults was 52.1 ms. Similarly a TE of 30 ms is typically used in the adult population, which is much shorter than the reported T2* values. Therefore, both results demonstrate that while a longer TE is required during studies in infants, TEs above 50 ms do not appear to improve signal intensity detection.

Rivkin *et al.* scanned participants on a 1.5 T scanner and as BOLD signal is also dependent on the strength of the initial magnetisation it was important to investigate whether these

results held at 3 T. Overall the $T2^*$ values obtained at 3 T were shorter than those reported by Rivkin *et al.*, at 1.5 T, as expected from the literature [233]. Moreover the difference between the adult and infant transverse relaxation times at 1.5 T was greater than that reported at 3 T (difference at 1.5 T = 76 ms, difference at 3 T = 43 ms), which is in agreement to observations from Peters *et al.*, in adults [233]. Both these observations are contributed to by the increase in the strength of the scanner. By increasing the strength of the B_0 field, the net magnetization is also increased thereby recruiting more protons into the net magnetic field and greater signal is available when the RF pulse excitation occurs. $T2^*$ values are reduced at increasing field strengths [233] due to a greater difference between the high and low energy state (i.e. B_1 or transverse and B_0 planes), therefore the time for acquiring signal and consequently the optimal TE would also be reduced. Hence the differences at greater field strengths explain why it was necessary to investigate these parameters at 3 T in newborn infants.

3.4.3. Recommendation that a TE of 50 ms is used in future fMRI studies investigating nociceptive processing in infants.

The data from this study suggests that a TE of 50 ms should be used for future studies in order to achieve maximal BOLD signal sensitivity. The data collected for this study was collected in parallel with the data reported in Chapter 5. Therefore, as limited data was available to suggest which TE would be optimal at the start of that study, a TE of 40 ms was used for data acquisition. The increased percentage water content and reduced macromolecular concentration reported in the infant brain compared to adults justified this longer TE. fMRI studies investigating somatosensory evoked activity in both premature and term infants reported using a longer TE of 45 ms and reported that this “produces good-quality images...between 23 weeks and term” [179]. Additionally Rivkin *et al.* showed that the $T2^*$ values reduce between premature and term infants but the difference is not significant [231] suggesting that the use of a similar TE would be appropriate.

Although this value is lower than the optimal 53.1 ms reported by this study, it is notable that the percentage increase in signal observed between TEs of 30 and 40 ms was 0.21 %. The increase from 40 to 50 ms added a further 0.09 % increase in signal. Therefore overall this data suggests that a TE of 40 ms would also provide greater sensitivity compared to a TE of 30 ms. However given that further gains in signal contrast can be obtained at a TE of 50 ms, it is recommended that this is adopted for future studies investigating nociceptive processing in infants at 3 T.

3.5. Conclusion

In conclusion, this investigation provides evidence for the use of a TE of approximately 50 ms in future fMRI studies in newborn infants at 3 T. This finding is supportive of investigations at 1.5 T, also demonstrating that longer TE values are employed during fMRI studies in newborn infants in order to capture maximal changes in BOLD signal. In addition, the mean T2* value in the whole brain in infants was 86 ms, which is longer than the optimal TE and illustrates that optimal BOLD signal contrast is not achieved at a TE that is equal to the T2*, as suggested by theoretical studies. Finally, the mean T2* values obtained for the infant brain at 3 T are longer than those obtained for adults, confirming that the underlying behaviour of the relaxation rates in the two populations vary considerably, further implying that an optimised approach is vital in order to obtain the maximal sensitivity towards BOLD signal in this population.

Chapter 4

Responses to acute noxious stimulation in newborn infants recorded using EEG and EMG.

4.1. Introduction

Assessment of pain in newborn infants is complex. The current best method for assessing pain in children and adults is by verbal report, however infants are not able to tell us when they are in pain. Therefore in such non-verbal populations, it is essential to find reliable surrogate measures that allow the successful detection of pain and discomfort. Clinical pain assessment tools such as the Premature Infant Pain Profile (PIPP), Neonatal Infant Pain Scale (NIPS) and Behavioural Indicators of Infant Pain (BIIP) have been developed for use in the assessment of pain in infants undergoing neonatal care [59,67,69,70]. The main surrogate measures currently adopted by these tools include monitoring of behavioural and physiological changes, such as changes in heart rate, oxygen saturations and facial expression. However despite the validation of such tools in a clinical setting [243-245], there is increasing evidence to suggest that the main behavioural and physiological indicators being measured are not reliably detecting pain [51,63].

Craig and colleagues reported that facial behavioural responses showed specificity for pain because they were significantly greater following a heel lance procedure compared to innocuous control events, such as touching the infant and swabbing the region before heel lancing [63]. In contrast, changes in physiological responses including changes in heart rate were similar across all events and were not deemed to show specificity for pain [63]. Multiple studies have also reported that the same lack of concordance between facial expression change and heart rate particularly applies to infants of extremely low gestational age [51,65]. In addition, behavioural and physiological responses are often

combined to provide a composite, multidimensional pain assessment score but the two dimensions are inconsistent with one another [71,73,246]. It is the presence of such discordance that calls into question what assessment strategies are best suited for successfully assessing pain in this non-verbal population.

The use of non-invasive brain imaging tools such as NIRS and EEG have opened up the possibility of observing patterns of evoked brain activity in response to nociceptive stimulation. Slater and colleagues used EEG to characterise a nociceptive-specific pattern of brain activity [3]. The specificity of the response was established because the activity evoked by a noxious heel lance was not evoked by non-noxious touch stimulation [3]. Following the characterisation of this nociceptive-specific brain activity, the sensitivity of the response was investigated further by comparing the evoked brain activity in prematurely born infants who had undergone at least 40 days of neonatal care and were now at term equivalent age, to age-matched term infants [203]. Premature infants exhibited a greater magnitude of nociceptive-specific brain activity following a heel lance, which the authors related with higher exposure to painful procedures compared with the term-born infants [203]. Consequently, it is possible that this functional change underlies greater sensitivity to noxious inputs seen in infants exposed to high levels of pain in early life [247-249]. Thus measuring brain activity could provide a method for detecting differences in sensitivity between patient groups.

Processing of nociceptive inputs within the brain is deemed essential for the perception of pain [29]. So it is possible that these nociceptive-specific evoked responses could provide a more accurate surrogate measure of pain in infants. While changes in haemoglobin concentration in the somatosensory cortex contralateral to the site of stimulation show strong positive correlation with clinical pain scores (measured using PIPP) following a heel lance, 30 % of infants who mounted a cortical haemodynamic response, showed no concomitant change in facial expression [73]. So measurement of brain activity might increase sensitivity towards nociceptive processing and could be used for detecting pain

in a greater number of infants, including those who do not demonstrate facial expression reactions to noxious stimuli.

However, despite the benefits of using brain-imaging techniques to help characterise nociceptive activity in the immature infant brain, these methods require specialist knowledge and training in order to be set up and hence are not necessarily appropriate for use in clinical pain assessment. Hence, it has been proposed that reliable surrogate pain-related measures that correlate with nociceptive-specific brain activity could be used to inform the development of new clinical pain assessment tools [71,72].

Grunau and colleagues have suggested that body movements could be a useful surrogate measure of nociception in infants. They demonstrate that certain actions including finger splay, brow bulge and leg extensions show significantly increased frequency during intrusive endotracheal suctioning compared to control events such as diaper change (tactile and low stress) and nasogastric feeds (non-stressful) [250]. In addition, in 1993, Craig *et al.* also investigated body movements (scores for movement of hands, arms, legs, torso and head were generated using the Infant Body Coding System (IBCS)) together with facial expression change and reported that although body movements were not reliably produced in response to a noxious heel lance event, when a response was observed, it was greater following the heel lance procedure compared with a control swab [63]. Interestingly, this measure did not show variability with gestational age with all premature infants mounting similar levels of responses, compared with facial expression change where the response increased in magnitude with age [63]. However, despite this neither group investigated the relationship between brain activity and body movements.

To date, much of the research investigating noxious-evoked responses in infants has primarily focussed on observing clinically required acute noxious procedures. It is therefore not possible (unless medically essential) to apply repeated stimulation to the same participant; making it extremely difficult to investigate within-subject variability with regards to noxious-evoked responses to clinical, skin breaking procedures. It is also

the case that healthy infants are often not able to participate, as they do not require painful clinical interventions. In order to better understand the relationships between brain activity and other surrogate measures of pain, it is beneficial to be able to present and record responses to multiple stimuli within a single subject. This approach also allows the variability observed both within and between infants to be explored.

One approach to the outlined problems is to use an experimental method for assessing and testing nociceptive sensitivity in infants. The experimental noxious stimulators (PinPricks, MRC Systems) applied in this study were designed to be used for testing nociceptive sensitivity in chronic pain patients [4] meaning they are suited towards use in sensitive populations. They are specially designed to apply varying forces without piercing the skin. The flat-tipped, small diameter ending allows high-pressure force to be applied to the surface of the skin, preferentially activating neuronal endings within the upper epidermal layers [155]. The application of the stimulators predominantly activates type I-AMH (type 1 A-fibre mechano-heat) A δ nociceptors responsible for signalling high-threshold mechanical stimulation. The experimental stimulators described have been used in adults during numerous pain studies including neuroimaging studies using functional MRI and EEG [141,175]. These studies demonstrated that PinPricks evoke both BOLD and electrophysiological brain activity within similar brain areas to laser-evoked brain activation [175,251] (a heat pain stimulus that selectively activates superficial A δ and C-fibre nerve endings in the epidermis [143]). In addition it has been shown that the magnitude of evoked brain activity increases with subjective pain report, although this has only specifically been investigated using thermal laser stimuli [252]. Ianetti and colleagues recently showed that an evoked potential (coined the PinPrick evoked potential (PEP) after the stimulator's trade name), could be recorded bilaterally in healthy adults and unilaterally in a single patient who had their right spinothalamic tract compromised following spinal surgery [141]. PEPs were also shown to be sensitive enough to assess capsaicin-induced mechanical hyperalgesia (a good model of mechanical hyperalgesia in patients with neuropathic pain). This method proved more sensitive in comparison to laser-evoked potentials (LEPs) that preferentially assess capsaicin-induced thermal

hyperalgesia - a much less frequent phenomenon in patients. It was therefore proposed that PEPs provided a useful tool for assessing central nociceptive mechanisms [141].

It has been possible to test mechanical sensitivity in newborn infants using calibrated von Frey hair stimulators [91,92,96,102], as well as PinPrick stimulators up to a force of 64 mN [200]. The forces applied in this experiment (32 - 128 mN: equivalent of 3.3 - 13.1 g forces) are all within range of those previously applied in studies using von Frey hair stimulators [91,96,102]. The studies using von Frey hair were designed to assess flexion reflex response thresholds in infants and did not investigate the relationships between brain activity and other surrogate measures of pain that may be useful for a clinical setting. Cornelissen *et al.* report that the mean threshold to elicit a reflex withdrawal response in term infants is 2.1g (approximately 21 mN force) [91]. Therefore the range of stimuli used in the experiment was expected to evoke reflex withdrawal.

Before the relationships between brain activity and surrogate measures could be investigated, it was necessary to demonstrate that the stimulators evoked nociceptive-specific brain activity in infants. Despite the stimulators previously being used during an fMRI study [200], the nociceptive-specificity of the evoked brain activity in infants had yet to be demonstrated. Recent work by Hartley *et al.* [94] has built on the previously characterised nociceptive-specific evoked potential [3] and utilised the sensitivity afforded by this measure. Hartley *et al.* first recorded the evoked brain activity in a group of term-born infants requiring a heel lance, and after confirming that a nociceptive-specific response could be identified, used this activity to develop a template of the nociceptive evoked-potential. The template can be projected onto either individual or averaged group responses in order to (i) identify whether a nociceptive evoked response is present and (ii) weight the response observed in comparison to the template. Therefore in this chapter, the template was projected on to the brain activity evoked by the experimental stimuli in order to investigate nociceptive specificity of the response and compare it to that evoked by a heel lance.

In this chapter, a series of experiments were conducted in which acute experimental noxious stimulation was applied to a group of healthy term-born infants within the first few days after birth. The aims were to establish whether the stimuli could be applied to the infants without causing behavioural distress or clinical concern; and investigate the relationships between evoked changes in facial expression, reflex withdrawal and brain activity, recorded using EEG.

In this chapter it is demonstrated that the highest force presented to infants (128 mN) can be applied without causing clinical concern or behavioural distress. The stimulators evoke spinally-mediated reflex withdrawal activity, with greater magnitudes of reflex withdrawal observed with increasing force of stimulation. Noxious-evoked brain activity, with a similar morphology to that evoked by the heel lance [3], is also observed following all intensities of experimental noxious stimulation. Furthermore the magnitude of noxious-evoked brain activity is sensitive to stimulus intensity and the level of reflex withdrawal activity correlates with the magnitude of the observed noxious-evoked brain activity.

4.2. Methods

4.2.1. Subjects

26 healthy term infants (average GA at birth: 39.6 weeks, range 37-42 weeks; average PNA at study 3 days, range 0-9 days) were recruited to take part in this study and were separated into the following 3 groups:

- Group 1:** 10 infants had clinical pain scores calculated in response to the 128 mN force of stimulation,
- Group 2:** 4 infants had an EEG and EMG recorded during a clinically required heel lance procedure,
- Group 3:** 12 infants had EEG and EMG recorded during three different

intensities of acute experimental noxious stimuli (applied forces: 32, 64, 128 mN).

4.2.2. Protocol

Parents of all the infants in the study were approached and recruited as described in section 2.2.

4.2.2.1. Infant clinical pain scoring

Infants in Group 1 had facial expressions, heart rate and oxygen saturations recorded during a period of background and following the first and last stimuli in a train of 10 acute experimental stimuli (applied force: 128 mN). Video recordings of facial expression change and changes in heart rate and oxygen saturation were epoched into independent sections with 15 seconds before and 30 seconds after each event of interest (i.e. background, first stimulation, last stimulation). Clinical pain scores (PIPP [59]) were calculated by a trained scorer who was blinded to the stimulus type in the epoch.

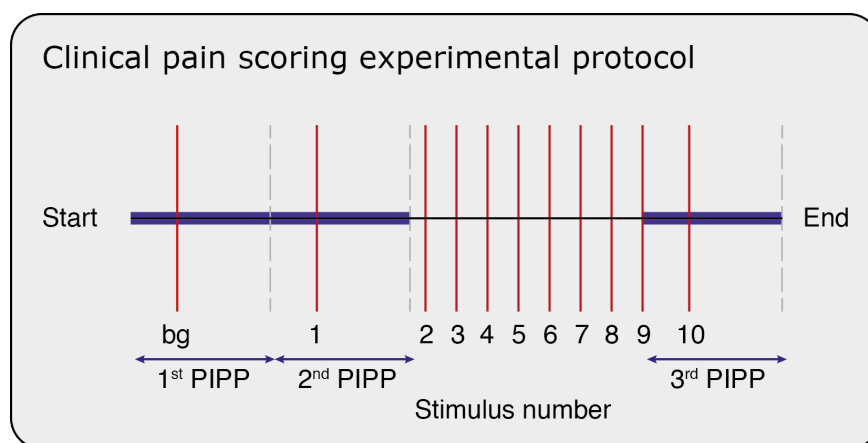


Figure 4.1: Schematic of the protocol for infants in the clinical pain scoring group. Numbers indicate the stimulus number. Blue boxes identify the stimuli for which a PIPP score was calculated. A minimum of 15 seconds before and 30 seconds after was required for the background (bg), first and last stimuli to allow enough time to calculate a pain score using the PIPP. There was a minimum inter-stimulus-interval (ISI) of 10 seconds for stimuli 2-9 as no PIPP score was calculated for those events. Red lines indicate onset of stimuli and grey dashed lines represent the end of a PIPP scoring period.

4.2.2.2. EEG and reflex withdrawal to a heel lance

Infants in Group 2 were approached and recruited if they were identified as requiring a heel lance for a clinically required blood test. Once all the equipment was set up and recording (as described in section 2.4.1) and the infant appeared settled, a period of background EEG activity was recorded. This was followed by recording of brain activity in response to a control heel lance (a heel lance rotated by 90 degrees and held against the heel before being released so the blade fires into the air, not piercing the skin) and an actual heel lance. A control heel lance was always done before the actual heel lance. The heel lance was performed last due to clinical considerations such as enabling uninterrupted blood collection and return of the infant to the parent for comforting. Bipolar EMG electrodes placed on the ipsilateral biceps femoris are used to record reflex withdrawal activity from the infant's leg.

4.2.2.3. EEG and reflex leg withdrawal to experimental noxious stimulation

Infants in Group 3 had a similar equipment set-up to that described for the infants in Group 2, however this time three different forces of acute experimental noxious stimulation (and no clinical heel lance stimuli) were presented. Each force was applied 10 consecutive times to the sole of the left foot. The order in which the stimuli were presented was randomised in blocks (i.e. once a force was randomly selected, it was delivered 10 times before the next force) and a minimum ISI of 10 seconds was allowed between each stimulus. A video camera was used to record the exact point at which the experimental noxious stimulator was applied (described in section 2.4.1). The point of stimulation was identified (precision of 9 ms and accuracy of 4.5 ms) as when the barrel of the stimulator was first depressed following contact with the skin. This point corresponded with when the full force of the stimulator was applied to the skin on the sole of the foot. All stimulators were held perpendicular to the skin when being applied so that the full force was placed on the point of skin contact. In order to apply the stimuli in this way, the experimenter was required to hold the infant's foot. The foot was held

loosely so as not to restrict any reflexive movement. The videos used to identify the point of stimulation were also used to observe and record whether a reflex withdrawal response was visible following application of the stimulus.

All EEG data epochs were visually inspected and rejected if gross movement artefacts were present. This resulted in 9 % of epochs being rejected. 7 % of EMG data were excluded from further analysis (for example, due to movement artefact) according to the following definitions: post-stimulus mean > population mean plus twice the standard deviation; pre-stimulus mean > population mean plus twice the standard deviation; or the fold increase (post-stimulus mean divided by the pre-stimulus mean) > population mean plus twice the standard deviation.

4.2.3. Statistical methods

Comparison of PIPP scores between background, first experimental noxious and last experimental noxious stimuli were carried out using a repeated measures ANOVA. Sphericity was checked using Mauchly's Test for Sphericity. A one-way ANOVA was used to compare the nociceptive-specific brain activity evoked by the heel lance, experimental noxious stimulation and background activity. As the data was not normally distributed, a Kruskal-Wallis test was used to compare the EMG RMS responses.

