

**THE EFFICACY AND MECHANISM OF PERIPHERAL
OPIOIDS IN PAEDIATRIC INFLAMMATORY PAIN**

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ABSTRACT

Background:

The management of pain in children is a common but difficult symptom to treat and the use of strong analgesics is often limited, for a variety of reasons, including fear of inducing side effects. Morphine is normally considered to be a centrally acting analgesic but recently, the beneficial effects of peripheral opioids have been demonstrated in adults for a number of painful inflammatory conditions. When administered in this non-invasive way, opioids provide analgesia without achieving significant plasma concentrations and therefore it can be assumed that many of the adverse effects associated with oral or systemic opioids are avoided.

Methods:

A) The first part of the thesis describes the mechanism of action of peripheral opioids through development and particularly their effect during inflammation. This work was conducted using different ages of Sprague Dawley rat pups and skin inflammation was induced using carageenan.

B) The second section is a description of a double-blinded, randomised-controlled, placebo-controlled trial with crossover design, to assess the efficacy of peripheral opioids in paediatric inflammatory pain. This was conducted in children with a diagnosis of Epidermolysis Bullosa (EB), as a model for acute and chronic inflammatory pain

Results:

The laboratory study demonstrates that mu opioid receptor (MOR) expression is up regulated in neonatal plantar skin and significantly up regulated in neonatal lumbar dorsal root ganglion (DRG) four hours post hind paw inflammation, MOR protein levels in the rat hind paw plantar skin are significantly up regulated post-natally, and MOR protein levels are significantly up regulated in both neonatal and young adult plantar skin four hours post hind paw inflammation. Clinically, pain reduction was most significant with background pain

Conclusion:

The developmental regulation of peripheral MOR both in naïve and inflamed cutaneous tissue may have implications for the use of topically / peripherally applied opioids in infants and children.

“I, Gillian Watterson confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis”.

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CHAPTER 1

1.1 GENERAL INTRODUCTION

Pain is a complex and multi-dimensional symptom, which we all experience at some time in our lives. Under-treatment of pain can lead to long-lasting catastrophic effects both physiological and psychological. Fortunately, much has changed in the field of paediatric pain research since the initial reports of inadequate pain management in infants and children documented well over 25 years ago (Swafford and Allan 1968). Although laboratory research has now revealed the precise pain mechanisms, demonstrating that even the youngest infant is capable of painful experiences and therefore in potential need of analgesia (Fitzgerald et al 1988), there is still a gap between this knowledge and implementation in the clinical setting. Strict ethical processes exist, particularly for clinical trials involving children and yet there is a pressing need for research, especially in the areas of pain assessment, management of neuropathic and persistent pain states in children as well as the introduction of novel analgesics.

Traditionally opioids act through the central nervous system but studies in the adult population have now characterised definite peripheral actions of opioids upon peripheral morphine receptors (MOR), providing both anti-nociceptive and anti-inflammatory effects (Stein et al 1991, Krajnik and Zylicz 1997; Flock et al 2000; Stein et al 2001). This exciting development of opioid receptor pharmacology could potentially lead to effective analgesia when opioids are applied to peripheral tissues, but without the centrally mediated and often intolerant adverse effects.

My research, both a laboratory and clinical project, has attempted for the first time to provide evidence for the existence of peripheral opioid receptors throughout development in skin tissue, from the neonatal period onwards. It also explores whether inflammation might have an effect on the quantity of receptors (chapter 3). The second part of the thesis is clinical work exploring the efficacy of a novel route of peripheral opioids in a paediatric population (chapter 4).

Layout of thesis:

The thesis is divided to two main sections:

A) Laboratory study The first part of my thesis describes various studies carried out in the Department of Anatomy and Developmental Biology laboratory, University College London. This work focuses on the existence of peripheral opioid receptors throughout development and whether there is any alteration in receptor number following an inflammatory painful insult, using immunofluorescence and light microscopy, as well as quantification of the MOR protein using Western Blot technique. The skin tissue of rat pups are used in these studies.

B) Clinical study The second part explores the efficacy and use of topical (peripheral) opioids in children and young people with Epidermolysis Bullosa (EB). EB is a group of inherited mechano-bullous disorders involving blistering of the skin and mucous membranes, in response to minor frictional trauma. I focus on one of its main symptoms namely pain, because different sub-types of pain are experienced in EB patients and it is therefore an extremely useful model for the purpose of this work (Herod et al 2002). I have not included details of the patho-physiology or management of the disorder. Previous studies investigating peripheral opioids, have only been carried out in the adult population and have recruited mainly a population of patients who are in the palliative phase of their illness and who have experienced a varying range of cutaneous lesions, triggering episodes of either acute inflammatory pain, acute incident pain, chronic inflammatory pain, neuropathic pain or indeed a combination of these pain types (Krajnik and Zylicz 1997; Twillman et al 1999; Flock 2003; Zeppetella et al 2003). This study focuses on the use of topical morphine and its efficacy particularly in incident pain, background inflammatory pain and post-procedural pain in a paediatric patient group.

In order to understand the neurobiology, physiology and classification of pain in the developing human, I will first document the commonly used definition of pain and summarise how important historical theories have provided a foundation for our current knowledge, of pain and its mechanisms in the human adult.

1.2 PAIN DEFINITION

The International Association into the Study of Pain has defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage.”

This definition encompasses both the physical and emotional perception of pain, rather than considering the actual amount of tissue damage leading to the pain i.e nociception (IASP Taskforce on taxonomy 1994).

However considering the diverse and individual nature of pain experiences, the above definition is not suitable to be applied in all situations; e.g. in the preverbal child or the cognitively impaired. The relationship of the perception to the stimulus is variable and depends on the patient’s previous experiences, emotional state and cognition. In particular relation to pain in neonates and infants, it is also necessary to consider the development of sensory pathways as well as development of consciousness and the mind.

1.3 PAIN THROUGH THE CENTURIES

The first mechanism of pain perception was suggested by Descartes almost four centuries ago 1664 - he stated that pain was “*fast moving particles of fire.. the disturbance passes along the neurofilament until it reaches the brain*”. This simply described the concept that the pain system was a direct channel from the skin to the brain. He stated that a flame sets alight particles in the foot into motion up the leg and back and into the head where an alarm system is then set off, causing the person to feel pain (Descartes 1664).

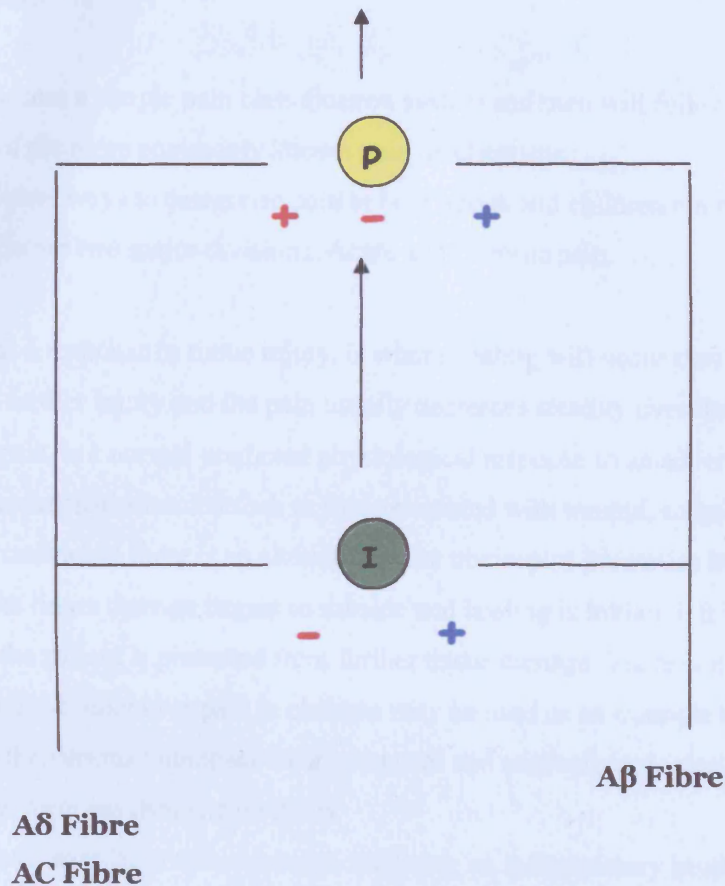
In the nineteenth century, Max Von Frey, physician proposed that specific pain receptors in the body project via pain fibres to a pain centre within the central nervous system. His second suggestion was that below each sensory spot in the skin lay a specific receptor, linked to each of the four sensory modalities but this has never been proven.

However a final proposal which was accepted was the pattern theories of pain, which suggests that painful information generated by peripheral skin receptors is coded in patterns of nerve impulses (von Frey 1894).

In 1965 the *Melzack and Wall Gate Control Theory* was published in *Science* entitled a “A New Theory of Pain”. Simply speaking it stated that pain transmission from the peripheral nerve through to the spinal cord was subject to both intrinsic controls and controls originating from the brain. Melzack and Wall proposed that the perception of pain depends not only upon pain signals in small fibres, but also upon the balance of activity in large myelinated fibres (A-alpha and A-beta fibres) and small myelinated and unmyelinated nociceptive fibres (A-delta and C fibres). They hypothesised that under normal conditions, any stimulus which increases the activity of the large mechanoreceptive fibres tends to reduce pain, and anything that increases the activity of small nociceptive fibres tends to increase pain (Melzack and Wall 1965), (See Fig 1.1). However they failed to include the peripheral processes involved in pain.

For example, when there is tissue damage and inflammatory pain ensues, there is a peripheral inflammatory soup of chemical mediators released which sensitise the peripheral sensory nerve endings and following neuropathic pain, excitability changes occur within the peripheral nerves themselves. Both of the above peripheral mechanisms then cause change within the central pain systems (McMahon et al 1993). Pain as a symptom continues to emerge as a complex sensory modality, initiating from Descartes’ basic linear theory, to the current theories based on Wall’s Gate Theory.

Higher Centres in Brain



Key: Inhibitory Interneurone + inhibition - excitation
 Projection Interneurone

1. Explanation: When there is no stimulation, all pain fibres are inactive and the inhibitory interneuron blocks the signal in the projection neuron. The gate is therefore closed and no pain is experienced.
2. With a noxious stimulus, A δ and AC fibres are activated, which in turn stimulate the projection neuron and blocks the inhibitory neuron. The gate is open and there is pain.
3. When a non-painful stimulus such as touch is experienced, the A β fibres are stimulated and activates the projection neuron as well as the inhibitory neurones which in turn blocks the projection neuron which connects to the spinothalamic tract ascending to the pain centres in the brain and no pain is experienced

Fig 1.1 Basic Diagram explaining the Gate Control pain Theory

1.4 TYPES OF PAIN IN CHILDREN

1.4.1 Classification

I will first discuss a simple pain classification system and then will follow on with a description of the more commonly known pain mechanisms.

There are several ways to categorise pain in both adults and children but broadly speaking there are two major divisions; Acute and Chronic pain.

Acute pain as a response to tissue injury, is where healing will occur thus protecting the patient from further injury and the pain usually decreases steadily over time. Acute nociceptive pain, is a normal predicted physiological response to an adverse chemical, thermal or mechanical stimulus such as that associated with trauma, surgery, and acute illness. It occurs when there is an alteration in the nociceptor properties but usually resolves as the tissue damage begins to subside and healing is initiated. It is a protective pain, in that the patient is protected from further tissue damage. There is normal neural transmission. Post-operative pain in children may be used as an example to demonstrate the various immediate neurochemical and neurophysiological responses, as well as longer term psychological effects.

Acute pain may arise from tissue damage following an inflammatory insult, such as in burns. Juvenile rheumatoid arthritis is a classic example of inflammatory pain, where an influx of immunocytes, such as mast cells and macrophages release excitatory neurotransmitters in response to a noxious stimulus and subsequent tissue damage.

Chronic or persistent pain was previously defined by the International Association for the Study of Pain (IASP 1986), as pain which is constant for 6 months or longer, but it has now become clear that there are elements of chronic pain which may actually arise much earlier and the revised classification is now pain less than 1 month, 1 to 6 months or longer than 6 months (IASP Subcommittee on Taxonomy 1994).

This may be a result of prolonged acute pain secondary to an underlying organic disorder such as a tumour, a skeletal dysplasia or in arthritis, where there are repeated inflammatory episodes such as in those patients with EB.

It can be distinguished from recurrent pain syndromes where there are alternate periods of painful and pain free episodes, for example in chronic recurrent headaches or in the recurrent abdominal pain syndrome.

Chronic pain may also exist where there is no underlying pathological cause. Possible predisposing factors are female gender, hypermobility, maladaptive coping strategies, and parental modelling of pain behaviours.

Unfortunately this pain may go unrecognised for years and may progress to a pain disability syndrome affecting the child's physical, emotional and psychological coping mechanisms (Zeltzer et al 1997). Sleep disturbance is often a huge problem for these patients as well as school absenteeism. It is believed that for some of these patients, there is reinforcement of the sick role and apparent pain amplification (Eccleston 2003).

Neuropathic Pain is a characteristic type of pain which persists independent of ongoing tissue damage or inflammation. It may be acute or chronic and is due to an altered excitability of the peripheral, central or autonomic nervous system (Bennett 1997). It is generally not protective and usually persists even though the original noxious stimulus has subsided. There are a range of particular sensory disturbances such as allodynia (a pain response secondary to a non-noxious stimulus) and hyperalgesia (heightened pain response to a painful/thermal or mechanical stimulus) as well as motor disturbances such as weakness and spasms and autonomic symptoms such as cyanosis, sweating and swelling of the affected site (Devor 1984). This subtype of pain occurs in such conditions as the Complex Regional Pain Syndromes (Wilder et al 1992), secondary to tumour compression or tumour therapies, in phantom limb pain (Wilkins et al 1998) or in diabetic patients who have ulcerative lesions as well as peripheral neuropathy.

Breakthrough Pain The terms, breakthrough /episodic and incident pain are often confused. Breakthrough pain was initially described by Portenoy and Hagen (1990) following a study on cancer patients, as a "transient increase in the intensity of moderate or severe pain, occurring in the presence of well-established pain."

Just over a decade later, the American Pain Society (2005) consolidated the definition as “intermittent exacerbations of pain that can occur spontaneously or in relation to specific activity; pain that increases above the level of pain addressed by the ongoing analgesic; includes incident pain and end-of-dose failure.”

Incident pain is the description traditionally given to pain which may occur on movement, for example coughing/vomiting or whilst walking.

There is also a subtype of incident pain unrelated to motion and occurs when the patient is at rest with no known triggering factor. These pains are episodic in nature such as secondary to spasms. Incident pain may therefore be acute or chronic depending on the disease progress and treatment given. Management of breakthrough pain is straightforward and requires regular review and alteration of medication and dosage. However it is more difficult to achieve adequate control of incident pain and requires an individualised approach of balancing efficacy of each drug against adverse effects (McQuay 1989).

1.5 MECHANISMS OF PAIN

Several types of pain are now recognised and the complex mechanisms behind such pains are becoming clearer. The following mechanisms will now be discussed in more detail:

- A) nociception
- B) peripheral sensitisation
- C) phenotypic switches
- D) central sensitisation
- E) neuropathic pain mechanisms

A) Nociception

Nociception is the detection of a painful noxious stimulus and it encompasses the entire process from the actual site of active tissue damage, through to the cortical perception of pain via a complex array of physiological and biochemical events. It involves four major processes:

- transduction
- transmission

- modulation
- perception

Transduction

Transduction involves relaying the specific noxious physical and chemical stimuli to the spinal cord, once they have been converted into electrical impulses, at the peripheral terminals of nociceptor sensory fibres.

These terminals are known as primary afferent nociceptors and give rise to sensory fibres, which possess characteristic properties, distinguishing them from other sensory nerve fibre. The table below, (table 1.1) summarises the properties of these nociceptors:

Adelta (Aδ)	Abeta (Aβ)	C
finely myelinated, small fibres	large , myelinated	unmyelinated, small cell bodies
rapid firing – 10-40 m/s	rapidly conducting	slow rate < 2 m/sec
sharp ; first acute pain	transmit innocuous information	dull; second pain
mechano-thermal mechanosensitive	respond to touch, vibration, deep pressure	polymodal receptors- respond to thermal, mechanical and chemical noxious

Table 1.1 Properties of Primary Afferent Nociceptors

Transmission

Transmission is the propagation of an impulse from neuron to neuron throughout the sensory nervous system, by primary afferent neurons which synapse in the dorsal horn of the spinal cord. Afferent fibres enter the spinal cord and travel via Lissauer's tract before sending processes into the dorsal horn. A δ fibres synapse in laminae 1 and 5, C fibres synapse in laminae 1 and 2 (substantia gelatinosa) and A β fibres synapse in laminae 3, 4, 5 and 6. (See Table 1.1 for the properties of these fibres).

Then this process activates projection neurons, either directly or via interneurons, which then cross over the midline and ascend to the brainstem and thalamus. These fibres along with the second order neurons, form the spinothalamic tract, the major ascending pathway for pain and temperature.

Modulation

Modulation is the process of both attenuation and amplification of the initial noxious signal. It occurs between pathways of descending inhibition originating within the somatic sensory cortex, the hypothalamus, the periaqueductal gray matter of the midbrain, the raphe nuclei and other nuclei of the rostral ventral medulla. Complex modulatory effects occur at each of these sites as well as in the dorsal horn. Both excitatory and inhibitory neurotransmitters are involved.

Descending pathways release transmitters such as 5HT, noradrenaline, and endogenous opioids to produce inhibitory control. At the spinal cord level, there are classes of interneurons which contain one or more peptides such as enkephalins or inhibitory amino acids such as GABA and glycine. Overall these neurotransmitters aim to act upon inhibitory interneurons, thus causing a decrease in pre and postsynaptic activity, or excite the second order neuron, producing descending excitation.

Perception of Pain

Pain is a complex phenomenon not only involving the transduction of a noxious stimulus but also cortical processing, which then determines the individual perception of pain.

Pain perception is largely a cognitive process influenced by physiological, neurochemical, contextual, emotional and behavioural variables.

Cognitive factors affect pain perception at all ascending neural levels of pain, from transmission of nociceptor input, through to thalamic and cortical pathways. Perception of pain depends not only on the extent of the physical injury, but also on a series of complex interactions, where modification of the nerve impulses which have been generated by tissue damage and it occurs by means of ascending systems and descending pain suppressing systems.

B) Peripheral sensitisation

Tissue damage at the periphery, following a noxious stimulus, triggers the release of peptides such as substance P as well as serotonin, histamine, in combination with the involvement of inflammatory cells, which then forms an “inflammatory soup”. This directly activates and sensitises peripheral nerve endings to cause pain, swelling and tenderness, secondary to vasodilatation and plasma extravasation (Dray 1997).

Primary hyperalgesia may develop from the above process around the area of tissue damage so non-noxious stimuli seem painful (allodynia) and noxious stimuli produce an even more heightened pain response (hyperalgesia). The entire process of chemical signalling, which arises from local damage, not only protects the damaged area, but also promotes healing and helps to prevent infection, due to the increased blood flow and inflammation.

C) Phenotypic changes

Following tissue injury and inflammatory pain, transport of neurotransmitters from the periphery to the dorsal root ganglion activates intracellular pathways, which then alter the properties of sensory neurons, e.g. in neuropathic pain, changes in the phenotypic expression of sodium and potassium channels post nerve injury lead to hyperpolarisation of the membrane (Amir et al 2006).

D) Central Sensitisation

This is characterised by reduction in threshold and an increase in responsiveness of dorsal horn neurons, as well as enlargement of their receptive fields and sensitivity to non-noxious stimuli (allodynia and secondary hyperalgesia). During central sensitisation in the dorsal horn, there is a repeated constant C fibre stimulus which can induce an augmented response to subsequent C fibre input - "wind up." This leads to an intense acute pain secondary to closely repeated stimuli.

The NMDA (N-methyl-D-aspartate) receptor is required for this amplification, as well as activation of other ion channels such as the AMPA (amino hydroxy methyl isoxazole propionic acid) receptor, the neurokinin receptor and tyrosine kinase receptors (Dickenson and Beeson 1997). The release of peptides such as substance P into the spinal cord, removes the magnesium block from the channel of the NMDA receptor and permits glutamate to activate the receptor into a persistent pain state (De Felipe et al 1998). Also activation of the NMDA receptor allows calcium to enter the neurone, which leads to further production of other mediators from spinal neurones contributing to the overall process.

E) Neuropathic Pain mechanisms.

A combination of the above pain mechanisms may exist in the syndrome of neuropathic pain. Human studies have demonstrated that A fibre activity leads to neuropathic pain, more specifically the touch evoked allodynia, while burning spontaneous pain is probably maintained via C nociceptors. Peripheral sensitisation has been shown in certain neuropathic pain states such as post-herpetic neuralgia, where C fibre sensitisation has been shown to induce touch evoked A β allodynia (Chabal 1989). Axon damage in the adult also leads to a change in the function, structure and phenotypic expression of cell bodies within the dorsal root ganglion including opioid peptide receptor and glutamate receptor expression (Alvares and Fitzgerald 1999, Woolf and Mannion 1999). This may be due to loss of peripheral neurotrophic influences.

It has also been demonstrated that spontaneous ectopic discharges within the DRG can occur after nerve section, as well as accumulation of sodium channels at the axotomy site, which then may lead to a hyperalgesic, persistent pain state (Amir et al 2006). Centrally, following adult axotomy, there is a synaptic rearrangement whereby A β fibres, which normally occupy Laminae 3 and 4, sprout up to Laminae 1 and 2 and thus contributing to allodynia (Shortland and Woolf 1993).

Central sensitisation including the wind-up phenomenon are the hallmarks of neuropathic pain but advances in molecular studies as well as functional imaging have led to the introduction of novel clinical therapies in an attempt to manage this complex pain state.

1.6 THE DEVELOPMENT OF PAIN PROCESSING IN NEONATES AND INFANTS

1.6.1 Introduction

Table 1.2 documents the neurodevelopment of human pain pathways.

Until very recently, it was the common belief that neonates and even infants did not have the ability to experience or even to remember early painful events. Under treatment of pain in children occurs because of the following common misconceptions and views held about infant and paediatric pain which include (Schechter and Allen 1986, McGrath and Finley 1996).

- a) The nervous system of the infant is immature.
- b) Active children are not in pain.
- c) Children always report their pain.
- d) Children cannot reliably describe or locate their pain
- e) Parent's are the best reliable source of their child's pain
- f) Analgesics are not safe in infants and children.
- g) Psychological intervention such as cognitive therapy is not effective in reducing children's pain.
- h) Fear of adverse effects using stronger analgesics such as opioids.

A major source of under treatment is lack of knowledge and awareness amongst medical professionals, concerning the mechanisms of children's pain. Until fairly recently, there have been a lack of paediatric textbooks and other teaching materials, which focus specifically on the management on pain in children (Rana 1987). In view of these factors, pain in the clinical setting has been under recognised and under treated and coupled with inappropriate fears of increased adverse effects from strong analgesics such as opioids, even many surgical and other invasive procedures, such as chest drain insertion and removal have been performed without analgesia (Barker and Rutter 1995).

However now, due to well conducted laboratory and clinical research, it is more widely accepted that infants not only experience pain, but possibly even more acutely than the adult population (Fitzgerald and Koltzenburg 1986; Anand 2000).

1.6.2 Neurodevelopment of pain pathways (Table 1.2)

It is practically impossible to study pain in children, particularly the neonatal age group mainly due to ethical reasons. Animal models have been therefore been developed and because of the similarities in the pain systems of the rat and human the neonatal rat pup is the most frequently accepted model for understanding the neurodevelopment of the pain pathways. Some of the principle similarities between the two species include;

- neurotransmitters released in response to pain (Anand 1999; Marti et al 1987)
- large receptive fields in the dorsal horn (Yi and Barr 1995)
- presence of functional C polymodal receptors (Fitzgerald 1987)
- immature descending inhibitory systems (Fitzgerald and Koltzenburg 1986)
- exaggerated cutaneous reflexes (Fitzgerald et al 1988)

Table 1.2 Neurodevelopment of human pain pathways

(Puchalski et al 2002)

The following **Table1.3** gives a guide to the age equivalents of rats and humans (Anand 2000) but it is still important take into account the differences in complexity between the human and rodent central nervous system , in particular the cognitive and emotional development of the human neonate.

Table 1.3 Age of rat pups and equivalent human age

Age of rat pup	Equivalent human age
P0	24 weeks
P7	Full term
P14	1 year old
P21	Toddler

The nervous system at birth exhibits a much greater hypersensitivity to various stimuli in comparison to the adult. The developing human neonate's central nervous system (CNS) demonstrates plasticity, that is the ability to be moulded or reformed. This may be beneficial, as an enriched environment may assist the neonate to remodel his brain with a regeneration of neurons and consequently they have the ability to recover more readily from cortical results. Conversely, persistent pain during the neonatal period may have permanent detrimental consequences on long- term nociceptive neuronal development (Woolf and Salter 2000).

It is now known that pain experienced in the neonatal period will be remembered by this ever-changing nervous system for a long time and lead to permanent alterations within the neural circuits, therefore strategies aimed at reducing or abolishing these early pain experiences are essential (Ruda et al 2000).

1.6.3 Long -term effects of neonatal and infant pain

There are now a series of elegant laboratory studies which illustrate the long-lasting changes (cellular, neurophysiological and behavioural) that ensue both centrally and peripherally, following neonatal noxious insults (Fitzgerald and Swett 1983; Fitzgerald 1985; Fitzgerald and Koltzenburg 1986; Alvares et al 2000).

Following repeated painful insults, it has also been demonstrated there are changes in the central and peripheral pain systems in the neonate rat pup and it is becoming more evident that these early pain experiences will have an impact on the developing nervous system and lead to permanent alterations (Anand 1999).

1: Spinal and supraspinal changes. Repetitive stimulation leads to “wind up”, where there are lower thresholds to any further stimuli and due to the larger receptive fields, any weaker noxious stimuli actually produce exaggerated responses. It has also been demonstrated that there is an immature descending inhibitory pain system as well as lack of neurotransmitters (Fitzgerald and Koltzenburg 1986) and even some inhibitory mediators may actually be excitatory in the neonate such as glycine and GABA contributing to the increased susceptibility of neonates and infants to experience an heightened pain response.

2: Peripheral changes : The number of sensory neurons responding to pain depends on neurotrophic factors such as nerve growth factor (NGF), which also determines skin innervation. If there has been a series of noxious insults to the skin at birth, then an increase in NGF exists leading to skin hyperinnervation and sprouting of cutaneous nerve fibres (Reynolds et al 1997). The laboratory studies conducted to study the effects of long- term pain include neonatal rat pups who had 4 daily needle pricks during the first week of life and were more hypersensitive to pain at 16 and 22 days (Anand 1999).

Neonatal rat pups who had local inflammatory pain for 5-7 days, had an increase of sensory fibres which formed connections with the laminae 1 and 2 as well as caudally L5/S1, where there is usually no sensory input. The dorsal horns were then noted to be hyperexcitable at rest and led to an overall increased pain response (Ruda et al 2000). Behavioural studies showed that neonatal rats who were exposed to repeated noxious stimuli grew up to be adults who had withdrawal behaviours, increased alcohol preference as well as decreased latencies to heat showing that they may have an altered ability to cope and deal with stress (Anand 1999).

In human infants, following a series of brief noxious stimuli (heel lances), it has been demonstrated, using the cutaneous withdrawal reflex, that responses are not as discriminative, more exaggerated, with larger receptive fields, lower threshold and therefore increased sensitivity and with a tendency to hypersensitivity (Andrews and Fitzgerald 1994). This threshold was half that of the contralateral heel which led to hypersensitivity but that this could be prevented with the local anaesthetic cream EMLA (Fitzgerald et al 1988). Those infants who have been in the neonatal intensive care unit requiring repeated painful tests displayed an immature facial response following each procedure (Johnson and Strada 1986).

It has been shown that full-term neonates, who underwent circumcision without any anaesthetic, had an associated heightened pain response at 4-6 months post operation during their routine immunisations. This hypersensitivity was partially reduced with application of a local anaesthetic during circumcision (Taddio et al 1997).

