
THE DEVELOPMENTAL NEUROBIOLOGY OF OPIOIDS

Reema Nandi

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Department of Anatomy and Neurobiology

University College London

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ABSTRACT

Opioid analgesia is widely used in neonates. There is evidence that morphine requirements may be low in the youngest patients. Opioid receptors are developmentally regulated, but there are no studies of opioid receptor function in the immature sensory nervous system.

The aim of this MD was to determine (i) postnatal changes in opioid receptor distribution and function in rat sensory neurons (ii) whether alterations in morphine efficacy, consistent with changing opioid receptor function, occur over the postnatal period.

Methods

Mu opioid receptor (MOR) immunostaining combined with selective neurofilament labelling to distinguish large A fibres from small C dorsal root ganglion (DRG) neurons was used to study the postnatal regulation of MOR across these two subpopulations of sensory neurons in cultured rat DRG cells. Calcium imaging was used to examine MOR function using the same model. In order to determine whether changing functional MOR expression with increasing age has an effect on the intact nervous system, behavioural sensory thresholds measurements to mechanical and noxious thermal tests were conducted in neonatal and young adult rats following morphine administration. Dose response curves of morphine potency in sensory tests over the postnatal period were generated from these tests.

Results

Calcium imaging showed that significantly more functional mu opioid receptors are expressed in neonatal DRG neurons compared to adult. This over expression is confined to the large, A sensory neurons, while expression in small, C neurons remains unchanged. In behavioural tests, dose response curves demonstrated that the analgesic potency of morphine to mechanical cutaneous sensory stimulation is significantly greater in the neonate and declines with postnatal age. This was not observed in thermal nociceptive tests.

Conclusion

Functional MOR are more widely expressed in the neonatal DRG, particularly on large A neurons. These MOR are subject to postnatal developmental regulation. This changing functional receptor profile is consistent with greater morphine potency in mechanical sensory tests in young animals. These results have important clinical implications for the use of morphine in neonates and provide a possible explanation for the differences in morphine requirements observed in the youngest patients.

For my family

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List of Abbreviations

AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CAP	compound action potential
CGRP	calcitonin gene related peptide
CNS	central nervous system
CTAP	D-Phe-Cys-Tyr-D-Tryp-Lys-Thr-Pen-Thr-NH ₂
CV	conduction velocity
DAMGO	D-Ala ² ,N-Me-Phe ⁴ ,Gly ⁵ -ol]-enkephalin
DLF	dorsolateral funiculus
DOR	delta opioid receptor
DRG	dorsal root ganglion
EAA	excitatory amino acids
ER	endoplasmic reticulum
GABA	γ -amino-butyric acid
GDNF	glial cell line-derived neurotrophic factor
GIRK	G protein-activated inwardly rectifying potassium
GPCR	G-protein coupled receptors
HRP	horseradish peroxidase
HTM	high-threshold mechanoreceptive
HVA	high-voltage-activated
IB4	Isolectin B ₄
KOR	kappa opioid receptor
LL	large light
LTM	low-threshold mechanoreceptive
M3G	morphine 3 glucuronide
M6G	morphine 6 glucuronide
MOR	mu opioid receptor
NF200	neurofilament 200

NFR	nociceptive flexion reflex
NGF	nerve growth factor
NK1	neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NT	neurotrophin
ORL	opioid receptor-like
PAG	periaqueductal gray
PET	positron emission tomography
PG	prostaglandins
RVM	rostroventral medulla
SD	small dark
SG	substantia gelatinosa
SP	substance P
TrkA	tyrosine kinase A
TTX	tetrodotoxin
VDCC	voltage dependent Ca^{2+} channel
vFh	von Frey hairs
VIP	vasoactive intestinal polypeptide

1 Introduction

1.1 Pain and Development: General Introduction

We have all experienced pain at some time or another but it is not always possible to relieve it. Pain experience begins in childhood associated with health, injury and disease. Increasing understanding in recent years of pain processing in the central and peripheral nervous systems has brought about exciting breakthroughs in analgesic treatments. However, for various reasons, from ethical difficulties to a lack of economic imperative, this research has neglected children and focused mainly on adults. The developmental immaturity of the nervous system in neonates and infants means that there are important differences in pain processing between adults and these young patients, which warrants greater investment.

1.2 Why consider pain in the neonate and infant?

Until about twenty years ago, it was assumed that infants did not experience pain. Term as well as premature neonates were likely to undergo essential but traumatic medical or surgical procedures with minimal anaesthesia and no analgesia (Lippmann et al 1976). Even young children's analgesic requirements were considered to be much lower than adults. Surveys in the 1970s and 80s reported that infants and children were less likely to receive postoperative analgesics than adults (Schechter et al 1986). At that time little was known about the development of somatosensation or the substantial differences in structure and function between developing and adult pain pathways (Fitzgerald and Anand 1993; Fitzgerald et al 1994).