Linear regression, with subject taken as a factor, was used to calculate the relationship between reflex withdrawal and stimulus intensity, and the relationship between the nociceptive-specific brain activity and stimulus intensity (across responses to 32, 64 and 128 mN experimental noxious stimuli and background activity). Linear mixed-effects modelling, with subject taken as a factor, was also used to calculate the correlation between nociceptive-specific brain activity and reflex withdrawal response, and Pearson's linear correlation coefficient was calculated. The relationship between the stimulus number and evoked responses (at each stimulus intensity) was investigated using a Friedman's test (due to the relatively low subject numbers in comparison with the number

of trials). Where a single missing trial occurred, the missing value was included by regression substitution. Subjects with more than one missing trial (due to artefact rejection or where the camera angle did not allow the onset of the stimulus to be determined) were excluded from this analysis. When necessary, post-hoc comparisons were conducted using t-tests and Mann-Whitney U tests with Holm's method used to correct for multiple comparisons.

The work reported in this chapter was done in collaboration with three colleagues and members of the research team. I led collection of EEG and EMG data for the study and I assisted with data analysis. Data analysis was conducted together with Dr C. Hartley.

4.3. Results

The main results presented in this chapter have been published in Hartley, Goksan et al., 2015 (see [94]).

4.3.1. Experimental noxious stimulation can be applied to infants without causing behavioural distress.

A small feasibility study in 10 infants investigated whether the 128 mN force of PinPrick stimulator could be applied without causing clinical concern or behavioural distress. In this group of infants the Premature Infant Pain Profile (PIPP) clinical scoring tool was used to calculate a pain score following application of the 128 mN stimulus to the sole of the infant's foot. Examples of the observed changes in facial expression, heart rate and oxygen saturation following application of the 128 mN stimulus are shown in figure 4.2 A-C. The responses in the figure are examples, representative of the population studied. From the photographic stills (obtained from the video used to score facial expression response, figure 4.2 A), it can be seen that the infant is quietly awake and active. A fleeting brow bulge was observed following application of the stimulator but this would need to be sustained for a minimum of three seconds to result in a PIPP score of 1 or

more. No overall change in heart rate (HR) or oxygen saturation can be seen following the application of the stimulus (figure 4.2 B-C).

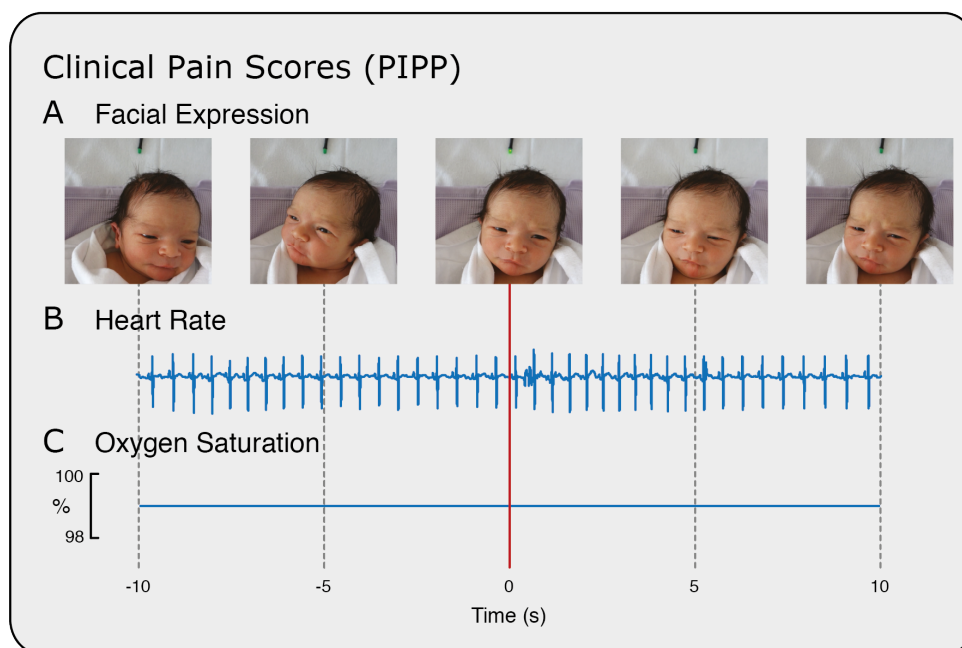


Figure 4.2: Data used to calculate the PIPP score in response to a 128 mN PinPrick in one infant. Examples of changes in (A) facial expression, (B) heart rate and (C) oxygen saturation in one healthy term infant in response to a single 128 mN stimulus (presented at time = 0 and shown by the red line).

In order to assess whether repeated stimulation was causing any sensitisation to the stimulus, PIPP scores were also calculated following a background period (where no stimulus was applied), and in response to the first and last stimuli in a train of 10 128 mN stimuli. The PIPP tool specifies “scores equal or lower than 6 indicate the absence of pain or minimal pain”. The mean PIPP score was 2.8 ± 1.6 (mean $\pm$ standard deviation) during a background period; 3.9 ± 3.4 after the first experimental noxious stimulus; and 3.7 ± 2.0 after the last stimulus. It is of note that an infant can receive a score above zero during the background period for being quietly awake or asleep (see table 1.1). There was no significant difference between PIPP scores across the 3 stimulus conditions (repeated measures ANOVA, $p = 0.55$, figure 4.3). The total score from the 3 facial expression components was also low (background period: 0.2 ± 0.4 , first experimental noxious

stimulus: 1.6 ± 2.9 , and last experimental noxious stimulus: 0.7 ± 2.0) and was not significantly different across stimulus conditions (repeated measures ANOVA, $p = 0.56$).

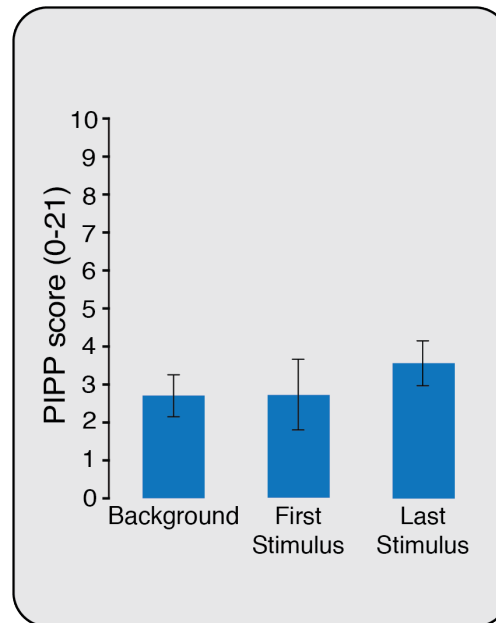


Figure 4.3: Average PIPP scores during different stimulus conditions. The average PIPP score ($n = 10$) is reported in a background period, and following the first and last in a train of 10 128 mN stimuli. Error bars represent the standard error of the mean.

4.3.2. Reflex withdrawal activity can be observed in response to experimental noxious stimuli.

Reflex withdrawal activity was quantified using EMG electrodes placed on the biceps femoris and was recorded in response to all intensities of noxious stimulation. Reflex withdrawal activity was visually observed in response to all forces of applied experimental stimulation (32, 64 and 128 mN forces). This was in agreement with the observation that the threshold for reflex withdrawal response in term infants is approximately 21 mN [91]. The frequency with which a reflex withdrawal was visually observed increased with increasing stimulus force (visible withdrawal: 32 mN: 32.6 %; 64 mN: 48.0 % and 128 mN: 72.5 %).

Figure 4.4 demonstrates average reflex withdrawal responses and average EMG root mean square (RMS) at each force of experimental stimulation (4.4 A-F) and the reflex withdrawal response and average EMG RMS elicited by the heel lance (4.4 G-H). The magnitude of the reflex withdrawal muscle activity (quantified using the average RMS) in response to the lance stimulation is more than twice as large as the maximal average RMS muscle activity evoked by the 128 mN stimulus. In addition, a decrease in the latency to the peak average RMS activity is observed between the 64, 128 mN and heel lance stimuli (see figure 4.4 C-H). The observations described here support the findings of Andrews and Fitzgerald who used von Frey hair stimuli to investigate cutaneous reflex withdrawal thresholds across development in newborn infants, and showed a decrease in the latency to peak EMG activity with increasing force as well as greater evoked EMG activity at greater forces [102].

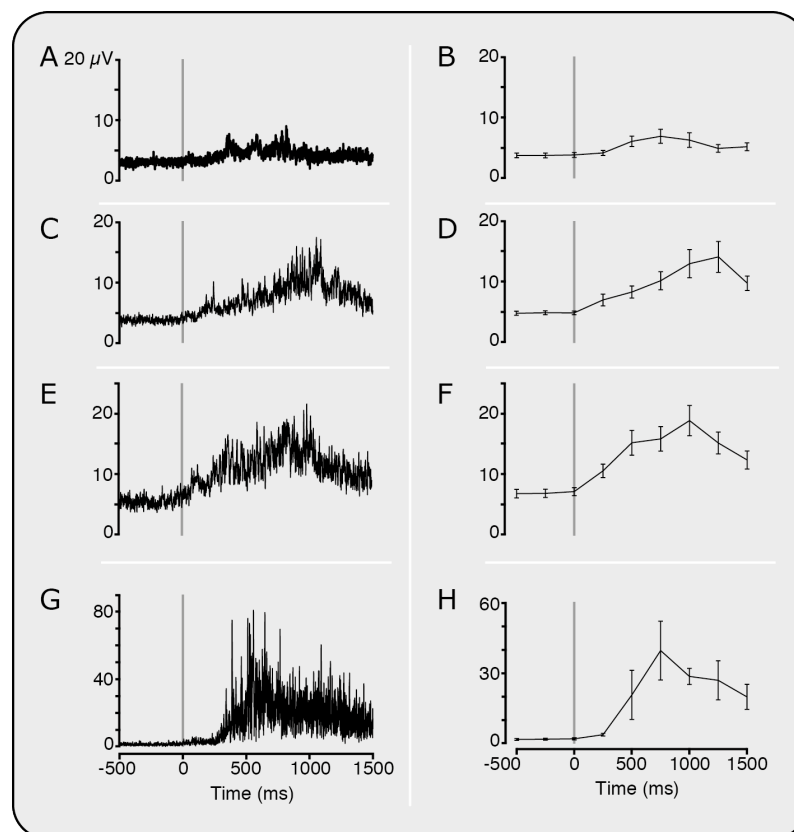


Figure 4.4: Average EMG muscle activity in response to experimental and clinically required noxious stimuli. Average EMG response (A, C, E, G) and average EMG RMS (B, D, F, H) to the (A, B) 32 mN, (C, D) 64 mN, and (E, F) 128 mN PinPrick stimuli and (G, H) clinically required heel lance.

4.3.3. Magnitude of reflex withdrawal is significantly correlated with stimulus intensity.

The magnitude of the reflex withdrawal elicited by all stimuli was significantly different from background ($p < 0.001$, Kruskal-Wallis test; post-hoc comparison of background and 32 mN: $p=0.01$; 64 mN, 128 mN and heel lance: $p<0.005$, Figure 4.5 A). The magnitude of reflex withdrawal response recorded following noxious stimulation increased with increasing stimulus intensity ($p < 0.001$). The average EMG RMS activity following the lance is almost twice that evoked by the greatest experimental stimulus force (128 mN). In turn the average EMG responses to the 128 mN stimuli was 1.5 and 2.5 times greater than that evoked by the 64 mN and 32 mN forces respectively (mean EMG RMS: lance = 23.23, 128 = 15.23, 64 = 8.83 and, 32 = 5.86). The average EMG RMS activity elicited by all experimental stimulus forces was lower than the response to a heel lance, again providing evidence that the range of experimental stimuli applied were appropriate for testing nociceptive sensitivity. It is also notable that following repeated use of the experimental stimuli, there was no significant difference in the average RMS across any of the stimulus trials (Friedman test, $p > 0.05$, figure 4.5 B). Greater experimental forces demonstrated more variability across subjects (greater standard deviations at higher stimulus forces).

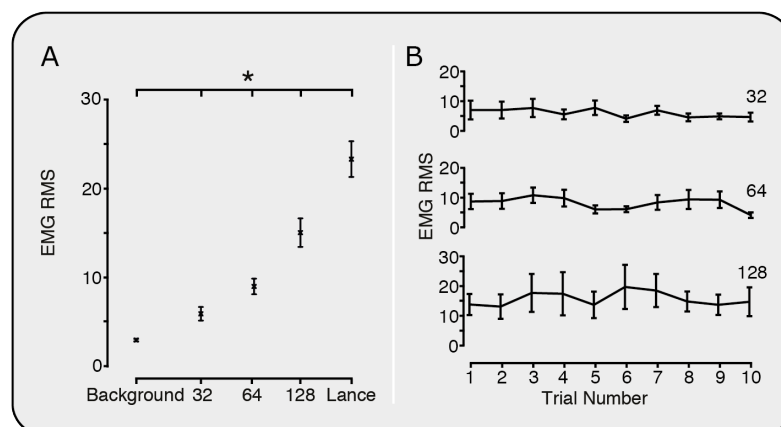


Figure 4.5: Average EMG RMS in response to experimental and clinically required noxious stimuli. (A) The reflex withdrawal was graded with stimulus intensity and for all stimuli the response was significantly different to background activity (*: $p<0.05$). (B) The reflex withdrawal response was not significantly different across stimulus number for any of the experimental noxious stimuli. Error bars indicate standard error of the mean.

4.3.4. Nociceptive brain activity can be evoked by acute experimental noxious stimulation.

Noxious-evoked brain activity was observed following all experimental noxious stimuli (applied forces: 32, 64 and 128 mN) and following a clinically required heel lance. Figure 4.6 shows the average EEG brain activity for the experimental noxious stimuli (4.6 A-C) and the heel lance (4.6 D). The nociceptive-specific evoked potential is characterised by a positive deflection (conventionally plotted with an inverted y-axis so that positive responses are identified as deflections from the more negative (top) to the more positive (bottom) region of the y-axis), occurring at approximately 400 - 500 ms following the heel lance. Visual observation of the data suggests that a similar positive deflection can also be observed following the application of the experimental noxious stimuli and that the magnitude of the deflection increases with increasing stimulus intensity (figure 4.6 A-C).

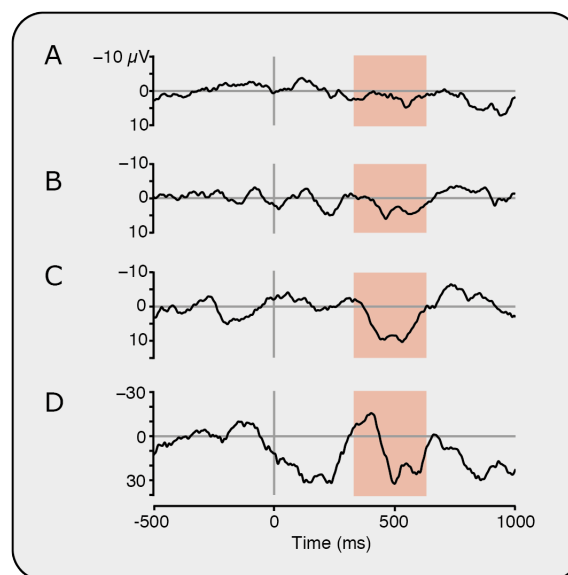


Figure 4.6: Average evoked brain activity in response to experimental and clinically required noxious stimuli. Average traces (without Woody filtering jitter) in response to the (A) 32 mN, (B) 64 mN, and (C) 128 mN experimental noxious stimuli, and (D) clinically required heel lance. The coloured boxes highlight the window in which an evoked response is observed following a stimulus.

4.3.5. Brain activity evoked by experimental noxious stimulation is correlated with stimulus intensity.

Projection of the nociceptive-specific template [94] onto the data allowed the magnitude of the noxious-evoked brain activity elicited by a heel lance and experimental noxious stimulation to be investigated. The heel lance and the experimental stimuli evoked nociceptive-specific activity that was significantly greater than activity recorded in the background period ($p < 0.001$, one-way ANOVA; post-hoc comparison of background and 32 mN stimuli: $p = 0.040$; 64 mN stimuli: $p = 0.007$; 128 mN stimuli: $p = 0.0002$; heel lance $p = 0.0005$, figure 4.7 A). The nociceptive-specific activity was significantly higher in response to the heel lance than any of the experimental noxious stimuli (post hoc t-test with Holm's correction for multiple comparisons, $p < 0.03$). Similar to the reflex withdrawal activity, the magnitude of the nociceptive-specific brain activity increased with increasing stimulus intensity ($p < 0.001$). In contrast to the EMG activity however, there is less of a difference in the observed response (i.e. in this case the weight of the nociceptive-specific brain activity) between the three experimental stimulus forces.

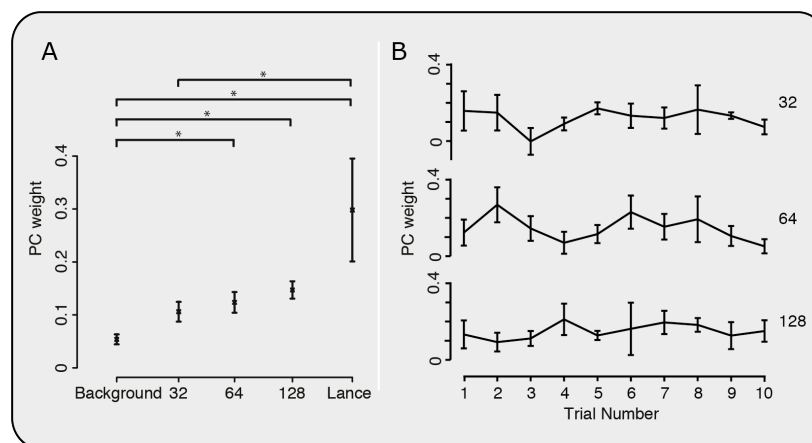


Figure 4.7: Average PC weights in response to experimental and clinically required noxious stimuli. (A) PC weight of the nociceptive-specific brain activity was graded with intensity and was significantly different to background data following all the experimental noxious stimuli and heel lance (*: $p < 0.05$). (B) Average PC weight plotted per stimulus number for the experimental noxious stimuli. Error bars indicate standard error of the mean.

The PC weights from projecting the template onto the evoked responses also demonstrates that, similar to the observations reported from the EMG activity (figure 4.5 B), the average EEG response following each trial was not dependent on stimulus number (Friedman's test, $p > 0.05$, see figure 4.7 B). Therefore no sensitisation or habituation can be detected in the evoked brain activity using this experimental force and at this inter stimulus interval.