A subsequent patient group which included full term neonates of diabetic mothers, who required repeated heel lances in the first 36 hours of life, demonstrated a greater pain response than normal infants and learned to anticipate pain, with subsequent noxious stimuli (Taddio et al 2002). Even though they may not consciously recall their early painful experiences, it has been suggested that they may be more likely to display abnormal behavioural patterns or altered sensory processing in later life (Grunau and Craig 1987).

In summary, repeated noxious stimuli experienced in the fetal or neonatal period may lead to permanent changes within the adult pain sensory system such as;

- skin hyperinnervation
- increased skin nerve sprouting
- hyperexcitable sensory neurons within dorsal horns which cause adaptive changes causing an overall hypersensitivity and susceptibility to pain

1.7 MANAGEMENT OF PAIN IN CHILDREN

Recently, there has been an increase research into the study of the safety, efficacy, pharmacokinetics, pharmacodynamics and clinical outcomes associated with a variety of analgesics for children. (Berde and Sethna 2002).

The best method of managing acute pain is with a combination of both pharmacological and non-pharmacological approaches using protocols for specific use in the paediatric patient group.

The World Health Organisation guidelines recommend an analgesic ladder approach, which emphasises the initial use of simple analgesics such as paracetamol, then proceeding to stronger analgesics, such as codeine and then finally strong opioids, although the terms “weak opioids and adjuvants” are rather ambiguous in the current environment of advancing analgesic pharmacology (WHO 1990). It is intended to provide a basic pathway for pain management. This ladder was initially introduced for management of pain in adult cancer patients with little adjustment for paediatric or non-malignant pathology.

There are 4 basic concepts summarising the ladder approach in relation to children;

- by the ladder
- by the clock
- by the appropriate route
- by the child

There are questions surrounding the use of the ladder in other diagnoses such as non-malignant pain other than cancer and although it has not been formally validated for these conditions, it is generally widely accepted that the principles of the step wise analgesia ladder may be applied (**Fig 1.2**).

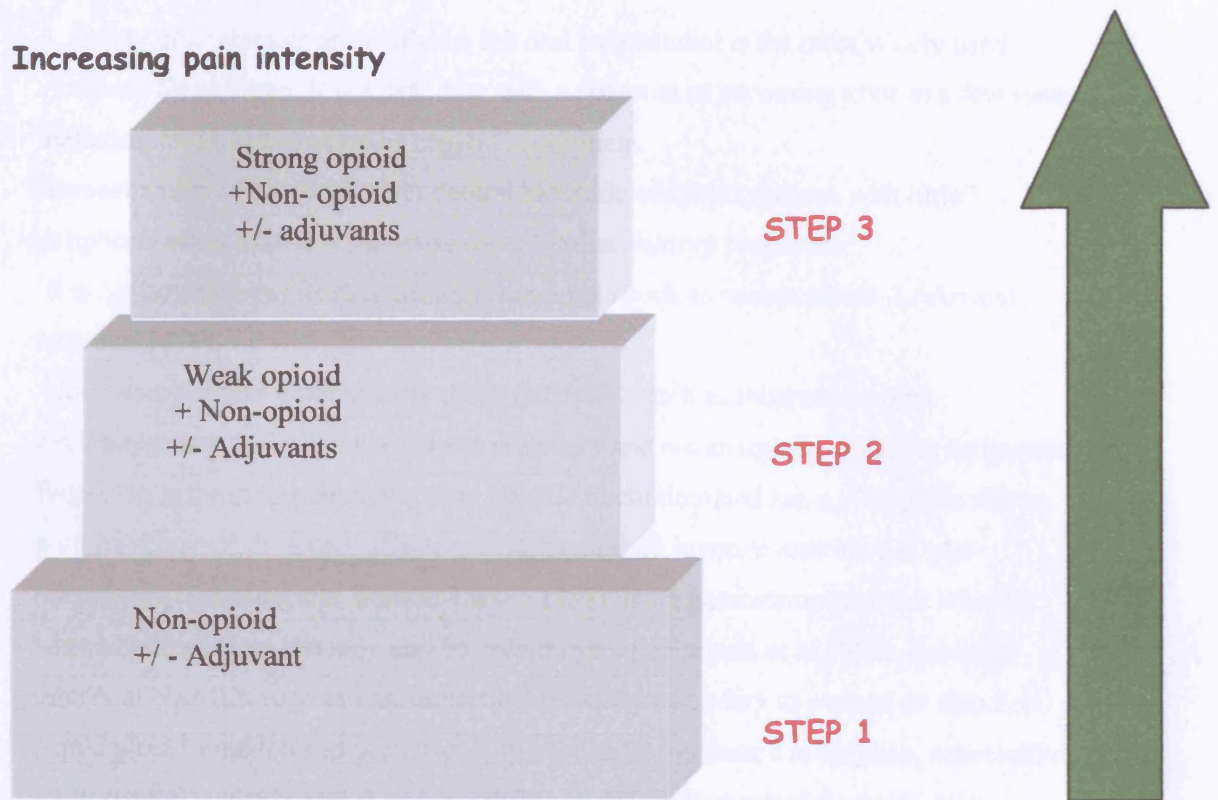


Fig 1.2 WHO Analgesic Ladder: The World Health Organisation Analgesic Ladder has provided an excellent framework for the escalation of analgesia for many years. A more flexible approach is now advocated, particularly for children's pain with management strategies customised according to the child's pain, the mechanism involved, the child's particular needs and response to previous treatments. The entry level onto the ladder will be influenced by these factors. Treatment should be frequently reassessed and modified. An adjuvant analgesic agent can be added at any point on the ladder if indicated by the clinical circumstances eg. bone pain, neuropathic pain, inflammatory pain. (The World Health Organization 1998). The addition of non-steroidal anti-inflammatory drugs (NSAIDs) and co-analgesics should be considered at any stage throughout the treatment process.

A variety of analgesics are available, but oral paracetamol is the most widely used analgesic for children. It is a safe drug with a potential of becoming toxic in a few states including fever, dehydration or hepatic impairment.

Its mechanism of action involves central blockade of cyclooxygenase with little peripheral effect and so it possesses no anti-inflammatory properties.

It is not however as effective for procedural pain such as venepuncture (Lesko and Mitchell 1999).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen inhibit cyclooxygenase in the spinal cord and periphery and are analgesic as well as antipyretic. Ibuprofen is the most commonly used NSAID in children and has a synergistic effect with paracetamol. It is very effective in inflammatory juvenile arthritis and peri-operatively (Romsing and Walther-Larsen 1997). Both paracetamol and the NSAIDs have a ceiling effect and may also be opioid sparing (Korpela et al 1999). Adverse effects of NSAIDs such as gastrointestinal bleeding secondary to platelet dysfunction, peptic ulcer formation and nephrotoxicity need to be monitored in children, especially those requiring regular use (Lesko and Mitchell 1999). Paracetamol and NSAIDs such as ibuprofen are particularly useful in combination with each other for mild to moderate pain especially if there is bony pain, such as arthritis (Williams et al 1986).

Paediatric protocols have now been devised for the safe use of opioids and experience with analgesic use in children is improved. Opioids are discussed in greater detail in the following chapter.

For acute procedural pain in children, local topical anaesthetic agents, EMLA / Ametop are effectively used for short procedures such as venepuncture (Gunter 2002).

The combination of therapeutic agents and cognitive behavioural strategies such as guided imagery or visualisation should always be encouraged in children undergoing acute procedures (Kuttner et al 1988). Patient controlled analgesia may be used in children greater than 5 years for administering opioids or ketamine and in adult patients, local nerve blocks and epidural anaesthesia for acute pain post-operatively, have been studied, confirming their safety and efficacy (Monitto et al 2000).

Most research in chronic pain syndrome management in the paediatric and adolescent population has focused on the use of a multidisciplinary rehabilitation programme.

There are different models worldwide; for example some require the child and a family member to be residential for a period of up to three weeks (Bennett et al 2000).

It has been demonstrated that patient education and cognitive behavioural therapy as well as regular physiotherapy and occupational therapy with minimal medical intervention is successful in reducing symptoms of chronic pain syndrome improving school attendance (Eccleston et al 2003). Care should be provided in a way, which approaches all of the dimensions of the chronic pain and after an initial assessment with the child and family, the management plan can be individualised (Perquin et al 2000). Non-pharmacological options include, relaxation techniques such as visualisation and guided imagery; physical therapies such as transcutaneous electrical stimulation (TENS) (Chabal et al 1998), heat and cold therapies are particularly useful if there is a defined neuropathic element and alternative therapies such as acupuncture, aromatherapy and massage are currently being trialled for use in paediatric chronic pain as adjuncts to conventional treatments (Rusy and Weisman 2000; Kemper et al 2000; van Epps et al 2007).

Drug treatments which are currently used clinically in adult patients with chronic pain, include the sodium channel blockers: amitriptyline, the antiepileptics: including carbamazepine, lamotrigine and gabapentin (although the precise target of action is still unclear) and NMDA antagonists: the most commonly used being ketamine (McQuay et al 1995, 1996). Although there have been various randomised controlled trials (RCTs) in adult patients which suggest the efficacy of gabapentin in painful neuropathies such as in diabetic patients and in post-herpetic neuralgia (Rowbotham et al 1998), there are no such trials in paediatric patients with neuropathic pain and in fact there are limited case reports only to suggest there may be a use for gabapentin and amitriptyline (Wheeler et al 2000; Collins et al 1995).

Most evidence for these therapies is anecdotal in children. Also there are potential limitations to the use of these drugs due to the known adverse effects such as anticholinergic effects and life threatening cardiac events with amitriptyline (Varley 2001) and severe hallucinogenic episodes with ketamine.

Local nerve blocks have been used in children with neuropathic pain and there is some evidence for their benefit for complex regional pain syndrome (Lloyd-Thomas and Lauder 1995). They are usually used when conventional therapies have failed and are beneficial in combination with physiotherapy in a combined rehabilitation setting.

1.8 PAIN IN EPIDERMOLYSIS BULLOSA

1.8.1 Clinical Background

Epidermolysis bullosa (EB) is a genetically inherited skin disorder, characterised by extreme fragility of the skin and mucosa and its susceptibility to blister and separate from the underlying tissue in response to minimal every day friction and trauma. Children with the more severe forms of EB may lead a very disrupted life and may even be excluded from daily physical activities or be unable to attend school on a regular basis mainly due to the severe pain they are experiencing. Broadly speaking there are three main categories, with over 25 sub-types of varying severity depending on the affected level within the skin: simplex, junctional and dystrophic, (Fine et al 2000) **(Table 1-4)**.

The lesions may be localised or generalised. Each form has a specific cleavage plane within the epidermal-dermal basement membrane zone, as well as specific clinical manifestations and has distinguishing pathophysiology, mode of inheritance and prognosis (Tidman and Garzon 2003).

It is likely to affect 1 in 17,000 live births and it is estimated that there are currently 5,000 people with the condition in the UK (Dystrophic Epidermolysis Bullosa Research Association Research Association-Debra).

Dystrophic EB (DEB) affects 25% of all sufferers and may be dominant or recessive in its inheritance. The clinical severity of DEB as well as in all the other major variants of EB is based on the type of mutation.

The blisters in DEB usually heal with scarring and are usually associated with some degree of nail dystrophy, joint contractures, microstomia, narrowing of the oesophagus and pseudodactyly. Those patients with the recessive type may also go on to develop squamous cell carcinoma.

EB Simplex is the most common type of EB and affects around 75% of all patients with EB; it is dominantly inherited or may arise as a spontaneous mutation. Currently there are approximately seven subtypes of EBS, each with specific clinical manifestations. EBS Weber-Cockayne is a variant with blistering confined primarily to the palms and soles. EBS Koebner is a different variant with widespread blistering at sites of friction.

EBS Dowling-Meara is a form of the disease with neonatal widespread blistering progressing to blistering in characteristic clusters. EBS is inherited in an autosomal dominant manner but with a few exceptions. The Weber-Cockayne and Koebner variants account for the majority of EBS cases.

Junctional epidermolysis bullosa (JEB) is the rarest form of EB with only 5 % of all EB patients affected. The usually lethal form of JEB is called JEB-Herlitz. All types of JEB are inherited in an autosomal recessive manner.

Table 1.4: Molecular Classification of EB (Uitto and Pulkkinen 2001)

1.8.2 Pain in EB: which model exists?

As the bullae may affect both internal and external mucosal surfaces, there is a huge potential for both acute and chronic pain in patients with EB. Although there is little research discussing the symptom of pain in EB, it was evident from my involvement in the EB multidisciplinary teams that pain exists as a major symptom. Many discussions with parents, young people and children both at Great Ormond Street and in other centres within the UK, confirmed this. There has been one case study relating to the use of amitriptyline, in combination with cognitive behavioural therapy, in an 11- year old with junctional EB (Chiu et al 1999) and there is ongoing research at Great Ormond Street investigating the efficacy of amitriptyline in EB in neuropathic pain, sleep and mobility (Howard et al). There have been very few review articles providing information on best clinical practice in relation to anaesthesia management in EB patients (Herod et al 2002; Iohom and Lyons 2002; Lin and Golianu 2006). I have summarised potential sources of pain in EB in **Table 1.5**.

ACUTE PAIN	CHRONIC PAIN
superficial bullae	inflammatory pain from scars
corneal ulcers	contractural pain
dental	dental
gastro-oesophageal reflux	osteoporosis
anal fissures	constipation
procedural pain	

Table 1.5 Possible sources of Acute and Chronic Pain in EB

It is believed as mentioned above that as well as acute and chronic pain, **neuropathic** pain may exist in these patients as anecdotally there appears to be a beneficial response to low dose amitriptyline which is often used as an analgesic for neuropathic pain in adults. The pathophysiology behind this proposed neuropathic element to pain in EB may be due to the sustained C fibre discharge occurring within the early neonatal period as a result of constant painful stimuli which this patient group experiences , inducing “ wind –up phenomenon”- the hallmark of central sensitisation. However this needs further investigation.

Incident pain occurs in EB patients especially secondary to dressing changes, daily activities such as bathing and in some cases as a result of any slight movement such as waving “bye.” Control of the symptoms such as constipation and osteoporosis is possible with appropriate medication such as laxatives and calcium supplementation, bisphosphonates (Fewtrell et al 2006) and anti-reflux medication (Fine et al 2008).

Regular dental and ophthalmological assessments (Wright et al 1994 ; Tong et al 1999) are necessary and treatment of superficial skin infections, which seem to exacerbate acute pain, with topical or oral antibiotics is essential. Procedures in patients with EB are a major source of pain, for example both dressing changes and baths which form a large part of EB management, may induce both actual pain due to anticipatory fear.

In some of the children, bathing may be difficult because of the simultaneous exposure of all wounds and pain from being lifted in and out of the bath. Due to the physically disfiguring nature of EB and the shortened life- span for the patients, psychological anxiety and distress are common especially in those with dystrophic EB (Landsown et al 1986). Older children are aware that their condition is progressively disabling and they may even lose previously mastered skills. They become anxious about school and socialising and this may contribute to reduced compliance especially during the adolescent period. Management of dystrophic and junctional EB is symptomatic and eventually palliative, as no cure is currently available.

Why I chose patients with EB as the pain model for my research study.

Having now discussed the various types of EB, I would like to explain why I chose this patient group to be the pain model for my clinical research. It is evident now that neonates born with a diagnosis of EB, especially those with DEB and JEB will be susceptible from birth to a variety of pain sub-types.

Above in (section 1.6.3, Long term effects of neonatal pain, page 26), I have summarised the findings both in laboratory and clinical studies, occurring as a result of repeated painful noxious injuries, e.g. lower sensory thresholds causing a hypersensitivity in the wounded area, delayed facial reaction in response to future pain and stronger pain response to future painful stimuli leading to an altered ability to cope with stress and pain in adulthood. Particularly in relation to skin wounding and even perhaps in the intrauterine period, these patients will have an increase in expression of nerve growth factor which in turn leads to hyperinnervation , sprouting of cutaneous nerves and therefore hypersensitivity in the surrounding area of skin.

Consequently the patients may have a combination of not only acute pain but also have a susceptibility to chronic persistent pain.

In addition to this, peripheral sensitisation and central sensitisation resulting from repetitive and sustained C - fibre discharge is likely to be present , secondary to the noxious inflammatory stimuli in patients with EB, therefore leading to possible neuropathic pain. In chapter 2, I will explain why this patient group may also have an increase expression of peripheral mu opioid receptors enabling the use of peripherally applied morphine.

1.8.3 Current pain management strategies used in EB

There are a few reviews providing guidelines for mainly the anaesthetic management in EB but there are no studies investigating analgesic management in children and young people (Lin and Golianu 2006; Iohom and Lyons 2002). When analgesia is required for mild pain e.g. small blisters requiring a quick dressing change, combining simple analgesia such as paracetamol with an NSAID e.g. ibuprofen is usually sufficient. However with more severe pain associated with dressing changes or baths, opioid analgesia and sedation such as midazolam is usually necessary. In this case, doses of oral morphine should be in the range of 0.3 – 0.6mg/kg and buccal midazolam, 0.5mg/kg given around 30 to 45 minutes prior to the procedure (Herod et al 2002). Procedural pain has also been managed by novel methods of analgesia such as Entonox and fentanyl lozenges (Kalach et al 2002; Schechter et al 1995). In both of these cases, the child should be able to self-administer the method of pain control.

In order to manage the chronic background pain administration of long-acting, slow release morphine (MST or Zomorph) on a daily basis may be required if milder analgesics are insufficient.

One case study has demonstrated the efficacy of amitriptyline (Chiu et al 1999) and there is anecdotal evidence from its use on a subset of children and young people with EB at Great Ormond Street Hospital, where at low dose, 0.5mg/kg, those with pain causing restriction to mobility and sleep interference have benefited further strengthening the proposal that there is an neuropathic element. There is currently a much larger open labelled trial to further assess its use.

In the terminal stages of their disease, higher doses of opioid analgesics may be required via subcutaneous or intravenous route, in combination with anti-emetics and sedatives together in a syringe driver for children.

As well as the above pharmacological therapies, physical and psychological treatments such as physiotherapy, hydrotherapy, visualisation and guided imagery should be offered, in combination with conventional analgesics, to assist in the management of the patient's pain.

1.9 PAIN ASSESSMENT IN CHILDREN

Accurate pain assessment is essential as it allows pain to be recognised and managed promptly. The assessment of pain is a broad term to describe not just the measurement of degree of pain but also other dimensions such as the family's understanding of pain and cultural differences. There is a variety of pain assessment approaches in paediatrics and their appropriateness of use depends on the age of the child, the child's cognitive awareness, the nature of the underlying pathology and whether the pain is acute or chronic.

1.9.1 Pain Assessment tools

Pain perception is individual and children and adolescents vary greatly in their cognitive and emotional ability to conceptualise their pain experience. A combination of the uni-dimensional, multi-dimensional and composite measures, which can identify the sensory, psychological and emotional aspects of pain, provide a more accurate and realistic estimation. Self-report measures are regarded as the gold standard of pain assessment and in order to decide which scale is the best for a particular patient, the level of cognitive understanding should initially be determined by the clinician. **Table 1.6** is a guide to the cognitive levels at different ages based on the well-known Piaget's levels of cognitive development. (Piaget 1972).

Pain perception is also based on other factors including, genetic, psychological, socio-cultural and previous pain experiences (Bursch et al 1998). Children exhibit individual reactions to pain e.g. one child may withdraw silently, whilst another may display fighting behaviour in an attempt to alleviate pain.

AGE GROUP	COGNITIVE DEVELOPMENT
Infants	No verbalisation.
Toddlers	Use simple words for pain Stranger anxiety – more distressing if parents not present during procedure
Preschool	Concrete language to assess pain at sensory level only No cause / effect concept Tries to delay painful procedures Believe they have pain as a punishment Magical thinking
School age	Fear body mutilation – more blood = greater injury. Cause / effect understanding Understands time Logical reasoning – still related to concrete ideas. Rely less on parents for coping
Adolescents	Understand abstract Need to maintain self esteem May not display pain behaviour due to embarrassment / stoical Lack of compliance with pain treatments

Table 1.6: Understanding of pain perception in relation to cognitive development in children based on Piaget's Theory (1972).

Important factors to consider when choosing a pain assessment tool

Age of child

Cognitive awareness

Acute vs chronic pain assessment

Underlying pathology of pain

Understanding of parents / nurses completing assessment

Length of time of pain assessment – i.e. daily assessment required then a simple quick tool should be used.

Physical ability of child / adolescent completing assessment

The reliability, validity and discriminatory power of the test.

There are numerous tools presently available to measure infant and paediatric pain, and there is no one uniform technique but the various methods can be broadly classified.

- Self – report
- Physiological
- Behavioural
- Multi-dimensional

1: Self-report measures of pain

Self-report of pain is a description of the child's subjective experience of pain and does not consider the nociceptive aspect of pain. It is regarded as the gold standard measurement and several self-report tools have been validated and are reliable methods of pain assessment.

There are three factors, which determine which particular tool should be selected:

- 1) The cognitive/developmental maturity of the child
- 2) The category of the pain; acute vs chronic
- 3) Whether the pain ratings are required for research or for clinical purposes

Self-rating scales may be divided into, visual analogue scales, category rating scales and numeric rating scales.

Visual Analogue Scales (VAS)

A VAS is a straight line either horizontal or vertical and usually measures 10 centimetres. A horizontal line has been shown to produce a more uniform distribution of scores than a vertical line (Huskisson 1983).

The two end points are no pain and worst pain and the patient is asked to place a mark along the line corresponding to their level of pain. The distance in centimetres to this mark is used as a numerical pointer of the pain severity.

The main advantage of a VAS is its ease of use and it takes a very short time to complete. The results are generally reproducible and it can be applied in a variety of clinical settings. It is also sensitive to pharmacological interventions and it has been used in children as young as aged 5 (Goodenough et al 1999).

Category Pain Scales

Category or verbal rating scales use words to describe the severity of pain. It was the earliest type of pain scale devised and most use the 4 -word category, from none, to slight, moderate and then severe. Later pain relief scales were constructed with a 5 -point scale ranging from none, to slight, moderate, good and then complete relief. Children choose the word, which describe best their pain.

Face scales are the most common form of categorical pain scale used in children, as they do not depend greatly on cognitive or language skills (Wong and Baker 1988). There are considerable variations of the face scale, ranging from simple line drawings, to photographs of children's faces and cartoon like faces. Some of the scales have 5 points, some have 6 or even 7. The worst face may or may not have tears and the no pain face may be a smiling or a neutral face. There has now been some work validating the different face scales (Bieri et al 1990). Face scales are easy to use and are appealing to young children and many do possess good psychometric properties (for example reliability and validity) (Chambers and Craig 1998).

Numeric Pain Scales

These are ordinal scales using numbers to measure the increasing degree of pain. They are easy to use but the child must understand the concept of numbers. Work on numeric scales has shown good correlation between pain diaries and behavioural components of pain. It is important to note that the intervals cannot be assumed to be equal, e.g. a difference from 2-4 is not necessarily the same change in pain as 4-6. A pain thermometer is a variation of the numeric pain scale, graduated from 0 (no hurt) to either 10 or 100 (most hurt). These scales have been introduced and developed by Hester and colleagues who detected a good correlation between concurrent diary reports of pain (Hester 1979).

Other examples of self-report tools

The Poker Chip Tool allows children to concretely describe their pain using 4 red pieces of "hurt" and one white piece of "no hurt". The validity of this measure is questionable and children should have number concept as more red chips equates to more severe pain (Hester et al 1990).

The Eland colour scale allows a child to choose different colours of crayons and then to devise a key of corresponding pain severity. The child draws the location of the pain on a line drawing. This scale however is difficult to validate due to inability to convert the drawing into numerical data, but is very useful for those children from different ethnic cultures and it also helps to differentiate between separate pains (Eland and Anderson 1977).

2: Physiological methods of pain assessment

Following a painful stimulus, the metabolic, endocrine and autonomic systems interact to initiate the fight, flight and fright response (Anand and Carr 1989). Variables such as heart rate (Johnston and Strada 1986), blood pressure (Jay et al 1987) and oxygen saturation fluctuate (Williamson and Williamson 1983). These variables are used in neonates and infants and those critically ill children who are in the intensive care environment, as an objective measure of their acute pain.

However at present there is no one perfect physiological measure of pain and heart rate appears to be the most promising, but by no means is specific (Owens 1986). It is easy to record and observe using non-invasive techniques (van Dijk et al 2001).

There are some major reasons why physiological measures should not be used in isolation to assess pain, e.g. the variability of the measures is not necessarily related to pain, but in fact they reflect an overall response to pain associated with stress and there is habituation over time of the physiological indicators to pain, following repeated or persistent pain. The physiological responses produced secondary to a noxious stimulus reflect the intensity, location and duration of that stimulus but may in fact be independent of the final sensation produced by the stimulus. They are not specific and may occur for many other reasons including, anxiety, handling, general malaise, for example pyrexia.

Consequently they are most useful in acute incident pain, or as an aid to measurement of pain in those children who are preverbal or who have an element of cognitive impairment. Clinicians usually associate changes ranging from 10- 20 % in the physiological parameters, which are measured non-invasively, to a painful experience, however there are no validated assessment scores based on these parameter changes. During the initial phase of a painful experience the sympathetic nervous system is activated producing the changes otherwise known as the fight, flight or fright response. It is the measurements of these changes, which provide the most useful information related to pain.

However following this acute phase, due to homeostatic mechanisms, the parasympathetic nervous system comes in to play, resulting in opposing physiological alterations, which do not serve as an aid for pain assessment (Sweet and McGrath 1998).

3: Observational / Behavioural Pain Scales

These pain rating scales are most often used in the preverbal child or critically ill patients, or children undergoing painful procedures in the hospital setting. They may also be useful as an adjunct to self-report scales. The behaviours most often related to pain include cry, facial expression, posture and rigidity of torso and body movements.

The following are 4 examples of observational methods of assessing children's pain:

1) Behaviour rating scales: e.g. the "procedural behaviour rating scale" which was originally devised for children aged 6-18 years old, undergoing bone marrow aspirations (Jay et al 1987). It is made up of 13 defined behaviours, which are suggestive of pain prior during and after bone marrow aspiration. However the scale just documents the presence or absence of these behaviours and does not quantify them.

2) It is extremely difficult to assess a neonate's pain, changes in the facial expression is the most consistent pain-related behaviour and the Neonatal Facial Coding System comprises of an observational scale based purely on the neonate's facial response to pain including bulging brow, eyes squeezed tightly, open lips, taut tongue etc (Grunau and Craig 1987).

3) Premature Infant Pain Profile (PIPP) is a multi-dimensional tool to assess pain in preterm infants > 28 weeks and combines both the neonate's behaviour, as well as physiological parameters. It has been validated for use in post-op pain and other non-pharmacological procedures in preterm infants (Stevens et al 1996).

4) In older children, the Children's Hospital of Eastern Ontario Pain Scale (CHEOPS) scale has been validated for use in aged 1- 7 years post-operatively (McGrath et al 1985). It consists of 6 pain indicators such as crying, facial expression, torso activity, which are described in great detail and are scored by the patient's carer.

Behaviour pain scales are beneficial in the preverbal and post-operative patient, but they have limitations. It is difficult to differentiate between pain related and anxiety related behaviour. Also children and adolescents have individualised and inconsistent behaviour related to their pain. Some children may exhibit withdrawal of behaviour rather than increased expression, in an attempt to achieve their own coping mechanisms. It is essential that the observer documenting the behavioural ratings is skilled in pain assessment. Therefore behavioural rating scales should be used cautiously, as many factors contribute to an alteration in a child's behaviour.

1.9.2 Chronic Pain Assessment

Despite the recent advances in children's pain assessment, there are several populations of children where pain assessment is not optimal including children with cognitive impairment or neurodevelopmental delay and in chronic pain states (Rucker et al 1996). When pain becomes chronic, it influences many aspects of a patient's life, increasing the complexity of the patient's perception of pain and subsequently the prescribed treatment. There are a few comprehensive pain questionnaires, which have been designed for use in children with chronic illness and associated pain (Jenson et al 1996):

a) The McGill Pain Questionnaire – originally designed for use in adults, this scale has been used with older adolescents and consists of 78 items describing the quality and intensity of the pain as well as affective dimensions. These are represented as 20 clusters of between 3 and 5 similar descriptors ordered for least to most painful. The patients circle the clusters, which describe their pain experience.