In recent years, however, studies of the developmental neurobiology of pain have revealed a very different picture of pain processing in the immature. They have indicated that neonates undergo considerable maturation of peripheral, spinal and supraspinal afferent pain transmission over the early postnatal period (Fitzgerald and Jennings 1999), but are able to respond to tissue injury with specific behaviour and with autonomic, hormonal and metabolic signs of stress and distress (Anand et al 1987).

Since treatment of pain and suffering is a priority for all clinicians, all paediatricians should be aware of our increasing knowledge of the development of somatosensation and understand the implications for their clinical practice.

The issue of foetal pain remains a contentious and extremely controversial one.

The question of when, in foetal development, pain processing begins is very relevant to medical practice since interventions during pregnancy (Giannakouloupoulos et al 1994), foetal surgery (Flake and Harrison 1995) and abortion, which may expose the foetus to noxious stimuli, have become commonplace. Opinions, however, are divided. Some scientists feel that foetuses do not feel pain, as they do not have a conscious appreciation of pain (Derbyshire and Furedi 1996). Others have argued that foetuses cannot appreciate pain before 26 weeks of gestation as the thalamo-cortical connections are not established before then (Lloyd-Thomas and Fitzgerald 1996). Yet another group has suggested that foetuses as young as 10 weeks may be able to perceive pain and that the thalamus may play a role in this perception (Saunders 1997). Although the issue of whether or not a foetus can feel pain remains open to debate it cannot be denied that, just before the last trimester, the fetal nervous system mounts protective responses to tissue injury. Noxious stimulation may not need to penetrate consciousness to produce potentially harmful physiological responses and to substantially alter the course of sensory development (Anand 2000). A more relevant question, therefore, would be to ask whether the stress response secondary to noxious stimuli can and should be avoided or treated.

Pain in neonates is now well established. Considering analgesia in this group is essential as more and younger and younger preterm term and term neonates are being treated on intensive care units. Little objective evidence is available regarding the exposure to pain or treatment of pain, by paediatric patients receiving intensive care. A few studies of the incidence of pain in infants in the neonatal intensive care unit indicate that the number of invasive procedures (up to one an hour) has not reduced over the past decade despite the increased use of non-invasive monitoring. The table below summarises the findings of studies investigating the incidence of pain in the neonatal intensive care unit (Porter et al 1998).

Author	n	No. of Procedures	Observation period	Procedure
Pohlman and Beardslee 1987	164	1025	2 hours	Invasive
Werner and Conway 1990	11	177	2 hours	Treatment
Barker and Rutter 1995	54	3283	To discharge	Invasive
Johnston et al 1997	239	2134	1 week	Invasive
Stevens and Franck 2001	122	10-821	1 week	Noxious and invasive

In the Stevens' study the mean number of procedures for the total sample was 134 (SD 144) within the first 2 weeks of life. For each infant approximately one third of the procedures were tissue damaging (heel stick, intravenous line insertion) and the remainder were non-tissue damaging (lavage tube insertion, endotracheal suctioning). Approximately 10% of the youngest and/or sickest infants received over 300 procedures each. These results give us some idea of the magnitude of the problem of pain on neonatal intensive care units and the challenge to alleviate this suffering.

1.3 Definition and history of pain

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

The diversity of pain experiences makes it incredibly difficult to label and the above definition by Merskey et al (1979) is not entirely satisfactory, particularly to describe infant pain. Pain is a private perception that arises in a conscious brain, typically in response to a noxious provoking stimulus, but sometimes in the absence of a stimulus. The relation of the perception to the stimulus is variable, and depends on the individual's prior expectations and

beliefs, and on his/her cognitive and emotional state, not just on the nature of the stimulus itself. The nervous system may react to noxious stimuli with autonomic changes (e.g. in blood pressure), and even with adaptive behavioural responses, in the absence of conscious pain perception. Because of the necessity for an association with possible damage to tissue, pain may not be possible for a being unaware of its own body and existence. Thus, there are circumstances in which the presence of pain is ambiguous, such as when the individual is unable to report on his/her conscious perception, or with reference to animals. In these situations, the word 'nociception' is used instead of the word 'pain' to express that the nervous system has detected the noxious stimulus without necessarily implying that a pain percept was evoked.

The above definition does recognise the loose association between injury and pain and includes the emotional dimension of pain as well as its sensory dimension. However, the qualities of 'unpleasant' are complex and have yet to be determined.

Intense stimuli or tissue damage trigger activity in sensory receptors and central circuits and pathways. However, pain is always a subjective experience that is constructed in the mind. It is a perception, rather than a sensation in the way that vision or hearing is, and cognition is involved in the formulation of this perception. There are emotional consequences as well as behavioural responses to the cognitive and emotional aspects of pain. So, in order to understand pain development it is necessary to consider the development of sensory pathways as well as the development of consciousness and the mind. In addition, pain has been considered as the perceptual counterpart of the body's response to stimuli that threaten the integrity of the tissues. It thus functions as a sensory warning system (Treede et al 1992).