4.3.6. The magnitude of reflex withdrawal is strongly correlated with the magnitude of noxious-evoked brain activity.

In order to identify whether reflex withdrawal activity was a sensitive surrogate measure of nociceptive processing in the infant CNS, evoked reflex withdrawal activity was correlated with evoked brain activity. The data showed that the magnitude of reflex withdrawal strongly predicts the amount of evoked brain activity in response to increasing levels of noxious stimulation ($r = 0.53$, $p = 0.037$, see figure 4.8). This correlation suggests that reflex withdrawal may be a sensitive surrogate measure of nociceptive brain activity.

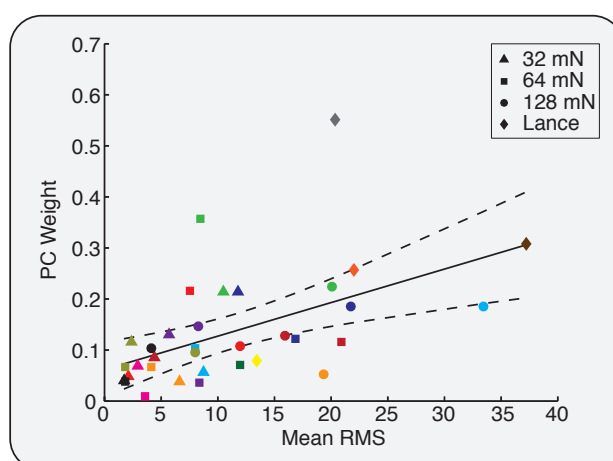


Figure 4.8: Correlation between nociceptive brain activity and reflex withdrawal activity. PC weights of nociceptive brain activity are plotted against EMG RMS for all stimuli across all subjects (triangles, squares and circles represent the 32 ($n = 11$), 64 ($n = 12$) and 128 mN ($n = 9$) experimental noxious stimuli respectively. Diamonds represent the heel lance stimulation ($n = 4$)). All data points have been colour coded to represent individual subjects. Linear regression (black line) was used to calculate the Pearson's correlation coefficient ($r = 0.53$), and a linear mixed effects model was used to test the slope of the regression line, taking subject into account as a factor ($p = 0.037$).

4.4. Discussion

4.4.1. Nociceptive spinal cord and brain activity can be recorded in the absence of facial expression change.

In this chapter, it has been demonstrated that both reflex withdrawal activity and nociceptive-specific brain activity can be recorded in the absence of facial expression change. The greatest force of experimental noxious stimulation (128 mN force) presented to newborn infants in this study produced no change in facial expression while still eliciting both nociceptive spinal cord and brain activity that was lower than the clinically required heel lance (see figures 4.5 and 4.7). This observation is important as it provides evidence suggesting that the applied stimuli are suitable for investigating nociceptive sensitivity in infants. In addition this suggests that infants may only mount coordinated facial expression responses to higher intensities of noxious stimuli.

Changes in facial expression used in the Premature Infant Pain Profile (PIPP) (brow-bulge, eye squeeze and nasolabial furrow) demonstrate specificity for nociception, particularly in term infants [59,63,65]. Despite this specificity, Slater and colleagues have demonstrated that 30 % of infants who respond with a nociceptive-specific pattern of brain activity following a heel lance show no significant change in facial expression [73]. Therefore observing facial expression changes alone lacks sensitivity in a high number of infants. The data collected in this study suggests that reflex withdrawal muscle activity and brain activity are sensitive surrogate measures of low-level nociceptive inputs and may provide more reliable measures of nociceptive sensitivity in newborn infants.

4.4.2. Magnitude of nociceptive spinal cord and brain activity increase with stimulus intensity, and are strongly correlated.

In this data a strong positive correlation is observed between spinal cord and brain activity in newborn term infants.

Evidence from a combination of infant human and animal studies suggests that the neural components forming the spinothalamic and thalamocortical pathways; required for nociceptive processing in the brain, are fully developed and functional by early postnatal age. The development of primary afferent fibres begins in the dorsal root ganglia and from there, diverging neuronal endings grow in two separate directions: toward the dorsal horn of the spinal cord and the peripheral skin and muscles. The development and migration of these terminals has been shown to occur *in utero*, and peripheral nociceptive afferents have developed terminals that innervate the epidermal layers by birth in newborn rat pups [86,253-255]. Similarly, projections towards the dorsal horn of the spinal cord, generate action potentials that can be observed using electrophysiological recordings, following stimulation of A and C-fibres, in the superficial lamina (I and II) from postnatal day 0 [256]. Projection neurons from the superficial lamina of the rat lumbar dorsal horn are present and produce excitatory postsynaptic potentials from P18 in rat pups [257]. While P18 has been suggested to correspond with the postnatal period in human infants [258], recording of somatosensory-evoked brain activity using NIRS, EEG and fMRI in both premature and term born human infants suggests that ascending fibres are functionally active from early in the third trimester of pregnancy [1-3,211]. The recording of nociceptive-evoked brain activity with EEG has demonstrated that a nociceptive-specific response is observable by 35-37 weeks gestation in newborn human infants [204]. Sensory and cognitive-affective components of pain can only be processed within the brain; therefore the presence of nociceptive-specific patterns of brain activity in an infant's brain provides a direct, quantitative measure of nociceptive processing. Together this evidence suggests that the spino-thalamo-cortical pathways required for cortical nociceptive processing in human infants are developed and functional before an infant reaches term age.

In adults, reflex withdrawal is a useful measure of central pain processing. Adults are able to discriminate nociceptive intensity well, observable in both increasing reflex withdrawal amplitudes [259] and increasing responses in the cortex to greater intensities of

stimulation [32,39,260,261]. Indeed, in adults, greater cortical brain activity correlates with increased pain report [39,262]. Therefore increased reflex withdrawal activity is likely to represent greater levels of pain perception, supporting the suggestion that this could be a useful surrogate measure of nociception.

In contrast to infants, reflex withdrawal activity in adults is observed in response to activation of nociceptors within the noxious range. It has previously been shown that infants exhibit reflex withdrawal responses to low-level punctate stimulation [91,96]. Two observations from studies in rat pups have led to a hypothesis regarding these lower nociceptive thresholds in infants. Firstly the newborn rat dorsal horn has both cutaneous and nociceptive afferent terminals present in the superficial laminae (lamina I and II) [99,263]. This is in contrast to the adult where only C-fibre afferents terminate in lamina II. At birth, input to the superficial dorsal horn is dominated by cutaneous A-fibres and this gradually reduces over two postnatal weeks until C-fibres take over [255]. Prior to the withdrawal of A-fibres from lamina II, activity can be elicited in the superficial laminae by low-level cutaneous as well as noxious stimulation [98]; an observation also reported in preterm human infants, who indiscriminately reflex to both noxious and non-noxious stimulation [91,96]. This switch also coincides with the development of more specific reflex withdrawal of the stimulated limb in response to noxious stimulation [92]. The proper pruning and differentiation between cutaneous and noxious stimulation is reliant on sensory input [99,113]. Consequently it is hypothesised that the lower thresholds to noxious stimuli observed in infants are developmentally regulated and subsequent refinement of termination of noxious and innocuous afferents in the dorsal horn are activity-dependent. As well as this overlap in afferent fibre terminals, the delayed maturation of inhibitory interneurons as well as GABA mediated inhibition of the spinal cord has also been observed in the newborn rat spinal cord [115]. Both these phenomena are believed to render the newborn dorsal horn more excitable [248,264]. Although these observations are from neonatal rat pups, numerous medical research studies use the rat as their primary model due to the animal's size, ease of breeding and the close relation of its genome to the human genome [265]. Therefore if the observations described above

hold true for human infants, the nociceptive sensitivity to low level inputs observed in this study and others investigating nociceptive withdrawal thresholds [91,96] could be a result of both immature dorsal spinal cord organisation and delayed maturation of descending inhibitory control.

The significant increase in the amplitude of brain activity with increasing reflex withdrawal intensity from this data suggests that reflex withdrawal provides a sensitive measure of higher levels of nociceptive processing in infants. This could be of particular clinical importance in this pre-verbal population. This is also in line with observations of adult responses and suggests that infant's brains are capable of encoding nociceptive stimulus intensity.

4.4.3. A clear neurobiological rationale could underpin the development of new clinical pain assessment measures.

Observation of behavioural responses is the quickest and simplest way for clinical members of staff caring for newborn infants to assess pain. The immaturity of the newborn infant's central nervous system means that behavioural responses differ from those seen in adults [264] or indeed older infants; for example the lower nociceptive reflex withdrawal thresholds visible in preterm infants and the more generalised responses observed following noxious as well as non-noxious touch stimulation [91,96,266]. This evidence is supported by observations of noxious-evoked brain activity since non-modality-specific neuronal bursts of EEG activity are more commonly produced across the brain in preterm infant's less than 35 weeks gestational age compared with older infants who exhibit localised nociceptive-specific evoked brain activity [204]. Therefore the systematic investigation and characterisation of behavioural correlates that reliably predict central nociceptive processing could lead to improvements in the development of pain assessment tools for use in infants. The requirement for such measures has been emphasised by the most recent summary of the evidence-based

knowledge on newborn infant pain from the Neonatal Pain-Control group [67]. In addition it has been a recurring area of focus in recent review papers [71,72,248].

Iannetti and colleagues used the PinPrick stimulators to demonstrate that PEPs can be reliably recorded in adults using EEG and the potentials show increased amplitudes following the application of capsaicin to the skin (an accepted experimental model of hyperalgesia during neuropathic pain [267]). The stimulators could therefore be used in a clinical setting to assess mechanical sensitivity thresholds, a commonly reported phenomenon in neuropathic pain patients and one that had thus far proven difficult to assess. The clinical utility has also been extended to the assessment of analgesic efficacy since opioids attenuate the amplitude of the noxious-evoked brain activity observed in adults [43,268,269]. Analgesics are infrequently used in newborn infants for acute procedural pain due to concerns over adverse effects [6]. However their necessity is vital due to the identification of adverse cognitive and developmental outcomes associated with increased exposure to pain including reduced attention, executive function and motor abilities in infants born premature [249,270,271]. Altered brain microstructure has also been demonstrated using MR diffusion tensor imaging; with reduced white matter structural integrity (inferred by lower fractional anisotropy (FA) values) and reduced development of subcortical grey matter (indicated by lower N-acetylaspartate-to-choline ratio (NAA/choline)—a marker of metabolism and density) measured in infants and correlated with higher exposure to repeated skin-breaking procedures throughout an infant's stay in neonatal intensive care [272]. Future directions to improve the use of analgesics include the conduction of clinical trials to establish safe analgesic doses for newborn infants. Therefore in addition to using these experimental noxious stimulators to extend our neurobiological understanding of central pain processing in infants, it may also be reasonable to use these stimulators to assess analgesic efficacy.

4.4.4. Strengths and Limitations

This experimental protocol allowed the simultaneous recording of noxious-evoked electrophysiological brain activity and reflex withdrawal muscle activity in newborn infants. BOLD responses have been observed in infants using fMRI in response to multiple applications of noxious PinPrick stimulation in a single infant [200], however simultaneous recording of muscle activity were not obtained so the relationship between evoked muscle and brain activity was not described. The forces used in this study were all higher than the mean forces required to elicit reflex responses in term infants [91] and included a single force (128 mN) that was double the strength of the greatest force used in the paper by Williams and colleagues [200]. In this study we used a range of experimental noxious stimuli, which produced responses that were lower than, but still approximate to the responses elicited by a clinically used heel lance stimulus. The experimental stimuli were also applied at a level that did not cause any clinical concern or behavioural distress.

The stimuli used in this experiment do not pierce the skin making them well suited for use in studies investigating central nociceptive processing in healthy participants as well as more sensitive patient populations [141,175]. Experimental studies in humans using PinPrick confirm that the stimuli predominantly activate A δ nociceptors as well as activating a small number of C-fibre nociceptors (where directed blockade of these fibres caused an 82 % and further 14 % reduction in the observed stimulus-response function respectively [155]. Acute tissue damage causes two waves of activation (the fast “first-pain” response and the on-going slower “dull, throbbing pain” that follows tissue insult in order to facilitate protection of the wounded site). PinPrick stimuli predominantly activate A δ -fibres responsible for the fast pain response and will therefore likely produce a feeling of transient pain. Although this is unlike that produced following tissue insult, the mechanical nociceptive sensory input produced by these experimental stimuli result in a sensation that is more commonly experienced and identifiable compared to, for example, the heat pain evoked by laser stimuli. This stimulus has also been suggested to hold more

clinical relevance as sensitivity to mechanical stimulation is a common phenomenon amongst neuropathic pain patients [141]. The predominant activation of A δ nociceptors is therefore a suitable experimental paradigm for assessing nociceptive sensitivity.

The experimental noxious stimuli did not evoke any sensitisation or habituation at the intensity and ISI used in this study protocol however, this does not rule out the possibility that central mechanisms of nociceptive sensitivity could be observed if either a greater stimulus force (such as those potentially provided by clinically painful procedures such as heel lance or venepuncture) or a shorter ISI were to be used. Such questions have clinical utility, for example in the case of clustered care in neonatal units. Clustering of procedures during neonatal care has been recommended by The American Academy of Pediatrics and The Canadian Paediatric Society [68] in an attempt to reduce the amount of daily pain exposure and provide maximum periods of rest for growth and recovery. Further investigation of the underlying mechanistic properties of sensitisation and habituation in infants can provide greater knowledge for scientists and clinicians to draw upon in order to continue to guide and improve future clinical care. As these non-skin-breaking experimental stimuli can be applied numerous times, they could also invoke a more thorough investigation of how environmental factors influence surrogate measures of pain.

The number of infants who had an EEG and EMG recorded in response to a heel lance was very low ($n = 4$), however the morphology of the recorded evoked responses were a convincing reproduction of those described in previous publications [3,91,140,202-204] therefore it was not considered necessary to include more infants in this group. While increasing the number of events could have decreased the variability observed in the evoked brain activity and spinal reflex withdrawal responses to heel lance, as discussed previously, it can be more difficult to control for factors that effect the variability of responses that occur following a clinically required procedure, compared with an experimental noxious stimulus for example. It has been shown that behavioural responses such as facial expression can be highly influenced by factors such as the frequency of

handling and recent pain exposure [273,274]. Therefore it is plausible that such factors may also influence brain activity and reflex withdrawal responses to heel lance. Indeed premature infants display increased noxious-evoked brain activity in response to a heel lance compared with term born infants [203].

The infants in this data set together form a relatively homogeneous group of term born infants with no neurological problems. Infants were only included if a clinician confirmed they were well enough to take part in research (i.e. clinically stable), were born at term (≥ 37 weeks gestation) and were within nine days of birth. Although prior pain exposure was not specifically controlled for, half the infants in the group had no additional pain exposure between delivery and the study. The remaining infants included in the study had heel lances for blood collection for reasons varying from jaundice to suspected meningitis. Overall 6 infants were delivered by caesarean section, of which four were emergency caesareans and two were elective. The remaining infants were delivered vaginally. When taking the method of delivery into account, no infant can be considered to be completely pain naïve, however as far as possible, this group of infants formed a uniform group, with all being exposed to infrequent pain prior to the study. Given the small numbers of infants in this study, it was not possible to investigate whether factors such as the method of delivery could have altered the observed responses.

EEG has a very high temporal resolution, which is important to allow recording of evoked potentials that occur in the order of milliseconds. In order to accurately detect event-related potentials it is necessary to record the timing of the stimuli with precision. Heel lance stimuli were time-locked to the EEG and EMG recordings using an accelerometer and an event-detection interface developed and used previously [140]. Experimental noxious stimuli needed to be time-locked to the EEG in a different way since there was no spring-loaded release mechanism for the accelerometer to detect. The presentation of these stimuli were therefore recorded using a hand-held high-speed camera (recording 220 frames per second) which had the recordings synchronised with the EEG recording. This allowed the point of stimulation to be reviewed post-acquisition and manually marked on

the recordings (accuracy: precision of 9 ms and accuracy of 4.5 ms). The point of stimulation was taken to be “the point where the barrel of the stimulator was first depressed, i.e. the time at which the force is first applied”. Another method for time-locking PinPrick stimuli has been developed by Iannetti and colleagues who use an optical detector placed just above the weight inside the PinPrick probes [141]. They program the optode to produce a trigger when the weight is first moved up the barrel (concurrent with the moment that the full weight is resting upon the tip of the stimulator and consequently when the barrel of the PinPrick is first depressed). Despite taking different approaches, both methods described are consistent with one another, and the methodology adopted in this experiment was the most accurate one available at study design.

4.5. Conclusion

In this chapter, it has been demonstrated that a range of PinPrick stimuli (32 - 128 mN) can be used to assess nociceptive sensitivity in newborn infants. The non-skin-breaking stimuli can be applied to the heel of an infant’s foot and used to investigate both evoked brain activity and spinally-mediated reflex withdrawal responses in the absence of a change in facial expression. All forces of PinPrick stimuli evoked nociceptive-specific brain activity. In addition a significant correlation was observed between the magnitudes of reflex withdrawal and evoked nociceptive-specific brain activity, suggesting that reflex withdrawal may be a sensitive surrogate measure of nociceptive brain activity. In the next Chapter, the same forces of PinPrick stimuli are used in an fMRI study with the aim of investigating the cortical and subcortical brain regions that are involved in the generation of the noxious-evoked responses.

Chapter 5

Nociceptive-evoked brain activity recorded using fMRI in infants and adults

5.1. Introduction

The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [10]. Investigations of nociceptive processing in adults have identified a widely distributed network of brain regions involved in the detection and processing of noxious stimuli [28]. As is eluded to by IASP’s definition, pain is described with both sensory and emotional context and neuroimaging studies have demonstrated that different brain regions can be associated with different aspects of pain perception [14,29]. The different aspects or qualities of pain perception referred to include sensory, cognitive, and affective dimensions of the experience (described in sections 1.3.5 and 1.6.3). Uncovering the subtle dynamics involved in nociceptive processing in adults has significantly improved our understanding of the particular functions that different brain regions play.

An example of such differentiation of the roles of different brain regions in nociceptive processing is demonstrated in a study by Wiech *et al.*, who show that the level of perceived threat towards a physically identical stimulus modulated the amount of activation in the anterior insular (AI) cortex, which in turn positively correlated with the verbal report of stimulus intensity [44]. This demonstrated that activation of AI can be manipulated by context and is therefore involved in the cognitive-affective dimension of pain perception [44]. Similarly, Winston *et al.* used a psychological “willingness to pay” paradigm in order to assess how much participants would pay to avoid low, medium and high intensity pain stimuli [45]. When comparing brain activation evoked by a cue representing the stimulus intensity to be avoided, they found that activity in the OFC

corresponded with the evaluation of the stimulus in its current context (i.e. how it compared with lower or higher pain intensity and therefore how much it is worth paying to avoid it) [45]. During a PET neuroimaging study, Rainville *et al.* used hypnosis to manipulate the degree of unpleasantness associated with a given pain stimulus [39]. With this paradigm, the authors reported that activity within the ACC was positively correlated with perceived unpleasantness while activity in the somatosensory cortex remained unchanged [39]. This therefore provides a demonstration of sensory processing in the somatosensory cortex compared with affective processing within the ACC. Winston and colleagues also reported that the somatosensory cortex and thalamus were amongst brain areas responding to the intensity of the noxious stimuli they presented [45]. The complexity of pain perception is augmented by this interaction between sensory and emotional dimensions. While neuroimaging studies have been used to unravel these functional correlates, verbal report of pain remains the gold standard in assessment of individual pain experience. However, as newborn infants cannot communicate verbally, we are more dependent on alternative measures to successfully detect and treat their pain.