The questionnaire has 4 subscales; the first and second are the sensory and evaluative and measures the intensity and type of pain; the third measures the emotional response and the fourth, captures a number of miscellaneous items. It takes approximately 15 minutes to complete (Melzack 1975).

b) The Varni / Thompson Paediatric Pain questionnaire, has been used to assess chronic pain in juvenile rheumatoid arthritis. It takes into account both the child's and parents perception of the child's pain experience and includes a combination of visual analogue scales, colour coded rating scales, and verbal descriptors which provide information about the sensory, affective, and evaluative dimensions of children's chronic pain as well as information about the child's and family's pain history, symptoms and pain relief interventions and socio-environmental situations which influence pain. It contains 10 pages to be completed each separately by parent, physician and child and may take up to 20 minutes to finish (Varni et al 1987).

c) Douleur Echelle Gustave – Roussy; DEGR This was developed and validated for its use in children aged 2 – 6 years with cancer. It is composed of 3 main categories, signs of pain, voluntary expressions of pain and psychomotor atonia. This scale requires observation for 4 hours and a rating of 17 behavioural items. It is also quite time consuming to complete and has not been validated for use on other patient populations (Gauvain – Piquard et al 1987).

d) Pain Diaries Previous work with children who have juvenile rheumatoid arthritis, has illustrated the benefits of using a daily pain diary, with a high compliance and indeed daily pain measures may be more sensitive than periodic measures, in the assessment of subtle, day – to day changes, which occur following an intervention (Schanberg et al 1997).

e) The Paediatric Pain Profile (PPP) Children and adolescents who have a neurodisability or who are cognitively impaired often have many reasons to be in both acute and chronic pain and are unable to easily communicate their pain, e.g. medical problems such as hip dislocation, scoliosis and gastro oesophageal reflux procedural pain inconsistent signs to suggest pain. This profile is a 20 item rating scale specifically for this group of children. It is validated for use in both clinical and research situations (Hunt et al 2004).

(f) The Brief Pain Inventory The Brief Pain Inventory This tool was originally devised for use in adult patients, who had a diagnosis of cancer and based on The Wisconsin Brief Pain Questionnaire (Daut et al 1983) where patients with a diagnosis of cancer and rheumatoid arthritis were interviewed regarding the differing parameters of pain they were experiencing as well as impact of enjoyment of life. The long form BPI was then introduced (Cleeland and Ryan 1994).

It provides a quick and easy method of assessing pain intensity as patients note their worst, least, average and current pain as well as the impact of their pain on their daily function such as sleep, mobility and mood. The BPI has also now been validated for its use in those with chronic or a disability related pain (Tan et al 2004). The short form only takes 5 minutes to complete.

1.9.3 What tools I chose for the pain assessment in my research study and why

VAS

I chose the VAS as the tool to measure the pre, during and post dressing changes, as it was the quickest for the patients to complete. Many of the children wanted to complete the assessments themselves in order to have some control and ownership of part of the study and as some had problems with manual dexterity, the completion of the VAS was most achievable for them. It was completed on a daily basis or on the days when dressings were changed.

BPI

I chose this assessment in order to have an evaluation of the multidimensional aspects of the patients' pain. I used the short form and focused on the 7 daily functions of sleep, mood, mobility, school, friends, general activity, enjoyment of life and schoolwork. With permission I modified these slightly in order to be age appropriate. It was completed on a weekly basis throughout the study.

CHAPTER 2: OPIOIDS AND THEIR RECEPTORS

2.1 BACKGROUND

“Among the remedies which it has pleased Almighty God, to give man to relieve his sufferings, none is so universal and so efficacious as opium” (Sydenham, seventeenth century).

Opium, the dried poppy juice from the unripe seed capsules of the opium poppy contains several alkaloids of which only a few; morphine, codeine, noscapine and papaverine are clinically useful.

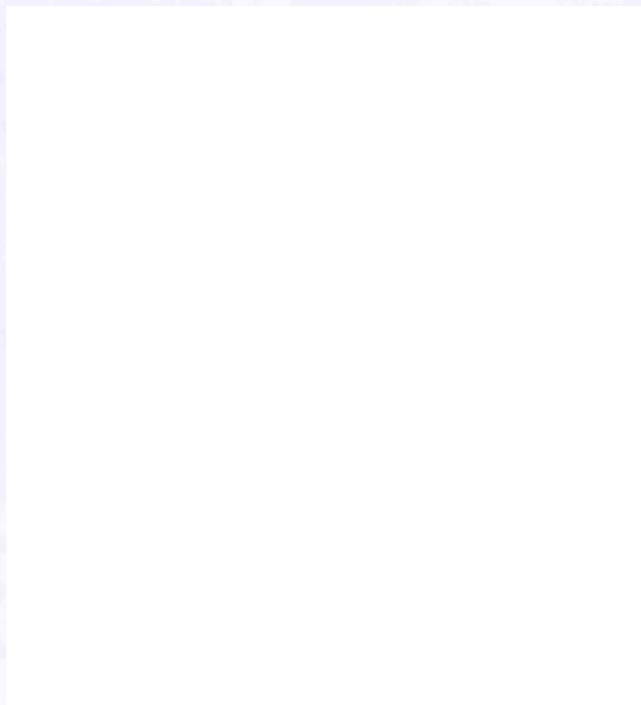


Fig 2.1 The opium poppy (www.opioids.com/images/opiumpoppy)

In the nineteenth century, a German pharmacist, Friedrich Serturmer isolated the component of opium with the most marked analgesic activity and named it morphium after the Greek god of sleep, Orpheus. Morphine remains the most widely used, even though a variety of synthetic opioids have emerged such as pethidine and fentanyl.

As there is now a more liberal approach to the use of opioid analgesics especially in cancer patients, but also in non-malignant pain such as in sickle cell crises (Jacobson et al 1997), the total consumption of opioids appears to be increasing worldwide. Over the last twenty years, considerable progress has been made in the understanding of the various endogenous opioid peptides, their anatomical distribution and the receptors with which they interact (Nagasaka et al 1996, Harrison et al 1998). In particular, the identification of peripheral opioid receptors through which locally applied opioids could interact, would provide advantages for paediatric patients, in whom the use of centrally administered morphine continues to produce troublesome adverse effects (Stein et al 1993, Antonijevic et al 1995). Multiple receptor sites, which recognize opioid drugs as well as endogenous peptides, exist (Standifer and Pasternak 1997; Satoh and Minami 1995). The classical subgroups are the mu (μ), kappa (κ) and delta (δ) receptors and subsequent activation leads to a variety of effects mediating modulation of nociception, locomotion, respiration and motivation. The μ receptors have been further sub classified into two distinct subtypes, μ_1 and μ_2 (Pasternak et al 1993).

2.1.1 Location and action of opioid receptors in adult (rat) CNS

All three opioid receptors mu, (MOR), kappa (KOR), delta (DOR), mediate pain inhibition and are found throughout the nervous system, in somatic and visceral sensory neurons, spinal cord projections and interneurons, midbrain and cortex (Pasternak 1993; Nagasaka et al 1996) (**Table 2.1**). The actions of OR in the adult CNS will be briefly reviewed before focusing on the developmental aspect of OR.

Table 2.1 Location of opioid receptors in adult (rat) CNS

	<i>LOCATION</i>	<i>ACTION</i>
Spinal	<ul style="list-style-type: none"> • Superficial horn of spinal cord • Highest density in lamina 1 and substantia gelatinosa • 50-70% presynaptic location • MOR – 70% • KOR & DOR – 30% <p style="text-align: center;">(Dickenson 1994)</p>	<ul style="list-style-type: none"> • Presynaptic: hyperpolarisation of C fibre terminals • Suppress release of excitatory transmitters • Attenuation of pain transmission entering the spinal cord • Postsynaptic: unclear action <p style="text-align: center;">(Lombard and Beeson 1989)</p>
Supraspinal	<ul style="list-style-type: none"> • Brainstem, cortex, thalamus, amygdala, hippocampus • MOR – highest in cortex, hypothalamus, raphe, locus coeruleus <p style="text-align: center;">(Atweh and Kumar 1977)</p>	<ul style="list-style-type: none"> • Behavioural and mood effects linked with pain • Endocrine functions • Autonomic reflexes • Role in descending mechanism of pain <p style="text-align: center;">(Mansour et al 1995)</p>

2.1.2 Opioid receptor binding

Opioid receptors are G protein coupled receptors that are activated by opioid peptides and alkaloids (Cruciani et al 1993). Both classes of these agonists have analgesic effects in the central and peripheral nervous system. G proteins all have a similar structure, with an extracellular N terminal region and 7 transcellular domains as well as an intracellular C terminal tail structure. G proteins are heterodimers of α , β , and γ subunits.

Opioid receptors are coupled with 6 members of the Gi family. Once a ligand has bound to the receptor, G proteins can be activated by coupling and GDP is replaced by GTP, with dissociation of α subunit from the $\beta\gamma$ dimer (see Fig 2.2). The GTP α and $\beta\gamma$ unit then interact with the effectors producing for example, inhibition of adenylyl cyclase which in turn results in diminished pain (Woolf and Salter 2000). GTP then reconverts back to GDP and the α unit re-associates with the $\beta\gamma$ unit. There are potentially many steps within this process, as one receptor may activate one or many G proteins, conversely several receptors may activate an isolated G protein.

It has been demonstrated that with the application of an opioid agonist, G protein activation was significantly enhanced in animal models with peripheral painful inflammation, thus providing one possible explanation why topical opioids may be effective where peripheral inflammation is present (Zollner et al 2003).

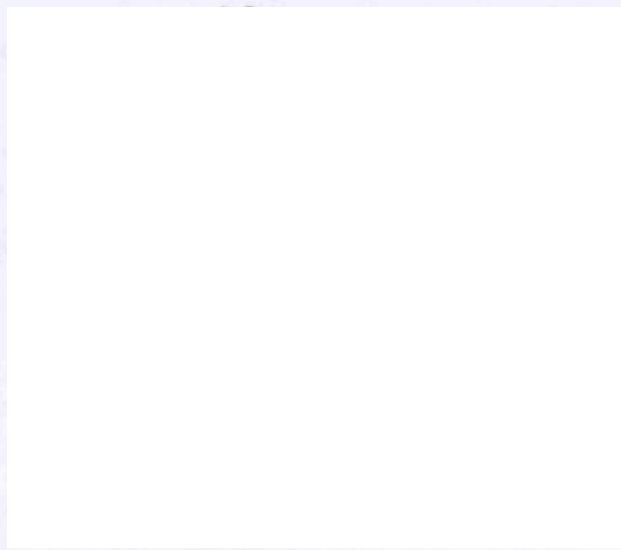


Fig 2.2 Morphine as a G protein coupled receptor showing α β γ subunits.

(<http://mosberglab.phar.umich.edu/projects/pictures/proj7pic1.gif>)

There are around 20 endogenous peptides, which are mostly derived from three precursor proteins: pro-opiomelanocortin (POMC), prepro-enkephalin (PPE) and prepro-dynorphin (PPD). Endorphin, enkephalin and dynorphin are the 3 derivatives and are relatively selective for the μ , δ and κ opioid receptors respectively.

Opioid binding and subsequent receptor activation to the agonist, initiates a cascade of biological events such as analgesia, bradycardia, miosis, sedation, hypothermia and depression of flexor reflexes. A series of the following events all resulting in diminished pain sensation, occur:

- 1) Inhibition of adenylyl cyclase
- 2) Activation of potassium conductance
- 3) Inhibition of calcium conductance
- 4) Inhibition of transmitter release (Duggan and North 1993, Moises et al 1994)

And more recently, the following observations have extended the actions of opioids to include:

- 1) Activation of protein kinase
- 2) The release of calcium from extracellular stores
- 3) The activation of the mitogen activated protein kinase cascade

(Connor and Christie 1999)

2.1.3 Development of the opioid receptors and their function in CNS

The presence and distribution of opioid receptors, the G protein messenger system and their endogenous ligands are developmentally regulated (Szucs and Coscia 1990).

Opioid receptors are present from early fetal life in rat and human in the brain, spinal cord and exhibit pre and postnatal maturational change (Kar and Quirion 1995). Studies have investigated the development of opioid receptors using tissue homogenate binding methods, which provide information relating to the overall receptor density but not to the exact localization within the tissue (Attali et al 1990).

However it is difficult to predict from the density of receptor binding sites, the actual degree of function of the receptors.

MOR and KOR binding sites have been found diffusely throughout the spinal cord from P0 (see chapter 1 page 26, **Table 1.3, Age of rat pups and equivalent human age**) in rat pups, using in vitro autoradiography and selective ligands (Rahman et al 1998).

Binding peaked at P7 and decreased to mature levels with the MOR binding sites becoming denser in the superficial dorsal horn.

DOR binding was initially seen at P7 with no difference seen in the distribution between superficial and deeper laminae. The same study, using in vivo electrophysiological methods, showed that rat pups at P21 were more sensitive to spinal morphine compared to adults and P14 pups even though in P14, the MOR binding was greater.

The conclusion was that receptor binding sites were not the only factor in determining function, but other factors such as coupling of the receptors should also be considered as important.

Within the primary afferent neurons, it is now clearly established, with immunohistochemistry, using neurofilament antibody (NF200 which stains the large non-nociceptive sensory afferents) and MOR and DOR antibody staining of the lumbar dorsal root ganglia in neonatal rat pups, that both MOR and DOR are developmentally regulated in these afferents during postnatal development.

In fact, they are down-regulated as the neonatal animal progresses throughout development, in the large diameter sensory neurons which are the cell bodies of the non-nociceptive A β proprioceptors and mechanoreceptors (Beland and Fitzgerald 2001). This results in a more abundant expression of MOR expression in the neonatal rat pup in comparison with the adult.

In the neonatal rat, opioids can depress the A β fibre mediated responses as well as A and C nociceptive inputs in contrast to adults, when only the A and C fibre responses are selectively depressed. Also, the neonatal withdrawal reflex is known to be mediated via the A β fibres (Fitzgerald and Jennings 1999).

Subsequent work using immunohistochemistry on dissociated neurons from cultured lumbar dorsal root ganglia in neonatal rat pups has confirmed that there is an over-expression of MOR in the neonatal primary sensory afferents. Using calcium imaging, these receptors were shown to be functional concluding that this over abundance of MOR may be a vital factor in the developmental responses to opioids (Nandi et al 2004).

2.2 PERIPHERAL OPIOID RECEPTORS

2.2.1 Introduction

Opioids are used in both severe acute and chronic pain and provide analgesia via activation of their receptors within the CNS. In the nineteenth century Wood first reported that morphine induced analgesia occurred when it was applied topically to peripheral tissues (Wood 1885). More recently, the presence of peripheral opioid receptors in various tissues, which respond to both endogenous and exogenous opioids, has enabled effective analgesia without any centrally mediated side effects. As well as possessing a potent analgesic role, peripheral opioids are thought to induce an anti-inflammatory action, which would be an additional benefit for acute and chronic inflammatory pain (Stein et al 2001).

2.2.2 Background: Location and possible mechanism of action

Opioid receptors have been detected using immunohistochemistry, simple light microscopy and electronmicroscopy techniques, on the peripheral processes of primary afferent neurons in laboratory animal studies. Dorsal root ganglia house mRNA for all 3 opioid receptors and following their synthesis they are transported centrally and peripherally via axonal transport (**Fig 2.3**). They have been located in various tissues including lung, colon, immunocytes and skin as well as on sympathetic postganglionic neuron terminals (Stein et al 1993). More specifically 29% of MOR and 38 % DOR have been located on the unmyelinated cutaneous sensory nerve fibres in adult rat tissue, which also express substance P, calcitonin gene related peptide and isolectin B4 (Coggeshall et al 1997).

The binding properties of both central and peripheral receptors are similar (Hassan et al 1993). Modulation of calcium currents appears to be the main mechanism of action of opioids acting upon peripheral receptors at peripheral sensory terminals. These effects are also mediated by the G protein system.

Also, similar to their action at the soma and central terminals, opioids have the following actions at peripheral opioid receptors

- 1) attenuate the excitability of peripheral nociceptor terminals (Russell et al 1987)
- 2) attenuate the propagation of action potentials (Andreev et al 1994)
- 3) reduce the release of excitatory neuropeptides such as substance P (Yaksh et al 1980)
- 4) decrease the vasodilatation, which arises by C fibre stimulation (Stein et al 2001)

Fig 2.3 The transport of opioid receptors and signalling in primary afferent neurons



OP = endogenous OR: EO = exogenous receptor:
G i/o = inhibitory G proteins
Sp = substance P

(Stein et al 2003)

2.2.3 Inflammation, analgesia and peripheral opioid receptors-possible mechanisms of action

The action of peripheral opioid receptors is not evident when there is uninjured tissue, but under inflammatory conditions the peripheral analgesic effects of exogenous opioids are enhanced, most likely due to an up regulation of receptors at the periphery (Antonijevic et al 1995).

As the analgesic effects of exogenous opioid agonists are seen as early as 24 hours following acute inflammation secondary to a peripheral injection of complete Freund's adjuvant (CFA) into an adult rat hindpaw, it suggests that opioid receptors are pre-existent on the peripheral terminals of primary neurons (as CFA normally induces acute on chronic inflammation at 12 hours post injection reaching its peak effect at 96 hours). It has also been demonstrated that neither the mRNA for mu opioid receptors in the DRG, nor the number of peripheral MOR increases in early inflammation, so other mechanisms must come into play at the start of the inflammatory process (Stein et al 1993).

In early inflammation, the tight perineurium, which normally surrounds the peripheral nerve fibres, has been shown to be disrupted, facilitating passage of opioids and agonists to the receptors (Antonijevic et al 1995). In later stages of inflammation, higher doses of OR antagonists have been required at 96 hours in comparison to 12 hours, to diminish their effect, suggesting an a probable increase in the axonal transport of the receptors to the cutaneous nerve fibres later during the process of inflammation (Zhou et al 1998).

How does this relate to my laboratory work?

The above work was demonstrated in adult rat models and my study aims to demonstrate the presence of peripheral mu opioid receptors in the skin tissue of rat pups at various age groups and whether there is developmental regulation and an increase in number of receptors following a painful inflammatory insult, quantified by Western Blot technique (see chapter 3, page 87).

2.2.4 Peripheral opioid receptors and immunosuppression

In parallel with the increase of peripheral opioid receptors during inflammation, endogenous ligands at opioid receptors are manufactured in circulating immune cells and then migrate to the site of tissue injury. During the inflammatory process, memory T lymphocytes migrate towards the inflamed tissue and dose dependent release of the β endorphin triggered by inflammatory factors such as corticotropin – releasing factor (CRF) and interleukin IL-1, as well as environmental stressful stimuli, such as viruses, endotoxins and cytokines (Cabot et al 1997).

Migration towards the site of tissue injury is mediated by cell adhesion molecules, which are up regulated and located on immune cells and vascular endothelium. The opioid peptides also bind to the OR on peripheral sensory nerves inhibiting pain. This process is abolished if there is immunosuppression, (Machelska and Stein 2002) thus enhancing the production of endogenous opioid peptides and promoting the use of peripheral opioid receptors in patients who are immunosuppressed would be particularly beneficial.

2.2.5 Tolerance at peripheral opioid receptors

There are conflicting reports as to whether there is tolerance at peripheral OR. Studies reveal that repeated peripheral administration of opioids in mice produced tolerance, which could be reversed using an NMDA antagonist, but there was no initial tissue inflammation present (Kolesnikov and Pasternak 1999). There was no tolerance after repeated application of loperamide, which is a μ agonist in animal thermal inflammatory model (Nozaki-Taguchi and Yaksh 1999). Also in two further studies where morphine is administered peripherally over time, no tolerance developed (Tokuyama et al 1998; Ueda et Inoue 1999). The overall evidence currently is more supporting lack of tolerance at peripheral OR.

2.2.6 Developmental regulation of peripheral opioid receptors

In contrast to the work demonstrating the developmental regulation of opioid receptors in the CNS (Beland and Fitzgerald 2001, Nandi et al 2004, Rahman et al 1998, Kar and Quirion 1995), there is very little research to investigate the developmental regulation of peripheral opioid receptors during an inflammatory process.

There are however a number of developmental processes in neonates, such as the perineural membrane maturation, the functional regulation of central nervous system opioid receptors and the maturation of the immune system, all of which would suggest there might be potential differences in the analgesic and anti-inflammatory actions of opioids upon peripheral opioid receptors.

The analgesic effects of peripheral opioids have been tested in infant rats in two studies (Barr 1999; Barr 2003).

In the first of these two behavioural studies, peripheral inflammation was induced by the intraplantar injection of formalin in p3, p10 and p21 ratpups (**see chapter 1 page 26, table 1.3, Age of rat pups and equivalent human age**). Three different doses of morphine were also injected into the same area using controls of saline or subcutaneous morphine injections. Expression of fos protein in the dorsal horns was measured using immunohistochemistry (Barr 1999).

The results showed that, in the P3 pups, local injection of morphine using the middle dose was more effective than the same dose given subcutaneously. However the higher dose in this age group was equally effective given by both methods probably due to the immature permeable blood brain barrier. In the other 2 ages, the intraplantar injections of the morphine doses were significantly more effective than the subcutaneous injections of the same doses.

The group's subsequent study explored which of the receptors mediated peripheral analgesia. Unexpectedly the kappa agonist was the most effective at local pain reduction in both phases of the formalin test in P3 and P21 pups.

Consistent with previous work on opioid receptor development, the δ agonist was inactive in both P3 and P21 most likely due to the later postnatal development of the δ opioid receptor (Rahman et al 1998).

However the μ opioid receptor agonist was ineffective at P3 but effective at P21 contradicting the results of the previous study (Barr et al 2003).

Recent work has concluded that MOR and DOR within the brainstem are developmentally regulated (Kivell et al 2004). My following work describes whether there is a similar developmental regulation of MOR within cutaneous tissue and if acute cutaneous inflammation alters the MOR expression at different ages in the rat.

These preliminary studies investigating the development of peripheral opioid receptors in the neonatal rat provide a mechanistic basis for the potential use of low dose locally applied opioids in human neonates and older children who experience painful cutaneous lesions.

2.3 PHARMACOKINETICS OF MORPHINE THROUGH DEVELOPMENT

2.3.1 Introduction

Although morphine has now been used for many years, there is still a lack of knowledge surrounding its pharmacology, both kinetic and dynamic. There are marked interindividual differences as well as variability in the kinetics of morphine and its metabolites related to age, route of administration, and duration of treatment and presence of renal impairment (Chay et al 1992).

In neonates and young children, there is a marked variation between dose requirements of opioids and the subsequent response. Many factors contribute one of which is the pharmacokinetic handling of opioids (Kart et al 1997). Establishing pharmacokinetic differences during development is vital in order to enable dosage guidelines to be established for neonates, infants and children. However pharmacokinetic handling is only one factor that impacts upon the analgesic efficacy of opioids and age and inter / intra patient variability must always be considered. Many pharmacokinetic studies involving morphine have been performed in order to evaluate the handling of opioids in neonates, infants and children-see below.

Although overall, these studies are helpful in providing a basic understanding, there are variabilities in the studies due to design, indication for analgesia, and analytical methods (Choonara et al 1989; Bouwmeester et al 2003, 2004).

The major differences in pharmacokinetic handling at various ages are due to differences in clearance and elimination half-life.

2.3.2 Main differences in pharmacokinetic handling of opioids in neonates and infants

- Protein binding is decreased in preterm and term infants (18-22%) in comparison to adults (20-35%) (Koren et al 1985; Kart et al 1997).
- The elimination half- life of morphine is twice as long in neonates less than 1 week of age, than in older infants and adults (Kart et al 1997).
- Infants from 2 months of age have similar values to adults (Kart et al 1997; Lynn and Slattery 1987) or from 6 months in some studies (Olkola et al 1995).
- Clearance is similarly decreased in the neonate (McRorie et al 1992; Bhat et al 1990; Kart et al 1997).
- Immature cytochrome P450 at birth may be a reason for prolonged clearance and elimination of some opioids and their derivatives in early life (Hakkola et al 1998). Also the neonate has little CYP2D6 (the most important enzyme substrate of the P450 system which is responsible for metabolism of greater than 40 drugs including morphine derivatives) and therefore newborns receive little analgesic effect from codeine in the first weeks of life (Treluyer et al 1991).
- Possible reduced metabolic capacity to produce morphine glucuronides in neonates, although there are conflicting views (Choonara et al 1989; Barrett et al 1996).

2.4 CLINICAL ACTION OF ORAL AND SYSTEMIC OPIOIDS AND THEIR ADVERSE EFFECTS

The clinical use of opioids needs to be titrated against actual pain, as whatever occurs whenever opioids are administered to someone in pain is different from when they are given to someone who is not in pain. (Table 2.2 shows the various physiological actions of opioids and consequent clinical effects).

For example, respiratory depression, which is seen with the acute use of morphine when someone is not in pain, is kept at bay when lower regular doses are given to patients with chronic pain (Borgbjerg et al 1996). The main approaches for dealing with opiate adverse effects are:

- Reduction of dose
- Specific therapy to treat the side effect
- Opioid rotation
- Change route of administration

Table 2.2 Actions of opioids

<i>ACTION</i>	<i>EFFECT</i>
Depress minute ventilation by reducing the sensitivity of the respiratory centre to hypercarbia / hypoxia	Respiratory depression Depress cough reflex
Peripheral vaso / venodilator	Hypotension
Inhibits the intestinal smooth muscle	Decreases peristalsis Increases the tone in pylorus, ileo-caecal valve and anal sphincter
Stimulation of chemoreceptor trigger zone	Nausea, vomiting
Increase tone of detrusor muscle	Urinary retention
Triggers release of histamine	Pruritus

Tolerance is the requirement for a higher dose of drug to be administered to achieve the same desired pharmacological effect. Patients vary greatly in their ability to handle opioids. When the need for a drug escalation arises, a variety of processes may be involved.

True pharmacological tolerance is a much less common reason than disease progression or increasing psychological distress.

Pharmacological tolerance may be in part caused by the down-regulation, internalization and desensitization of opioid receptors, but it is more likely thought to be related to mechanisms at the sub-cellular and intracellular level such as in regulation of secondary and tertiary messengers e.g. cyclic AMP (Bohn et al 2000). Clinically tolerance to the non-analgesic effects of morphine may occur at different rates, for example tolerance to respiratory depression occurs rapidly and constipation, slowly. Escalating pain in a patient receiving opioids may be due to disease progression rather than true tolerance.

Physical dependence is the term used to describe the phenomenon of withdrawal when an opioid is abruptly discontinued or if an opioid antagonist is administered. Some of the typical signs of a withdrawal syndrome are nervousness, anxiety, hot flushes, lacrimation, and sneezing. Gradual weaning to 75% of the previous daily opioid dose is recommended and patients should be reassured that physical dependence is not synonymous with addiction.

Psychological dependence and addiction refer to a syndrome characterized by a constant craving for an opioid mainly to achieve the effects, which are mediated in the CNS. It is now known that the risk of developing addictive behaviour secondary to the medical use of opioids is low; patients, family and health care professionals commonly over estimate the risk of addiction in the clinical setting; physical dependence and addiction are often confused and together these concerns contribute to some physicians' reluctance to prescribing opioids (Mortimer and Bartlett 1997).

2.5 ROUTES OF ADMINISTRATION OF OPIOIDS

The route of administration should be the least invasive and safest route of providing the best analgesia

- **Oral** preparations are best for those patients who can swallow and tolerate them. Morphine is available either as liquid or tablets and may be short or sustained release preparations. The aim for a child with chronic malignant pain is to achieve adequate analgesia with the long acting preparations, which only require twice-daily administration. The standard intravenous dose for a child is 0.1 mg/kg and because only 20-30% of an orally administered opioid reaches the circulation, when converting from an oral dose, it should be divided by three times. However opioids when given orally, vary substantially with respect to the analgesic potency.
- **Parenteral administration** is used for those patients with impaired swallowing, who require rapid onset of analgesia or with gastrointestinal obstruction. Continuous infusions of morphine are used for the more acute post-operative patients' analgesia and may be achieved using doses of 0.02-0.03mg/kg in a PCA (patient controlled device where a steady background dose of morphine is administered with frequent mini doses initiated by the patient. The time intervals between boluses ("lock-out period") are programmed in order to minimize overdose. A child as young as 5 may be capable of this method otherwise a NCA (nurse controlled analgesia) system is set up (Monitto et al 2000). The subcutaneous route is the usual and most appropriate method of administering analgesia in combination with sedation, in children who are at the terminal stages of their disease and who are unable to tolerate oral medication.