Although physicians have sought to alleviate pain for centuries, the history of pain research and modern pain treatment begins in the 1800s. The enigma of pain, which Albert Schweitzer called 'the most terrible of all the lords of mankind,' has yielded slowly to determined investigators and clinicians. Four centuries ago, in 1664, Descartes gave a classical description of the traditional 'specificity theory' of pain. He suggested that the pain system was a straight through channel from the skin to the brain. He proposed that a flame sets

particles in the foot into activity and the motion is transmitted up the leg and back and into the head where an alarm system is set off. The person then feels pain and responds to it. This theory underwent little change until the nineteenth century when Max von Frey, a physician, suggested that specific pain receptors in body tissue project via pain fibres to a pain centre in the brain. He assumed that receptors are specialised. This is physiological and seems to be a genuine biological law. He also believed that a single morphologically specific receptor lay beneath each sensory spot on the skin and he assigned a definite receptor type to each of 4 sensory modalities. However, histological examination of the skin under mapped temperature spots has never supported von Frey's anatomical correlations. Finally he suggested that each psychological dimension of somaesthetic experience bears a one-to-one relationship to a single stimulus dimension and to a given type of skin receptor. This is essentially similar to Descartes' concept of pain proposed 300 years earlier. This specificity concept, which attributes a direct, invariant relationship between a psychological sensory dimension and a physical stimulus dimension was rejected, however, and gave way to 'pattern' theories of pain. These suggested that the information generated by skin receptors is coded in the form of patterns of nerve impulses and that temporal and spatial patterns of nerve impulses provide the basis of sensory perceptions. The emphasis on central summation mechanisms is supported by the clinical observations of extraordinary temporal and spatial summation in pathological pain syndromes. Theories of a spinal reverberatory system that persist in the absence of noxious input provide a satisfactory explanation of prolonged pain and the concept that large fibres inhibit activity in small fibres is supported by evidence that pathological pain is often associated with a loss of large myelinated fibres. These theories, however, lack unity and fail to integrate the diverse theoretical mechanisms of pain.

Then in 1965 Melzack and Wall published their paper in Science entitled a 'New Theory of Pain'. The theory simply stated that the transmission of pain from the peripheral nerve through the spinal cord was subject to modulation by both intrinsic neurons and controls originating from the brain. The theory was then added to and refined to include changes in afferents, prolonged central excitability and changes in these systems after nerve damage. Gate control showed that excitations and inhibitions are independently controlled and that

different types of convergent afferent activity may be turned on and off. The new theory had far reaching consequences – the concepts of convergence and modulation reduced the emphasis on destruction of pathways and led to the idea that pain could be controlled by modulation, by reducing excitation or increasing inhibition. However, the Gate Theory did not emphasise peripheral processes. It is now known that, other than physiological pain, the main clinical pains arise from damage to tissue (inflammatory pain), whereas neuropathic pain results from changes in damaged nerves. However, both can cause profound changes in the spinal cord and the brain. It is now believed that all persistent pains exhibit plasticity in that the peripheral and central signalling mechanisms can alter. Peripheral changes drive central compensations and adaptations so that the mechanisms involved in the pain are likely to be multiple and located at a number of sites. When tissue is damaged, peripheral chemicals sensitise the sensory endings and after neuropathic pain, excitability changes occur within the nerve itself. These peripheral changes then alter activity in the central systems (McMahon et al 1993).

The picture of pain and its perception has emerged as an increasingly complex one, from Descartes' straightforward linear theory to current theories based on Wall's gate theory. When considering pain in the immature infant we need to take into account the plastic nature of the developing nervous system in addition to the huge intricacies of pain mechanisms. The postnatal period is a critical time in the development of spinal sensory systems. It is a time of structural and functional reorganisation of sensory connections accompanied by marked changes in expression of molecules, receptors, and channels associated with sensory transmission (Alvares and Fitzgerald 1999; Fitzgerald and Jennings 1999). In addition it is becoming increasingly evident that these postnatal events are dependent upon neural activity and that synaptic development requires defined patterns of afferent input (Ben-Ari 2002; Debski and Cline 2002; Fox 2002).

This makes the investigation of pain in neonates and young infants particularly challenging and interesting.

1.4 Pain in children

There have been significant changes in pain management in children over the last 20 years. There seems to have been little serious clinical interest in the subject before the late 1980s. The first report of the under treatment of postoperative pain in children came from Eland in 1977 (Eland and Anderson 1977) to be shortly followed by others (Mather and Mackie 1983; Beyer et al 1983; Schechter et al 1986). Analgesics were given on demand, when pain was proven, but opioids were only seldom used and then only in mechanically ventilated children. Analgesics were not prescribed or administered as standard practice in children as a result of concerns over a perceived increase in susceptibility to adverse effects, particularly of opioids, as well as ignorance of pain perception in children.