In adults, the magnitude of nociceptive-evoked brain activity has been correlated with verbal report of pain, suggesting that recording brain activity may provide a reliable surrogate measure of pain perception [252]. The inference of pain perception from a surrogate response uses a method of reasoning, termed “reverse inference” [275,276]. Yarkoni and colleagues recently reported their development of an algorithm that provides a quantifiable method for reverse inference from brain imaging data [277]. The automated approach utilises the recent surge in available neuroimaging data in the scientific literature, to identify coordinates, brain regions and subsequent patterns of neural activity that are specifically associated and reported with particular words or phrases of interest. Yarkoni *et al.* then used their algorithm to investigate “pain”, “emotion”, and “working memory” as “broad psychological terms” [277]. They consequently demonstrated that the loci of brain activity associated with the term pain could be extracted from the currently available neuroimaging literature. In doing this,

the aim was to identify brain regions that were selectively (and not just consistently) activated during pain. When the data were input into their algorithm (NeuroSynth), patterns of brain activity following intense and mild thermal pain were identified with a sensitivity and specificity of 65 % and 98 % respectively [277]. Classification of pain was also highly discriminable in comparison to 24 other broad psychological terms tested suggesting that pain is a distinctive state that can reliably be identified using reverse inference methodology [277]. Wager and colleagues subsequently used this methodology to report that these patterns of brain activity could provide a useful clinical tool for inferring pain experience, particularly in vulnerable, non-verbal populations [278].

In infants, little is known about the cortical and subcortical structures involved in nociceptive processing. This is primarily because infant pain research was relatively uncommon until the late 1980's, due to an overarching belief amongst scientists and clinicians that infants could not feel pain. While this is no longer the prevailing view, better understanding regarding the experience of pain in newborn infants is fundamental to paediatric care because pain continues to be under-treated in current clinical practice [6]. Presently an infant requiring surgery would be provided with pain relief both during and following their surgery, however many infants still undergo an average of 11 invasive, acute painful procedures per day during their stay in neonatal care for which pain relief is infrequently provided [6]. A recent observational study on a neonatal unit in the Netherlands reported that pharmacological analgesia was only being provided to infants for 36.6 % of painful procedures [6].

Using NIRS, Slater and colleagues show that an infant as young as 25 weeks gestation showed an increase in haemodynamic activity in the contralateral somatosensory cortex following a clinically required heel lance [2]. Heel lances are one of the most frequent acute painful procedures required in NICUs [7,9,279], and one for which no national or international guidelines exist recommending pharmacological intervention. This becomes all the more important when considering that repeated exposure to stress/pain has been correlated with altered brain development (smaller brain size and lower

apparent diffusivity and relative anisotropy parameters indicating less structural integrity have been reported) [271]. Part of the problem surrounding the lack of guidelines for pain management is that it is still incredibly difficult to assess pain in this population and we still understand very little about the kind of experience these young infants may have.

In Chapter 4 it was demonstrated that nociceptive-specific brain activity could be recorded in response to all forces of PinPrick stimuli applied in infants. In addition, it was shown that increases in brain activity and reflex withdrawal activity could be observed in newborn infants in response to increasing forces of noxious stimulation using PinPrick stimuli. It was also emphasized that these stimulators provide a useful experimental method for assessing nociceptive sensitivity in newborn infants as they do not cause any behavioural distress or clinical concern and can be provided to infants numerous times (i.e. do not cause sensitisation or habituation at a minimum inter stimulus interval of 10 seconds). Moreover, the PinPrick stimulators are appropriate for use during fMRI studies as they can be designed to be MR-safe. To date these stimulators have been used extensively during fMRI studies investigating nociceptive processing in adults [37,174,175,280] and the feasibility of their use in infants has also been demonstrated [200]. The fact that they can be applied multiple times is important for obtaining adequate signal to noise in fMRI experiments. These noxious-stimulators therefore provided an ideal opportunity to exploit fMRI's high spatial resolution in order to investigate the cortical and subcortical brain regions involved in nociceptive processing in newborn infants.

In this chapter, a series of experiments were conducted using PinPrick stimulators in a group of adults and two groups of infants, term born infants (> 37 weeks gestation) and premature born infants (35 - 37 weeks gestation), within 11 days of birth. All infants and adults had an fMRI scan in a 3 T scanner and all adults additionally took part in a pain psychophysics experiment. The objectives of the experiments were to:

- Investigate PinPrick-evoked verbal pain reports describing the sensory qualities of the stimulation experienced by adults.
- Investigate the pattern of noxious-evoked brain activity in infants and compare it to that seen in adults.
- Investigate intensity encoding within brain regions significantly activated by noxious stimulation.
- Investigate the development of noxious-evoked BOLD responses within the somatosensory cortex in premature and term born infants.

As the infant brain has yet to undergo a significant period of postnatal growth and development, it was hypothesised that the pattern of nociceptive brain activity evoked by PinPrick stimulation would be different to that observed in adults. Cortical brain regions, in particular the frontal cortex increase in volume during infancy as new neuronal connections develop and neural pathways mature and are refined [234,241]. Hence it is plausible that reduced activity will be observed in frontal brain areas in infants compared with adults. In addition, the somatosensory cortices were of particular interest because an increase in total haemoglobin concentration in the contralateral somatosensory cortex has previously been measured in infants aged between 25 and 45 weeks gestation, following a noxious heel-lance (measured using NIRS optodes placed bilaterally above the somatosensory cortices) [2]. Anatomical studies have also shown that the somatosensory and motor cortices begin synaptic pruning early at 24-28 week gestation [281] while the rest of the brain continues synaptogenesis which has been shown to peak at approximately 34 weeks gestation [282]. Myelination of the precentral and postcentral gyrus is also detected from 35 weeks gestation [283]. During the ensuing late gestational period (> 35 weeks) the layered cortical plate, increases in density with the formation of local circuitry, elaboration of dendritic processes and connections [234,235,284] coupled with an increase in the gyration of the cortical surface [178]. It was therefore my prediction that an increase in BOLD would be observed across the whole age range studied and that the response in the sensorimotor cortex would increase with age.

5.2. Methods

5.2.1. Participants

10 adults were recruited to the study and took part in two separate experimental sessions: the first involved an fMRI brain scan and the second was a psychophysics session. 23 infants were recruited to the study and of those, 22 had an fMRI brain scan at 3 T. One infant was too restless and could not be settled for the scan so the study was aborted. In addition data from one infant had high stimulus correlated movement throughout and so had to be rejected (see section 2.6.4.2.). The remaining 21 infants formed two groups:

- Group 1** (n = 10) term born infants who had noxious-evoked brain activity recorded and were compared to the adult group, and
- Group 2** (n = 21) premature and term born infants were included together with those in Group 1 and the activity within the somatosensory cortices was investigated across age.

5.2.2. Adults

5.2.2.1. Subjects

10 adults (5 males, mean age = 28.3 years; range: 23-36) took part in an MRI study at FMRI and were scanned on the 3 T scanner. Adult participants were members of staff or postgraduate students at the University of Oxford. fMRI of the brain was performed on all participants in response to five intensities of PinPrick stimulators (32, n = 9; 64, n = 10; 128, n = 10; 256, n = 9; and 512 mN, n = 10). Time restrictions during two of the sessions meant that in one subject no 256 mN force and another subject no 32 mN force was applied and therefore no functional data recorded at those intensities.

On a separate test occasion in the same participants, the experimental protocol was repeated outside the scanner. During this session participants were asked to verbally rate pain intensity using a numerical scale [0 - 10] and to describe the type of pain they experienced using the McGill pain questionnaire (see Appendix 3).

5.2.2.2. Adult psychophysical session

5.2.2.2.1. Protocol

The psychophysical session was conducted in a quiet experimental lab containing a reclining patient bed. The experimental protocol that had been conducted during the MRI session was repeated (i.e. the order in which PinPrick stimuli were presented was not changed). Participants were asked to lie down on a patient bed (in an attempt to emulate the scanner environment as much as possible), and throughout the experiment, were asked to verbally state a pain score following each individual stimulus. Stimuli were applied in a random order and each force was applied 10 consecutive times to the dorsum of the subject's left foot. A minimum ISI of 25 seconds was used. The pain scoring system was described to the participants just before the experiment began, using the following statement:

“Please describe the level of pain you feel following each individual stimulus on a scale between 0 to 10, where 0 is no pain and 10 is the worst pain imaginable.”

Once all stimuli had been presented to the participants they were asked to indicate descriptors that corresponded with the type of pain they experienced by completing the McGill Pain Questionnaire [285] (for example of questionnaire, see Appendix 3).

5.2.2.2.2. Statistical analysis

Each train of 10 verbal pain scores (following each individual stimulus applied at a particular force) were averaged to produce an overall numerical score per stimulus intensity for each subject. Average scores were input to *GraphPad Prism*, where a linear

regression model was generated with pain ratings as the response variable and adult number and stimulus force taken as explanatory variables. The coefficients of the linear regression model were tested to investigate applied stimulus force versus average adult verbal pain scores.

5.2.2.3. MR study protocol

10 adult participants had fMRI of the brain performed during a single scanning session at FMRI. All participants were prepared for the scanning session as described in section 2.5.1 and were provided with ear protection (foam ear plugs, 3M, attenuation 36 dB) before being positioned comfortably on the MRI scanner bed.

MR imaging was acquired on a Siemens Magnetom Verio 3 T scanning system using a Siemens 32-channel adult head coil. Adult's heads were positioned inside the head coil and ear padding was used to restrict head movement and provide additional ear protection. Pulse and oxygen saturations were monitored throughout scanning using a 3T MRI compatible monitoring probe placed on the right foot (NONIN pulse ox). All studies began with collection of a T1-weighted structural image (for details of image acquisition parameters see section 2.5.1). This was followed by 5 EPI acquisitions lasting approximately 6 minutes each, during which five intensities of PinPrick stimulation (applied forces: 32, 64, 128, 256 and 512 mN) were applied in a random order to the dorsum of the participant's left foot. Each stimulus force was applied 10 consecutive times with a minimum ISI of 25 seconds during a single acquisition sequence.

5.2.2.4. MRI data analysis

All data preparation and first level analyses of adult functional EPI images are described in detail in section 2.6.1 and 2.6.3. EPI scans were processed in order to identify brain regions significantly activated in response to the stimuli applied during each scan, i.e. separately for each stimulus force (threshold at $z = 2.3$ with a corrected cluster

significance level of $p < 0.05$, contrast EVs used: +1). These analyses were then passed into higher group level analyses.

5.2.2.4.1. Group analysis

Multiple group level analyses were run. Firstly, all subject data were grouped by stimulus intensity in order to investigate significant brain increases in BOLD per stimulus force (threshold $z > 2.3$, $p < 0.05$). A second analysis was then used in which all data from all subjects ($n = 48$ datasets) were grouped to define activity masks within the brain regions identified as being significantly active ($z > 2.3$).

5.2.2.4.2. Identifying active regions

Active brain regions were defined by adding the significant activity from each group intensity analysis and, following a transformation of the activity to EPI space, identifying regions where two or more voxels of positive BOLD were present (threshold $z > 2.3$, $p < 0.05$). In order to achieve this, a mask of the sum of significant activity was input into the *FSL* function *atlasquery* which generated a list of active brain regions, based on the Harvard-Oxford cortical and subcortical atlases [220]. This approach of summing grouped activity across stimuli was used as it (i) avoided biasing the identification of active regions towards the higher intensity stimuli (ii) was the simplest available method due to the large number of stimulus conditions used in the adult group. The use of *atlasquery* ensured that all active voxels were identified in any brain region, and *atlasquery* also reported the percentage of the region that was active. Following this, a conversion of the activity from the standard template brain to individual subject's functional EPI space was conducted and used to identify regions in which there were two or more active voxels. This transformation into EPI space was used to allow consistency between the adult and infant groups, given that both populations had fMRI data collected using 3 mm cubed voxels.

The decision to use a threshold of two or more voxels within EPI space was introduced following the observation that large regions of activity sometimes resulted in a single active voxel at the edge of an adjacent region. As this was not the main core of the activation, increasing the threshold provided a more accurate reflection of the significantly active brain regions.

The resulting list of active regions was then taken and used to generate a final list of active brain regions as defined in section 2.6.5, so that significant brain activity could be compared to the regions identified using the infant atlas. Finally, the *FSL* function *Cluster* was used to identify the peak *z* statistic and MNI coordinates in each active region (see table 5.2).

5.2.2.4.3. Obtaining parameter estimates

Once the active brain regions had been identified, “activity-cluster-masks” were created for each region of interest (ROI). The masks were created based on the group analysis of all inputs for all intensities (threshold $z > 2.3$). By overlaying the group activity onto the same atlases used to identify active brain regions (see 5.2.2.4.2.), the active areas in each ROI were isolated and used to create the so-called “activity-cluster-masks”. These masks were then input into *Featquery* [208], which calculated the mean percentage BOLD increase within the mask for each individual functional data set per subject.

5.2.2.4.4. Intensity analysis

Regression analyses were carried out in the software package *Graphpad Prism*. Mean percentage increase in BOLD signal was plotted against stimulus force and regression analysis was used to test the null hypothesis that no intensity encoding was present across the masked activity within a brain region. Correction for multiple comparisons (Bonferroni) was used to determine the *p*-threshold required in order to reject the null

hypothesis. Parameter estimates for the gradient of the regression were used to rank significantly intensity encoding, active brain regions.

5.2.3. Infants

5.2.3.1. Subjects

21 infants (12 males, mean gestational age at time of study = 39.8 weeks, range: 35.7 - 42.7 weeks) participated in the study. At the time of study all infants were less than 11 days old (mean postnatal age = 4.2, range: 1-11 days). Infant participants were eligible for inclusion in the study if they were healthy, had no history of neurological problems, self-ventilating in air, and clinically stable.

5.2.3.2. MR protocol

Infants were accompanied and transported to FMRIB by a member of clinical staff from the Neonatal Unit trained in neonatal life support. The clinical member of staff remained with each infant throughout the study to ensure the infant's safety and wellbeing. Full details of the preparation of all infants for an MRI Study can be found in section 2.4.3

All MR imaging was acquired on a Siemens Magnetom Verio 3 T scanning system using a Siemens 32-channel adult head coil, the same as that used for the adult scans. Pulse and oxygen saturations were monitored throughout scanning using a 3 T MRI compatible monitoring probe placed on the right foot (NONIN pulse-oximeter or Expression monitor, Invivo Corporation).

All scans began with the collection of a T2-weighted structural image (for details of image acquisition parameters see section 2.4.3). Following this, three EPI scans, lasting approximately 6 minutes each were acquired. During the EPI scans three intensities of

PinPrick stimulation (applied forces: 32, 64 and 128 mN) were applied to the sole of the left foot. Each force was applied 10 consecutive times with an ISI of 25 seconds.

Once the study was completed, the infant and parents were transported back to their ward by the research team and clinical member of staff.

5.2.3.3. MRI data analysis

All data preparation and first level analyses of infant functional EPI images are described in detail in section 2.6.2, 2.6.3 and 2.6.4. First level analysis identified brain regions significantly activated in response to the stimulus for each EPI acquisition, i.e. for each stimulus force separately (threshold at $z = 2.3$ with a corrected cluster significance level of $p < 0.05$, contrast EVs used: +1). These analyses were then passed into higher group level analyses.

5.2.3.3.1. Group analysis

MRI data from 10 newborn infant subjects were grouped by stimulus intensity (as described for adult data). *Feat* was used to determine the voxels in which significant increases or decreases in BOLD could be observed at a particular stimulus force ($z > 2.3$, $p < 0.05$). In addition a second separate group analysis was run in which functional data from all subjects and all intensities of stimulation were grouped ($z > 2.3$).

5.2.3.3.2. Identifying active regions

Wherever possible, the methods adopted were the same as that described above for adults (see section 5.2.2.4.2). The creation of the summed activity mask consisted of the addition of three groups of significant activity (32, 64 and 128 mN) as opposed to five groups used in adults. Active brain regions were identified using an infant atlas [221]. This image was then imported into *MATLAB* and used to list all the brain regions for

which two or more voxels in EPI space were active (defined by the list in section 2.6.5). As in adults, a conversion from standard to functional space was used in order to identify brain regions where two or more significantly active voxels were present. The *FSL* function *Cluster* was used to identify the peak z-statistic and coordinates in neonatal template space for all active regions (see table 5.2).

5.2.3.3.3. Obtaining parameter estimates

Using the same approach described for adults, “activity-cluster-masks” were created for each ROI identified as active following the above method (section 5.2.2.4.3.). Masks of the regional brain activity were input into *Featquery* and mean percentage BOLD increase within the mask for each individual functional data set per subject was calculated. Mean percentage BOLD activity was used for subsequent regression analyses.

5.2.3.3.4. Intensity analysis

Regression analysis was identical to that described for adults (see 5.2.2.4.4.).

5.2.3.3.5. Development analysis

In order to investigate developmental changes in the BOLD response, *MATLAB* was used to run linear regression analysis with gestational age of the infant at the time of the study as the independent variable and the mean percentage BOLD within an activity mask of the somatosensory cortices used as the dependent variable.

5.3. Results

The main results presented in this chapter have been published in Goksan et al., 2015 (see [286])

5.3.1. Adults describe PinPricks as painful, “sharp and pricking”.

A psychophysical session was conducted in order to ascertain how adults described the PinPrick stimuli, in both numerical and descriptive scales. 10 adult participants took part in a psychophysical session during which 5 intensities of PinPrick stimuli were presented to the dorsum of their left foot. Participants were asked to verbally score the pain following each stimulus application. On average participants rated forces of 128 mN or greater as painful (mean pain score of applied force: 32 = 0.4 ± 0.4 , 64 = 0.5 ± 0.5 , 128 = 1.1 ± 0.8 , 256 = 1.7 ± 1.2 , 512 = 2.7 ± 1.3). All adults reported a pain score of 1 or greater, at least once, during the presentation of the 128 mN stimulus force. When comparing this to the verbal pain rating scale, a score of between 1 and 3 is equivalent to a “mildly painful” sensation [287]. All adults reported PinPrick stimuli between 128 - 512 mN as “mildly-painful”. Linear regression analysis was used to investigate the relationship between applied force and average verbal pain score (figure 5.1). There was a significant increase in the verbal pain score with increasing stimulus force ($n = 50$, r -squared = 0.85, $p < 0.0001$). A linear regression analysis was also run across a subset of the stimuli (32 - 128 mN: the same range used in infants) and this also showed a significant relationship between force and verbal pain scores ($n = 30$, r -squared = 0.80, $p < 0.0001$).