- **Spinally administered** opioids. The delivery of low dose opioids near the direct site of action at the opioid receptors located in the dorsal horn of the spinal cord provide longer spells of analgesia with lower supraspinally mediated adverse effects. They are used for post-operative acute pain or in some cases of chronic pain. Epidural opioids are effective after thoracic, abdominal and cardiac surgery even if given caudally. Facial and segmental pruritus, nausea, urinary retention and respiratory depression are the possible adverse effects with epidural morphine.
- **Transdermal fentanyl** patches in children are beneficial for those children with chronic pain who require steady pain relief and they are thought to have reduced side effects especially constipation and nausea (Haazen et al 1999). Fentanyl is highly lipophilic and so can be easily absorbed across any membrane. A reservoir immediately below the patch in the stratum corneum is established over a 12- 16 hours once a system is applied and then constant blood fentanyl concentrations are maintained for up to 3 days. It is contraindicated for acute pain management because of its long onset of action, inability to rapidly adjust drug delivery and long elimination half-life and it continues to be released for almost 24 hours after the patch is removed (Grond et al 2000). Transdermal buprenorphine is a partial opioid antagonist and it has x60 the potency of morphine. It needs 24 hours to establish its full effect and this patch can be cut.
- The **transmucosal fentanyl lozenge** is effective for acute procedural pain. It looks like a lozenge and is fruit flavoured. The child rubs the lozenge on the inside of the cheek for around 20 minutes when it is rapidly absorbed (around 25%-33% of a given dose) into the systemic circulation. It lasts around 2 hours and has been licensed for use in children as a premedication pre-operation or for procedural pain such as in lumbar punctures. It is best used in non - opioid naïve patients because of the main side effects of nausea and respiratory depression (Schechter et al 1995).

- **Intranasal/ buccal opioids** are also mucosally absorbed and have been used in adults when there is no intravenous access or when they cannot tolerate the oral preparation. An RCT in children aged 3-16 years who presented with fractures to Accident and Emergency department concluded that the use of intranasal diamorphine had a quicker onset than intramuscular morphine, had fewer side effects and was a more acceptable method of administration for the patients. The major side effect of intranasal routes is the localized irritation as seen with midazolam (Kendall et al 2001).
- **Rectal administration** of opioids is a route available for those patients who are unable to swallow or have persistent vomiting in spite of anti-emetic therapy. The absorption from the rectal mucosa is hugely variable depending on which formulation is used as this route may have limited absorption due to partial bypassing of the presystemic hepatic metabolism.
- **Nebulised opioids** have been used in palliative care patients who suffer with dyspnoea at the end stages of life (Chandler et al 1999). The mechanism of action of inhaled opioids is thought to be targeted at the opioid receptors situated in the lung tissue. However this route has low bioavailability of around 5%-30% and morphine administered in this way may even cause bronchospasm patients with underlying airway disease.

When using different routes of administration or rotating the opioids used, equivalent doses will be needed and this needs to be carefully calculated. Unmanageable side effects, which are due to opioid action on a particular opioid receptor, will not be abolished by switching to a different opioid, which acts upon the same receptor.

2.5.1 Difficulties with administering opioids via the above routes in children with EB

Patients with EB may tolerate oral preparations of morphine, but if they experience dysphagia and pain on swallowing due to internal blistering then the morphine may be given via a gastrostomy tube. Children with EB are usually unable to use a PCA as they have very limited finger dexterity due to fusion of fingers from their scarring and contractures. Transdermal fentanyl is contraindicated as children with EB due to their extensive skin damage. It has been anecdotally documented that transmucosal fentanyl can be rather painful to use in children with EB as it is necessary to rub the lozenge for 20 minutes on their blistered inner cheeks for any analgesic benefit. The rectal route is not ideal for paediatric patients and especially those with EB due the common occurrence of rectal blistering preventing any drugs to be administered by this method.

2.6 THE USE OF PERIPHERAL OPIOIDS IN ADULT PATIENTS

2.6.1 Introduction

Historically, opioids have been thought of as having a central action through their respective receptors distributed throughout the central nervous system.

However even as long ago as the late 19th century, the English Physician William Heberden, included in his renowned Commentaries on the History and Cure of Disease (published 1802) that as pain relief for haemorrhoids, he previously advised to apply a *“mixture of an ointment and the soft extract of opium for immediate analgesia”* (Heberden 1774).

2.6.2 Peripheral (locally injected) opioids in clinical models of acute inflammatory pain

The first controlled clinical trials investigating peripheral (intra-articular) (IA) morphine were performed in patients with arthritis and those patients requiring knee surgery.

A systematic review was completed in 1997, containing information of 31 RCTs with 1500 patients (Kalso et al 1997)). Those reports were included if they fulfilled the following criteria:

- 1) randomised comparisons of I.A. morphine to saline
- 2) Dose studies of IA morphine
- 3) IA morphine compared to systemic morphine
- 4) Use of intra -articular bupivacaine as a marker of internal sensitivity

The Jadad scale was used to assess the validity of the study as a RCT i.e. to ensure correct method of randomisation, double blinding and reporting of withdrawals. The outcomes of the RCT were whether extra analgesia was required or any change in the measures of pain intensity.

The effectiveness of the treatment was defined as a significant difference in pain intensity between drug and placebo, covering two periods, up to 6 hours or from 6 hours to 24 hours; or total consumption of rescue analgesics required.

Only six of the RCTs investigated groups comparing saline, morphine and bupivacaine (active control) were valid, but only 4 of these groups demonstrated internal sensitivity.

In these 4 studies, IA morphine was significantly analgesic in comparison with placebo at both early and late time points, therefore fulfilling the desirable criteria set out in 1-4 above. There was no dose response evident in the range of 0.5 mg – 5mg used in the studies. It was difficult to conclude, due to the low numbers of trials assessed, in this systematic review, whether this route of peripheral morphine was efficacious.

However a subsequent systematic review performed by the same authors was designed to include the majority of RCTs, aiming to simplify the study sensitivity (Kalso et al 2002).

The review included those studies, which fulfilled the criteria as set out previously, except 3 time periods were evaluated; 0-2 h, 2-6h and 6-30 hours. Sensitivity for each of these time periods was accepted if pain on the VAS was 30% of 100 in the placebo group. Effectiveness was the difference between the quantities of rescue analgesics required via patient controlled anaesthesia, when saline or morphine was administered. For any secondary outcomes, effectiveness of the RCT was defined as a statistically significant difference in analgesic effect between different doses of IA morphine or IA morphine providing a better analgesia than systemic morphine.

In this review, 28 RCTs were included and in the early test period, 14 trials were sensitive (7 positive), in the intermediate period, 11 trials were sensitive (8 positive) and in the late period, 12 trials were sensitive (10 positive). 5 mg of IA morphine provided the optimum analgesia in any one of the three time periods and provided analgesia up to 24 hours, which would be advantageous for the post operative arthroscopic day case.

Randomised controlled trials have also assessed the benefit of peripherally injected morphine in dental patients and concluded that they were effective where there was evidence of local inflammation and in low doses which were ineffective, when administered systemically (Likar et al 2001).

2.6.3 Topical opioids in chronic inflammatory pain

Opioids appear to be efficacious if topically applied to areas of local painful inflammation. Topical morphine and diamorphine have been researched in adult patients suffering from a number of inflammatory skin disorders such as pressure sores and fungating lesions (Krajnik and Zylicz 1999). Pressure sores or ulcers arise as a direct consequence of prolonged periods of immobilization or secondary to an underlying organic condition such as sickle cell disease and diabetes.

Usual malignancies progressing to such lesions include breast carcinomas, squamous cell carcinomas and in children, lymphomas and rhabdomyosarcomas. The emergence of such a painful symptom additionally causes psychological distress due to altered body image.

The first case reports using topical diamorphine mixed with Intrasite gel was reported in 1995 and the 3 patients were all being treated in palliative care units (Back et al 1995). Two of the patients in Back's case reports had pressure sores and 1 had a fungating malignant ulcer. They were already on long acting morphine sulphate or intravenous diamorphine as (doses ranging from 240mg – 500 mg in a 24 hour period) as well as a non-steroidal anti-inflammatory drug. Diamorphine 10 mg was mixed with the gel and applied to the wounds with a documentable effect after the first application, which lasted the duration of 24 hours. Intrasite, is a colourless transparent aqueous gel, which contains modified carboxymethylcellulose (CMC) polymer together with propylene glycol. It has the following beneficial properties:

- creates a moist environment to promote wound healing
- aids debridement
- absorbs exudates at wound surfaces
- the propylene glycol has bacterostatic properties
- generally non-adherent to dressings

The Intrasite /morphine gel mix has shown to be stable for up to 28 days at room temperature (Zeppetella et al 2005).

A subsequent isolated case report illustrated analgesia in a palliative patient suffering with Hodgkin's lymphoma, who complained of intractable pain due to an inflammatory lesion on her scalp. The patient's pain interfered markedly with her sleep despite large doses of ibuprofen and her altered self-image caused much psychological distress. After an application of just 3.2 mg mixed with 4 grams of Intrasite gel, to a surface area of 100cm on her scalp, her pain score decreased from 7 to 1, 2 hours post administration of the gel. The gel was daily applied with continuous benefit and without adverse effects (Krajnik and Zylicz 1997).

Further case series documented an exciting beneficial analgesic effect in the majority of patients without any central opioid adverse effects (Twillman et al 1999). In this series of n = 9, patients with painful skin ulceration secondary to a variety of diagnoses such as diabetes, Crohn's disease and fungating breast cancer, documented benefit using this method of analgesia. Morphine infused gel (MIG) was made in a uniform manner, so as to contain an equianalgesic dose of morphine to constitute a preparation of 0.1% weight to weight solution; i.e. approximately 1 mg of morphine mixed with 1 ml of Intrasite gel on a 4 x 4 cm gauze dressing.

The earliest time to achieve initial analgesia was 15 minutes. The longest time to maintain analgesia was 45 hours but on average the pain free period was 12 hours. The topical morphine was efficacious in all of the patients except one man, whose skin lesion differed from the other patients in that the epidermis was intact and there were no definitive signs of inflammation.

This supports the hypotheses from animal studies, that in order for the peripheral morphine to provide effective analgesia, there needs to be epidermal and consequently perineural membrane disruption, presumably for the peripheral opiate receptors to be exposed (Antonijevic et al 1995).

In the above case series, the maximum dose of morphine used for topical application was 5mg, which if applied systemically, would be unlikely produce such a long-lasting analgesic effect. Apart from the size of the dressing used and the % of morphine mixture, there was no matching in the wound type or size or in the subject characteristics. What is illustrated in this study, is the variety of cutaneous and mucosal lesions which respond to the analgesic effect of peripheral morphine / diamorphine.

In a different case series (Krajnik and Zylitz 1999), the morphine gel was used as a mouthwash for oral mucositis, a frequent adverse effect secondary to chemotherapy. This patient gained almost immediate relief, within 30 minutes of administration. The only patient who experienced adverse effects in this report was an elderly woman with renal failure and who, after receiving the highest dose of morphine (80 mg) topically in this case series, experienced mild constipation.

Similarly, in this report a variety of doses of morphine and therefore strength of morphine gel was administered ranging from 1.6 mg – 80 mg and applied to a range of sizes of wounds. The frequency of application differed from patient to patient and the length of use and time to documented initial analgesic effect widely varied.

Other examples of such published case reports but each with a slightly different emphasis, illustrated the use of diamorphine mixed with an antibiotic gel, metronidazole and this combination was used on a female patient whose underlying diagnosis was end-stage ovarian carcinoma and associated widespread, necrotic leg ulcers due to her immobility (Flock et al 2000).

She was having a combination of analgesics including paracetamol, diclofenac and oral morphine in order to attempt to control the resulting severe pain but without success. Remarkably, having stopped all analgesics and after one application of topical diamorphine / metronidazole gel (0.1%) this patient remained pain-free for 48 hours and the opioid toxicity from the oral morphine actually began to subside.

Two case reports of patients with sickle cell disease, who presented with deep penetrating leg ulcers not relieved by conventional analgesics, were trialled with topical opioids (Ballas 2002). In the first patient, an ankle ulcer caused excruciating nocturnal pain to a degree of 10/10 on VAS. The woman took up to x 18, 5mg oxycodone tablets mainly at night in an attempt to reduce the pain, but without success.

However application of a mixture of her usual debridement ointment, with water and one 5mg oxycodone tablet, provided immediate analgesia to such a degree that the patient required to take only a maximum of x 2, 5mg oxycodone tablets daily. The second patient again had severe chronic bilateral ankle ulceration requiring a variety of measures to treat including, 2 hourly pethidine, topical xylocaine gel, grafting, debridement, hyperbaric oxygen and growth factor, all of which did not provide healing. Again use of the current oral opioid, pethidine, dissolved in water and mixed with topical xylocaine gel, produced almost immediate analgesia, reducing the requirement for the huge doses of oral opioids consumed.

The above case reports are useful in demonstrating the potential use of new pharmacological therapy in patients, but ultimately, randomised case controlled trials (RCT) provide the strongest evidence.

There have been two recent small RCTs, which have been carried out in a similar population of adult palliative patients treated in 2 different hospices (**Table 2.3**, Flock 2003; Zeppetella et al 2003). One of the groups showed no significant difference between placebo and morphine while the other demonstrated the efficacy of topical morphine without adverse effects. In Flock's study, the Intrasite gel acted as the placebo and was demonstrated to have no analgesic effects.

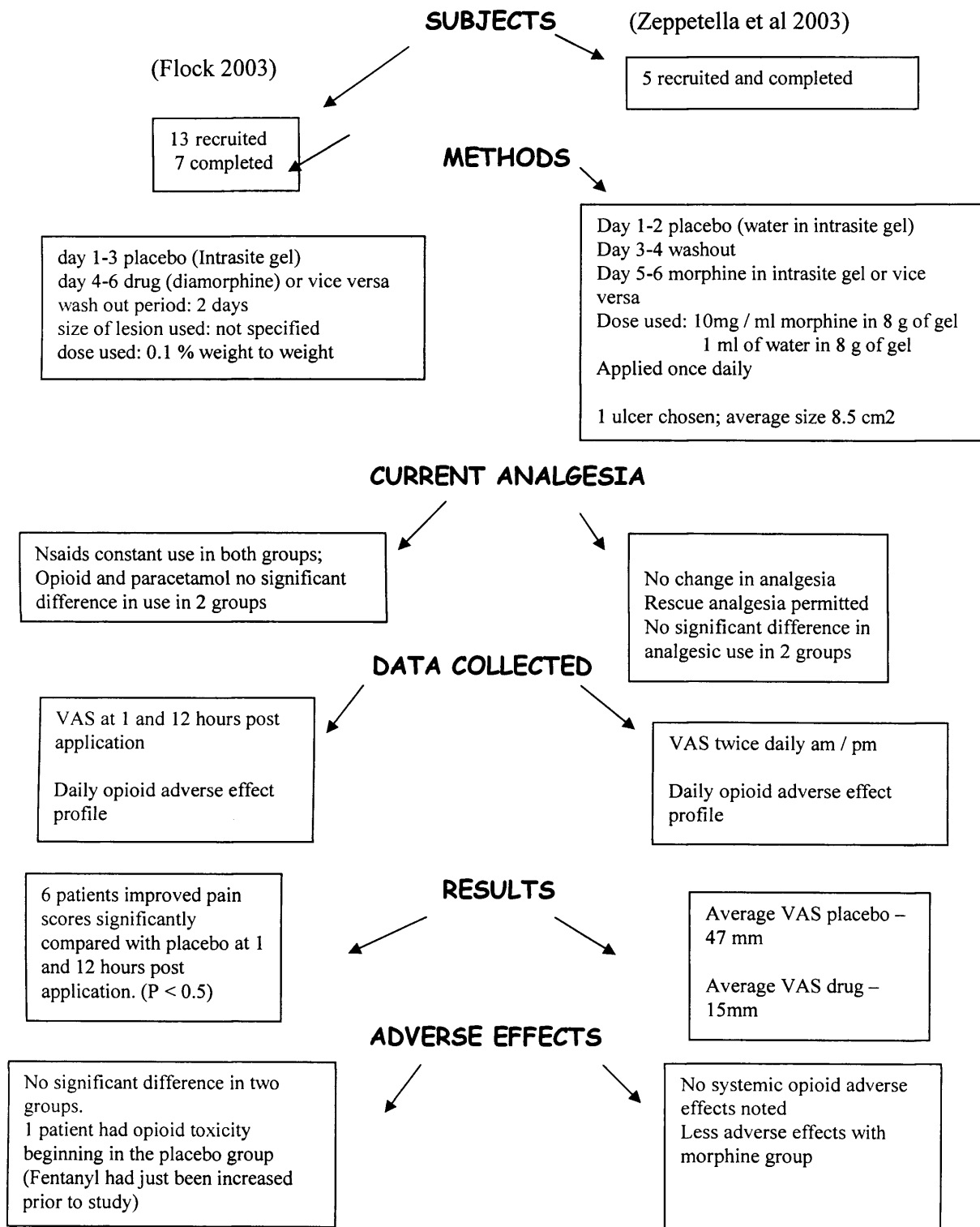
Both of the following RCTs comprise very small trial numbers due to a high withdrawal rate from the patients. This is typical of such a patient population who are managed in a palliative setting as they may deteriorate very quickly.

It may be difficult to obtain an accurate pain intensity measure due to the severity of ill health and subsequent inability to complete serial pain scores vital for data analysis. The majority of palliative care patients will not be opioid naive and this may interfere with clarity of results. Also during the study period, symptoms such as the pressure ulcers may indeed alter, for example heal or deteriorate both of which may be independent or indeed dependent on the actual trial.

2.7 KEY GOALS FOR USE OF PERIPHERAL OPIOIDS IN PAEDIATRIC ACUTE AND CHRONIC INFLAMMATORY PAIN

Neonates, infants and children with acute incident pain and cutaneous inflammatory pain such as burns, blistering lesions in EB, mucositis post - operative surgical wounds and fungating malignancies such as lymphomas and rhabdomyosarcoma have pain which may be severe and intractable. Topical application of morphine either as the sole analgesic agent or as an adjunct, may provide a life - changing advantage for these children, (who are also immunocompromised and therefore possess a minimum endogenous opioid response), if proven beneficial. The concept of peripheral analgesia with minimal or no systemic adverse effects is an additional beneficial property to this novel route of analgesia. The current study is the first to investigate peripheral and topical opioid use in paediatric inflammatory pain.

Table 2.3 Randomised controlled double blind trials



CHAPTER 3: LABORATORY STUDY

3.1 BACKGROUND

To understand the actions of peripheral opioids in children, it is important to investigate their possible mechanisms and sites of actions. Application of local opioids to the skin will lead to activation of opioid receptors in the cutaneous tissue. In Chapter 2 the expression of mu opioid receptors in peripheral sensory neurons in the dorsal root ganglia and their peripheral terminals and in peripheral tissues such as skin, colon, joints and cornea in adult rats, was described (Stein et al 2003).

Less is known about opioid receptor expression in young rats but expression in dorsal root ganglia is known to be postnatally regulated, being more widespread in neonates than in older animals (Beland and Fitzgerald 2001; Nandi et al 2004). A greater number of cells in the DRG express opioid receptors at birth than later in life (Nandi et al 2004) as well as greater binding in the newborn spinal cord (Rahman et al 1998). Expression in skin and subcutaneous tissue has not being examined in young animals but it is reasonable to hypothesise that this may also be greater than in adults.

In adults, both tissue and sensory neuron opioid receptor expression has been reported to be up regulated in the presence of local inflammation (Hassan et al 1993; Coggeshall et al 1997; Sengupta et al 1999), and this suggests that peripheral opioids could be more effective under these conditions. Quantitative evidence for this is somewhat patchy in the literature and it is not known if the above also applies to young animals.

3.1.1 Aims of study

The aims of the study were to test the following two hypotheses:

- 1) Mu opioid receptor expression is denser and more widespread in immature cutaneous tissue than in adult tissue and is down regulated postnatally.

- 2) Mu opioid receptor expression in cutaneous tissue (most probably located in the sensory neurons that supply this tissue), is up regulated in the presence of inflammation in neonatal rat pups

To test these hypotheses, we have used immunohistochemical and Western blot analyses of mu opioid receptor expression in both hindpaw skin and dorsal root ganglia in normal and peripherally inflamed rats of different postnatal ages. Although there are now mouse models for Recessive Dystrophic Epidermolysis Bullosa (RDEB) (Professor Leena Brucker – Tuderman, Germany) which are currently being used in research to investigate squamous cell carcinoma and a separate mouse model of Epidermolysis Bullosa Simplex (EBS) (Professor Dennis Roop, Houston), because the primary focus of my work was pain management in children, using peripheral analgesia, I wanted to use an animal model, the rat, which is the most commonly investigated model worldwide, in the field of neonatal and infant pain research.

3.2 GENERAL METHODS

3.2.1 Animals

All experiments were performed under personal and project licences in accordance with the United Kingdom Animal (Scientific Procedures) Act, 1986.

Sprague Dawley rat pups of both sexes were used for this study and were obtained from the Biological Services, Central Animal Facility at University College London.

3.2.2 Induction of Inflammation

Inflammation was induced in rat pups aged 3 days (P3), 7 days (P7) and 21 days (P21) under brief halothane anaesthetic (2-4 % in oxygen).

2% carageenan (λ Sigma, St Louis, USA) was 50:50 mixed with sterile water and injected intradermally into the plantar surface of the left hindpaw using a 30-gauge needle.

The above human equivalent ages are P3, P7, and P21 are 28 weeks gestation, term and toddler and above (see table 1.3 page 26, “Age of rat pups and equivalent human age in previous chapter”). The volume injected was 5 μ l for P3 and P7 and 20 μ l for the P21 pups. This dose has been previously used to induce inflammatory pain in neonatal P0-P5 rat pups (Walker et al 2003). In each animal, a single slow injection was administered with an aim to spread the carageenan evenly across the subcutaneous plantar surface and minimise leakage. These pups were earmarked and on recovery from anaesthesia, returned to their dam and litter for four hours with free access to food and water. After four hours, the 5 earmarked rat pups were taken from their litter and the paw diameter was measured across the midpoint of both hindpaws using a calibrated calliper across the dorsal to plantar surface. There were clinical signs of acute inflammation i.e. redness and swelling in all of the injected paws. The ratio of the injected paw to the contralateral paw was determined in order to standardise for variability between animals and changes with growth. The measurements were used to demonstrate the presence of acute inflammation.

3.3 PROTOCOL 1: IMMUNOFLUROESCENCE

3.3.1 Preparation of tissue

Pups were administered a lethal dose of sodium Phenobarbital (100 mg/kg, intraperitoneal injection). This was followed by a transcardial perfusion with heparinised saline (0.9%) and cold (4⁰C) 4% paraformaldehyde 0.1M in phosphate-buffered saline (PBS). The skin from the plantar surface of both the inflamed (ipsilateral) and non-inflamed (contralateral) paws along with the dorsal root ganglia, (DRG, both ipsi and contra) from the L4 and L5 spinal cord regions were removed from each animal. In some cases, the lumbar section of spinal cord was removed as a positive control for the MOR antibody. The tissue was post fixed in 4% paraformaldehyde for 2 hours and then stored in 30% sucrose in 0.1M PBS at 4⁰ C.

Picric acid was also tried as a fixative, as it assists in binding of antigens to their respective antibodies, by making the epitopes more accessible. However this method seemed to cause abnormal morphology of both the skin and dorsal root ganglia. The fixed tissue was mounted in Cryo-M-Bed freezing compound (Bright Instrument, Huntington, UK).

Cryostat sections of the plantar surface of the hindpaw skin were cut at 40µm thickness, dorsal root ganglia at 10µm thickness (Beland and Fitzgerald 2001) and spinal cord at 20 µm thicknesses (Raman et al 1998) and mounted serially on gelatinised slides. 100 µm skin thicknesses have been used in neonatal models of skin wounding (Reynolds et al 1997) but having tested various skin thicknesses with MOR and immunostaining, I decided to choose 40 µm as the thickness most suitable for the antibody application. These slides were air-dried overnight before storage at -20⁰ C.

3.3.2 Establishing an appropriate immunohistochemical method

Immunohistochemistry is a technique to detect, visualise and localise antigens at the cellular level, most commonly using primary and secondary antibodies, which bind to the antigens embedded in paraffin or frozen tissue sections. A number of different methods of immunohistochemistry staining were performed in this study to evaluate which technique was the most suitable for both the tissue and antigen being studied.

- Basic immunofluorescence, where a secondary fluorescent antibody binds to the primary antibody and detects the epitope of the antigen, was the first approach used. However, this technique was not suitable, as a lot of background tissue fluorescence was obtained making localisation of the mu opioid receptor hard to discriminate. Different concentrations of the mu antibody were tried but with similar results each time.
- The diaminobenzidine (DAB) method is another form of immunohistochemistry, involving an enzymatic reaction, which assists in amplification of the mu opioid receptor antigen signal. The ABC technique is then employed to detect the antibody / antigen complex. ABC stands for avidin, biotinylated horseradish peroxidase, macromolecular complex, which forms complexes for immunoperoxidase staining.

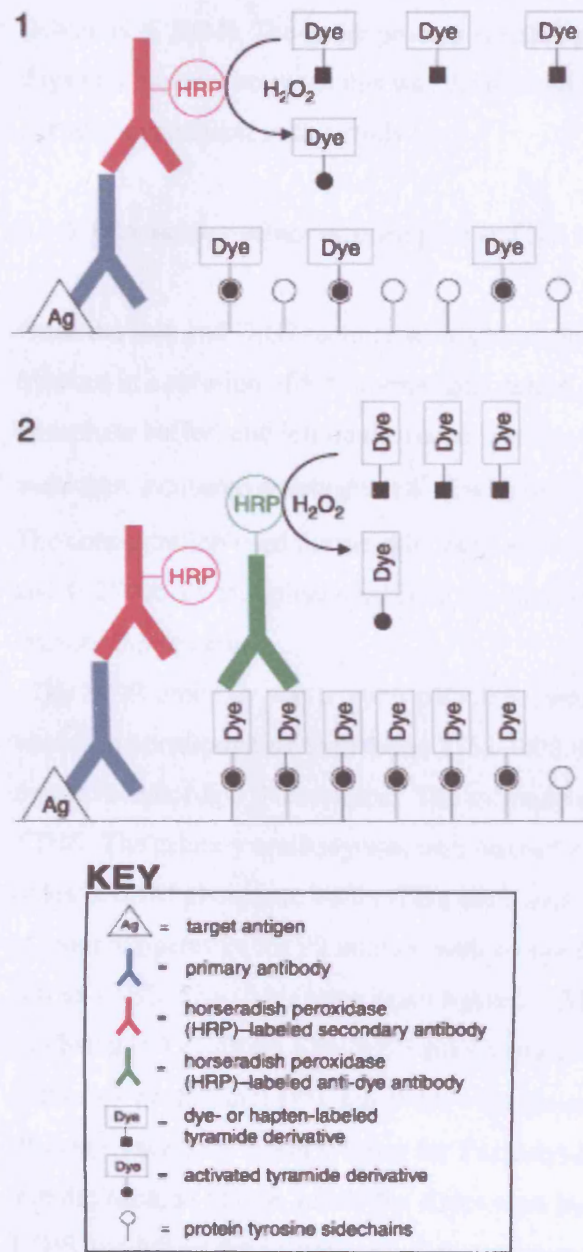
- This method involves an additional step, using an avidin- biotinylated, horseradish peroxidase complex and the final stain is developed, following a reaction between a detection kit, which is composed of DAB, hydrogen peroxide and nickel.