What has made management in children so difficult? The answer to this is a variety of reasons ranging from problems of assessment to lack of economic imperative and resistance to change.

Several constraints have governed pain management in children and these are still very much a part of the discipline today. These constraints have resulted in a slow change in attitudes to analgesia, despite reports of the under treatment of pain in children. Firstly, it is impossible to test directly pain perception and memory in neonates and infants. As a result, historically, it has been concluded that pain in neonates is not perceived because of cognitive immaturity or not remembered and therefore of no consequence. Secondly, the gold standard of pain assessment used in adults, self-report, is unavailable to those treating infant pain. While infants are quite sensitive to minimal disturbance, they lack specificity in their behaviour. So pain assessment must depend on tools based on a variety of physiological, metabolic and behavioural parameters. Many of these variables can be confounded by alterations in other systems e.g. cardio respiratory and endocrine and by pathologies unrelated to the pain being measured e.g. sepsis. There is an extensive literature on paediatric pain assessment and in the neonate and infant alone 14 different pain assessment tools have been devised and validated (Buttner and Finke 2000; Franck et al 2000). There is little consensus on which tool is superior for different groups or situations.

Reports of the deleterious effects of inadequate analgesia during and after surgery in preterm neonates (Anand et al 1987; Anand and Hickey 1992) confirmed the need for adequate analgesia in neonates. Patients without analgesia exhibited not only significant stress responses, characterised by increased adrenaline, noradrenaline, glucose levels and decreased insulin levels, but also increased morbidity and mortality. Despite these findings the fear of adverse effects of opioids and the lack of efficacy data meant that doctors were still reluctant to administer large doses of morphine in small children (Purcell-Jones et al 1988). And this is the third difficulty –a lack of evidence based information about appropriate dosing regimens at different ages, with regard to both safety and efficacy. There are some useful studies investigating the pharmacokinetics and safety of opioids in neonates and infants. These have demonstrated that opioids are not harmful in these groups, even in spontaneously breathing patients (Lynn and Slattery, 1987; Lynn et al 1993; Singleton et al 1987; Greeley and de Bruijn 1988; Choonara et al 1989; Chay et al 1992; McRorie et al 1992; Hartley et al 1994). They suggested that opioids should be administered to protect infants from the adverse effects of surgical stress (De Lima 1996). Pain management after major surgery has moved from 'on-demand' to regular doses or continuous infusions, in the hope of benefiting from 'pre-emptive' effects. Balanced analgesia in children remains the most popular approach on both empirical and theoretical grounds despite the lack of systemic evaluation. Balanced analgesia encourages the addition of new agents in order to improve pain relief rather than increasing the dose of single drugs, thereby exploiting synergistic effects and minimising unwanted effects.

Finally a lack of economic imperative to treat paediatric pain has meant that the drive to research in this area is not great. A recent search of the Cochrane Central Register of controlled trials revealed 703 randomised trials of analgesics in children compared to 5323 in adults. There are few systematic reviews and guidelines, mainly as a result of inconsistent measures and outcomes to compare trials.

Clinical research on the use of analgesics in children needs to be directed to improving the evidence for use of existing agents as well as research on new drugs or novel modes of delivery. As much as possible research should be done as controlled comparative trials with

clearly defined pain and functional outcomes and include rigorous evaluation of side effects so that the risk benefit for each intervention can be clearly determined.

There is a lack of theoretical knowledge as well as evidence based information in many areas of paediatric pain management. Current information must be carefully balanced with the clinicians' duty of care to prevent unnecessary suffering in this group of vulnerable patients. The difficulties in assessing and measuring pain in younger children means that we must be as confident as possible of the efficacy and safety of analgesics we use. We must continue to investigate the effect of development in all areas of pain and realise that infants are not scaled down adults but that pain in infancy is a special case.

The present challenge is to understand more clearly the developmental regulation of pain processing and the mechanisms through which the acute and long-term effects of pain occur, in order to identify treatments that are most effective and have the fewest side effects.

1.5 Measuring infant pain

There are currently over 30 published assessment scales for measuring pain in infants and children in hospital. However, there is no widespread agreement on which pain scales should comprise standard measures for clinical or research use. The degree of sensitivity and specificity of pain measures with which to recognise and treat pain in children remain unsatisfactory for the comparative testing of the efficacy of pain interventions.

Children's pain assessment techniques can be classified as self-reports, behavioural observation, or physiological measures (Franck 2000). Clearly self-report is not applicable to infants and young children. Observational pain assessment tools have been developed to rate the intensity of children's behavioural distress associated with painful procedures and postoperative pain using behavioural cues. Validity and reliability related to procedural and postoperative pain assessment has been established (Franck 2000). However, healthcare professional using behavioural observation tools consistently underestimate children's pain compared with children's self-report (Romsing et al 1996). Furthermore, discriminating

between distress and agitation from causes other than pain, for example fear of needles, is difficult. Observational methods measure global behavioural distress that may be pain related.