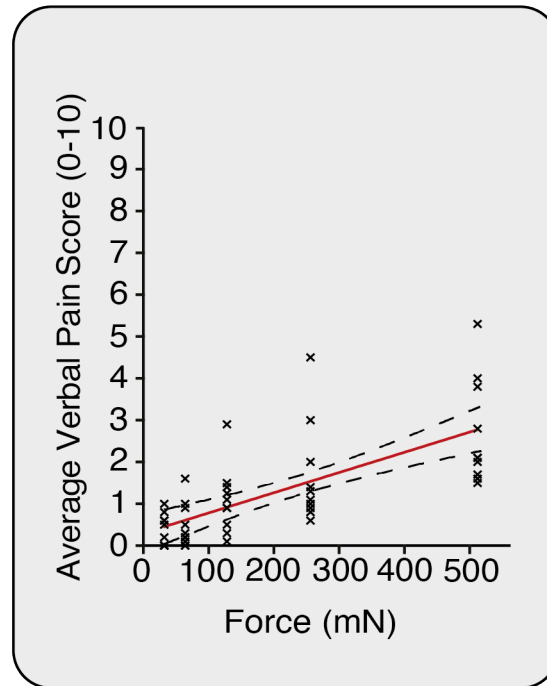


Figure 5.1: Regression of force and verbal pain scores in adults. Force is plotted against average verbal pain score for each adult at increasing stimulus intensity ($n = 50$, applied forces: 32, 64, 128, 256 and 512 mN). Red line shows the linear regression (r -squared = 0.85, $p < 0.0001$).

Adults were asked to choose words that best described their overall experience of the experimental noxious stimulators from the McGill Pain Questionnaire (see Appendix 3). Adults most frequently described the pain sensation as “pricking” ($n = 8$ of 10) and “sharp” ($n = 6$ of 10) (table 5.1). Both of these descriptors fell into the “sensory” category and interestingly were the words with the lowest “rank-value” within their word sets; meaning they provided the mildest description of pain within the set, consistent with the description of a “mildly painful” stimulus. However higher ranking descriptors were also occasionally chosen including “pulsing” and “throbbing”, which ranked as 3 and 4 respectively (see table 5.1). The next most frequent descriptive word was “brief” ($n = 5$ out of 10), best describing the temporal sensation of the stimulus. “Annoying” and “piercing” were the only descriptors chosen from the “evaluative” and “miscellaneous” categories respectively. It is notable that adults did not choose any descriptors from the “affective” category, which included descriptors such as “tiring”, “sickening”, “fearful”, “punishing”, “cruel” and “wretched” (see Appendix 1). Given the descriptions provided

by adult participants, it is reasonable to assume that infants also experience these noxious stimulators mildly painful and “sharp and pricking” sensory qualities.

McGill Pain Questionnaire results			
Descriptor Category	Descriptors chosen from category	Frequency	Rank value
Sensory	Pricking	8	1
	Sharp	6	1
	Tingling	4	1
	Pressing	2	2
	Pulsing	1	3
	Throbbing	1	4
	Itchy	1	2
	Tender	1	1
Evaluative	Annoying	2	1
Miscellaneous	Piercing	3	4
Temporal	Brief	5	1
	Transient	2	3
	Rhythmic	3	1
	Periodic	2	2
	Intermittent	3	3
	Steady	1	2

Table 5.1: Overview of the results from McGill Pain Questionnaires. All descriptors chosen across all questionnaires are listed together with their category, frequency (out of 10; i.e. once for each participant) and “rank value”. Each word has an associate rank value, which indicates the intensity of the pain within the word set. Low rankings indicate a more mild final pain rating. Rank values range from 0-6 though not every word set has 6 descriptors (see questionnaire in Appendix 3).

5.3.2. Cortical and subcortical brain regions identified as active in response to noxious stimulation

20 of the 25 brain regions listed in our simplified atlas were identified as active in response to noxious stimulation in adults. Active brain areas included the primary somatosensory cortices (which were identified as activity within the superior postcentral gyrus), ACC, bilateral thalamus and all divisions of the insular cortices (see table 5.2). 18 of the 20 brain regions identified as active in adults were also active in infants. All brain regions are listed in table 5.2. All active brain areas were consistent with previously reported pain literature [14,28,29]. Contrary to adults, noxious stimulation in infants did not evoke activity in the bilateral amygdala or OFC (see table 5.2). In addition, while activity was present across all divisions of bilateral insular cortices in adults, visual inspection revealed that activity in the anterior division was not present in infants (figure 5.2).

The activity observed in infants displayed some striking similarities to the activity seen in adults. The ipsilateral cerebellum was significantly activated in adults, and although the activity was bilateral in infants, the ipsilateral response was more pronounced (figure 5.2). In addition both groups had strong contralateral activation in the superior regions of the pre- and post-central gyri - corresponding to the primary somatosensory and primary motor cortices (figure 5.2).

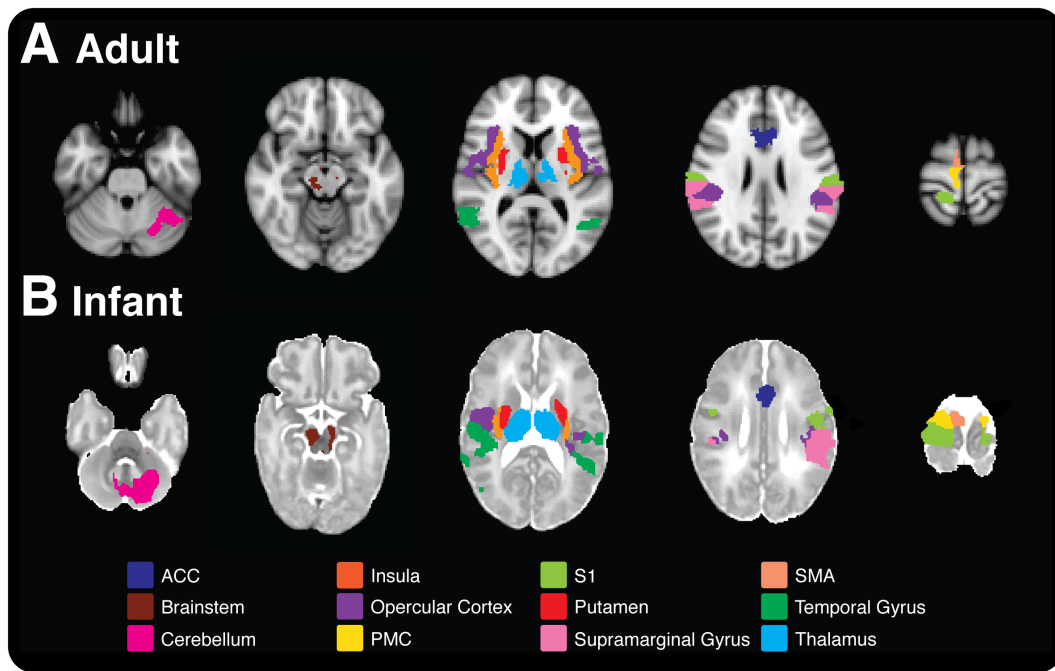


Figure 5.2: Schematic of commonly activated brain regions in adults and infants. The schematic shows activity-cluster-masks in example brain regions that were commonly activated in adults (A) and infants (B). The activity in each brain area has been colour coded and matched in the two groups. Adult activity is overlaid onto a standard T1-weighted MNI template and infant activity is overlaid onto a standard T2-weighted neonatal template, corresponding to a 40-week gestation infant. Reprinted with permission from ELife Sciences [286].

While in adults the parietal lobule, pallidum and precuneous cortex were only active contralateral to the site of stimulation, in infants these regions were also active on the ipsilateral side. In infants, additional brain regions including the bilateral auditory cortices, hippocampus and caudate were also active (table 5.2).

				Adults						Infants							
		Anatomical area	Region	Peak Z within cluster	MNI coords			Rank	Slope of Regression (x10^-3)	P Val*	Peak Z within cluster	Neonate template coords			Rank	Slope of Regression (x10^-3)	P Val*
					x	y	z					x	y	z			
Active regions in both adults and infants	Intensity encoding regions (in adults)	Temporal Gyrus	Contra	3.92	64	-34	20	1	1.01	0.0002	3.05	32	-32	12	1	2.46	0.0083
		Cingulate Gyrus	Anterior	4.11	6	4	40	2	0.65	0.0005	2.58	-1	1	26	11	1.01	0.3971
		Opercular Cortex	Contra	5.60	40	6	10	3	0.63	0.0001	3.38	32	-13	19	2	2.23	0.0391
		Insula	Contra	4.18	34	14	6	4	0.61	0.0001	3.04	19	-22	23	3	2.15	0.0207
		Supramarginal Gyrus	Contra	4.33	64	-38	20	5	0.60	0.0008	3.29	25	-23	39	9	1.08	0.1749
		Postcentral gyrus	Contra	4.28	58	-18	22	6	0.60	0.0012	3.85	15	-22	52	10	1.01	0.2667
		Visual Cortex	Contra	3.62	44	-62	4	7	0.59	0.0004	3.25	21	-52	34	6	1.41	0.0814
		Putamen	Contra	3.68	22	6	6	8	0.55	0.0001	3.30	17	-17	18	8	1.20	0.1656
		Thalamus	Contra	3.51	14	-14	0	9	0.50	0.0010	3.58	6	-16	15	4	1.91	0.0592
		Insula	Ipsi	4.67	-38	-18	14	10	0.49	0.0001	2.59	-26	-14	14	5	1.69	0.1015
	Supplementary Motor Area	Contra	3.91	8	4	46	11	0.39	0.0008	3.50	6	-18	48	7	1.23	0.2315	
	Non intensity encoding regions (in adults)	Cerebellum	Ipsi	3.88	-20	-66	-44		0.35	0.0029	3.53	-3	-46	-6		3.57	0.0164
		Temporal Gyrus	Ipsi	3.72	-52	-56	10		0.18	0.5487	3.41	-32	-22	14		2.90	0.0196
		Supramarginal Gyrus	Ipsi	4.59	-64	-28	20		0.51	0.0035	3.13	-31	-24	30		2.79	0.0055
		Cerebellum	Contra	3.36	20	-70	-50		0.31	0.0246	3.16	2	-44	-6		2.72	0.1634
		Opercular Cortex	Ipsi	5.23	-50	-28	26		0.50	0.0018	2.69	-27	-12	13		2.23	0.0710
		Postcentral gyrus	Ipsi	4.71	-62	-18	24		0.44	0.0375	3.52	-31	-15	41		2.12	0.0845
		Thalamus	Ipsi	3.52	-12	-14	10		0.42	0.0018	3.48	-1	-20	13		1.67	0.1009
		Angular Gyrus	Ipsi	3.59	-58	-50	18		0.53	0.0107	2.98	-23	-39	33		1.56	0.0528
		Precentral gyrus	Ipsi	4.01	-58	0	10		0.43	0.0578	3.46	-23	-17	48		1.53	0.1247
		Frontal Gyrus	Contra	3.88	58	12	0		0.56	0.0212	3.11	11	-12	48		1.42	0.0646
		Cingulate Gyrus	Posterior	3.71	-14	-28	38		0.08	0.2480	3.18	-9	-23	35		1.42	0.1101
		Angular Gyrus	Contra	3.71	60	-46	18		0.54	0.0080	3.12	22	-51	35		1.42	0.0407
		Precuneous Cortex	Contra	3.60	16	-68	40		0.38	0.0714	3.70	5	-30	52		1.19	0.1623
		Visual Cortex	Ipsi	3.82	-52	-70	10		-0.09	0.3758	2.59	-7	-40	11		1.17	0.1657
		Brainstem		3.86	10	-26	-8		0.33	0.1710	2.99	-3	-27	-10		1.11	0.4350
		Parietal Lobe	Contra	3.10	20	-44	68		0.61	0.1097	3.10	27	-24	46		1.09	0.1271
		Putamen	Ipsi	3.63	-16	10	-2		0.45	0.0023	3.13	-14	-14	19		0.92	0.2813
		Supplementary Motor Area	Ipsi	3.55	-6	4	44		0.40	0.0219	3.16	-4	-10	46		0.91	0.3903
		Precentral gyrus	Contra	4.05	58	4	8		0.44	0.0276	3.76	6	-20	53		0.88	0.2672
		Frontal gyrus	Ipsi	3.57	-8	22	32		-0.24	0.1954	2.79	-13	-9	50		0.70	0.4820
	Pallidum	Contra	3.40	16	-4	-4		0.49	0.0071	2.84	13	-13	13		0.64	0.4863	
Active regions in adults only		Amygdala	Contra	3.49	20	-2	-14		0.69	0.0160	no activity						
		Amygdala	Ipsi	4.28	-20	-2	-12		0.43	0.0860							
		Orbitofrontal Cortex	Ipsi	3.40	-18	4	-16		0.42	0.0157							
		Orbitofrontal Cortex	Contra	3.57	34	30	-2		0.44	0.0460							
Active regions in infants only		Precuneous Cortex	Ipsi							3.80	-1	-26	52	1.26			0.17
		Pallidum	Ipsi							3.16	-8	-5	14	0.59			0.479
		Parietal Lobe	Ipsi							3.31	-28	-23	33	0.99			0.271
		Auditory cortex	Contra							2.89	26	-14	18	3.07			0.012
		Auditory cortex	Ipsi							3.34	-17	-29	19	2.56			0.03
		Caudate	Contra	no activity						3.61	13	-17	22	0.59			0.582
		Caudate	Ipsi							3.47	-7	-8	18	1.05			0.3415
		Hippocampus	Contra							2.61	21	-25	9	1.84			0.129
		Hippocampus	Ipsi							2.77	-15	-31	9	1.00			0.333
		Parahippocampus	Contra							3.02	11	-23	0	1.53			0.3740
	Parahippocampus	Ipsi							2.99	-7	-24	-8	0.19			0.901	

Table 5.2: List of all active brain regions in adults and infants. Active brain regions were defined as regions with two or more voxels of significant positive BOLD ($z = 2.3$; corrected cluster significance level of $p < 0.05$). Intensity encoding regions are reported with their corresponding p values and slope of the regression. The intensity encoding regions (in adults) are ranked according to the slope of the regression. * Threshold for significant intensity encoding was $p < 0.00156$ following a Bonferroni correction. Reprinted with permission from ELife Sciences [286].

5.3.3. Newborn infants exhibit more widespread brain activity in response to lower intensity stimulation compared to adults

The pattern of brain activity evoked by the 128 mN stimulus in infants was most similar to that evoked by the 512 mN stimulus in adults. 14 % of voxels across the whole brain were active following the application of the 128 mN stimuli in infants compared with 9 % of voxels following the 512 mN stimuli in adults (figure 5.3). In contrast, the 128 mN stimulus activated less than 1 % of voxels in the adult brain (figure 5.4).

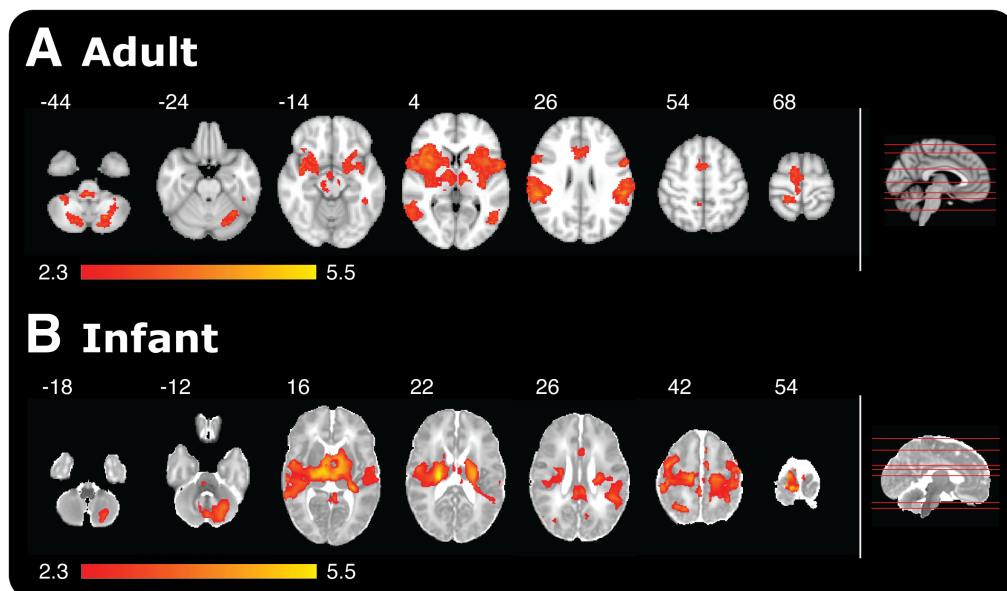


Figure 5.3: Noxious-evoked brain activity in response to the maximal presented stimulus force. Significant activity is shown in (A) adults (512 mN) and (B) infants (128 mN). Red-yellow coloured areas represent active brain regions (threshold $z \geq 2.3$ with a corrected cluster significance level of $p < 0.05$). An image of a midline sagittal brain slice (right panel) identifies the location of each example transverse slice. Adult activity is overlaid onto a standard T1-weighted MNI template and infant activity is overlaid onto a standard T2-weighted infant template, corresponding to a 40-week gestation infant. Reprinted with permission from ELife Sciences [286].

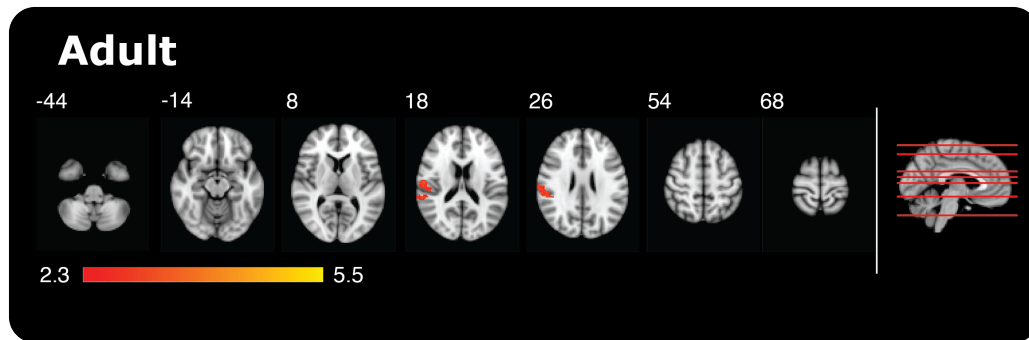


Figure 5.4: Noxious-evoked brain activity in response to the 128 mN stimulus in adults. Red-yellow coloured areas represent active brain regions (threshold $z \geq 2.3$ with a corrected cluster significance level of $p < 0.05$). An image of a midline sagittal brain slice (right panel) identifies the location of each example slice in the transverse plane. Activity is overlaid onto a standard T1-weighted MNI template.