Avidin is a 68, 000 molecular weight glycoprotein, which has an extremely high affinity for the small molecular weight vitamin, biotin and this affinity is actually x1,000,000 the binding of most antibodies for antigens and so this process is irreversible. Avidin has also 4 binding sites for biotin and most other proteins including antibodies can be conjugated with several molecules of biotin. Despite the increased sensitivity of this technique over classical immunofluorescence, it is better for the sections to be free floating, which means transferring the cut sections from the cryostat directly to a container with 0.1M Phosphate Buffer with azide and glucose. Any subsequent step in this procedure requires handling the sections with a fine paintbrush and with skin; it is very difficult to keep the fragile neonatal tissue intact throughout the entire process. This method also failed to detect MOR expression in the skin although MOR in the superficial dorsal horns was clearly stained.

- TSA Immunofluorescence TSA, Tyramide Signal Amplification. This method significantly enhances both chromogenic and fluorescent signals and was found to be the most successful method in these studies.

The principles of this method are shown in the diagram overleaf:

Fig 3.1 Tyramide Signal Amplification method



Taken from Molecular
Invitrogen Detection Tool TSA
information sheet.

1) The antigen is detected by the primary antibody, followed by a horseradish peroxidase labelled secondary antibody in conjunction with a dye-labelled tyramide, resulting in localised deposition of the activated derivative.

2) Further dye deposition and therefore higher deposition levels of signal amplification can be generated by detecting dye deposited in the first stage with a horseradish peroxidase labelled anti-dye antibody in conjunction with a dye-labelled antibody.

Because of the amplification, which occurs, increasing the dilution of the primary antibody by just 5 or 10 fold leads to better results.

This method has been used to detect opioid receptors both MOR and delta (DOR) in cultures of brainstem neurons throughout late fetal and early postnatal development (Kivell et al 2004). The entire process is rather prolonged and takes up to three or four days to complete, however this was the method which worked best for both the skin and dorsal root ganglion in this study.

3.3.3 TSA Immunofluorescence protocol for MOR

Once the skin and DRG sections were placed on the gelatinised slide, they were blocked in a solution of 5 % normal goat serum and TTBS (0.3% triton X- 100 in phosphate buffer) and left on the rocker at room temperature for 2 hours. The sections were then incubated overnight at 4^o C with mu opioid receptor antibody.

The concentration used for the skin sections was 1: 2500 and 1: 40,000 for the DRG's and 1: 25000 for the spinal cord (x10 the dilution which was used previously in simple immunofluorescence).

The MOR antibody was a rabbit polyclonal antibody raised against the immunogen sequence corresponding to residues 1359-1403 of the carboxy terminus of rat MOR (mu opioid receptor IgG, Neuromics). The antibody was diluted in 5% goat serum diluted in TTBS. The primary antibody was then washed off the slides the following morning 3 times in 0.1M phosphate buffer (PB), each wash for 10 minutes, before being incubated at room temperature for 90 minutes with biotinylated goat anti-rabbit at a dilution of 1: 400 in TTBS. The slides were again washed x 3 in 0.1 PB as before and then the incubated in Vectastain Elite ABC amplification kit solution for 30 minutes. After a further x3 washes in 0.1PB, the slides were placed in biotinylated tyramide solution (Perkin Elmer TSA biotin system) for 7 minutes before a further 3 washes, for 10 minutes each, as before. Lastly the slides were incubated with FITC avidin 1:600 in TTBS and left on the rocker at room temperature for 2 hours covered in foil. After washing the slides as before, the slides were cover slipped with gel mount (Sigma) and stored in foil at 4^oC.

All sections were run concurrently with **positive controls (the spinal cord)** and **negative controls (no primary antibody)**.

3.3.4 Analysis of immunofluorescent staining

Immunofluorescent slides were examined under a Nikon E800 fluorescence microscope through red coloured filter at 470/505nm excitation/emission wavelengths. To estimate the intensity of staining, microscope images (x10, x 20) were captured and analysed using MCID (Imaging research, GE Healthcare) image analyser software. For analysing MOR immunofluorescence, a threshold was set above background and the area of skin with staining intensity above that threshold was measured. The area analysed was a rectangle set by the computer programme as 500 x300 μm and fixed for all sections. The real size of the rectangle was calculated and is documented in all the figures. The threshold was also kept constant for all sections and the software measured the number of pixels above that threshold in each section. This method is not, therefore a measure of staining intensity but a measure of staining above threshold. For this reason it is called “relative staining density”. For each skin section, the relative staining density was measured in 3 separate regions of epidermal/dermal tissue and the mean intensity calculated. The rectangles were placed with one long side on the epidermal surface measuring along the section. Measurements were made on both ipsilateral and contralateral skin sections; there were 20 sections per animal. The observer was blind to whether the sections were ipsilateral or contralateral and the same observer performed all the analyses.

The same computer programme was used to analyse DRG immunostaining. For each DRG section, thresholds were set and numbers of neurons above the threshold counted. Approximately 500 cells per DRG were analysed and the numbers of cells positive for MOR immunostaining were expressed as a percentage of the total cell count. This was repeated for each of the L4 and L5 DRGs on ipsi and contralateral sides. Again to eliminate observer bias, the observer was blind to the experimental status of the animal and the same observer performed the analysis throughout.

3.4 PROTOCOL 2: WESTERN BLOTS

To obtain a more quantitative measure of the total amount of the MOR protein in our skin sample, western blots were carried out. In this method, protein bands of a particular antigen are obtained by gel electrophoresis.

The protein bands, which are charged ions, run at different speeds depending on their molecular weights and can be visualised for quantitative analysis.

3.4.1 Tissue Preparation for Western Blots

The skin of the plantar surface of both feet of P3 and P21 naïve and carageenan inflamed rat pups were dissected and snap frozen in liquid nitrogen and stored in eppendorfs at -80°C . Fresh tissue is essential for Western Blot analysis and should be kept frozen at all times to preserve the protein structure. The skin samples are then homogenised in RIPA buffer, which contains proteinases, phosphatase inhibitors in a solution of NaCl, NaF, EDTA, NP-40 and Hepes. This homogenises the protein and exposes the antigens. The homogenates are then kept on ice for 2 hours and then centrifuged (at 4°C , for 15 minutes at 12,000 rpm). The supernatants are then collected into sterile eppendorfs.

3.4.2 BCA Assay

The total protein extracted from the tissue was titrated using a BCA kit (Pierce, Rockford IL). This is an assay where the protein samples are normalised against a known concentration BSA (bovine serum albumin). It is an endpoint reaction, where the darker the colour of the samples, the more protein is present. The samples are then added to a volume of loading buffer in order to obtain a total concentration of $10\ \mu\text{g}$ of protein in $10\ \mu\text{l}$ of solution. The normal starting point for a western blot is to load $10\ \mu\text{g}$ of total protein per well.

3.4.3 SDS-PAGE and Loading of samples for the Western Blot.

All samples were analysed by SDS-PAGE gel (10% Tris –HCL gels, Biorad). Appropriate amounts of protein were added to each lane alongside a prestained broad range 10,000 – 250,000 kd Kaleidoscope marker (Amersham). They were then electrophoresed in running buffer (Tris base, SDS, glycine and distilled water) at 100V for 90 minutes.

Following activation using methanol and then rehydration, using transfer buffer (Tris base, methanol, SDS, glycine and distilled water), a PVDF (polyvinylidene difluoride) membrane was then used, onto which the proteins were transferred with 100 V for 45 minutes.

3.4.4 Immunostaining of Western Transferred Proteins

PVDF membranes were then transferred to blocking agent (5% skimmed milk powder in PBS Tween (1ml Tween in phosphate buffer) and left for 1 hour on the Rocker. The membranes were then incubated with 1:2500 MOR antibody (Neuromics as used for immunofluorescence) diluted in the blocking agent and left overnight on a rocker at 4⁰ C. The membranes were then washed 10 times for 5 minutes each in PBS with 0.1%Tween and then were incubated with Horse Radish Peroxidase (HRP) – conjugated secondary anti-rabbit antibody at 1:2000 for 45 minutes at room temperature. The membranes were washed as before.

3.4.5 Development of the protein bands

HRP- labelled proteins were then visualised using an enhanced chemiluminescent Substrate (ECL solution Amersham using 50:50 of each reagent) and developed using Kodak X- OMAT film in a dark room. The identified bands were labelled against the molecular weight of the kaleidoscope marker. The blots were then stripped using stripping buffer twice, for 10 minutes each and then washed with PBS tween as before. Following blocking as before, the membranes were then reprobed with GAPDH antibody at 1: 750 (Chemicon) and left overnight a 4⁰C.

The blots were again washed and secondary antimouse antibody was added (1: 2000) for 45 minutes, then again washed. They were then developed again in a dark room using ECL solution and Kodak film.

It is essential to control for protein levels and this is usually done using GAPDH, as a “housekeeping gene”. However anecdotal evidence has suggested that GAPDH is developmentally regulated and so an alternative method of controlling for protein levels is to use coomassie, a blue dye. The blot is placed in the dye for 5 minutes and then destained, using acetic acid. The bands of protein are then clearly visualised as dark blue against a lighter background. Using the MCID computer package, the relative optical density x the scan area was analysed for the blots and calculated as a percentage

of the GAPDH or Coomassie blue values.

Fig 3.2 Summary of Western Blot Procedure

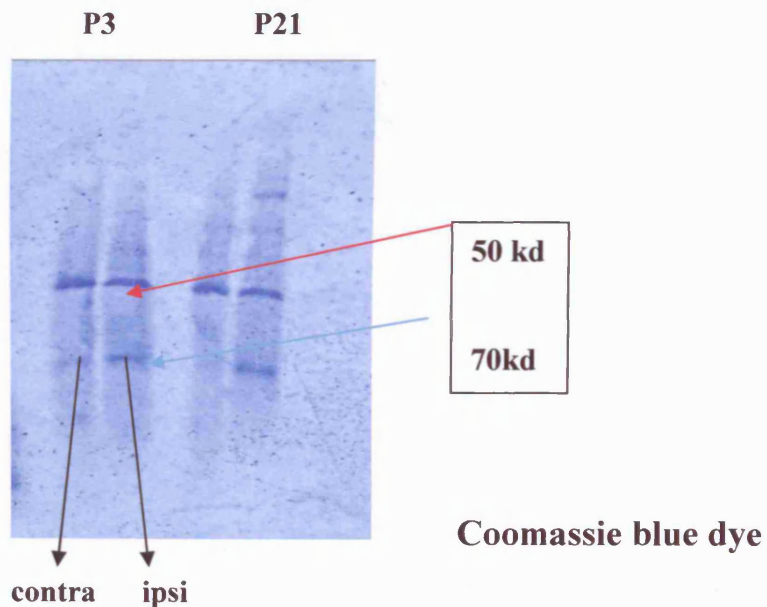
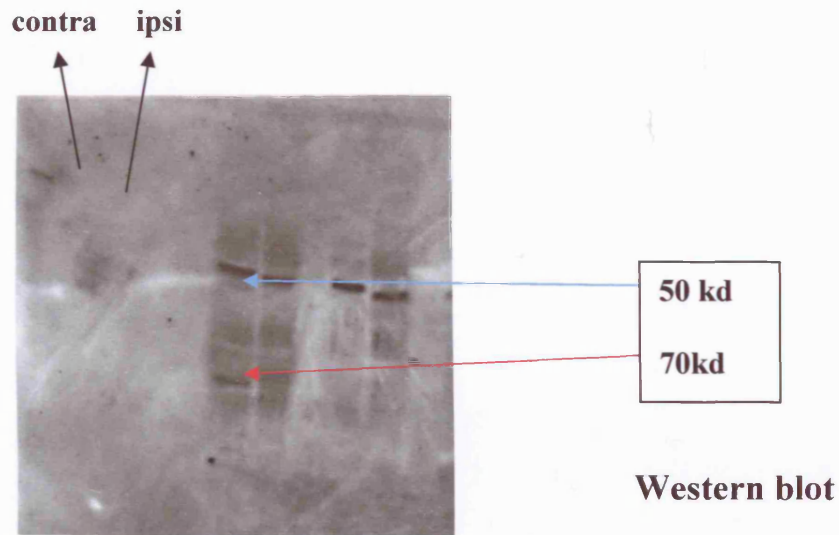
- 1) Proteins of interest are loaded into the wells are separated by gel electrophoresis, usually SDS-PAGE.
- 2) The proteins are transferred to a sheet of special blotting paper called nitrocellulose, though other types of paper, or membranes, can be used. The proteins retain the same pattern of separation they had on the gel.
- 3) The blot is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the nitrocellulose. An antibody is then added to the solution, which is able to bind to its specific protein. The antibody has an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) or dye attached to it, which cannot be seen at this time.
- 4) The location of the antibody is revealed by incubating it with a colourless substrate that the attached enzyme converts to a coloured product that can be seen and photographed.
- 5) To control for the amount of protein loaded, the blot is placed in coomassie blue dye and then destained

(Taken from www.bio.davidson.edu/COURSES/genomics/method/Westernblot)

3.5 RESULTS

3.5.1 The effect of postnatal age on MOR protein levels in plantar skin

Figs 3.3 and 3.4 show Western blots of MOR protein levels in rat plantar skin at two postnatal ages, P3 and P21. Two bands are observed, one at 50 kd and one at 70kd.



Figs 3.3 and 3.4 Typical Western blots of MOR expression in the plantar skin of P3 and P21 rat pups. Two bands, 50 kd and 70kd are shown.

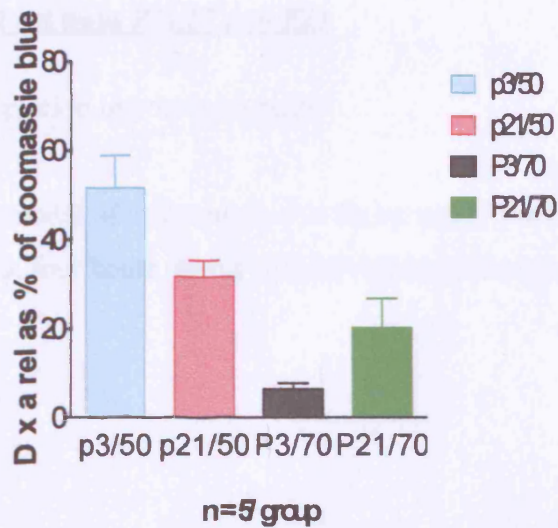


Fig 3.5 Mean expression of bands 50 kd (p3/50, p21/50) and 70 kd (p3/70, p 21/70) MOR at P7 and P21

(D x a is density of the band x area of band scanned)

The intensity of the two bands from 5 animals in each group is shown in **Fig 3.5**. A one way ANOVA showed that there was a significantly greater expression of 50kd MOR protein in the younger P3 animals, compared to P21 ($p < 0.05$). It also showed that there was a significantly greater amount of 50 kd MOR protein than 70kd MOR protein in the P3 animals ($p < 0.0001$). The 70 kd protein was more variably expressed. **Please see page 100 for possible explanation and significance of two bands.**

3.5.2 The effect of hindpaw carageenan inflammation on MOR immunostaining in plantar skin and DRGs of rat pups P3, P7 and P21

(i) Carageenan inflammation in young rat pups

Carageenan was used as a model of inflammation in the rat pups. Previous reports in adults have shown that at four hours strong clinical signs of inflammation are evident.

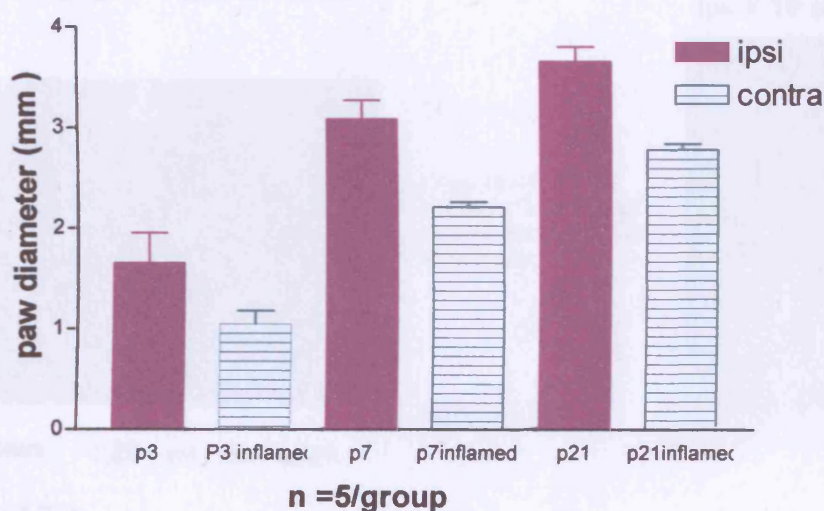
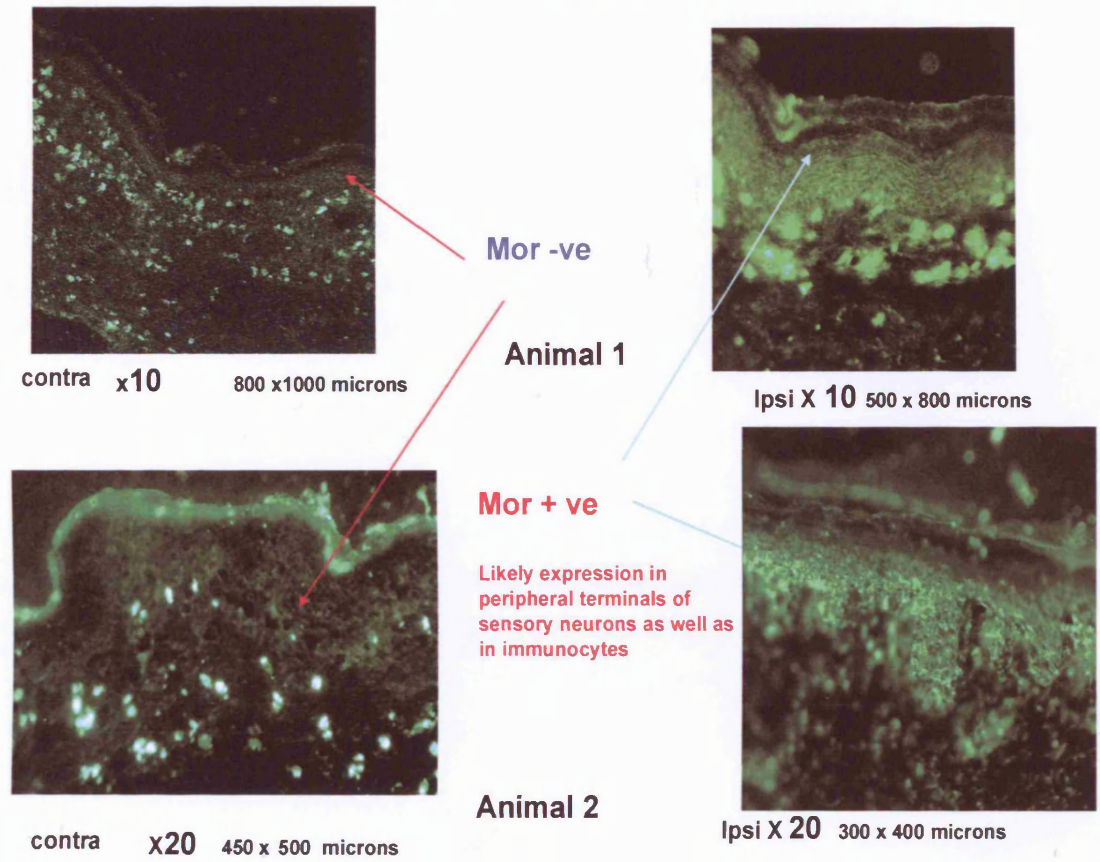


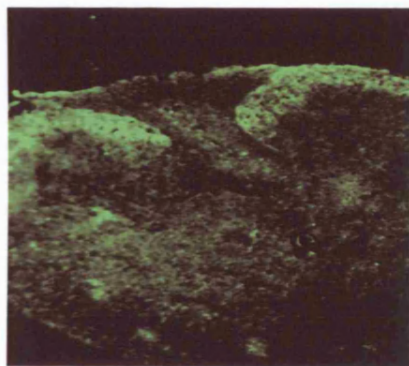
Fig 3.6 Mean (\pm SEM) paw diameter at P3, 7 and 21, 4 hours post carageenan injection. Data is shown for ipsilateral carageenan injected (ipsi) and contralateral control (contra) paws. **Fig 3.6** shows the clinical signs of acute hindpaw inflammation measured as increases in hindpaw diameter in neonatal rats. Using one way ANOVA, statistically significant inflammation was evident in the animals of p7 and p21 ($p < 0.05$) and clinically significant in the youngest pups. This time point was therefore used for all the subsequent studies.

(ii) Immunostaining of MOR in the plantar skin of control and inflamed hindpaws

Contra and ipsilateral images - TSA Immunostain in glabrous skin of the hindpaw for MOR in p3 rat pups



(Fig 3.7a)



(Fig 3.7b)

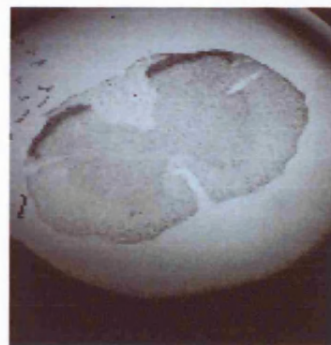


Fig 3.7a shows examples of typical immunofluorescent staining of MOR in naïve (contra lateral) and inflamed (ipsilateral, 4 hours post carageenan staining) plantar sections in the glabrous skin of the hindpaw in P3 rat pups.

Negative control showed no immunostaining without primary antibody application (fig not shown).

Fig 3.7b Positive control of MOR antibody present in the dorsal horn of the spinal cord of the control rat pups at all ages using immunofluorescent and DAB staining technique. In control tissue, the staining is relatively sparse in the epidermis and dermis but is concentrated in large cells in the subcutaneous tissue, which are likely to be immunocytes. Future work should then attempt to demonstrate this colocalisation with double-staining immunofluorescence. In contrast, there is abundant staining in the inflamed skin, concentrated largely in the dermis while the epidermis remains relatively free of MOR expression. Cellular staining is clearly increased but some of the cutaneous MOR expression is likely to be on sensory terminals, which are concentrated in the dermis. Again this should be the next step using an antibody such as PGP 9.5, which stains for nerve fibre within the superficial and deep dermis, and around adnexal structures such as hair follicles and sebaceous glands. Image analysis was performed in 20 contra lateral and 20 ipsilateral sections per animal at P3, in order to provide a quantitative analysis of this up regulation.

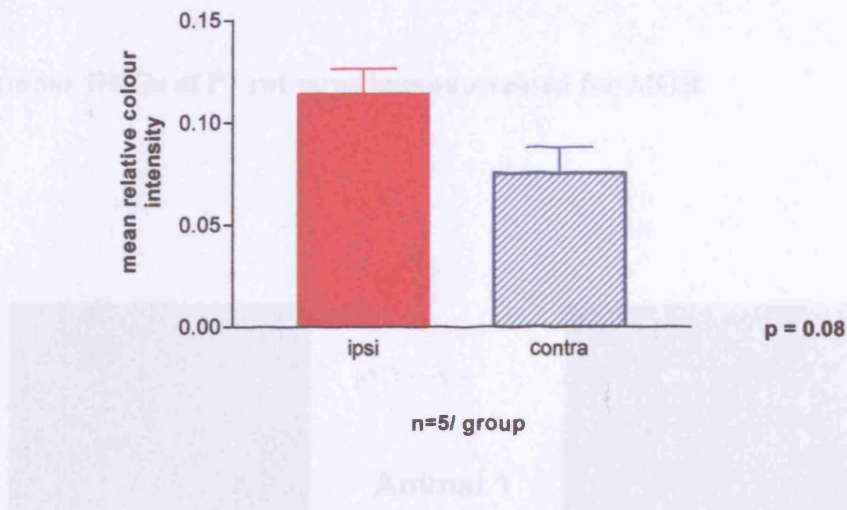


Fig 3.8 The mean relative density MOR immunolabelling in ipsi (inflamed, 4 hours) and contra (non-inflamed) plantar skin sections of P3 animals. The mean relative colour intensity was established for the contra, 0.07 ± 0.01 (SEM) and ipsilateral 0.11 ± 0.01 (SEM) respectively. Although this was not statistically significant; t test 0.08, the trend was consistent with visual inspection of sections (**Fig 3.8**).

(iii) Immunostaining of MOR in the L4 and L5 dorsal root ganglia of control and inflamed hindpaws.

Lumbar DRGs of P7 rat pups immunostained for MOR

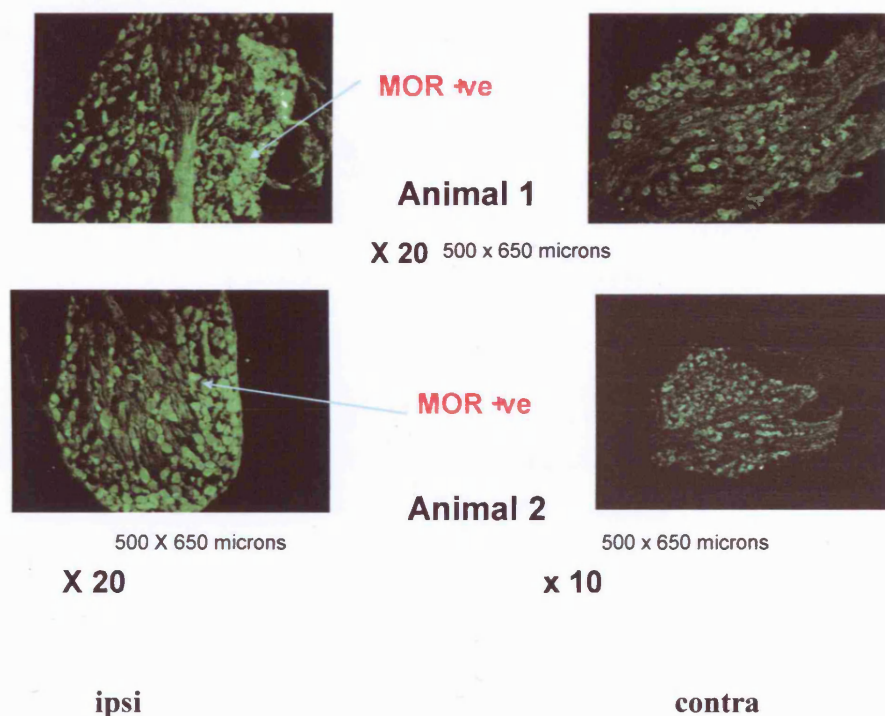


Fig 3.9 shows the typical immunofluorescent staining of MOR in non-inflamed (contra lateral) and inflamed (ipsilateral) sections of the P7 dorsal root ganglia. Negative controls were DRGs without primary antibody resulting in negative immunostaining (fig not shown). The DRG sections of the P3 animals were technically difficult to section intact and so immunostaining was not successful. The staining is localised to the cell bodies in the ganglia although some can be seen in the sensory nerve fibres and dorsal roots as well. It can be seen that the intensity of MOR staining is much greater on the inflamed side than the contra lateral control side. MOR immunoreactive positive cells were counted in 10 sections from each L4/5 DRG and expressed as a % of the unstained cells.

Again the mean values for the relative colour intensity of the contra and ipsilateral samples were calculated as 25.2 ± 2.1 (SEM) and 15.2 ± 4.7 (SEM) respectively and a t test showed that this difference was very significant with a value of < 0.009 (Fig 3.10).

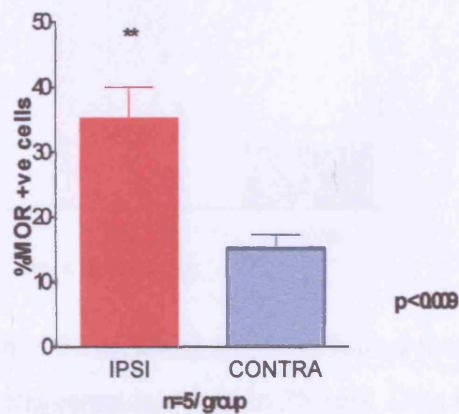


Fig 3.10 The mean relative density MOR immunolabelling in ipsi (inflamed, 4 hours) and contra (non-inflamed) L4 DRG sections of P7 animals.