Physiological measures of pain are sensitive to changes in pain intensity but they reflect a global response to pain-related stress and are not specific measures of pain. The precise measurement of physiologic and hormonal responses to pain is invasive, expensive and slow and therefore not appropriate for the clinical assessment of pain. Thus, although clinicians associate pain with changes of 10-20% in non-invasively measured physiologic parameters, for example heart rate, blood pressure and respiratory rate, no standard pain assessment tools exist at present that rely exclusively on these parameters (Franck 2000).

Multi-dimensional assessment tools that use more than one parameter in assessing pain experience are thought to be more accurate than single dimension measures. Physiologic and behavioural indicators are usually used for infants.

More research is needed to improve the validity and reliability of pain assessment tools across all age groups and settings.

1.6 Measuring infant nociception

An alternative approach to the problem of measuring infant pain using behavioural and physiological scales is to measure nociception using spinal reflexes, where the motor response is a measure of the noxious sensory input. The nociceptive flexion reflex reflects more directly nervous system processing of sensory stimuli particularly following tissue damage (Woolf 1984). To this end, the cutaneous flexion withdrawal reflex of the lower limb has been used to monitor the effects of injury on the developing nervous system in neonates (Fitzgerald et al 1989; Andrews and Fitzgerald 1999). Although it does not provide a direct measure of pain in this age group, as in the flexion reflex in the adult, its threshold is modulated by the presence of pain, being lowered in the presence of tissue injury (Fitzgerald et al 1989; Andrews and Fitzgerald 1999). The withdrawal reflex and its application in pain research are further discussed in chapter 5.

1.7 The neurobiology of pain

1.7.1 Physiological and pathological pain

One of the many ways that pain can be classified is the division into physiological and pathological pain.

Physiological pain is the sensation experienced in response to stimuli that threaten to damage tissue or cause localised injury insufficient to provoke an inflammatory response. It can be elicited by mechanical, thermal and chemical stimuli each of which have defined thresholds. It can be thought of as being 'protective' from a teleological perspective and is commonly accompanied by immediate and rapid reflexive action that removes the endangered tissue from the provoking stimulus.

Pathological pain is that sensation that accompanies substantial tissue injury (the inflammatory response) or damage to the nervous system. It differs from 'physiological' pain in several important ways:

- (a) the pain may occur in the absence of any apparent stimulus (spontaneous pain)
- (b) the response to suprathreshold stimuli may be exaggerated in either amplitude or duration (hyperalgesia) and commonly outlasts the period of stimulation or damage
- (c) innocuous stimuli may elicit pain as a consequence of a reduced pain threshold (allodynia)
- (d) the sensation of injury may spread from the site of injury to an uninjured or unaffected side (referred pain)
- (e) pathological interactions may occur between the sympathetic and somatosensory systems (sympathetic dystrophy)

Pathological pain can be said to result from the disruption of the normal selectivity and specialisation of the somatosensory system resulting in aberrant convergence, mismatch of stimulus to response, loss of thresholds and prolonged, excessive responses. In neuropathic pain states it is difficult to discern any adaptive value in these changes but in many inflammatory pain states these functional changes may be appropriate for survival of the organism (e.g. may help to protect injured tissue from further damage).

Hyperalgesia can be measured quantitatively in human psychosocial studies but can only be inferred from behavioural and electrophysiological studies in animals. In such studies, consequences of both neural and non-neural tissue events may be manifest as expanded receptive fields, decreased thresholds and increased gain of stimulus-response relationships. Two types of hyperalgesia can be distinguished. The exaggerated response to stimuli applied within the area of injury is termed primary hyperalgesia while secondary hyperalgesia is elicited by applying stimuli outside the area of injury (Raja et al 1984).

The central and peripheral mechanisms responsible for hyperalgesia have been intensely investigated. Woolf in 1983 demonstrated the important role of central mechanisms using a spinal-decerebrate rat model (Woolf 1983). He showed that a localised thermal injury induced an increase in the excitability of the flexion reflex and that this change persisted despite complete local anaesthetic block of the injured tissue. Significant changes in both central (Cook et al 1987) and peripheral (Kocher et al 1987) nociceptive systems have been documented since then.

1.7.2 Mechanisms of pain

1.7.2.1 Peripheral pain perception and primary sensory neurons

The peripheral nervous system in mammals consists of a great variety of different types of primary afferents sensing changes of the internal and external environment. Primary afferents have their cell bodies in the dorsal root ganglion (DRG) and serve as the main channel for transmitting information about these changes in the peripheral trunk and limb tissue to the spinal cord.