5.3.4. Intensity encoding within active brain regions

Regression analysis was used to investigate the relationship between stimulus force and the evoked percentage change in BOLD within active brain regions in adults and infants (examples of regression in figure 5.5). In adults, 11 of the brain regions identified as active also exhibited significant intensity encoding across the full range of noxious stimuli applied. For the Bonferroni correction, a p-value of 0.05 was corrected for 32 comparisons, equivalent to the total number of active brain regions in both adults and infants giving a Bonferroni significance threshold of $p = 0.00156$ (table 5.2). In infants, no brain region significantly encoded intensity following correction for multiple comparisons. Despite this a clear trend can be observed in many brain areas with the ipsilateral cerebellum showing the greatest trend in newborn infants (slope = 3.57; $p = 0.016$). This is in contrast to adults who show a much lower trend and no intensity encoding of noxious stimuli in the ipsilateral cerebellum (slope=0.35; $p=0.003$) (figure 5.5 & table 5.2). In addition it is notable that no reflex withdrawal response was visually observed following application of any stimulus intensity in the adults. This is in contrast to the results demonstrated in infants in Chapter 4.

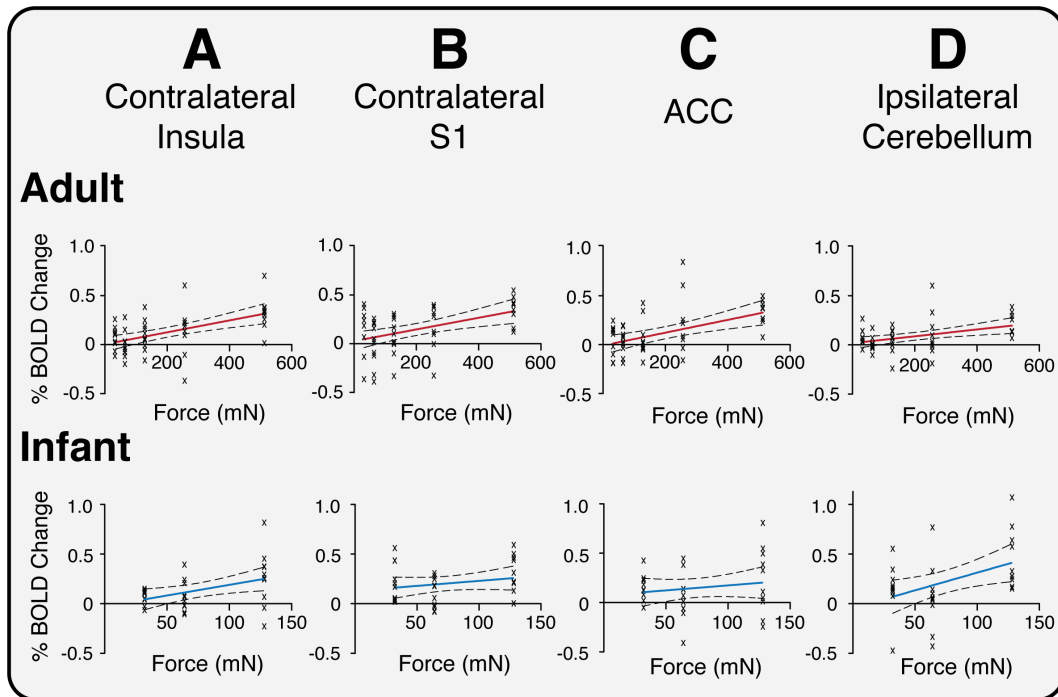


Figure 5.5: Relationship between stimulus force and percentage change in BOLD. Calculated regression lines are shown for four example active brain regions in adult and infant participants; (A) contralateral insula, (B) contralateral primary somatosensory cortex (S1), (C) ACC, and (D) ipsilateral cerebellum. The crosses represent activity in individual participants. Red and blue lines are fitted regression lines and dashed lines show 95 % confidence intervals. Reprinted with permission from ELife Sciences [286].

When considering adult brain regions that did significantly intensity encode, brain regions were ordered by the slope of their regression, and subsequently ranked. Three of the four highest ranked brain regions in adults, identified as the contralateral temporal gyri, opercular cortex, and all divisions of the insular cortex (ranked 1, 3 and 4 respectively), were ranked in the same order and constituted the top three ranked regions in infants (table 5.2). This highlights an interesting similarity between the way in which the immature brain and the adult brain encode nociceptive information. It is also notable that the ACC, which exhibited strong intensity encoding in adults ($p=0.0005$; rank=2), had the lowest ranked regression slope in infants ($p=0.40$; rank=11) (figure 5.5 and table 5.2).

Finally, regression analysis was used to investigate intensity encoding within a subset of the data collected from adults (applied force range: 32 - 128 mN). This approach showed that no brain regions exhibited significant activity in adults, which was comparable to

the results from the infant data. This suggests that investigation of intensity encoding in infants using a larger range of forces may provide greater sensitivity to investigate intensity encoding within active brain areas.

5.3.5. Development of BOLD response in the sensorimotor cortex.

The contralateral and ipsilateral somatosensory cortices (postcentral gyri) were chosen as regions of interest (ROIs) for a developmental analysis. These regions were significantly active in the analysis from Group 1. As mentioned the somatosensory cortices were of particular interest because an increase in total haemoglobin concentration in the contralateral somatosensory cortex has previously been measured in infants aged between 25 and 45 weeks gestation, following a noxious heel-lance [2]. Thus, it was hypothesised that noxious-evoked BOLD response would increase between 35 weeks and 43 weeks gestation.

In order to investigate this, an activity mask was defined from the group activity of 21 infants who had experimental noxious stimuli applied with a 64 mN force stimulator ($n = 21$, $z > 2.3$, $p < 0.05$, figure 5.6). The resulting group activity was used to define an activity mask for the ipsilateral and contralateral postcentral gyrus. The activity masks used for the analyses are depicted in green in figure 5.7 A. The data collected using the 64 mN stimuli were used to generate the group activity masks because high levels of noise were apparent in the 128 mN group analysis. The 64 mN group analysis had significant bilateral activation in the postcentral gyrus and was therefore sufficient to investigate the proposed hypothesis.

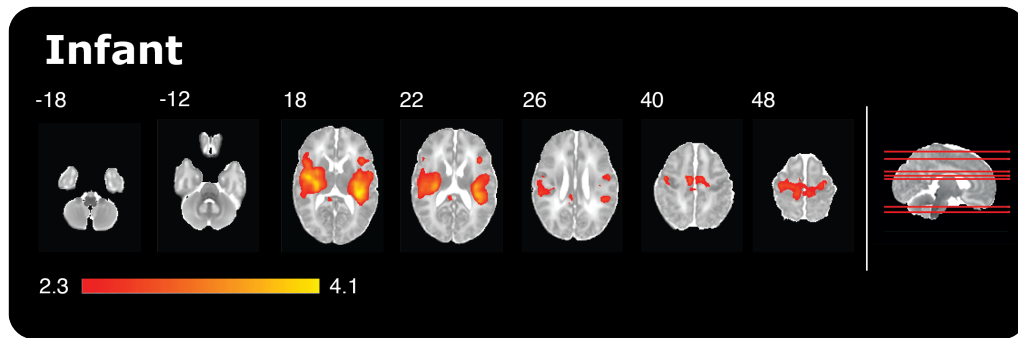


Figure 5.6: Noxious-evoked brain activity in response to the 64 mN stimulus in infants. Red-yellow coloured areas represent active brain regions (threshold $z \geq 2.3$ with a corrected cluster significance level of $p < 0.05$). An image of a midline sagittal brain slice (right panel) identifies the location of each example slice in the transverse plane. Activity is overlaid onto a standard T2-weighted infant template, corresponding to a 40-week gestation infant.

By projecting the activity mask onto each individual subject's functional image, it was possible to investigate whether the mean percentage BOLD response changed with age. A significant increase in mean percentage BOLD was observed with increasing age in the contralateral somatosensory cortex ($p = 0.04$, figure 5.7 B). In contrast, no such trend was observed in the ipsilateral somatosensory cortex ($p = 0.49$, figure 5.7 C). Moreover, activity observed in the bilateral somatosensory cortices was in the region of the primary somatosensory cortex, associated with sensory detection of stimuli from the foot [288,289] (see figure 5.8).

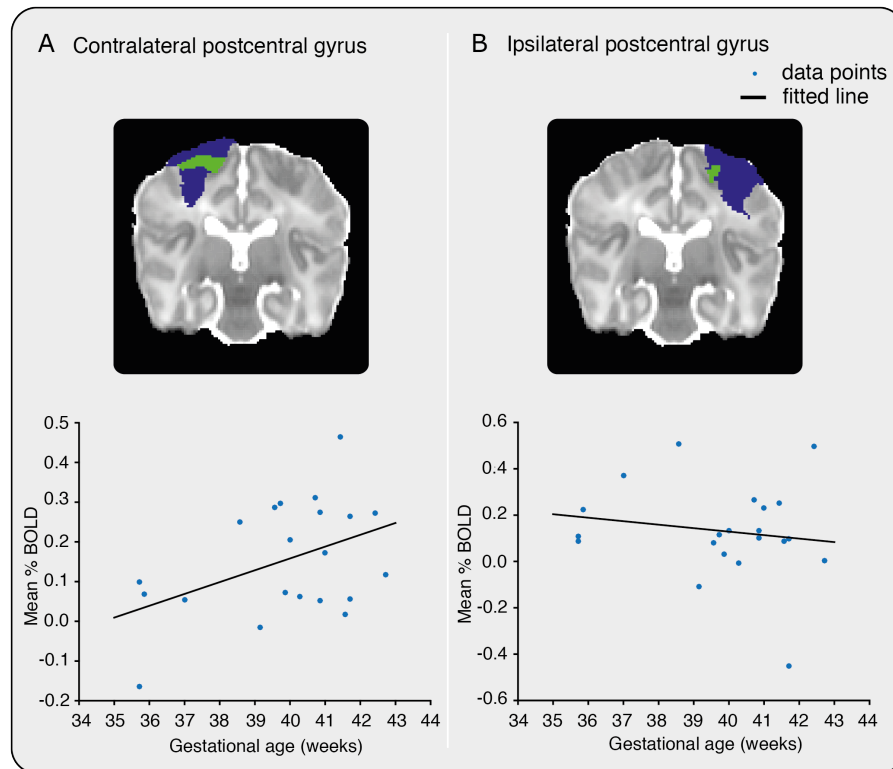


Figure 5.7: Investigation of changes in BOLD response across gestation. Mean percentage BOLD activity within active regions of the contralateral (A) and ipsilateral (B) postcentral gyrus are shown. Insets show the location of active voxels; blue areas depict the mask of the postcentral gyrus, and overlaid in green are areas that show significant activity within the region. Masks are overlaid onto a standard T2- weighted infant template, corresponding to a 40-week gestation infant. Data points are the mean percentage increase in BOLD plotted against the age of the participant at the time of the study (gestational age in weeks). Black lines correspond to the linear regression fits.

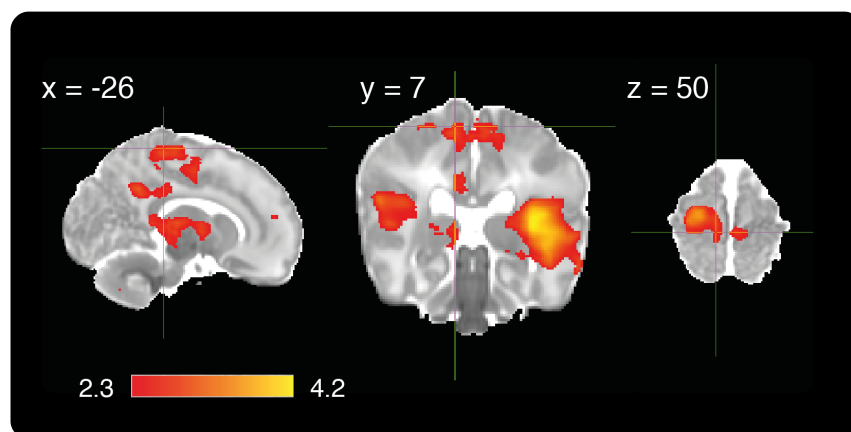


Figure 5.8: Noxious-evoked brain activity observed in the somatosensory foot regions in response to the 64 mN stimulus in infants. Red-yellow coloured areas represent active brain regions (threshold $z \geq 2.3$ with a corrected cluster significance level of $p < 0.05$). Cross hairs highlight a region of activity within the postcentral gyrus, contralateral to the site of stimulation, and corresponding to the somatosensory foot region. Activity is overlaid onto a standard T2- weighted infant template, corresponding to a 40-week gestation infant.

5.4. Discussion

5.4.1. Noxious-evoked brain activity overlap in infants and adults

18 of the 20 brain regions significantly activated by acute noxious stimulation in adults (range 32 - 512 mN) were also activated in infants (range 32 - 128 mN). All active brain areas reported were consistent with those previously described in the literature as active during nociceptive processing [29]. Active brain areas included those involved in sensory processing such as the somatosensory cortices and the thalamus but additionally included the ACC that has been shown to encode unpleasantness associated with noxious stimulation [39]. Unpleasantness is concurrent with the affective dimension of pain, therefore referring to the current IASP definition of pain as “an unpleasant sensory and emotional experience,” there is evidence to support the suggestion that affective pain processing may also be present in newborn infants. Although one should be cautious of inferring experience based on brain imaging data, Yarkoni and colleagues suggest that pain may be a system for which reverse inference is more successful than others [277]. So, it is with caution that I postulate that infants may be experiencing affective components of pain in a similar way to adults.

On average, adults verbally described experimental noxious stimuli between 128 - 512 mN as painful (mean pain score range: 1.1 - 2.7 out of 10, where the scale is from 0 = no pain to 10 = the worst pain imaginable). All adults reported that the 128 mN stimulus was “mildly painful” (pain rating = 1 - 3) during the psychophysics session. Therefore it is reasonable to consider that infants would also find this stimulus “mildly painful” and this is supported by the observed overlap in noxious-evoked brain activity in the two groups. Given that newborn infants are unable to verbally express their pain experience and it is possible to observe noxious-evoked brain activity in the absence of a concomitant behavioural response [73], brain imaging may provide an objective, quantitative method for validating and improving pain assessment in this population.

5.4.2. Amygdala and OFC activity was not observed in the infant brain

Although activation was observed in bilateral amygdala and OFC in adults, neither region was active in the infant brain. OFC has connections with the amygdala and anterior insula cortices and has been strongly implicated in decision making and assessment of threat value during neuroimaging studies of pain [45,290]. The amygdala is involved in cognitive processing and has been associated with fear *via* the investigation of aversive conditioning [291-294]. Neuroimaging studies have reported activation of the amygdala during pain experiments in humans and notably reduced activity has been associated with pain-coping strategies [295]. However, studies on rat pups have demonstrated that the converse is true in the early postnatal period and there is a critical time following birth, until postnatal day 10, when passive avoidance and conditioning behaviour are not elicited in response to the presentation of painful stimuli and this is associated with a lack of evoked activity in the amygdala [296,297]. Therefore this evidence may suggest that infants are too immature or inexperienced to evaluate the full range of aversive qualities that adults may associate with noxious stimulation.

5.4.3. Infant brain activity suggests greater sensitivity to noxious stimulation

In adults, a noxious stimulus force of 512 mN evoked a widespread pattern of neural activity that was most similar (in terms of magnitude of widespread activation) to the noxious-evoked activity observed from the 128 mN force in infants (adults 512 mN, 9 % of whole brain active; infants 128 mN, 14 % of whole brain active). Greater amplitude of neural activity has been positively correlated with higher verbal pain scores in adults [252], therefore it is possible that infants may demonstrate greater sensitivity to noxious inputs. This could be due to factors such as an infant's skin being very thin at birth. Premature infant skin is so thin that they cannot maintain thermoregulation and have high trans-epidermal water loss [298,299], and skin layers thicken with increasing age [300]. In addition it has been demonstrated using rat pup models that descending inhibition of noxious inputs undergo postnatal maturation [110,111]. Despite the

presence of GABA neurotransmitters within the spinal cord, it has been shown that these molecules appear to have the opposite effect in the infant CNS, in that they are excitatory rather than inhibitory [301]. This has also been confirmed for spinal cord neurons involved in nociception [302,303], and the introduction of a GABA antagonist to the neonatal rat pup's (P3) spinal cord has been observed to reduce reflex leg withdrawal thresholds, confirming GABA's excitatory action [109]. Therefore it is plausible that newborn human infants lack descending inhibitory modulation of nociceptive inputs rendering them more exposed to incoming nociceptive signalling [112]. Such a conclusion would require further investigation and may be confounded by factors such as variability of the HRF across individuals [304,305].

5.4.4. Encoding intensity of noxious stimulation

By investigating intensity encoding within brain regions demonstrating noxious-evoked activity it becomes possible to identify brain regions more specifically involved in encoding the force of nociceptive processing. Overall, no significant increase in BOLD response could be identified, within individual ROIs, in response to increasing experimental noxious stimuli. It is notable however, that the investigation using EEG did show a significant increase in nociceptive-specific evoked responses with increasing stimulus force. The responses recorded using EEG are likely to be the summation of activity from multiple brain regions, and, unlike in the fMRI analysis, no correction for multiple comparisons was needed, therefore the results are not directly comparable.

The results reported from the fMRI data demonstrate a trend for, but no significant increase, in brain activity to increasing stimulus force. Moreover, when the same analysis was run on the adult data with a reduced data set (applied forces: 32 - 128 mN), no significant trend was observable. Colonnese *et al.* also describe a similar result when investigating BOLD responses in the somatosensory cortex following three different intensities of electrical stimulation in the adult rat versus P13-17 rats [306]. Each stimulus was individually calibrated to the amplitude that evoked a reflex withdrawal

(threshold for infants = 1.5-2.5 mA, adults = 1 - 2 mA) when applied to the limb of the anaesthetised rat [306]. Following this, the amplitude of the BOLD response following stimulation, below, equal to and above threshold was investigated, and no change in the amplitude of the BOLD response was found in either age group [306]. Therefore it is plausible that in both rats and humans, despite the observation of a significant increase in reflex withdrawal activity, a wider range of noxious stimulation is required to investigate nociceptive intensity encoding within the brain.