3.5.3 The effect of hindpaw carageenan inflammation on MOR protein levels in plantar skin in rat pups at P3 and P21

- (i) Western blot analysis of MOR protein levels in the plantar skin of control and inflamed hind paws

Figs 3.11 & 3.12 show the effect of hindpaw carageenan inflammation upon the 50kd and 70 kd MOR protein bands in P3 plantar skin based on the data obtained for Western Blot analysis. **Fig 3.11** shows that it is the 50kd band that it is significantly up regulated in the presence of inflammation, whereas the 70kd band is more variable (**Fig. 3-12**). (see page100 of this chapter for possible explanations for the two different bands detected).

Although direct comparison cannot be made between Western blots, the up regulation at P3 was at least as great as that seen at in P21 animals (Figs 3.13 & 3.14).

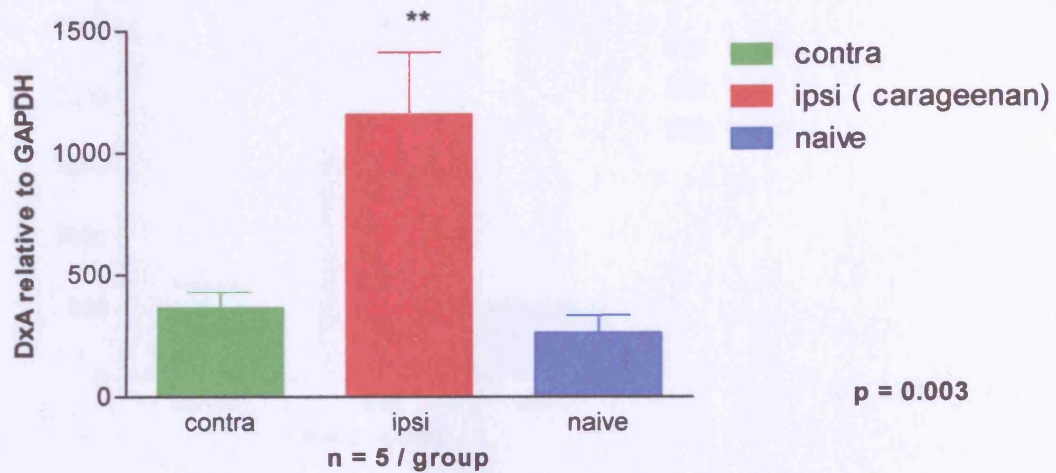


Fig.3.11 Mean expression of 50 kd MOR in non-inflamed (contra), inflamed (ipsi), and naïve skin 4 hours post carageenan injection in **P3 rats**. Data based on Western Blot Analysis of the sections obtained. Y axis shows the density times the area of band scanned as a percentage of GAPDH to control of amount of protein loaded, using the MCID image analyser

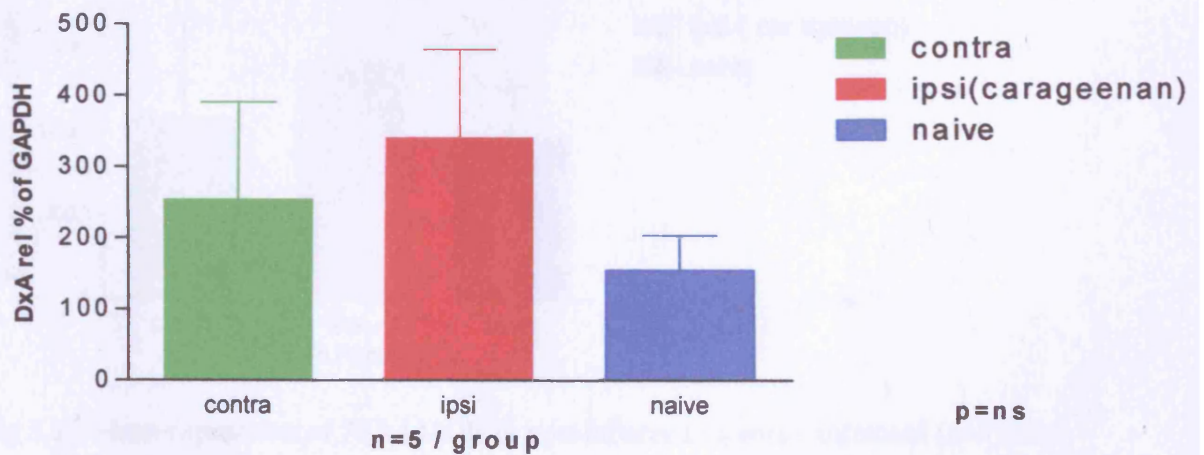


Fig 3.12 Mean expression of 70kd MOR in non-inflamed (contra), inflamed (ipsi) and naïve skin 4 hours post carageenan injection in **P3 rats**. Data based on Western Blot Analysis of sections obtained.

Y axis shows the density times the area of band scanned as a percentage of GAPDH to control of amount of protein loaded, using the MCID image analyser

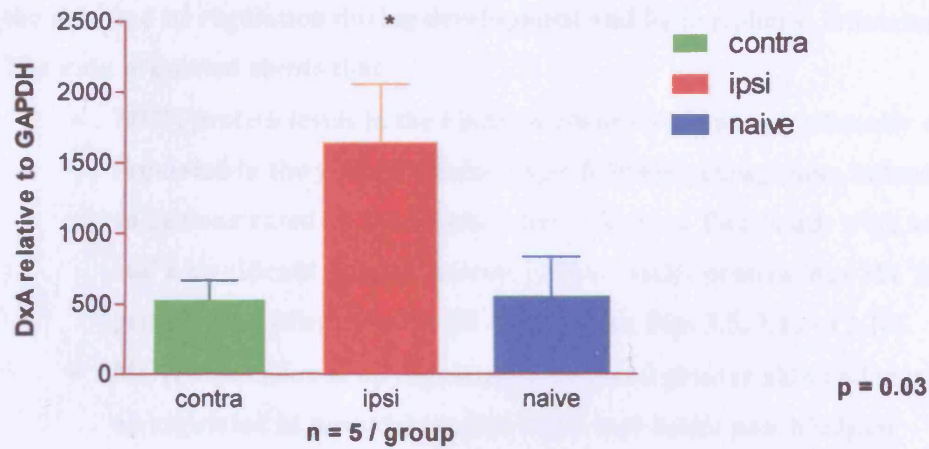


Fig 3.13 Mean expression of 50 kd MOR in non-inflamed (contra), inflamed (ipsi), and naïve skin 4 hours post carageenan injection in **P21 rats**. Data based on Western Blot Analysis of the sections obtained. Y axis shows the density times the area of band scanned as a percentage of GAPDH to control of amount of protein loaded, using the MCID image analyser

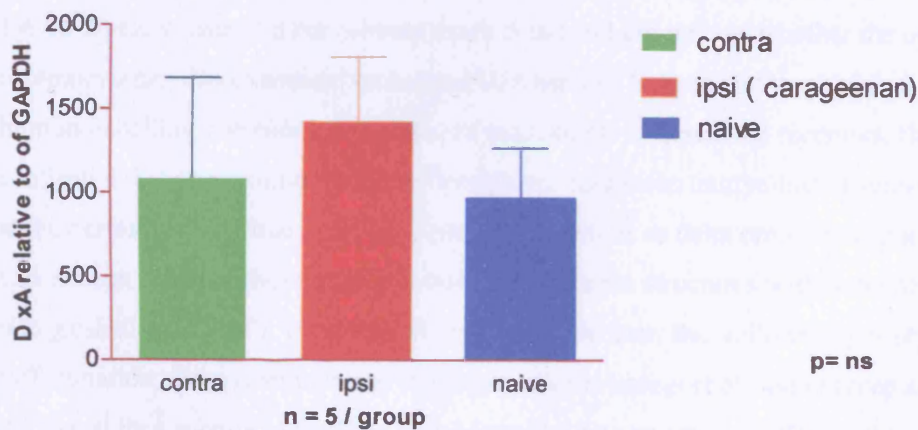


Fig 3.14 Mean expression of 70 kd MOR in non-inflamed (contra), inflamed (ipsi), and naïve skin 4 hours post carageenan injection in **P21 rats**.

Data based on Western Blot Analysis of sections obtained. Y axis shows the density times the area of band scanned as a percentage of GAPDH to control of amount of protein loaded, using the MCID image analyser

3.6 Discussion

In this chapter I have quantified, for the first time, peripheral MOR expression in the skin and its regulation during development and by peripheral inflammation.

The data presented shows that

- **MOR protein levels in the hindpaw plantar skin are significantly up regulated in the youngest animal and following carageenan inflammation as demonstrated by the Western Blot Analysis. Two bands were visible and a significant greater amount of 50kd MOR protein than the 70kd protein was present in the P3 animals (see Figs 3.5, 3.11 to 3.14)**
- **MOR expression is up regulated in neonatal plantar skin and significantly up regulated in neonatal lumbar DRG four hours post hindpaw inflammation as demonstrated by analysis of immunofluorescence staining (see Fig 3.7 to 3.10).**

The up regulation of peripheral MOR by carageenan inflammation and the postnatal regulation of peripheral MOR

As discussed in chapter 2, previous work, using light microscopy has demonstrated that MOR is expressed on the fine cutaneous nerves in the inflamed adult paw (Hassan et al 1993). These studies did not provide much detail and did not say whether the opioid receptors were also expressed on normal skin nerves. Subsequent work, using immunolabelling combined with electron microscopy to detect the receptors, then confirmed that approximately 30% of peripheral cutaneous unmyelinated sensory fibres at the dermal – epidermal junction express either MOR or delta opioid receptors, but it was unclear whether these were membrane bound or in structures within the nerves (Coggeshall et al 1997). It is generally accepted however, that following peripheral inflammation, there is an increase of the intraaxonal transport of opioid receptors from the dorsal root ganglia toward the periphery providing an up regulation in the receptors at the sensory nerve terminals (Stein et al 1993).

Our first aim was to confirm whether MOR is expressed in cutaneous tissue young rat pups and whether postnatal age and effects of carageenan inflammation affected this expression.

Our first aim was to confirm whether MOR is expressed in cutaneous tissue young rat pups and whether postnatal age and effects of carageenan inflammation affected this expression.

The results of MOR immunostaining in the skin of neonatal rats verify the existence of the receptors within the epidermis, but in the absence of electronmicroscopy, it is not possible to state the exact location. It is likely however that they are located within the peripheral sensory nerve endings as demonstrated previously in adult animal models (Stein et al 1990).

Stander et al 2002 has shown immunoreactivity for the isoform of MOR, MOR-1A in large fibres in the superficial and deep reticular dermis, in small nerves in the papillary dermis, at the dermal-epidermal junction and within the epidermis, using double immunofluorescence staining for MOR 1-A and NF (neurofilament) and PGP9.5 antibodies. This could also be confirmed in neonatal rat tissue as a possible follow-on study, by performing double immunofluorescence with a panaxonal antibody such as peripherin or PGP 9.5. MOR may also be located on various cells of the circulating immune system such as macrophages and lymphocytes (Machelska et al 2002).

Previous work has studied the effects of different inflammatory agents administered to neonatal rat pups and has provided useful information, particularly with respect to the onset of acute pain and the long term effects of the different inflammatory models.

In this study carageenan was chosen as the inflammatory agent as it acts almost immediately at around two to five hours post injection (Walker et al 2003) without long-lasting consequences. Although the clinical inflammation it produces may last up to 14 days with this agent, there are no alterations in longer term mechanical or heat behavioural response, in comparison to CFA(Complete Freund's Adjuvant), which may produce clinical inflammation lasting into adulthood without resolution.

Formalin has also been shown to be more severe noxious stimuli if injected into the neonatal rat pup and may actually lead to sensory neuron death (Tsujino et al 2000). Up regulation of opioid receptors in the skin was evident on inspection of the immunostained sections but image analysis did not reveal significant results.

Some reasons to account for this flawed method may be due to the fixation method or preparation of the tissue or may be due to the chosen size of the cryostat sections. The up regulation was clear, however, on Western blot analysis.

The method used to analysing immunofluorescent staining of opioid receptors in the skin is not a precise method, as it defines the colour intensity of the staining and is not a quantification of the actual number of cells or structures which are positively stained. It provides a relative change in colour intensity and so may not be entirely representative of the actual up regulation, which occurred. The method did produce statistically significant data for the DRG up regulation perhaps because of the more homogeneous nature of the tissue.

Recent work in adults, investigating the mechanism behind the transport of opioid receptors to the periphery as well as, how the inflammation at the periphery relays its signal towards the DRG, with the following results (Puehler et al 2004):

- There is a biphasic up regulation of mRNA for MOR at an early stage of 1-2 hours and then at a later stage of 96 hours in the ipsilateral DRG, following inflammation, induced by a local injection of CFA in to the hindpaw of adult rats.
- There are no significant changes in the mRNA levels for the receptor on the non-inflamed side.
- At the initial stages of an inflammatory response (1-2 hours), there is an increase in opioid binding in the DRG suggesting that the MOR expression may be transcriptionally controlled following stages of peripheral inflammation.
- As early as 4 hours post-inflammation, an increase in MOR transport towards the periphery occurred, demonstrated in the ligated sciatic nerve.
- Local anaesthetic was shown to block the increase in MOR transcription, which suggests that the early up regulation is controlled by neuronal electrical activity.

The above explains that the early up regulation of MOR in the DRG is most likely due to increased transcription, presumably triggered by increased spike activity from the sensitized sensory nerve endings in the inflamed skin (time scales of the inflammatory response differ to that produced in my work , as CFA not carageenan was used in Puehler's research).

This is followed by increased transport to the nerve endings in the skin. The above was investigated using PCR (polymerase chain reaction technique) which should be performed on neonatal tissue as the one of the follow- on studies.

This might explain the less significant results for the immunoreactivity within the skin, which may have been clearer at a later time point. In order to provide a more precise quantification of MOR within the skin, western blots were performed.

Developmental regulation of peripheral MOR

The results show that was significantly more peripheral 50kD MOR expression in the skin of the P3 pup in comparison to the P21 rat. This is consistent with previous reports showing more widespread expression in the infant rat dorsal root ganglion (Beland and Fitzgerald 2001; Nandi et al 2004) and greater MOR binding in the infant spinal cord (Rahman et al 1998).

The postnatal developmental regulation of key receptors is a common feature of the developing nervous system and may well reflect the change from neural communication for the purpose of synaptogenesis and growth to mature synaptic transmission and modulation of neural signals (Pattinson and Fitzgerald, 2004).

Possible significance of two protein bands

Two visible bands were observed on the Western blot; the 50 and 70 kd bands.

According to Neuromics, the MOR antibody manufacturer, the 50kd band is the typical band, which has been detected in various tissues such as brain, spinal cord and spinal cord in the adult rat in previous work (Arvidsson et al 1995). A second less clear band was seen at the 70kd in all of the westerns.

Similar banding patterns were reported in a study looking at the expression of MOR in the brainstem of rats throughout development. Although there were only 3 animals per age group in this study, the 50kd band was more likely to be expressed in the younger animal and the 70 kd band in the older animal (Kivell et al 2004). The different immunoreactive bands at 50 and 70 kd may be due to the presence of distinct isoforms of the receptor.

MOR-1 was first cloned just over 10 years ago and more recently studies have identified splice variants of this clone. Evidence now suggests that there are differences in the location both intracellularly and regionally of the splice variants.

For example the MOR-1 or MOR- C are located within the cells of the DRG and spinal but not together. Also, MOR-1 is located both pre and postsynaptically and MOR-C is located is just located presynaptically (Abbadie et al 2000).

Other reasons to explain the different bands of MOR expression may be post translational changes, alternative promoters or splice variant differences. Focusing on the 50 kd band, there is a significant increase in expression of peripheral MOR in the younger naïve neonate as well as following inflammation in both the younger and older age animal. A second conclusion is that the 50kd band is more commonly expressed in the neonate and the 70 kd band in the older animal.

The next important step would be to determine the exact cellular location of the peripheral opioid receptors using a pan neuronal marker such as PGP 9.5 and whether the peripheral opioid receptors are functional – this could be analysed using calcium imaging.

Clinical Implications

This study had shown that there is an up regulation of peripheral MOR following inflammation and that this up regulation is significant in the youngest animal in parallel to the findings of Beland and Fitzgerald (2001), in the developmental regulation of MOR in neonatal DRGs. It is reasonable to hypothesize; therefore, that the application of topical morphine should be more effective when applied to inflamed skin in the young rat pups but this would be a potential subsequent study to perform in order to confirm or dispute this hypothesis

Clinically, the developmental regulation in the presence of peripheral MOR may have implications for the use of topically or peripherally applied opioids in humans. There are limitations to extrapolating data from animal studies to man, but the sequence of events which occurs in the developing CNS of the rat, has some similarities to that of the human; P0 in the rat is approximately equivalent to a 24 week premature human neonate; P7 to an infant and P21 to an child/adolescent.

The data predicts that opioids will be especially effective when applied topically to inflamed tissue as the number of opioid receptors is increased under these conditions.

The increased opioid expression on sensory nerve endings should lead to better pain relief and the increased opioid receptor expression on immunocytes may also relieve inflammation. The data also predicts that, since opioid expression is already higher in younger subjects, the effect may be even greater in young patients.

Chapter 5 describes the flaws noted in this work and essential follow on studies required to enable a clearer understanding of precise location of the MOR receptors as well as the specific clinical implications.

CHAPTER 4: CLINICAL STUDY

4.1 BACKGROUND

My previously published case report (Watterson et al 2004 - see appendix) discusses the small pilot study, which was conducted prior to the main research project. It is a small case series, which describes the use of topical morphine gel in two teenagers with dystrophic EB. The results showed benefit in reduction of acute pain, following the morphine application and this benefit continued after prolonged use (five months) in one of the cases. There were no adverse effects following the use of the gel in these two cases. Following this success, I felt it would be valuable to expand upon the case studies with a much larger, randomised controlled study, in order to evaluate further as to whether there is reduction of other pain types using EB as the pain model. First I am going to briefly discuss the difficulties in conducting essential clinical studies, in particular randomised controlled trials in children and young people.

4.2 CONDUCTING CLINICAL TRIALS IN CHILDREN

Clinical practice should be governed by sound evidence and clinical trials in paediatric population have led to significant improvements in their health care. However this improvement tends to be clustered around certain childhood conditions such as cancer, where well organised multi - centre trials based on internationally agreed protocols, with strict review processes, have successfully recruited large numbers of patients. Conducting research in children is more challenging than in adults for a number of reasons related to the small number of patients available for trials in comparison to adults. There may also be difficult consent issues, stringent ethical board review procedures, lack of incentive for funding, parental anxieties, or even doctors' perceptions that research involves extra work and time for recruitment (Menson et al 2004). Consequently there is a lack of comparable randomised controlled trials (RCTs) in pain research for children and within the Cochrane Central Register of Controlled Trials, there are approximately 700 randomised trials of analgesics in children compared to well over 5000 such trials in adults (Cochrane Collaboration).

Unfortunately as a result, clinicians may need to extrapolate results from adult research, which is highly inappropriate, especially in the field of pain research, where pharmacokinetic and pharmaco-dynamics vary so greatly with age. The risks as well as the benefits need to be carefully considered in this group of vulnerable young patients. It is a well - known phenomenon that there are inclusion benefits for all participants in RCTs, the “Hawthorne effect” i.e. people’s behaviour or performance is noted to be altered whilst been observed in a situation and this alteration in behaviour is usually noted to be an improvement (Adair 1984). This advantage may be volunteer bias, but could also be as a result of better monitoring of trial patients.

On a local level, better education for doctors and nurses is required, regarding the benefits of trials and the risks of using medications, interventions which have not been adequately trialled for use in the paediatric population. Researchers have to consider ways of enhancing recruitment of children and young people to trials, such as keeping hospital/clinic visits to a minimum and reducing the need for extra blood samples. On a more national (Royal College of Paediatrics and Child Health Guidelines for Good Practice 2001) and international level, the EU Medicines for Children legislation has ordered for more clinical trials involving paediatric medications to be conducted which are age specific, so that there is increased efficacy and safety of medicines used in children (Choonara 2000 ; Sammons et al 2004).

4.3 CLINICAL TRIAL DESIGN

I had the opportunity of conducting a RCT which was placebo-controlled, double-blinded and with crossover design, providing an ideal design for any clinical study.

- **Crossover** This study used the patients as their own controls therefore avoiding difficulties with mismatching. In a crossover trial, the response of intervention A is compared to that of intervention B. The major limitation is, whether a carryover effect exists between the two treatment periods and to avoid this, a wash-out period is necessary and it is essential to test the data for carryover and if this exists, then the outcome of the interventions will also be affected depending on their sequence (period effect).

- **Placebo-controlled** It is a huge benefit to design a trial with placebo control in order to minimise bias. This crossover study ensures that each patient however not only trials the drug but also the placebo thus removing any ethical risk from the research. It has been historically documented that the placebo effect may occur in up to 33% of subjects (Beecher 1955). Particularly in studies of pain research, functional imaging has detected a placebo response (Petrovic et al 2002), one mechanism of which is thought to be secondary to release of endogenous opioids, as the placebo response may be halted by a naloxone injection (Sauro and Greenberg 2005).

4.3.1 Objectives: To investigate the efficacy of peripherally applied (topical) morphine in a model of paediatric inflammatory pain using patients with EB.

4.3.2 Design: Randomised, placebo-controlled with crossover design and blinding of participants, investigators and assessors. The trial took place over a four-week period with, two weeks for placebo application and two weeks for morphine application.

4.3.3 Power calculations and sample size

It is conventional to have a pre-calculated power and sample size prior to commencing the study. Any research study relies on the results based on a random sample of the population and then inferences are made on the entire population. Power calculations are performed to calculate a sample size which may prevent a type I (that is wrongly rejecting the null hypothesis) or type II error (that is wrongly accepting the null hypothesis). Interim or post hoc calculations may also be performed but are less effective. My trial was a multi-phasic randomised controlled trial involving a rare condition in a paediatric population and following advice from statisticians it was considered not appropriate to calculate power or sample size as the numbers of patients who have the disorder even in the entire population are small.

4.3.4 Setting: The study took place at Great Ormond Street Hospital, London and within the patients' homes, throughout the UK.

4.3.5 Participants

Inclusion criteria:

- Patients in the UK, aged 1 to 18 years, with a diagnosis of dystrophic Epidermolysis Bullosa (DEB) were eligible for the trial.
- Patients must have all or part of their routine care at Great Ormond Street Hospital EB department.
- Patients should be experiencing either acute or chronic pain at time of recruitment as well as having at least 1 area of broken skin (**Fig 4.1a** shows a painful skin lesion in EB and **Fig 4.1b** shows the extent of skin lesions covered with dressings in this young patient with DEB).

Exclusion criteria:

- patients who had a previous adverse reaction to opioids
- those with renal or hepatic insufficiency
- known parental drug misuse.



Fig 4.1 a A painful skin lesion in EB



Fig 4.1b Typical dressings required in EB

4.4 RECRUITMENT

The project, including all documentation, received approval by the local research ethical committee. A list of all eligible patients was obtained from the Epidermolysis Bullosa patient list held in the dermatology office at Great Ormond Street Hospital. For each eligible child, it was then noted when the next inpatient visit had been arranged, so that approximately two weeks prior to this scheduled visit, a parental and child information leaflet was posted to the family, providing information regarding the study. A covering letter addressed personally to the child was also included.

One week after posting the letter, I followed this up with a telephone call with an aim of allowing the parent or child to ask any further questions regarding the written study.

At this stage I asked the family to consider participating in the study and if they were interested, I would prepare the materials required for the study, prior to their admission and would meet them on the ward. If the parents or patients declined to be involved in the study, I reassured them that this would have no impact upon their current or future management.

I also attended the EB outpatient clinics within Great Ormond Street Hospital to recruit any eligible patients and I also discussed the study details with the EB nurse specialist in Scotland and informed her regarding the trial. These patients had joint care with Great Ormond Street Hospital and were also all eligible to be involved in the study (See Fig 4.3 for recruitment details).

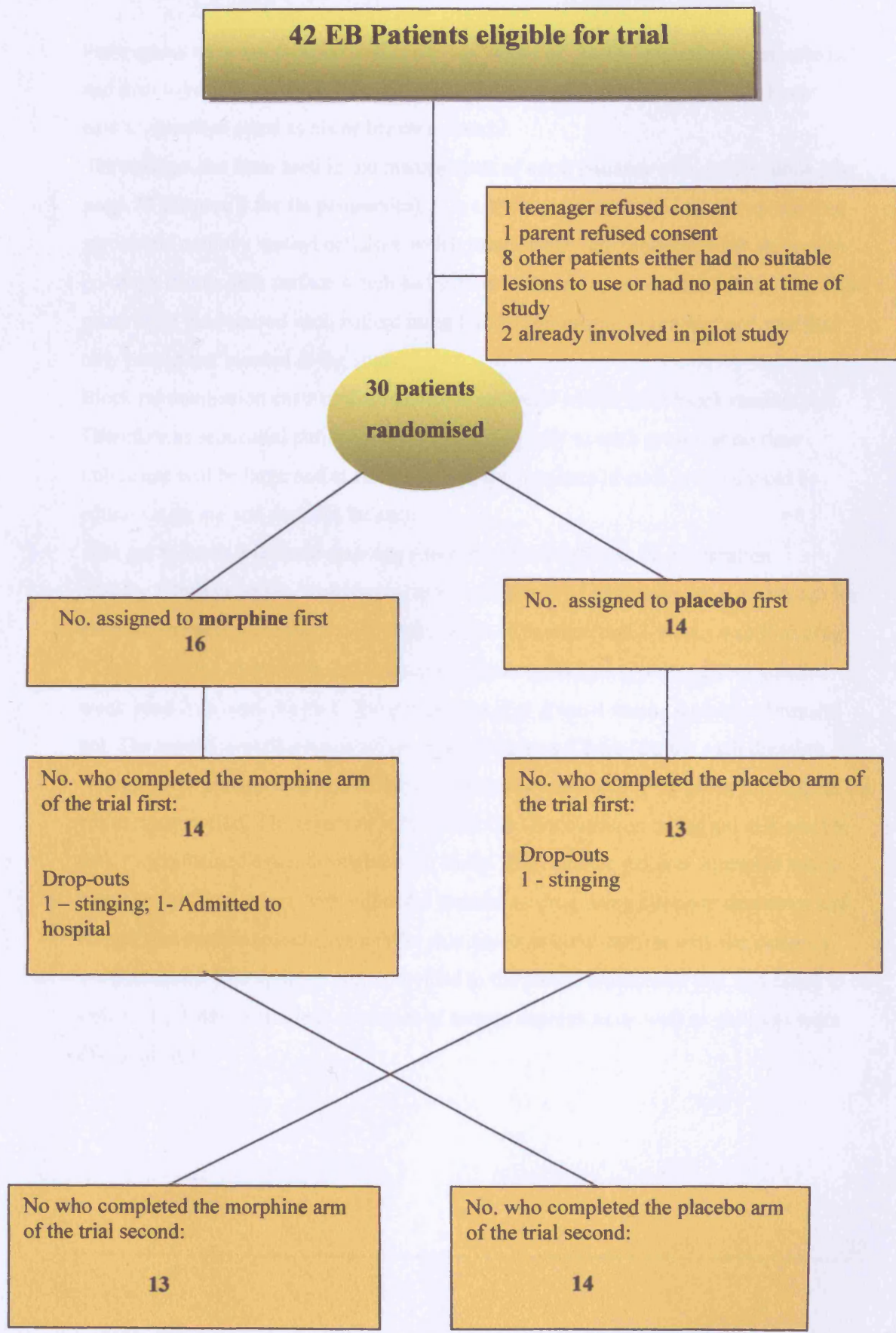
4.5 CONSENT AND FIRST APPLICATION OF GEL

The first application of the gel took place in hospital or at home in my presence. After the parents were fully informed, written consent was obtained according to LREC requirements and a general clinical examination took place to ensure the child was well enough for participation in the study. The child was asked to choose one particular painful skin lesion, which would be used for gel application throughout the study. This lesion was measured and photographed. The child was able to continue any regular analgesia as well as breakthrough medication and it was asked, that this should be documented by the parent throughout the study.



Fig 4.2 Mother applying the gel to a dressing

Fig. 4.3 Participant Flow Through Study



4.6 RANDOMISATION AND INTERVENTIONS

Participants were randomised to receive two weeks of placebo mixed with Intrasite gel and then to receive the drug mixed Intrasite gel for two weeks or vice versa. Each patient therefore acted as his or her own control.