The dorsal root ganglion (DRG) neurons are a heterogeneous population and differ from each other in several ways:

- (a) cell body sizes
- (b) fibre conduction velocities
- (c) surface and cytosomal molecular constituents
- (d) peripheral physiological receptor properties

- (e) membrane properties and discharge patterns
- (f) neurotransmitter and neuromodulator and receptor expression
- (g) organisation of central terminals in the spinal cord

These differences have led to various classifications of DRG neurons based on:

- (a) morphological type
- (b) conduction velocity (CV)
- (c) neuropeptide, neurotrophin and other chemical markers
- (d) sensory receptor type

I will briefly discuss these below.

1.7.2.1.1 Morphological types: large light (LL) and small dark (SD) neurons

Using histological staining techniques and light or electron microscopy two major subtypes of DRG neurons have been classified in mice, rats and other species. In rats, these populations have a distinct size distribution. The LL neurons extend across the whole size range of DRG neurons, whereas the distribution of SD neurons is limited to the much smaller range although the 2 subpopulations overlap (Lawson 1979; Lawson et al 1984; Perry and Lawson 1993).

This is further discussed in chapters 3 and 4.

1.7.2.1.2 CV of peripheral fibres: A α , A β , A δ and C fibre neurons

Neurons have been classified into several populations on the basis of their peripheral CVs. These have been determined using compound action potentials (CAP) recordings from peripheral nerve or dorsal root. In the 1930s Erlanger and Gasser recorded the CAPs of the whole saphenous nerve for the first time in cats and found a series of waves which correlated with the fibre cross-sectional area (for review see Perl 1994). From their work several fibre types, including A α , A β , A δ and C fibre neurons with CV fastest to slowest, were defined according to the relationship of CAP waves and CVs. Now it is widely accepted that A α and A β fibres are myelinated, A δ fibres are thinly myelinated and C fibres are unmyelinated.

CVs of DRGs have been found to show a weak positive correlation with neuronal cell size in rats (Harper and Lawson 1985 a, b). Harper and Lawson (1985a) showed that DRG somata with slow conducting fibres were within the size range of the SD population, whereas those

cells with fast conducting (A α and A β) fibres have a size range similar to that of LL neurons; neurons conducting in the medium range (A δ) had cell bodies with a mean size between that of LL and SD neurons. It was later shown that LL neuronal somata identified immunocytochemically by neurofilament antibody in rat DRGs had A-fibres, while SD neurons with neurofilament poor somata had C-fibres (Lawson and Waddell 1991).

1.7.2.1.3 Neuropeptide, Neurotrophin and other chemical markers

A large number of neuropeptides, neurotrophins and other chemical markers have been localised selectively within subpopulations of rat DRG neurons resulting in the definition of some biochemical properties of subclasses of DRG neurons (Prabhakar and Lawson 1995; Lawson et al 1997). These neuropeptides, neurotrophins and other chemical markers might contribute to the functional specificity of these sensory neurons.

(i) Substance P

SP is synthesised in certain primary afferent neurons (Duggan 1988; Perry and Lawson 1998) and released from their peripheral terminals. Noxious but not innocuous mechanical stimulation of skin could evoke detectable SP release in the spinal cord (Kuraishi 1989). SP-like immunoreactivity (SP-LI) fibres project centrally to the dorsal horn. In DRGs SP-LI was mainly in small and medium neurons, with C and A δ fibres (McCarthy and Lawson 1989) but also in a few large neurons (McCarthy and Lawson 1989). In guinea pigs all the cutaneous afferent somata with SP-LI whose sensory properties were identified proved to be nociceptive.

(ii) CGRP

CGRP is widely distributed in the nervous system including DRG neurons (Perry and Lawson 1998) and spinal horn. CGRP mRNA increased in the spinal horn of rats following skin ultraviolet irradiation and immunoreactive CGRP was released in the spinal horn in the cat by electrical stimulation of peripheral nerves and by application of noxious stimuli. 70% of the total population of small and medium diameter DRG neurons and 46%, 38% and 17% of neurons that give rise to C, A δ and A α/β fibres respectively contain CGRP (McCarthy and Lawson 1990) in the rat.

(iii) Neurotrophins and their receptors

Neurotrophins are a family of small proteins that support the survival and differentiation of neurons during their development and that maintain their phenotype during adulthood.

Several neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), neurotrophin-4/5 (NT-4/5) and glial cell line-derived neurotrophic factor (GDNF) have been identified. Among these, NGF and GDNF have been extensively investigated for their roles in the development and maintenance of sensory neurons.