5.4.5. Haemodynamic response in infants

The recent increase in fMRI studies in infants have generated opposing reports over the nature of neurovascular coupling in this population. Numerous studies have observed negative BOLD following sensory evoked stimulation [186,187,189], however, positive BOLD to the same stimuli have also been reported [187]. It has been suggested that age could be an important factor in the determination of the direction in which the response will occur [187,307]. It has been hypothesised that the observation of negative BOLD in the immature, developing brain could be due to greater oxygen demand related to increased density of neurons within the brain [307]. This, coupled with delayed development of vasculature, may reduce the brains ability to supply oxygenated blood to the active region, resulting in negative BOLD contrast [308].

During this investigation of noxious-evoked brain activity in infants, negative contrasts failed to detect any significant BOLD activity. In an attempt to clarify the nature of the developing haemodynamic response, Colonnese *et al.* investigated changes in the response with age in rats using both BOLD contrast and electrophysiological recordings [306]. The results of their study demonstrated that a BOLD response was first detected following electrical stimulation in P13 rat pups, and from this earliest detection, no negative BOLD activity was observed in any of the three age groups studied (P13 - P15, P20 - P30 and adult rats). Colonnese *et al.* also described the haemodynamic responses observed in the youngest rat pups as lower in amplitude and possessing a longer time to

peak response compared to adults, which was additionally supported by their electrophysiological recordings within the same brain region [306].

Arichi and colleagues have since demonstrated the same longer latency to peak and reduced amplitude of the haemodynamic response in human infants compared to adults (latency to peak BOLD: adults = 5.38 s, infants = 7 s; amplitude of percentage BOLD signal change: adults = 1.63, infants = 0.54) [211]. Arichi and colleagues also report however that the lower amplitude positive BOLD response observed in term infants is followed by an equal and opposite negative BOLD undershoot (median amplitude of negative to positive peak ratio = 0.49) [211]. This is much greater than the ratio of the negative BOLD undershoot seen in adults (median amplitude of negative to positive peak ratio = 0.23) [211], suggesting that the timing of BOLD contrast acquisition during fMRI in infants could influence the direction of observed signal change.

The fMRI studies reporting conflicting changes in the direction of the BOLD response do not provide any specific information on the HRF used. Arichi and colleagues, specifically mentioning this divergence amongst the literature, used a flexible method of modelling the HRF (the method is called F.L.O.B.S., for more information see [197]), which uses principal component analysis to estimate a sensible function that is then fit to the collected data [194]. Using this method, Arichi and colleagues describe significant positive BOLD responses in infants scanned at term age in response to somatosensory stimuli [194]. Moreover, a recent computational analysis from Cusack *et al.* suggests that mismatches in the HRF used could lead to inverted BOLD responses [196]. This evidence is in agreement with the data presented in this chapter, for which an age-appropriate infant HRF was used, and from which no negative BOLD responses were observed following noxious stimuli at any stimulus intensity.

5.4.6. Strengths and Limitations

As discussed in Chapter 4, PinPricks are ideally suited towards assessing noxious mechanical sensitivity in infants and adults (see section 4.4.4). Other strengths of this analysis include that all infants were newborn, healthy, self-ventilating, and 90 % were scanned within the first 7 days since birth. Only two infants were scanned at post-natal ages greater than 7 days. Those infants were 8 and 11 days old respectively. This is advantageous as it limits the infant's exposure to pain. In addition, many research fMRI studies of newborn preterm and term infants are opportunistically obtained following clinically required MRI scans, therefore sedatives are often provided to help keep infants from moving. In contrast no sedatives were used during any of the studies reported in this chapter.

Age was only included as a factor in the development analysis, however, it could be an interesting variable to consider in a larger study, since it has been demonstrated that younger infants lack descending inhibitory regulation of nociceptive inputs and therefore may demonstrate larger responses compared with older infants [109,302]. Furthermore, in fMRI studies that include the study of preterm and term infants, dynamic changes in the subplate need to be carefully considered [117,118,120,309]. The subplate is a brain region where the projections of thalamocortical neurons “wait” before they grow into the cortical layers to form their final synaptic connections. It is also a region of high neural density and blood supply [117,118,120,309]. As these projections are functionally active [310], it is possible that observed changes in BOLD activity may arise due to activity in the subplate region in prematurely-born infants.

Nevertheless, the studies conducted in this thesis were conducted on term-born infants, so the contribution of activity in the subplate is unlikely to be a significant factor. The subplate is a transient brain region, that is present during early development and reported to begin dissolution after approximately 34 weeks gestation (see section 1.5.3) [117,118,120]. Both thalamocortical and cortico-cortical connections are found to project

from the subplate and begin to form synaptic connections with the cortex between 26 and 34 weeks gestation [117,120,309]. Therefore, in the term-born infants included in this thesis, it is not likely that the reported activity is reflective of activity in the subplate regions. In the case of future studies in premature infants this would need to be considered further.

As demonstrated in Chapter 4, PinPrick stimulation at 128 mN did not cause habituation of nociceptive-specific brain activity at a minimum ISI of 10 seconds. The long ISI used in these fMRI experiments are therefore unlikely to result in habituation to noxious stimuli in either adults or infants [150,311,312].

The coverage and distribution of brain activity evoked by the 128 mN stimulus in infants, was most similar to that evoked by the 512 mN stimulus in adults, suggesting that infants may have increased sensitivity to noxious stimuli compared with adults. However, the data collected in this study, does not make it possible to ascertain whether differences in recorded brain activity are driven by differences in central or peripheral mechanisms. Fitzgerald and colleagues argue that the “immaturity of synaptic connections” in the CNS contributes to the “more diffuse” responses that infants display following noxious inputs and the authors even suggest that this may be indicative of a “potentially more powerful” experience of pain in infants [248]. However, it is also plausible that the observed differences in the evoked brain activity are due to differences in nociceptive transmission in the peripheral nervous system. For example differences in skin thickness could contribute towards an increased peripheral drive, or the immaturity of sensory afferent fibres and their receptors, could potentially result in lower thresholds for excitability. Fitzgerald and colleagues compared the thresholds required to elicit withdrawal reflexes to cutaneous mechanical stimulation in rat pups and human infants [92]. The average threshold in preterm infants (< 29.5 weeks PCA) was 0.5 g and this gradually increased to 1.7 g by term equivalent age [92]. Similarly the threshold in newborn rat pups at birth (comparable to preterm human infants) was “less than 1.0 g” compared with a threshold of 2.7 g observed two weeks later [92]

(comparable to a term infant according to [83]). Nevertheless, Fitzgerald argues that rat pup models are good models of human development and that the sensory fibres appear to be mature by term equivalent age. Therefore, I postulate that differences in central mechanisms are driving the differences in sensory function between adults and infants. Further neurophysiological studies would be needed to provide a more detailed mechanistic understanding of the factors that contribute to the differences observed in this study.

Simplified atlases were used during analysis of these data therefore some of the brain regions reported encompass large areas of the brain, for example the brainstem. This methodology did not allow for more detailed differentiation of specific regions of activity, however, where possible, attempts were made to account for this by describing the activity (or lack of activity) by way of visual observation. Nevertheless some brain regions do remain generalised such as the brainstem and cerebellum. We chose to take this approach because it was the simplest and most reliable way to compare the entire brain across adults and infants. Future studies will allow a more focussed approach to investigating the activity within larger brain areas.

Inferences on any subjective experience made based on surrogate measures, whether the measure is brain imaging data, behavioural responses or other autonomic or physiological observations are highly speculative. However, given that infants are incapable of communicating their own experience, it is necessary to investigate surrogate measures that could inform better treatment of pain in this population. Although ultimately we cannot infer an infant's subjective experience based on a given pattern of brain activity, the closer the pattern is to that observed in adults, who can report their subjective experience, the stronger the inference. The pattern of brain activity observed in both groups, following the same type of noxious stimulation, make it likely that the infant experience is similar to that described by adults. Thus, one could postulate that infants may have the capacity to experience pain.

5.5. Conclusion

By utilising fMRI's high spatial resolution, the cortical and subcortical brain regions involved in the generation of noxious-evoked brain activity in newborn infants have been investigated. This has demonstrated that the pattern of brain activity evoked by noxious stimulation overlaps considerably with that seen in adults. In addition there is a suggestion that newborn infants may be more sensitive to noxious stimuli as this overlap was observed in response to a stimulus that was 4 times smaller in infants compared with adults.

Pain is defined as an unpleasant sensory and emotional experience. This study provides the first demonstration that many of the brain regions that encode pain in adults are also activated in response to noxious stimuli in newborn infants. Since adults verbally reported that the stimuli were mildly painful, it is plausible that newborn infants also experienced this stimulus in this same way. The specific brain regions activated provide evidence supporting the hypothesis that infants are able to experience both sensory and affective aspects of pain. This hypothesis has strong clinical implications since infants undergoing neonatal intensive care are exposed to a high number of medically essential, acute painful procedures without the provision of pharmacological analgesia. Overall, the importance of developing new approaches for identifying effective clinical pain management is once again highlighted as a primary goal in this vulnerable population.

Chapter 6

Discussion

6.1. Thesis summary

The aim of this DPhil was to use brain-imaging techniques to investigate noxious-evoked brain activity in newborn infants. Although it has become accepted that neonates display physiological, behavioural, biochemical and cortical responses following painful procedures, little was known about the cortical and subcortical brain regions involved in pain processing. So fMRI was used to elucidate the brain structures involved in processing of noxious inputs in infants and to compare the pattern of activity to those observed in adults.

Before fMRI could be used to measure nociceptive processing in infants, it was necessary for a suitable MR-safe noxious stimulus to be identified. Until now, investigations of infant pain have primarily relied on observation of responses to clinically required acute noxious procedures, in particular heel lancing, injections and venepuncture. Since heel lancing was not an appropriate stimulus for use in the fMRI scanning environment, it was necessary to assess alternative experimental methods for investigating nociceptive sensitivity. In this thesis, it is demonstrated that noxious-evoked brain activity can be recorded in infants in response to experimental noxious PinPrick stimulation (applied forces: 32, 64 and 128 mN) using EEG and fMRI. The graded noxious stimulators are known to activate A δ fibres and were shown to evoke nociceptive-specific brain activity in infants using EEG. Furthermore, by measuring other surrogate measures of pain, such as nociceptive behavioural and spinal activity, the relationship between these measures was also investigated.

It was also established that BOLD responses could reliably be recorded in newborn infants in response to a range of PinPrick stimuli. The feasibility of this approach had been demonstrated in a single infant in a small pilot study [200]. In this project, fMRI was used to identify different cortical and subcortical brain regions involved in generating this noxious-evoked brain activity, and has demonstrated that 18 of the 20 regions activated following PinPrick stimuli in adults were also active in infants.

Overlapping active brain regions included the bilateral somatosensory (S1, S2), motor (M1, SMA), opercular, posterior insular (PI) and cingulate cortices (ACC, PCC) as well as the frontal gyri (prefrontal cortex), thalamus, putamen, cerebellum and brainstem. In a meta-analysis of neuroimaging studies investigating noxious-evoked brain activity, Apkarian *et al.* collated studies that used a wide range of nociceptive stimuli; including thermal, electrical, mechanical and chemical stimulation; and recorded noxious-evoked responses using a range of brain imaging methods including fMRI, EEG, MEG and PET [28]. From the investigation, Apkarian *et al.* identified a set of key brain regions (including those identified above) that were commonly activated in response to nociceptive stimuli and concluded that they demonstrated important involvement in pain processing [28]. This study also described how the network of brain regions involved in nociceptive processing included sensory (S1, S2, insula), affective (insula, ACC) and associative (frontal cortex) structures, supports our understanding of pain as a sensory and emotional experience [11,14,24,28].

As infants also demonstrated significant evoked activity in the same brain areas following noxious stimulation, this suggested that infants might have the capacity to experience pain in a similar way to adults. Indeed since this is the first study to report such a finding, further validation and reproduction of these results is necessary. However, this work has important implications for informing improvements in the management and treatment of pain in infants, particularly with regards to provision of analgesia. Recording of noxious-evoked brain activity is currently being used as an outcome measure for a clinical trial investigating the efficacy of morphine for

procedural pain in infants (www.controlled-trials.com/ISRCTN82342359). This provides an example of how the measurement of nociceptive-evoked brain activity can help inform the way that the measurement of infant pain is approached and treated. Studies using fMRI to investigate BOLD responses are becoming increasingly more common, however to date the work presented in my thesis represents the first time that nociceptive brain activity has been investigated in infants using a 3 T MRI scanner.

In the clinical trial mentioned above, it is proposed that a subset of infants receiving morphine will also have an fMRI scan, where PinPrick stimulators will be used to investigate how morphine modulates noxious-evoked brain activity. This provides an exciting demonstration of how quickly the methods reported in this thesis can be used to answer clinically relevant questions, which have the potential to change current clinical practice. In order to perform these studies, it is important to ensure that the fMRI data acquisition and analysis is optimised for the infant investigations.

The methods used to optimise the infant fMRI imaging parameters are also reported in this thesis. Of particular interest was the investigation of the optimum TE for use in infant studies, because use of a suboptimal TE results in lower sensitivity to changes in BOLD. Therefore, a series of experiments were conducted in order to investigate this parameter. The results demonstrated that if a TE of 30 ms, typically used in adult imaging studies, had been used, it could have resulted in no significant BOLD signal being observed. Overall, this work implies that future infant fMRI studies should adopt a longer TE of 50 ms to maximise sensitivity to the BOLD response.

The importance of carefully optimising imaging parameters can be illustrated by considering that positive and negative BOLD responses can be observed during the same fMRI study in infants [187]. Such conflicting reports of positive and negative BOLD response were common in the infant fMRI literature, which led Arichi and colleagues to use a flexible method of estimating the appropriate HRF from their data, therefore avoiding any prior assumptions about the response [194]. These methods demonstrated

that positive BOLD responses were observed in infants when flexible HRF estimation was adopted [194]. Subsequently, a modelling paper by Cusack and colleagues showed that use of an inappropriate HRF for infants results in reduced sensitivity to BOLD responses as well as inverted signal [196]. These examples further demonstrate the importance of using optimised parameters during infant fMRI scans.

Numerous epidemiological studies have reported under-treatment of pain in infants. A recent study reported that pharmacological analgesia was provided for less than 40 % of acute painful procedures during the first 14 days of neonatal intensive care [6]. Treatment of infant pain is extremely complex as infants cannot report their own pain. As verbal report is the “gold standard” for pain assessment in children and adults, there is currently no widely accepted best method for assessing pain in infants. Behavioural and physiological responses have been combined to form multidimensional tools to aid pain assessment, such as PIPP or BIIP [59,70]. Change in facial expression is an example of one of the behavioural responses that has been shown to demonstrate specificity for pain and correlate with noxious-evoked brain activity [60,73]. However, it is important to note that it has been demonstrated that 30 % of infants who mounted cortical haemodynamic responses following a heel lance, showed no concomitant change in facial expression [73]. This suggests that behavioural facial expression changes may not be sensitive enough for reliable pain assessment and highlights the need for improved surrogate measures to identify pain in infants.

Since the experience of pain requires higher cortical involvement, it has been suggested that, in the absence of verbal report, recording of brain activity may provide the most sensitive measure of nociceptive processing [278]. Pain processing is dynamic and variable such that no two individual's responses are the same and yet the same brain regions are activated when subjects experience different types of pain, such as thermal or chemical pain; psychogenic pain (i.e. pain with no nociceptive input); or pain induced by hypnotic suggestion [313]. The activation of these distinct brain areas has therefore been used to suggest that a neurological signature of pain perception could unlock the

key to individualised assessment and treatment of pain in a clinical setting. Wager and colleagues have taken this further by using machine learning techniques to provide a method of identifying pain in patients based on their neurological responses [278]. The authors demonstrate that multivariate pattern analysis can be used to discriminate between noxious- and non-noxious-evoked brain activity with a strikingly high rate of sensitivity and specificity (93 %) [278]. The authors therefore suggest that such methods could provide a tool for future pain assessment, particularly in populations of patients unable to communicate their pain experience, including patients in comas, neurologically impaired, the elderly and young infants.

Moreover, studies investigating pharmacologically and non-pharmacologically induced changes to activity within this network have demonstrated reduced activity in response to placebo [314], as well as opioids (including morphine and remifentanyl [315,316], for review see [317]). Improved characterisation of noxious-evoked patterns of neural activity in infants could provide a basis for investigation of safe and effective analgesia in this population [318]. A recent Cochrane review on opioid analgesia for ventilated infants included details on 13 studies which all used pain assessment tools such as PIPP for assessment of pain relief [82]. This review found insufficient evidence to recommend the routine use of morphine for pain relief in ventilated infants. However, trials incorporating measurement of noxious-evoked brain activity may provide new insights into such questions. Moreover, fMRI is also currently being incorporated into clinical trials testing analgesia in different adult patient groups [319]. Therefore this is also likely to be a promising future avenue for identifying potential efficacious analgesics in infants.

6.2. Limitations and further work

Many outcome measures were studied in this thesis, all investigating responses to experimental noxious PinPrick stimuli. Clinical pain scores were assessed, reflex withdrawal activity was recorded and brain activity was recorded using two different

brain-imaging techniques. Each technique comprised a group of at least 10 infants. It is arguably difficult to generalise across such small groups of infants and it would have been beneficial if all the outcome measures could have been recorded within the same infants, however this was not feasible in this vulnerable patient group, although may be possible in future studies. Nevertheless, while the data was collected from different study groups, the inclusion criteria for all the participants was the same and was very stringent; all infants were less than 11 days old when they took part in the study and did not have any neurological abnormalities. Although mode of delivery and exposure to infrequent acute noxious procedures were not controlled for, as far as possible the groups formed a homogenous group of infants with low levels of exposure to noxious stimuli.

During these studies, it was observed that the scanning environment encouraged infants to sleep and it was therefore reported that infants were either asleep or quietly awake. However this information was not video recorded at the time of the study. How sleep state can modulate responses to noxious inputs in infants has been investigated during studies examining behavioural and physiological responses. Grunau and Craig investigated the modulatory effects of sleep state on responses to pain, hypothesizing that infants that were quietly awake would be able to “reflect optimal orientation to environmental inputs” and therefore demonstrate the greatest responses to noxious inputs [60]. Indeed Grunau and Craig were able to show this result. It has also been demonstrated that sleeping infants have a lower cortical haemodynamic response to acute noxious procedures [2]. This evidence suggests that brain activity in sensory-motor cortical brain regions may be modulated during sleep. It would be of great interest to investigate this question further since the concept of clustered care for infants is recommended to allow longer periods of sleep and recovery for infants. Evidence that acute noxious procedures produce lower levels of brain activity during sleep could provide a simple way of reducing the impact of noxious inputs during early infancy.