Intrasite gel has been used in the management of adult patients with skin wounds (see **page 72 chapter 2 for its properties**). It is a hydrogel composed of water, propylene glycol and carboxy methyl cellulose which interestingly are thought to aid absorption of drugs from a skin surface which had suffered a burn (Aoyama et al 1984). The chief pharmacist randomised each patient using block randomisation method and was the only person not blinded in the study.

Block randomisation ensures that balance is enforced within each block randomised. Therefore as sequential patients are distributed equally to each group, at no time imbalance will be large and at certain points, the numbers in each group should be equal- i.e. group and periodic balance.

The gel mixture was made up using either morphine sulphate IV preparation (10 mg / 2ml) or saline, which acted as the placebo. The pharmacy gave a package for each patient which consisted of 2 weeks worth of placebo and 2 weeks worth of drug (0.3mg /kg), all identical in appearance and made up in 2ml syringes either labelled week 1 and 2 or week 3 and 4. The patient was also given 4 weeks supply of Intrasite gel. The agreed overall dose of the morphine mix was 0.3mg / kg for each dressing change so the same proportion of the total mix was instructed to be given throughout the entire study period. The essential point is that the concentration of the gel mix and the dose / kg remained equal throughout the study. The Intrasite gel was squeezed into a small gallipot and mixed with either the placebo or drug using a tongue depressor and the gel was applied to a chosen painful skin lesion in combination with the patient's usual dressing. The syringes were provided to the parent in a locked box and asked to be kept in the fridge. Sufficient quantities of tongue depressors as well as gallipots were also provided.

4.7 OUTCOMES AND DATA COLLECTION

4.7.1 Predefined primary outcomes

The primary outcomes were pain levels prior to, throughout dressing changes and one hour post dressing change.

- The pre dressing change pain scores were used to define *background pain* throughout the study period as the earliest time following a dressing change to the next pre pain score was 24 hours.
- The pain during the dressing changes was used to quantify *procedural or incident pain*.
- The post dressing change pain score (1 hour post) were used to define *post-procedural pain* through the study period.
-

4.7.2 Predefined secondary outcomes

The secondary outcomes were appearance of the skin lesion through the 4 week period as well as quality of life. An adverse effect profile was also recorded.

4.7.3 Data collection

Following consent, a patient history sheet was completed, which included demographic details as well as current analgesia, current symptoms associated with EB, any skin current infections. On a body outline diagram (see appendix), the skin lesion was documented with its dimensions. The patient was then shown their diary, which had written instructions regarding the making up of the gel mixture, and the researcher's contact details.

The patient or parent were asked to complete pain diary sheets (see appendix), which consisted of visual analogue scales pre, during and 1 hour post dressing change as well as documenting any skin infections, or incidents which could exacerbate pain such as a fall. Any emergence of adverse effects possibly related to the opioids such as nausea, vomiting, constipation were also documented on a daily basis and given a score out of 10 if present (10 = worst).

On a weekly basis the parent was asked to comment and document whether the lesion looked worse, the same or better than at the start of the study and using an amended (with permission) version of the Brief Pain Inventory (Cleland-see appendix).

Aspects of quality of life were documented such as schoolwork, relationships, and sleep. As the lead researcher, I recruited all the patients and followed up with weekly phone calls to ensure that there were no parental concerns and also to remind parents to complete all the diary sheets. In the majority of patients I made a home visit half way through the study, to encourage both the child and parent to complete the trial, as well as to document any extra, relevant information. The children lived throughout Great Britain including Wales, Scotland and Ireland. I gave the family a stamped addressed large envelope to return all the diary sheets after completion of the study.

4.7.4 Statistical Analysis

Due to the complex nature of this multi-phasic trial with composite outcomes, the statistical analysis was discussed and advice given by the statistics team, led by Professor Tony Cole, at the Institute of Child Health.

Each study day comprised of three assessment sessions:

- 1) Pre application of either morphine/placebo
- 2) During application of morphine/placebo
- 3) Post application of morphine/placebo

As the study was a crossover design, it was first essential to perform significance testing on the following:

- Carry-over effect
- Treatment effect
- Period effect (i.e. is there a change over time irrespective of treatment?)

(Jones and Kenward 1989)

The significance tests chosen to perform these analyses were two - tailed paired t tests and were performed for the background, incident pain and post-procedural pain scores. I also analysed separately, the pain reduction of the pre application scores (background pain) from the first baseline pain scores using paired t tests, and compared the results of those who had daily dressings with those who had less frequent dressings.

Period 1 is week 2 and period 2 is week 4. Two way ANOVA with post hoc Bonferroni test were also performed to analyse the confounding variables of size of lesion and age of child on the pain scores. Both Excel XP and Prism 3.0 were used to perform statistical analyses.

4.8 RESULTS (Fig 4-2 Participant flow chart)

At the time of the trial, out of 42 children at Great Ormond Street with DEB, there were a possible 42 patients who could have potentially been recruited for the trial. However, at time of recruitment, 8 of these children did not have appropriate lesions or did not complain of pain. 2 of the teenagers had already participated in the pilot study and so were not eligible. Only 1 teenager and 1 parent did not give consent for the trial. The parent who refused consent had a child who was just 1 year old and felt frightened about enrolling their son at such a young age. The teenager gave no particular reason for not consenting.

A total of n=30 patients were enrolled. A total of six assents were given by young people aged 12-18.

Of these 30, 2 of those patients randomised to morphine for weeks 1 and 2, did not complete the study as 1 patient was admitted to hospital because of an intercurrent illness and 1 complained of stinging from the gel on day 1 so no data was collected. 1 patient who was initially randomised to saline for weeks 1 and 2 did not complete due to stinging from the gel, again without any collected data.

Of the remaining 27 participants, a total of 24 completed data sheets were returned and analysed for the study. Fifty per cent of the subjects were female and fifty per cent male. **Table 4.1** shows the patients data for age, weight and the variation of surface areas of lesions used in the trial. **Fig 4.4** displays how frequently the dressings were changed in the patients and **Fig 4.5** shows which analgesics, either singly or in combination were taken by the patients prior to and throughout the study

	Lower range	Upper range	Mean	Median
AGE (yrs)	1.74	16.95	9.28	9.19
WEIGHT (kg)	12	70	26.7	25.5
SURFACE AREA OF LESION (cm ²)	18	1320	231.77	102

Table 4.1 Patient Characteristics n =30

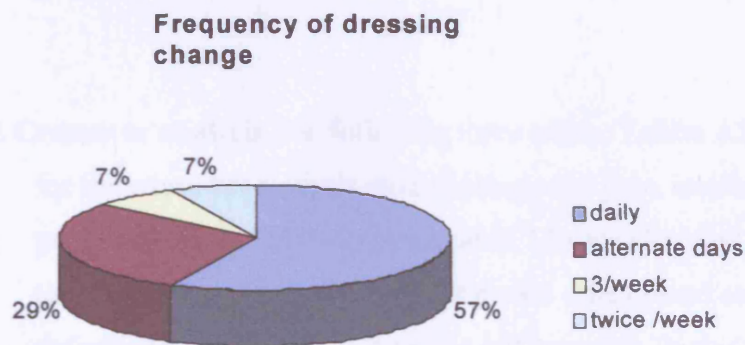


Fig 4.4 Frequency of dressings

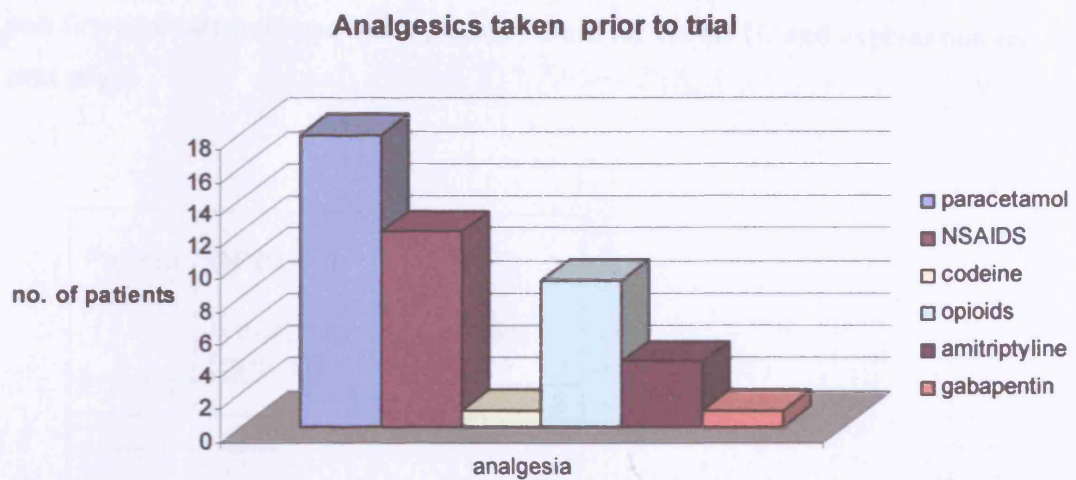


Fig 4.5 The number of patients who were prescribed a variety of analgesics prior to and throughout the study. Some patients consumed more than one type of pain relief medication.

4.8.1 Crossover analysis The following three tables, **Tables 4.2 to 4.4** provide the data for the crossover analysis of the background pain, incident pain and post procedural pain. Following each table, I have calculated and concluded whether there is a carry-over, treatment, or period effect based on Jones and Kenward definitions in *Design and Analysis of Crossover Trials* (1989) as recommended by the Institute of Child Health of London Statistics Department.

Table 4.2 Background pain (Values tabulated = VAS (mm / 100) day 8/22, 24 hours post first application of morphine / placebo). **Data for 1A 1B 1C and explanation see next page**

Patient no.	GROUP 1	
	A morphine (period1)	B placebo (period2)
1	6	3
2	0	27
3	7	29
4	3	5
5	0	0
6	29	10
7	5	11
8	6	9
9	0	0
10	9	63
11	0	15
12	0	0
13	27	14
	GROUP 2	
	B placebo (Period 1)	A morphine (Period 2)
14	0	0
15	14	4
16	0	0
17	79	47
18	84	56
19	43	1
20	16	10
21	9	0
22	60	27
23	6	8
24	3	20
25	15	0

1A) Carry over effect

The null hypothesis being tested is that the carry over effects are equal between groups one (A = morphine first) and groups two (B = placebo first). This is tested, by comparing the sum of the values over both treatment periods between groups 1 and 2. A 2-sample t test yields a p value of 0.18 and the conclusion is that the carry over effect is not significant.

1B) Treatment effect

We can only test this if the carry over effects are equal or not significant. The null hypothesis here being tested is that the treatment effects are equal. This is tested by comparing the difference, between period 1 and period 2 for the 2 groups. For group 1 this will be time spent on treatment A minus those after spent on treatment B. For group 2, differences will be for after time spent on time B minus those, after time spent on treatment A.

A 2-sample t test of the differences yields a p value of 0.0113.

The outcome of the 2-sample t test gives a 95% confidence interval for the difference - 20.46 of (-35.83, - 0.5093). To estimate the size of the treatment effect, we take half of the difference between the average differences for each group and hence a 95% confidence interval for this would be -10.23 (-17.41, - 0.254). **Thus it can be concluded that morphine has a significantly better effect on background pain reduction than placebo.**

1C) Period effect

It is of interest to formally test the period effect; i.e. is there a change over time irrespective of treatment. This is performed by testing the difference between treatment A and B for the two groups.

For group 1 the differences will be for after time period 1 minus those after time period 2; for group 2, differences will be for after period 2 minus those after period 1.

A 2-sample t test of the difference yields a p value of 0.46. The 95% confidence interval for the estimated period effect of 2.25 is (- 4, 10.45). This shows that there is not a significant period effect.

Summary of crossover analysis for background pain

Following the crossover analysis, there was a significant treatment effect with morphine on pain reduction of background pain, without carryover or period effect ($p= 0.0110$).

There is no such significant treatment effect with placebo.

It is clear from **Fig 4.6** that topically applied morphine gel provides significant pain reduction over at least a 24 hour period. This is not true of pain reduction with placebo.

However the reduction is only significant when the dressings are changed daily (**Fig 4.8**; $p<0.02$) and although there is a clinical improvement in pain for those children who have less frequent than daily dressings, this was not a significant result (**Fig 4.7**).

This group of children may need higher doses /kg of morphine applied to achieve a significant pain reduction but further dosing studies should be performed to decide this.

The age of the child or the skin lesion size does not effect the background pain reduction (**Figs. 4.9& 4.10**).

Table 4.3 Incident / Procedural pain (Values tabulated = Mean of total VAS (mm /100)

week 2 / 4

during dressing change).Data for 2A 2B 2C and explanation next page

Patient no.	GROUP1	
	A morphine (period1)	B placebo (period2)
1	59	39
2	28	14
3	19	16
4	4	5
5	36	16
6	20	10
7	60	77
8	77	60
9	0	0
10	68	83
11	27	19
12	35	0
13	42	31
	GROUP2	
	B placebo (period1)	A morphine (period2)
14	4	3
15	17	8
16	9	50
17	34	37
18	75	62
19	81	1
20	32	34
21	100	94
22	21	87
23	51.5	61
24	90	89
25	58	5

2A) Carry over effect

A 2-sample t test yields a p value of 0.228 concluding that the carry over effects are not significantly different.

2B) Treatment effect

A 2-sample t test yields a p value of 0.655 showing that the treatment effect is not statistically significantly different between morphine and placebo for procedural pain

2C) Period effect

A 2-sample t test gives a p value of 0.305 revealing that there is no significant period effect

Summary of crossover analysis for incident pain

The crossover analysis demonstrates that there is no significant treatment, carryover or period effect with reduction of incident pain with either morphine or placebo. Children with EB have severe pain at the moment the dressing is removed and anecdotal evidence suggests that, even higher doses of oral opioids do not reduce this pain. This may be due to a combination of the immense anticipatory fear as well as the pain and sedation is often more beneficial in combination with an opioid in this situation. Again the size of the skin lesion and age of child do not affect the above results (**Figs 4.11, 4.12**).

Table 4.4 Post procedural pain Values = mean VAS (mm /100) week 2 / 4, 1 hour post dressing change. Data for 3A 3B 3C see explanation next page

Patient no.	GROUP 1	
	A morphine (period1)	B placebo (period2)
1	7	0
2	0	0
3	0	0
4	4	5
5	1	0
6	0	0
7	9	16
8	6	4
9	0	0
10	6	3
11	9	
12	14	0
13	25	13
	GROUP2	
	B placebo (period1)	A morphine (period2)
14	0	0
15	10	3
16	0	6
17	36	23
18	15	18
19	2	0
20	14	10
21	6	3
22	50	70
23	8	9
24	3	1
25	0	0

3A) Carryover effect

A t test yields a p value of 0.175 concluding that the carry over effect for post procedural pain is not significant

3B) Treatment effect

A t test gives a p value of 0.64 showing that there is no significant treatment effect

3C) Period effect

A two-sample t test yields a p value of 0.0216, with an estimated size of this effect, giving a 95% confidence interval of 3.13 of (0.49, 5.6). Thus there is a statistically significant effect of whether morphine or placebo is given first or second for post procedural pain.

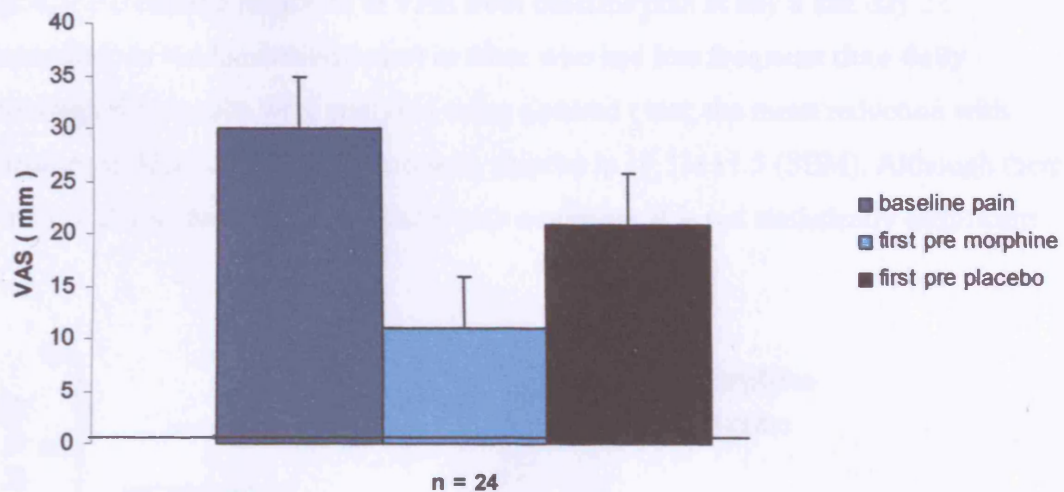
Summary of crossover analysis for post procedural pain

The crossover analysis demonstrates that there is no significant treatment, carryover or period effect with reduction of post procedural pain with either morphine or placebo and again the age of the child and the size of the skin do not affect the above results (**figs 4.15 and 4.16**). However analysing all the patients' pain scores together for both day 8/22 and the mean scores for period 1 and period 2, both morphine and placebo significantly reduced post procedural pain one hour after dressing change (**figs 4.13, 4.14**)

4.8.2 Overall background pain reduction from baseline pain on first application of morphine and in relation size of lesion and age of patient

Fig 4.6 is the data of all children analysed together but the data from those children who had daily dressings was also analysed separately from those who had less frequent dressings as it is not appropriate to compare one pre value after 24 hour application of gel (daily dressings) with a child who may have had a dressing 72 hours previously (less frequent dressings) (Figs 4.7 & 4.8).

The mean pre scores of period 1 (week2) and period 2 (week4) were analysed in relation to size of lesion and age of child, to investigate whether these were both potential confounding variables (Figs 4.9 & 4.10).



P<0.01

Fig. 4.6 VAS scores with morphine and saline (placebo) at day 8 and day 22 (depending on randomisation order). The data was analysed using paired T test showing that there is a significant difference in baseline pain reduction with morphine, $p < 0.01$ with a mean VAS of 11.96 ± 3.7 (SEM), compared to a mean VAS of 19.08 ± 5.12 (SEM) with placebo.

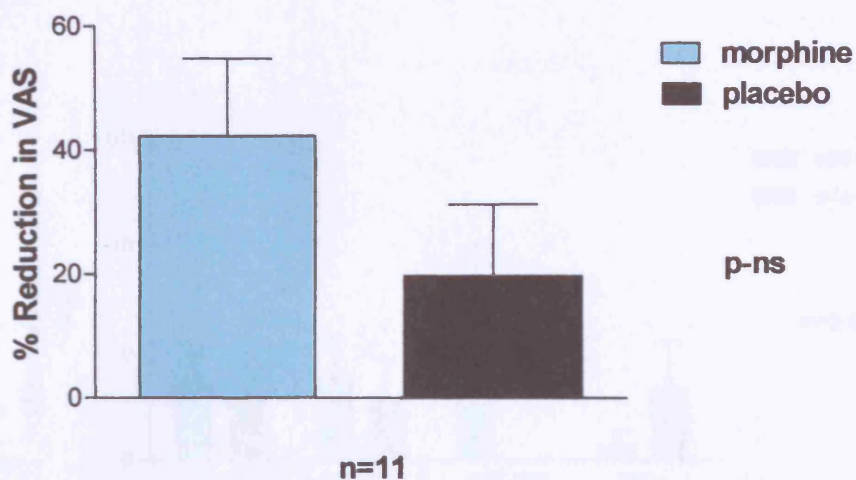


Fig. 4.7: Percentage reduction in VAS from baseline pain at day 8 and day 22 (depending on randomisation order) in those who had **less frequent than daily dressings**. The results were analysed using a paired t test; the mean reduction with morphine is 42.27 ± 12.5 (SEM) and with placebo is 19.53 ± 11.5 (SEM). Although there is a clinically a greater pain reduction with morphine, it is not statistically significant.

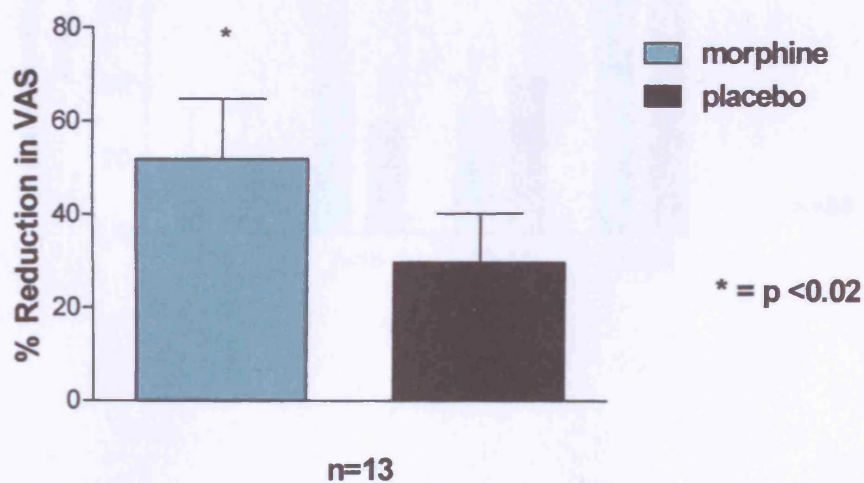
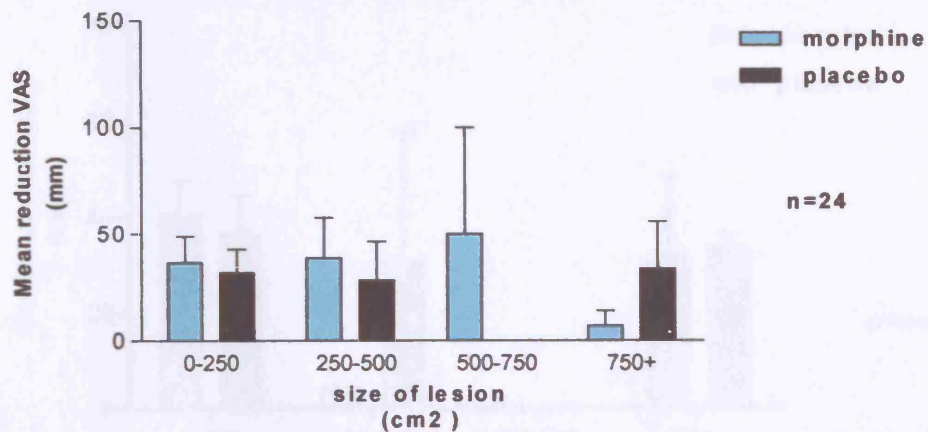
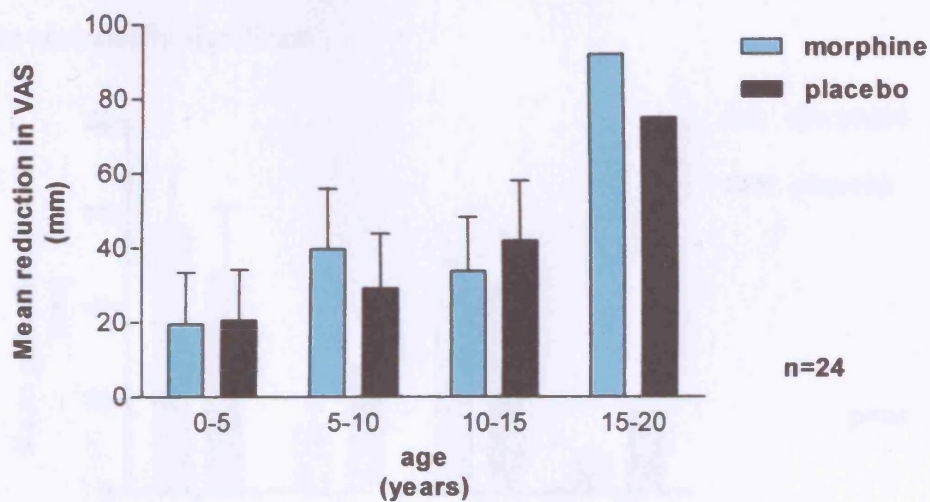


Fig. 4.8: Percentage reduction in VAS from baseline pain at day 8 and day 22 (depending on randomisation order in those who had **daily dressings**. The results were analysed using a paired T test and the mean reduction with morphine was 51.8 ± 12.9 (SEM), compared to mean reduction of 29.69 ± 10.57 (SEM) with placebo.



p=ns

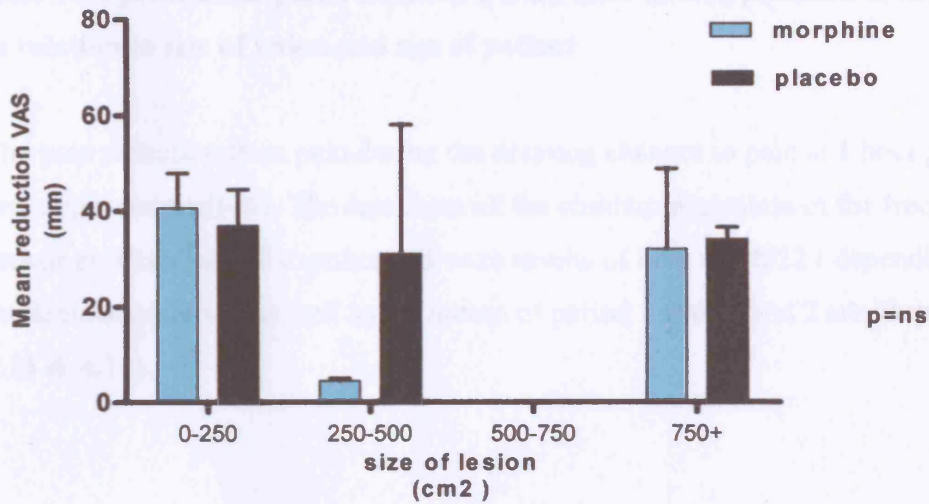
Fig 4.9: The mean reduction in VAS from baseline pain to the mean pre pain scores during period 1 and 2, with morphine and placebo in relation to size of lesion. The results were analysed using a two way ANOVA and were not statistically significant.



p=ns

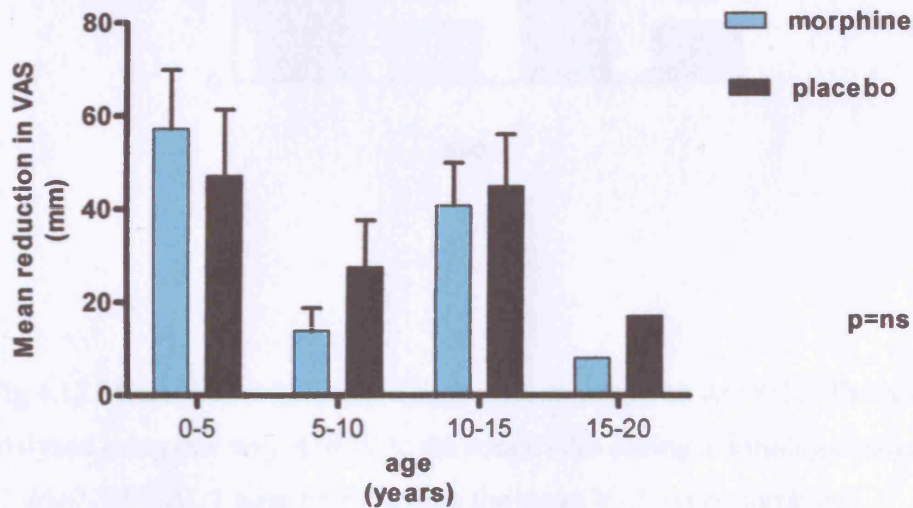
Fig 4.10: The mean reduction of VAS from baseline to the mean pre pain scores during period 1 and 2, with morphine and placebo in relation to age of patient. The results were analysed using a two way ANOVA and were not statistically significant.

4.8.3 Overall incident pain reduction in relation to size of lesion and age of patient



n=24

Figs 4.11 Mean reduction in VAS during a dressing change during period 1 and period 2 and in relation to size. The results were analysed using two way ANOVA and were not statistically significant.



n=24

Fig 4.12 Mean reduction in VAS during a dressing change during period 1 and period 2 and in relation to age.