During development NGF is important in the regulation of the density of innervation of a number of peripheral targets and in the survival of a subgroup of sensory neurons (Bennett 1996). Disruption of either the gene encoding NGF or that encoding its receptor in mice results in loss of most DRG neurons (Smeyne et al 1994). Other studies showed that in animals treated during development with trk A-IgG fusion molecule (a synthetic protein that sequesters endogenous NGF) or anti-NGF serum, the amount of neuronal survival decreased dramatically after birth (Ruit et al 1992; McMahon et al 1995). The vast majority of lost neurons were small-sized or unmyelinated and thinly myelinated. This subpopulation of neurons therefore appears to be NGF dependent for its development. In other studies the NGF receptor knockout mice displayed a wide array of sensory defects in reaction to noxious stimuli but showed intact normal basic motor functions (for review see Barbacid 1994) with a marked decrease in the number of neurons.

In the adult, deprivation of NGF does not cause any detectable decrease in the number of DRG neurons (Rich et al 1984). It has been suggested that NGF might be of great importance in regulating normal phenotype and function of certain subpopulations of sensory neurons, especially those expressing SP (Verge et al 1995) and CGRP (Winston et al 2001). It has been found that NGF regulates SP and CGRP gene expression in not only developing but also mature rat DRG neurons (Kashiba et al 1997). In the adult, NGF is also an important endogenous mediator in the generation of inflammatory hypersensitivity and the persistent pain associated with inflammation and nerve injury (Woolf 1994).

Two receptors for NGF have been identified. The first is the p75, low affinity receptor and the second is the high affinity tyrosine protein kinase receptor A (trkA). The trkA receptor is the

primary effector of NGF action in vivo. TrkA is expressed selectively in about 40% of small sized neurons in adult rat L4 and L5 DRG neurons and these have been assumed to be nociceptive (Averill et al 1995).

(iv) GDNF and its receptor components

Growing evidence indicates that GDNF has potent survival-promoting effects on midbrain dopaminergic neurons (Bowenkamp et al 1995), motor neurons (Oppenheim et al 1995) and sensory neurons (Forgie et al 1999). The survival of a distinct subpopulation of small DRG neurons, described as non-peptide expressing but labelled by IB4 (Molliver et al 1997) has been shown in vitro to be supported by GDNF suggesting developmental dependence on GDNF in these neurons (Gavazzi et al 1999). GDNF could also prevent the death of some axotomised developing sensory neurons in vivo (Bennett et al 1998, 2000).

The receptor for GDNF includes GDNF binding glycosyl-phosphatidylinositol (GPI)-linked GDNF receptor (GFR α)-1, GFR α -2, GFR α -3 and GFR α -4 (Bennett et al 1998, 2000) which acts as a ligand binding domain and a tyrosine kinase receptor component expressed by c-ret protooncogene (RET) which is the signal transferring domain (Homma et al 2000). Both small and large DRG neurons express mRNA and protein of GFR α -1 and RET while mRNA for GFR α -2 and GFR α -3 is expressed mainly in small neurons (Bennett et al 1998, 2000).

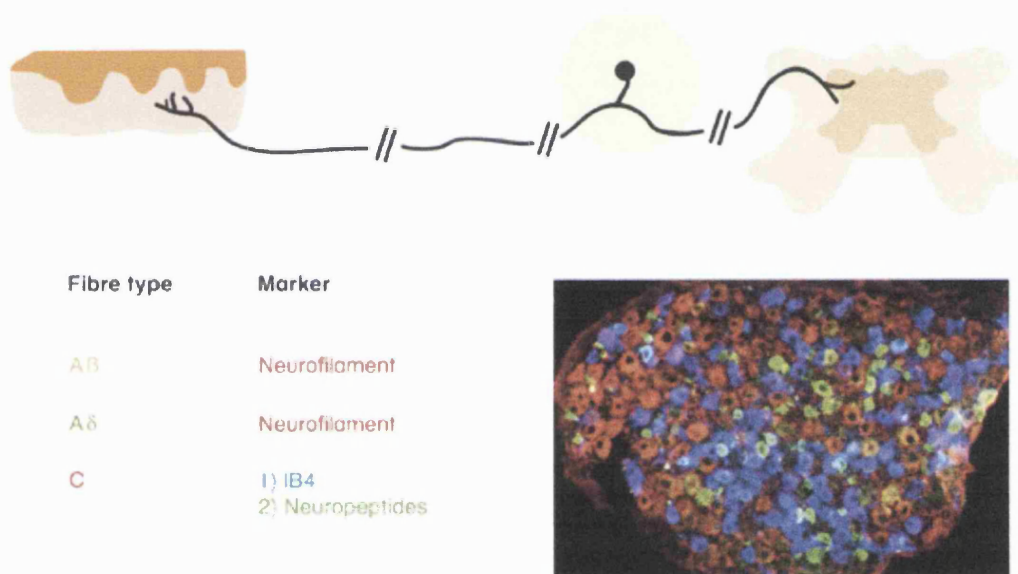
1.7.2.1.4 Other chemical markers

Isolectin B₄

Some lectins which bind specifically to glycoconjugates containing a terminal α -D-galactose selectively label a subpopulation of small DRG neurons and their central terminals in the spinal cord (Plenderleith and Snow 1990). IB₄ was found to selectively bind to about 25% of the total DRG neuronal profile (Ambalavanar and Morris 1992) which were thought to be nociceptive on the basis of their small size (Silverman and Kruger 1990), different electrophysiological properties (Stucky and Lewin 1999), and central termination to distinct laminae in the spinal cord (Ambalavanar and Morris 1992). Small IB₄ positive DRG neurons had longer somatic action potentials and higher densities of TTX resistant sodium currents than IB₄ negative small neurons (Stucky and Lewin 1999).