Another limitation of these studies is that the low group numbers do not allow for investigation of other factors such as gender or mode of delivery. Such factors were also not included in the regression analyses, however future studies directly assessing these questions will be of interest. For example, Wellman *et al.* show that the mode of delivery dramatically alters the levels of copeptin in neonatal cord blood, which is a marker of vasopressin levels [320]. Copeptin levels were elevated to approximately 100 times in infants born via vaginal delivery compared with caesarean section [320], and were also shown to correlate with exposure to neonatal stress [321]. Both vasopressin and oxytocin have been demonstrated to increase pain thresholds in rat pups and infants [322]. It is therefore possible that infants may be more vulnerable to noxious stimuli following a caesarean section due to reduced stress-induced analgesia mediated by vasopressin levels [322]. Gender has also been shown to influence sensitivity to pain, with females often demonstrated to have lower thresholds for pain but higher tolerance compared with males (for a review on gender differences and pain see [323]). Therefore it is possible that there are multiple factors requiring consideration when developing newer and more effective methods for managing pain in infants.

In this thesis, it is argued that, the similar pattern of brain activity, observed in response to noxious stimuli in both adults and infants, provides evidence that the infant experience is similar to that described by an adult. The assumption that infants feel sensory stimuli in a similar way to adults is not a new inference. To take a very simplistic example, the parent of a young infant will test the temperature of milk on their own skin before giving it to their child. The underlying assumption is that the child will detect the temperature in the same way. Similarly, when Carbajal and colleagues conducted their multicentre epidemiological study in France, they began by attempting to collect a list of clinical procedures that clinicians considered as painful for infants. For a clinician to make such an inference, they would have based their decision on what they themselves would describe as painful. The authors defined a painful procedure as follows:

“(A procedure that) invaded the neonate’s bodily integrity, causing skin injury or mucosal injury from the introduction or removal of foreign material into airway or digestive or urinary tract.”

It is however notable that this was not an extensive definition and “other procedures that were considered painful by clinicians at the bedside were also registered.” [9]

Tactile and noxious inputs produce overlapping somatotopic representation in the somatosensory cortices [324], and it is difficult to distinguish whether activity observed within a particular region of the somatosensory cortex is a result of tactile or nociceptive processing. There has been extensive debate with regards to the specificity of fMRI brain activity evoked by noxious stimulation (for examples of this debate see [325,326]). Mouraux and colleagues, demonstrated that the network of brain regions most commonly activated by noxious inputs, are also activated in response to other non-noxious salient stimuli, including touch, auditory and visual stimulation [327]. Therefore, it has been strongly argued that the brain activity observed following nociceptive stimulation is largely non-specific for pain. However, Wager and colleagues have recently been able to demonstrate that multivariate pattern analysis (MVPA) showed 93 % sensitivity and specificity in distinguishing between painful and non-painful thermal stimuli [278]. Thus, it may be possible that these techniques could be developed in the future such that they could be used to investigate nociceptive specificity in infants.

MVPA may also allow more precise inferences about perception to be made. For example Wager and colleagues also showed that MVPA could distinguish between physically induced and psychophysical or emotionally induced patterns of brain activity [278]. Further investigation of noxious-evoked brain activity in infants could potentially also be combined with MVPA. This method could provide particular benefits with respect to the investigation of analgesic drug efficacy, as MVPA could be used to extend the sensitivity of assessing modulation of nociceptive processing.

The investigation of changes in BOLD responses with age is a key tool for the non-invasive exploration of development of neural circuitry in humans. Using fMRI, increasing percentage BOLD response was observed in the contralateral somatosensory cortex with increasing gestational age. This result demonstrates the feasibility of using fMRI to investigate developmental changes across late gestation. In addition, functional connectivity between different brain regions provides a vital source of information about how developing networks interact. It is also possible to investigate whether this is similar to the way in which adult networks are connected. An example of such analysis is provided by Toulmin *et al.*, who investigated the relationship of functional connectivity between the thalamus and the somatosensory cortex, describing a network of connectivity to cortical brain regions in infants that was almost identical to that seen in adults [199].

6.3. Concluding statement

Historically it was believed that infants could not feel pain. Based on this belief, it was routine practice to perform surgery on infants using muscle relaxants and providing little or no pain relief. Although this practice has changed and analgesia and anaesthesia are now provided during surgery, infants are still receiving limited analgesia during routine clinical procedures. Research over the past 30 years has been integral in demonstrating that infants exhibit nociceptive behavioural (facial expression, bodily movements), physiological (heart rate, oxygen saturation, cortisol) and cortical (electrophysiological, haemodynamic) responses to noxious stimuli, however little was known about the brain structures involved in processing nociceptive inputs.

In this thesis noxious-evoked brain activity has been characterised in newborn infants using fMRI, and the potential for use of this pattern of brain activity in future studies assessing the safety and efficacy of analgesics in infants, has been discussed. This is particularly exciting because the outcome measures currently used to assess analgesic agents are limited in terms of their sensitivity to pain. This work therefore provides an

initial step towards identification of better outcome measures for testing analgesic agents. This has the potential to facilitate greater availability of safe analgesics for use in infants.

Chapter 7

Appendices

**Test certificate**

for the weights of the PinPrick stimulators

Examiner: H. Baum

Date: 02.08.2010

Test equipment: MRC 401 – Kern & Sohn PCB 250-3

(all measurements in mN)

weight	serial number	minimum	maximum	measured value	passed	failed
8	PP06-00015	7,2	8,8	8,09	☒	☐
16	PP06-00016	14,4	17,6	15,98	☒	☐
32	PP06-00017	28,8	35,2	31,80	☒	☐
64	PP06-00018	60,8	67,2	63,90	☒	☐
128	PP06-00019	121,6	134,4	127,95	☒	☐
256	PP06-00020	243,2	268,8	255,51	☒	☐
512	PP06-00021	496,6	527,4	511,66	☒	☐

Additional comments:

02.08.2010

Date, Signature Examiner

Release of product:

2.8.2010

Date, Signature PL

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Prüfprotokoll_Gewichte-MRC_Set06-003.odt	MG – 31.05.2010	page 1 of 1
	Version: 2	Status: approved

**Test certificate**

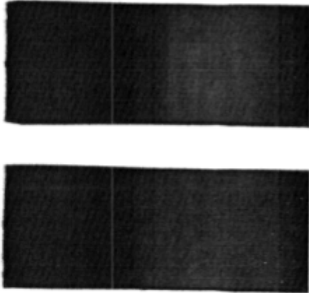
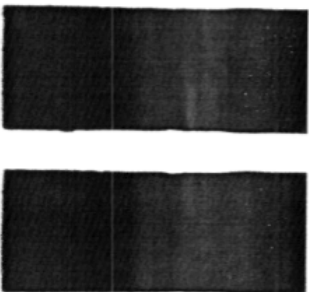
for the needle geometry of the PinPrick stimulators

Examiner: H. Baum

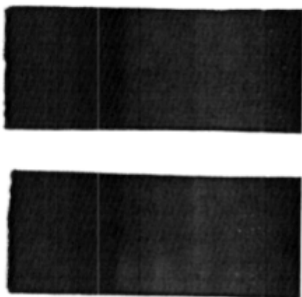
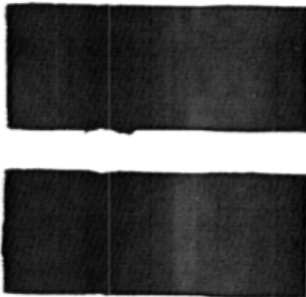
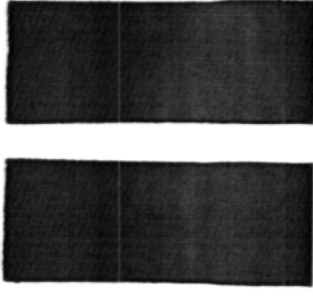
Date: 30.07.2010

Test equipment: Telecentric optical bench MRC 402

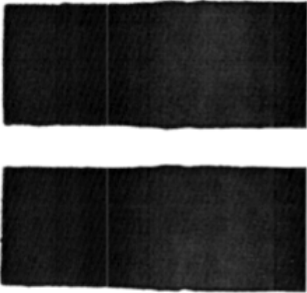
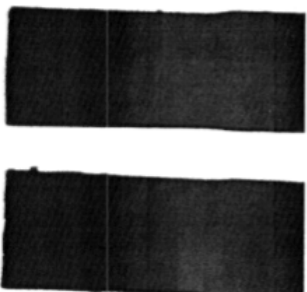
Target values: Radius of curvature: 5 μm , Tolerance: 3 μm ... 10 μm
 Diameter of contact area ($\varnothing$): 250 $\mu\text{m} \pm 15 \mu\text{m}$

weight [mN]	serial number	visual inspection	Radii / $\varnothing$ [μm]	passed	failed
8	PP06-00015		~5-8 / ~255	☒	☐
16	PP06-00016		~4-5 / ~261	☒	☐

Pinprick_Pruefprotokoll- Nadelgeometrie_en_PP06_Set3.sxw	MG – 31.05.2010	page 1 of 3
	version: 3	Status: approved

weight [mN]	serial number	visual inspection	Radii / Ø [µm]	passed	failed
32	PP06-00017		~5-10 / ~258	☒	☐
64	PP06-00018		~5 / ~260	☒	☐
128	PP06-00019		~4-5 / ~260	☒	☐

Pinprick_Pruefprotokoll- Nadelgeometrie_en_PP06_Set3.sxw	MG – 31.05.2010	page 2 of 3
	version: 3	Status: approved

weight [mN]	serial number	visual inspection	Radii / Ø [µm]	passed	failed
256	PP06-00020		~5-8 / ~250	☒	☐
512	PP06-00021		~5-10 / ~250	☒	☐

30.07.2010 Ba
Date, Signature Examiner

Release of product:

31.07.2010 B. PL
Date, Signature PL

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Pinprick_Pruefprotokoll- Nadelgeometrie_en_PP06_Set3.sxw	MG - 31.05.2010	page 3 of 3
	version: 3	Status: approved

Appendix 1: MRC Systems PinPrick Test Certificate - August 2010.

**Test certificate**

for the weights of the PinPrick stimulators

Examiner: Marcus Goetz

Date: 23.04.2015

Test equipment: MRC 401 – Kern & Sohn PCB 250-3

weight	serial number	measured value in g	measured value in mN	Minimum in mN	Maximum in mN	passed	failed
8	PP06-00015	0,825	8,09	7,2	8,8	☒	☐
16	PP06-00016	1,630	15,99	14,4	17,6	☒	☐
32	PP06-00017	3,241	31,79	28,8	35,2	☒	☐
64	PP06-00018	6,516	63,92	60,8	67,2	☒	☐
128	PP06-00019	13,047	127,99	121,6	134,4	☒	☐
256	PP06-00020	26,057	255,62	243,2	268,8	☒	☐
512	PP06-00021	52,189	511,97	496,6	527,4	☒	☐

Additional comments:

23.4.2015 
 Date, Signature Examiner

Release of product:

23.04.2015 
 Date, Signature PL

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Pinprick_Pruefprotokoll-Gewichte_en_v3_PP06-015-021.sxw	MG – 19.03.2012	page 1 of 1
	version: 3	Status: approved



Test certificate

for the needle geometry of the PinPrick stimulators

Examiner: Dr. Marcus Goetz

Date: 23.04.2015

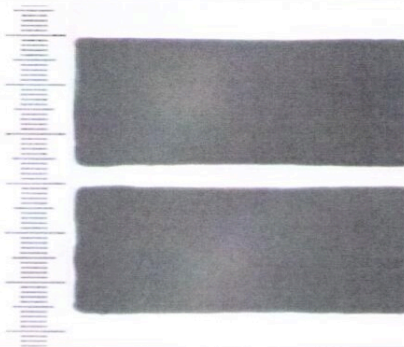
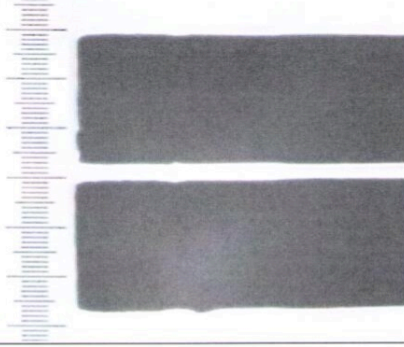
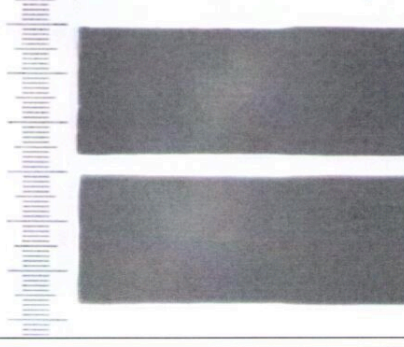
Test equipment: Telecentric optical bench MRC 402

Target values: Radius of curvature: 5 μm , Tolerance: 3 μm ... 10 μm

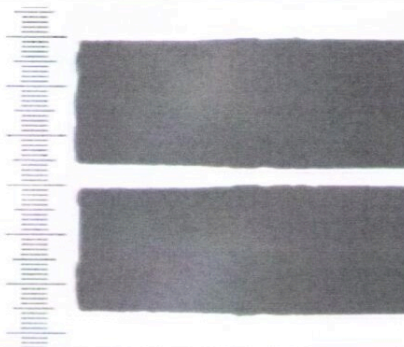
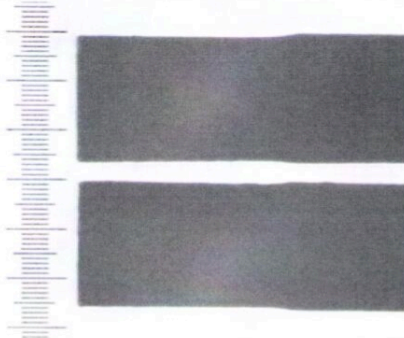
Diameter of contact area ($\varnothing$): 250 $\mu\text{m} \pm 15 \mu\text{m}$

weight [mN]	serial number	visual inspection	Radii / $\varnothing$ [μm]	passed	failed
8	PP06-00015		5-6 / ~256	☒	☐
16	PP06-00016		~6 / ~260	☒	☐

Pinprick_Pruefprotokoll-Nadelgeometrie_en_PP06-015-021.sxw	MG – 31.05.2010	page 1 of 3
	version: 3	Status: approved

weight [mN]	serial number	visual inspection	Radii / Ø [µm]	passed	failed
32	PP06-00017		7-10 / ~258	☒	☐
64	PP06-00018		8-10 / ~260 (rough surface)	☒	☐
128	PP06-00019		6-7 / ~260	☒	☐

Pinprick_Pruefprotokoll-Nadelgeometrie_en_PP06-015-021.sxx	MG – 31.05.2010	page 2 of 3
	version: 3	Status: approved

weight [mN]	serial number	visual inspection	Radii / Ø [µm]	passed	failed
256	PP06-00020		6-8 / ~250 (rough surface)	☒	☐
512	PP06-00021		5-6 / ~257	☒	☐

23.4.2015 
Date, Signature Examiner

Release of product:

23.06.2015 
Date, Signature PL

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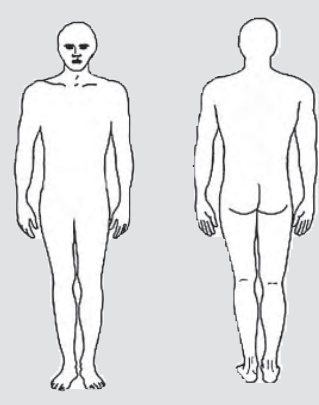
Pinprick_Pruefprotokoll-Nadelgeometrie_en_PP06-015-021.sxw	MG – 31.05.2010	page 3 of 3
	version: 3	Status: approved

Appendix 2: MRC Systems PinPrick Test Certificate - April 2015.

McGill Pain Questionnaire

PATIENT'S NAME _____		DATE _____	TIME _____	AM/PM _____
PRI: S (1-10) (11-15) (16) (17-20) (1-20)		A	E	M
		PRI(T)		PPI

1 FLICKERING _____ QUIVERING _____ PULSING _____ THROBBING _____ BEATING _____ POUNDING _____	11 TIRING _____ EXHAUSTING _____ 12 SICKENING _____ SUFFOCATING _____ 13 FEARFUL _____ FRIGHTFUL _____ TERRIFYING _____ 14 PUNISHING _____ GRUELLING _____ CRUEL _____ VICIOUS _____ KILLING _____ 15 WRETCHED _____ BLINDING _____ 16 ANNOYING _____ TROUBLESOME _____ MISERABLE _____ INTENSE _____ UNBEARABLE _____ 17 SPREADING _____ RADIATING _____ PENETRATING _____ PIERCING _____ 18 TIGHT _____ NUMB _____ DRAWING _____ SQUEEZING _____ TEARING _____	BRIEF _____ MOMENTARY _____ TRANSIENT _____ RHYTHMIC _____ PERIODIC _____ INTERMITTENT _____ CONTINUOUS _____ STEADY _____ CONSTANT _____
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E = EXTERNAL
 I = INTERNAL

COMMENTS:

2 JUMPING _____ FLASHING _____ SHOOTING _____ 3 PRICKING _____ BORING _____ DRILLING _____ STABBING _____ LANCINATING _____ 4 SHARP _____ CUTTING _____ LACERATING _____ 5 PINCHING _____ PRESSING _____ GNAWING _____ CRAMPING _____ CRUSHING _____ 6 TUGGING _____ PULLING _____ WRENCHING _____ 7 HOT _____ BURNING _____ SCALDING _____ SEARING _____ 8 TINGLING _____ ITCHY _____ SMARTING _____ STINGING _____ 9 DULL _____ SORE _____ HURTING _____ ACHING _____ HEAVY _____	0 NO PAIN _____ 1 MILD _____ 2 DISCOMFORTING _____ 3 DISTRESSING _____ 4 HORRIBLE _____ 5 EXCRUCIATING _____
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The descriptors fall into four major groups: sensory, 1 to 10; affective, 11 to 15; evaluative, 16; and miscellaneous, 17 to 20. The rank value for each descriptor is based on its position in the word set. The sum of the rank values is the pain rating index (PRI). The present pain intensity (PPI) is based on a scale of 0 to 5. Copyright © 1970 Ronald Melzack.

Appendix 3: McGill Pain Questionnaire [285].

Chapter 8

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