4.8.4 Post procedural pain reduction 1 hour after first application of morphine and in relation to size of lesion and age of patient

The pain reduction from pain during the dressing changes to pain at 1 hour post dressings was analysed. The data from all the children regardless of the frequency of dressings was analysed together and were results of both day 8/22 (depending in randomisation order) as well as the means of period 1 and period 2 are illustrated (figs 4.13 & 4.14).

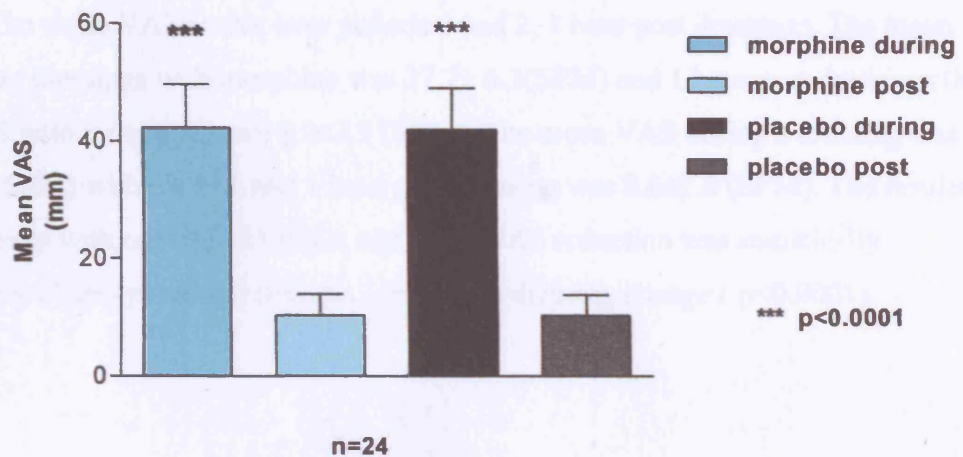


Fig 4.13 Mean VAS during and 1 hour post dressings on day 8/22. The results were analysed using one way ANOVA; the mean VAS during a dressing change was 42.46 ± 7.3 (SEM), 1 hour post dressing the mean VAS with morphine was 10.25 ± 3.5 (SEM). The mean VAS during dressings with placebo was 42.5 ± 7.0 (SEM) and 1 hour post dressings; the mean VAS with placebo was 10.29 ± 3.47 (SEM). The mean VAS reduction 1 hour post dressing change was statistically significant ($P < 0.0001$) with morphine and placebo.

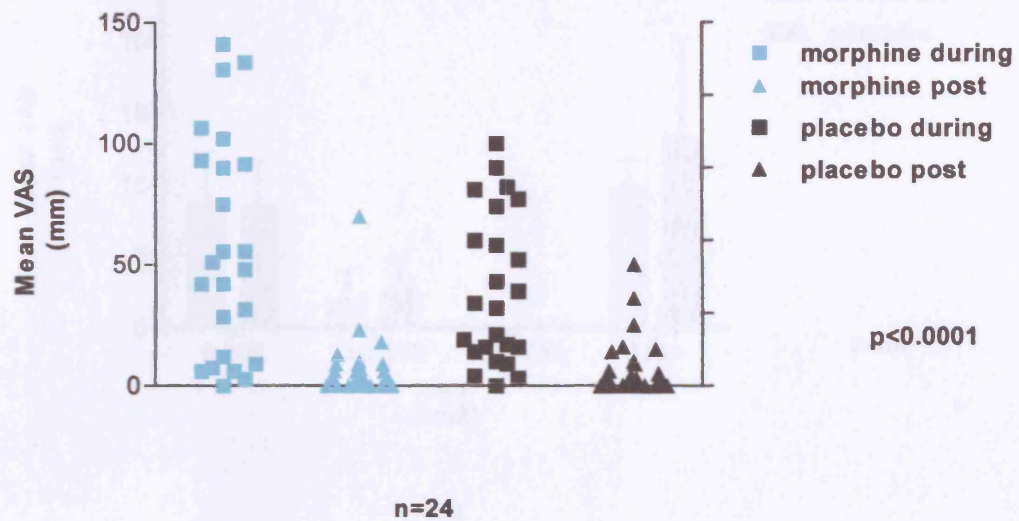
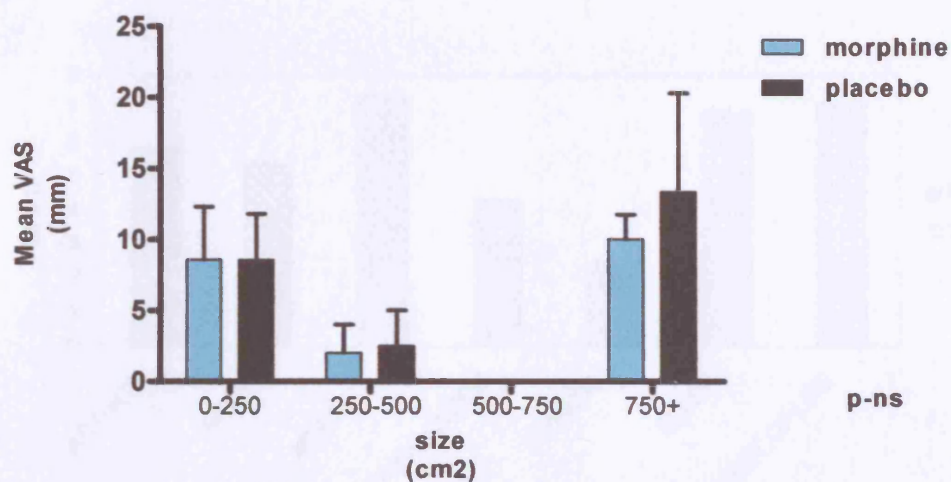
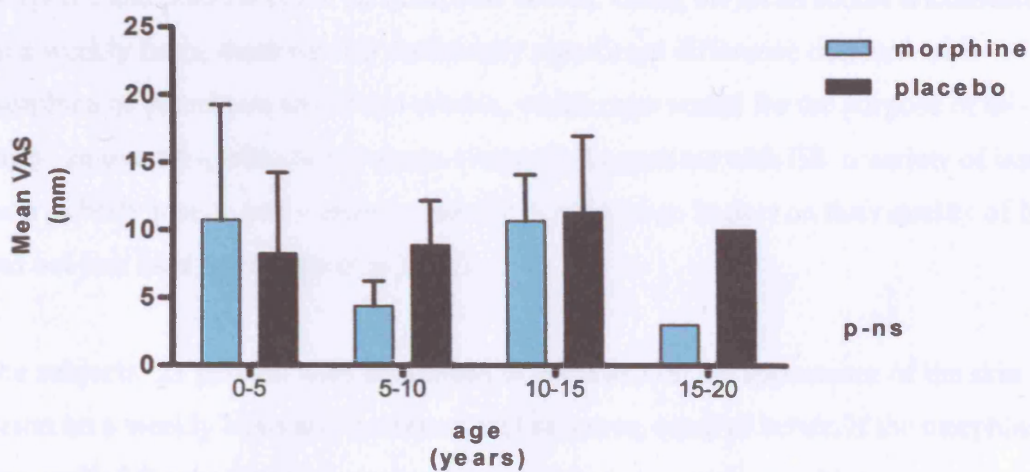


Fig 4.14 The mean VAS scores over periods 1 and 2, 1 hour post dressings. The mean VAS during dressings with morphine was 37.7 ± 6.1 (SEM) and 1 hour post dressings the mean VAS with morphine were 8.2 ± 2.9 (SEM). The mean VAS during a dressing was 39.9 ± 6.3 (SEM) with placebo and 1 hour post dressing was 8.6 ± 2.6 (SEM). The results were analysed with one way ANOVA and mean VAS reduction was statistically significant with morphine and placebo 1 hour post dressing change ($p<0.0001$).

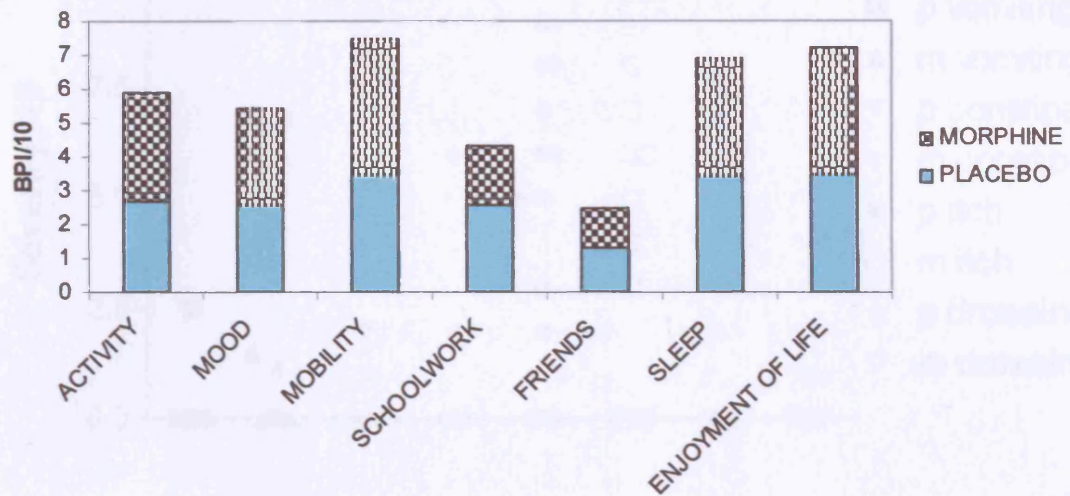


n=24



n=24

Figs 4.15 & 4.16 Mean VAS post dressing change in relation to size of lesion and age of patient during periods 1 and 2. The results were analysed using two way ANOVA and are not statistically significant.



n =20

Fig 4.17 BPI outcome scores. There was no statistically significant difference between morphine and placebo in the BPI outcome scores. Using the mean scores documented on a weekly basis, there was no statistically significant difference detected with morphine or placebo in any of the criteria, which represented for the purpose of this study, an overall quality of life score. Generally in patients with EB, a variety of issues such as body image, self – esteem, mobility have a huge impact on their quality of life and not just their pain (Fine et al 2004).

The subjects' 21 parents were also asked to comment on the appearance of the skin lesion on a weekly basis and document this as worse, same or better. If the morphine was applied for the first two weeks, the skin appearance with morphine was comparable with the placebo throughout. The morphine may have provided some initial healing of the lesion which continued throughout the placebo arm. If the patient was randomised in receiving placebo first, there was a much better improvement on skin appearance with morphine in weeks 3 &4 than with placebo in the first two weeks. Morphine is known to have anti-inflammatory properties and the application of peripheral opioids directly to the peripheral opioid receptors may induce skin healing which would be a great additional benefit for EB patients.

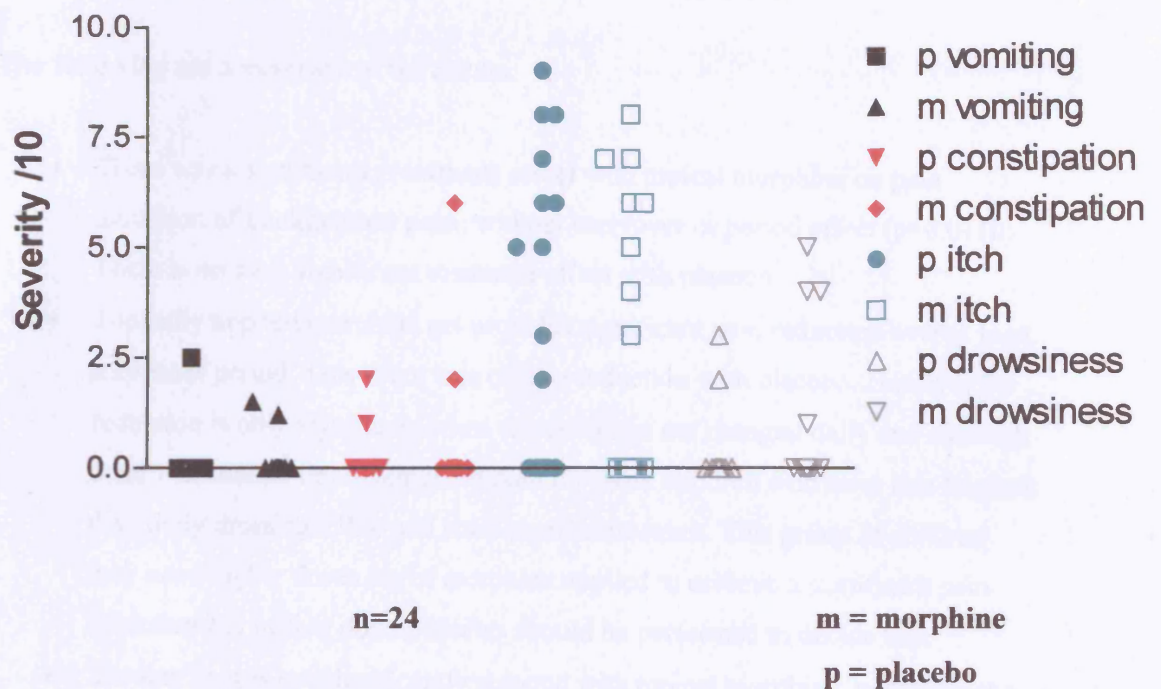


Fig 4.19 Possible opioid adverse effects

This shows the adverse effects graded out of 10, noted with placebo and morphine mixed with Intrasite gel. The most severe effect was itch, followed by drowsiness with both saline and morphine. These 2 symptoms are extremely common problems experienced by patients with EB and may not be to the topical opioid.

The differences in effects with placebo and morphine were not significant. 54% of the subjects scored 0/10 in relation to at least any of the three adverse effects asked to be recorded. The most frequent side effects noted were itch and to a lesser extent drowsiness; there was no significance difference with the placebo or morphine gel, which would firmly suggest that these symptoms were in no way related to the study. Itch is a very common symptom in EB as well as drowsiness secondary to anaemia, poor nutritional status and immunodeficiency. The relative low number of side effects suggests that the topical morphine is not systemically absorbed in sufficient quantities.

4.9 SUMMARY OF RESULTS

The following are a summary of the results.

- There was a significant **treatment effect** with topical morphine on pain reduction of **background pain**, without carryover or period effect ($p=0.0110$). There is no such significant treatment effect with placebo.
- Topically applied morphine gel provides significant pain reduction over at least a 24-hour period. This is not true of pain reduction with placebo. However the reduction is only significant when the dressings are changed daily and although there is a clinical improvement in pain for those children who have less frequent than daily dressings, this was not a significant result. This group of children may need higher doses /kg of morphine applied to achieve a significant pain reduction but further dosing studies should be performed to decide this.
- Incident pain is not significantly reduced with topical morphine, which is not a surprising result as it is evident that a dressing change is an acutely painful procedure
- Post procedural pain is significantly reduced by both placebo and morphine but this is most likely due to the fact that the acute pain from the dressing change is over and the patient experiences no significant relief at this time point with the morphine gel.
- There appears to be a consistent pain reduction with placebo and this required further exploration. Intrasite gel may in fact have healing and therefore pain relieving properties and this should be studied.
- The age of the child or the skin lesion size does not significantly affect the background/incident or post procedural pain reduction.
- Using the BPI, quality of life is not significantly improved by use of topical morphine
- The appearance of the skin lesion under the topical morphine is not enhanced by either topical morphine or placebo
- Itch and drowsiness were noted as the most common side effects experienced, but there were no significant differences between topical morphine and placebo

4.10 CONCLUDING REMARKS TO CLINICAL TRIAL

This is the first randomised control trial in children to investigate the use of peripherally applied opioids in children. It is also larger than the few similar trials, which have been performed, in the adult population (Zeppetella et al 2003; Flock et al 2003).

The benefits of topical morphine providing pain relief, are most evident in reduction of background pain, which is often the most difficult to control in this patient group.

However I noted a number of difficulties/pitfalls surrounding the trial design noted whilst conducting this trial, which I will discuss in the next chapter.

CHAPTER 5: CONCLUSION

Linking the two trials: Pitfalls noted during the studies: Future research.

Although my work consisted of two entirely different projects, this concluding chapter explains how the results of the laboratory study provide a basis for the clinical use of topical opioids. I will explain the association between the two projects. I will also discuss what the next stage should be in the laboratory work, in order to provide further crucial information. I will explore what pitfalls I have noted in the trial design of the clinical study and how the design could be improved, as well as what future clinical research should be carried out.

5.1 LINKING THE TWO TRIALS

The results I obtained following my laboratory work provided me with ideas of how to proceed with the clinical work. The fact that I detected the presence of MOR in neonatal, infant and young adult rat skin and that these receptors were regulated not only throughout development but also following a painful inflammatory stimulus, led me to concur that human infants and young people may indeed also possess identical receptors with similar properties.

I hypothesised that in accordance with the finding of the animal studies, MOR existed in the skin of human infants and children and therefore the application of peripheral morphine to the skin would be efficacious and regulated throughout development and post inflammation.

It is known that following neonatal cutaneous skin wounding such as a noxious inflammatory insult, there is hyperinnervation of the affected and surrounding skin tissue, leading to a greater number of peripheral sensory nerves sprouting up to the epidermis, in response to increasing nerve growth factor levels (NGF)(Reynolds et al 1997). This injury is thought to reduce pain thresholds and so produce a heightened pain response (hyperalgesia as well as allodynia in later life), as confirmed in a series of clinical trials by Taddio (Taddio et al 1997).

Therefore, infants and children with a diagnosis of EB, who are known to be susceptible to painful skin injury in the early neonatal period or even intrauterine period, should have hyperinnervation of the cutaneous sensory neurons in the skin of the affected and surrounding area based on the above research. Subsequently as the quantity of sensory neurons increase post inflammation, the quantity of MOR will also increase, as they are positioned on the peripheral sensory neurons (Stein 1993; Stander et al 2002). Also it is also known that MOR effects are enhanced during inflammation most likely secondary to perineural membrane disruption. The membrane is disrupted in early inflammation (Antonijevic et al 1995) and so provides evidence for the efficacy of peripherally applied morphine in disorders such as EB, where there are painful inflammatory lesions, thus enabling access of the topical morphine via the disrupted perineurium and interaction with MOR which are situated on the sensory neuron.

Therefore, in summary, following a painful inflammatory insult in skin tissue, there will be a proportionally increased efficacy of peripheral opioids for the following reasons;

- a) Hyperinnervation of region due to up-regulation of sensory neurons (Reynolds et al 1997)
- b) Proportional increase in MOR which are situated on the sensory neurons (Stander et al 2002)
- c) Perineural disruption allowing ease of opioid binding (Antonijevic et al 1995).

5.2 FURTHER LABORATORY STUDIES REQUIRED

The laboratory study demonstrated via direct immunofluorescence, the presence of mu opioid receptors (MOR) in cutaneous tissue of neonatal, infant and young adult rat models. The study also proved that there was significantly more staining for MOR in the youngest animals and post inflammation and this was postnatally regulated. This is in keeping with the postnatal regulation of central MOR, which have found to be located on cells of dorsal root ganglia (DRGs) of all diameter size in the first 3 weeks of life, but persist on the largest diameter cell bodies of the A β fibres in the youngest animals (Beland and Fitzgerald 2001).

These are the sensory neurons, which are sensitive to innocuous stimuli but in rat pups these neurons respond to both noxious and innocuous stimuli, thus further distinguishing between opioid function in the adult and in the immature central nervous system.

Again a significant number of these MOR expressed centrally on DRG neurons have been shown by calcium imaging to be more functional in the neonate rat in comparison to the adult (Nandi et al 2004).

Western blot analysis in my work, quantified the MOR protein levels and again demonstrated two distinct bands; the 50kd band expressed significantly in the younger age group and the total protein levels increased following inflammation. Interestingly this correlates with the changing receptor profile throughout development. I will now note the problems detected with the laboratory study with suggested follow up work.

Problem 1: Detection of precise location of opioid receptors. The exact location of the receptors was not investigated in this study but it was assumed that they would be located on the sensory nerve fibres, as well as the keratinocytes or immunocytes in the epidermis, dermis or sub-epidermal region, as previously demonstrated in adult human tissue from patients with atopic dermatitis and psoriasis (Stander et al 2002).

Follow on study required. The precise location should be demonstrated in neonatal and infant rat pups as a follow up study, using double immunofluorescence with MOR and a pan neuronal antibody such as PGP 9.5 or NF200, to demonstrate co-localisation both with and without and a post inflammatory insult such as carageenan.

Problem 2: Clinical effect of topical morphine in rat pups was not tested. Direct application of topical morphine was not tested on the rat pups due to time constraints and would have provided beneficial parallel information

Follow on study required. Further laboratory work should focus on application of the topical morphine to various ages of rat pups with an inflammatory wound and by using the techniques of electrophysiology/behavioural testing identification of the effects of the topical morphine at different ages.

Problem 3: No investigation of mRNA. Further work would help identify whether there is a similar biphasic up-regulation of m RNA for MOR in the neonatal and infant rat pup which has been studied in the adult rat model (Puehler et al 2004).

Follow on study required. This would involve performing Polymerase Chain Reaction.

5.3 FURTHER CLINICAL TRIALS REQUIRED

I noted a number of problems with the trial design on completion of the study which I would now like to highlight and then describe suggestions for future trials which could perhaps overcome these problems.

Problem 1: Application of morphine gel in those who have daily versus less frequent dressing changes was not trialled separately. It is not appropriate to compare analgesic effect of topical morphine for those who had daily dressings, versus those who had less frequent dressings, e.g. the same application of gel remained on some patients up to 4 days in those who had twice weekly dressings.

Follow on study required. Conduct separate trials to distinguish analgesic effect within groups who have same frequency of dressing changes per week.

Problem 2: No range of different doses trialled. Only one dose was trialled in this study and it would be worthwhile to repeat, using a range of doses, especially as the one dose was used on all sizes of lesions and it may be that larger lesions may in fact absorb the topical morphine more quickly than smaller lesions, therefore requiring a smaller dose or conversely larger lesions may require larger doses to achieve equitable analgesia to those with smaller lesions.

Follow up study required. Perform dosing studies using a range of doses and analyse against size of lesion.

Problem 3: The action of Intrasite gel was not separately studied on the skin lesions and may contribute to healing of skin lesions. It is unclear what action Intrasite gel alone may have had, if any upon the lesions in this patient group. A previous smaller RCT in adults used Intrasite gel as the placebo and showed that analgesia was not achieved when it was applied to painful ulcers, whilst the topical application of diamorphine gel/Intrasite gel resulted in significant pain relief (Flock 2003).

Further study required. It would be useful to have had a third arm in the trial using Intrasite gel alone and assessing analgesic effect. It would be also useful to identify during this work whether healing is occurring in either the Intrasite or topical morphine or even placebo arms of the trial.

Problem 4: There was no continuous documentation of concurrent use of analgesia throughout trial. This particular study did not specifically evaluate the concurrent use of the patients' usual analgesia as a confounding variable, as there was no significant difference in the amount of opioids/NSAIDs/or paracetamol used by patients in the two treatment arms. In my study, the patients continued taking their prescribed systemic analgesics when required.

Follow on study required. An essential follow on trial would be to analyse data on analgesic consumption throughout, to determine if similar results to my RCT would have been documented. One case study has demonstrated that topical morphine is best used as an adjunct rather than the sole analgesic in painful skin lesions (Tran and Fancher 2007).

Problem 5: There was no precise identification of the mode of action of peripheral morphine in the subjects in this study. Although my trial assumed that topical morphine acts on the peripheral opioid receptors as has been demonstrated in the adult population (Stein et al 2003), it was not proven in the paediatric patients studied in this trial.

Follow on study required. The detection of morphine and/or its metabolites in serum samples would be very useful to explore, even though studies in infants and children have verified that there is little correlation between systemic (although not peripheral) absorption and clinical effect (Bouwmeester et al 2004; Hansen et al 1996).

My research period did not allow time for this to be investigated but it is also very difficult to obtain serum samples from this patient group, as the fragility of their skin often prevents a straightforward venepuncture and so they become very needle phobic. Laboratory examination in the skin tissue of the patients for the actual presence, exact location and proposed up-regulation following painful inflammation would have provided exciting information to confirm the hypothesis.

Problem 6: Length of treatment arms. The length of each treatment arm may have been too long, and in fact the initial painful lesion may have begun to heal or in fact may have completely healed, thus allowing the researcher to have made inaccurate conclusions regarding the effect of the topical treatments.

The patients and their families in some incidences may also become uninterested in accurately completing the necessary dairy sheets.

Further study required It may have been more suitable to have shortened the treatment arm from two weeks to one week each with a few days washout period in order to avoid possible healing of lesions.

5.4 CONCLUDING REMARKS AND FUTURE RESEARCH

Although there are limitations in both my laboratory and clinical work I have attempted to contribute to pain research in children by investigating the presence of peripheral opioids in neonatal animal models and the effect of developmental regulation as well as any effect following a painful inflammatory insult. Although by no means ideal I have extrapolated the results from my laboratory work to propose that similar findings may exist in a human model of inflammatory pain along with similar developmental regulation. In this preliminary clinical work, I have provided initial evidence for clinical efficacy of this novel route of morphine in children particularly in background pain.

There should of course be further trials as suggested above, to clarify the precise mechanism and in what situations this route of morphine, may be most beneficially used in paediatric patients.

In conclusion, topical morphine may perhaps be used, most likely as an adjunct to conventional analgesia in order to manage background cutaneous inflammatory pain and following further research, may even in the be beneficial in a variety of other paediatric diagnoses where background pain is problematic, for example in post operative wounds, in burns, in ulcerating vascular haemangiomas or ulcerating tumours and in pressure ulcers.

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APPENDIX

- 1) Watterson G, Howard R, Goldman A. Peripheral opioids in inflammatory pain. Arch Dis Child 2004;89:679-671**

- 2) Patient Diary Sheet**

- 3) Brief Pain Inventory including body outline**



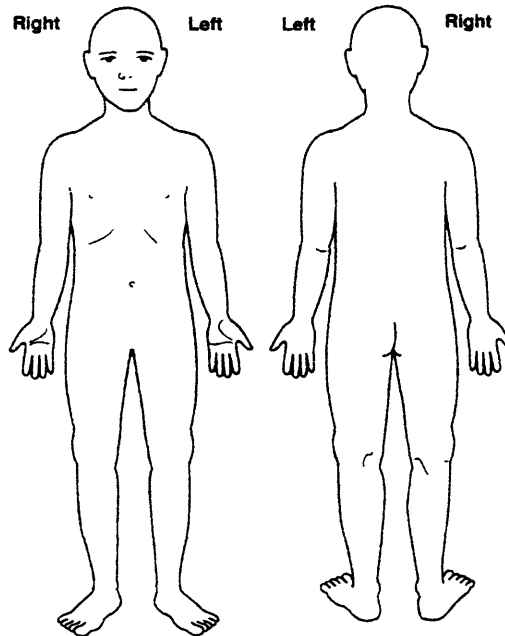
Brief Pain Inventory (Short Form)

Date

Time

Name

1. ♦ On the diagram, shade in the areas where you feel pain.
.. Mark the area where the gel is.



2. Please rate your pain by circling the one number that best describes your pain when it was at its WORST in the last week

No pain 0 1 2 3 4 5 6 7 8 9 10 Pain as bad as you can imagine

3. Please rate your pain by circling the one number that best describes your pain when it was at its LEAST in the last week

No pain 0 1 2 3 4 5 6 7 8 9 10 Pain as bad as you can imagine

4. Please rate your pain by circling the one number that best describes your pain on the AVERAGE

No pain 0 1 2 3 4 5 6 7 8 9 10 Pain as bad as you

can imagine

5. Please rate your pain by circling the one number that tells how much pain you have RIGHT NOW

No pain 0 1 2 3 4 5 6 7 8 9 10 Pain as bad as you can imagine

6. What other treatments or medications are you receiving for your pain?

7. Circle the one number that describes how, during the last week, pain has INTERFERED with your:

A. GENERAL ACTIVITY

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

B. MOOD

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

C. MOBILITY

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

D. NORMAL SCHOOLWORK

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

E. FRIENDSHIPS

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

F. SLEEP

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

G. ENJOYMENT OF LIFE

Does not interfere 0 1 2 3 4 5 6 7 8 9 10 Completely interferes

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