Different types of sensory neurons can be identified using tissue culture with antibodies against specific proteins present on their cell surfaces. In Figure 1-1, the green and blue cells represent two classes of nociceptors; the vast majority of these cells will respond to capsaicin, which excites only this class of cells.

Figure 1-1 Sensory neurons



(from the Wellcome Trust)

1.7.2.1.5 Sensory receptor types: nociceptive and non-nociceptive neurons

In order to fully understand the functional properties of DRG neurons it is helpful to classify them according to their receptive field properties. A large body of studies has shown that the large diameter, myelinated (A α/β) fibres are associated with carrying innocuous mechanical information from skin and muscle (Coggeshall and Galbraith 1978), while thinly myelinated (A δ) fibres transmit both nociceptive and innocuous information. The cutaneous unmyelinated C fibres signal information about noxious thermal and mechanical stimuli (for review see Perl 1996). On the basis of their peripheral cutaneous receptor types, sensory neurons can be divided into a number of groups as follows:

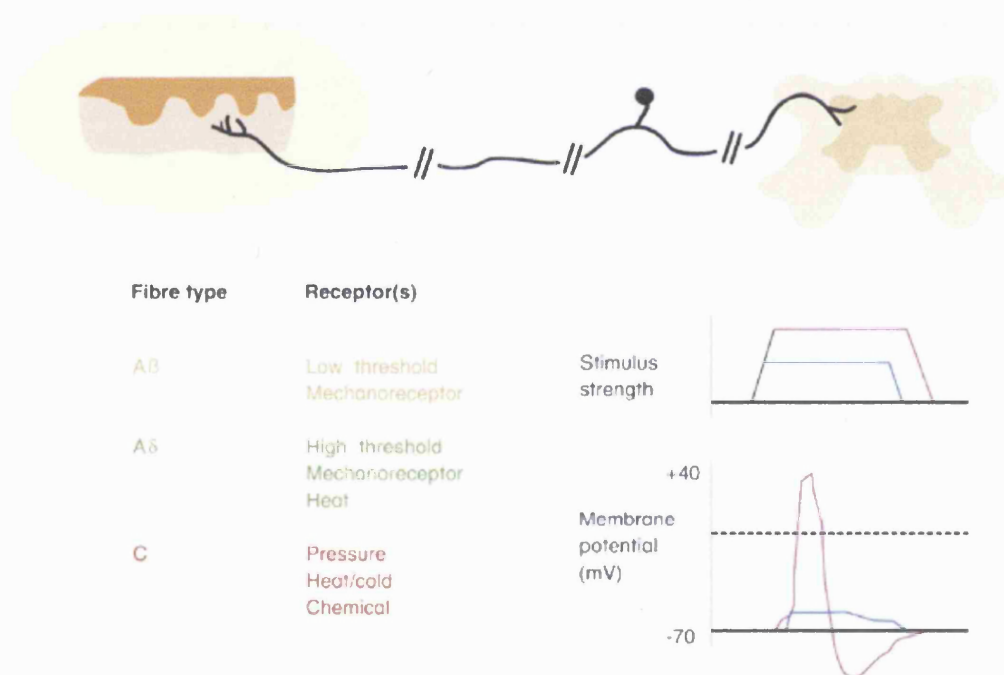
- (i) **Mechanoreceptive neurons.** Neurons responding with many impulses to innocuous or noxious mechanical stimuli applied to the receptive field and with few or no impulses to temperature change are defined as mechanoreceptive. These are divided into low-threshold mechanoreceptive (LTM) and high-threshold mechanoreceptive (HTM) neurons. LTM neurons respond to non-noxious mechanical stimuli with a short, rapidly adapting or long, slowly adapting discharge. Several subtypes of LTM neurons include hair follicle afferents, muscle spindle units, rapidly adapting and slowly adapting units. HTM neurons respond only to damaging mechanical stimuli with prolonged discharge. HTM neurons have CVs across the whole C and A fibre CV ranges (Djouhri et al 1998), while LTM neurons mainly have CV ranges of A fibres although some LTM neurons do have C fibre CV (Djouhri et al 1998).
- (i) **Polymodal nociceptive neurons.** Some C fibre neurons that are equally responsive to noxious mechanical, thermal or chemical stimuli have been termed polymodal nociceptive neurons. They have superficial receptive fields in the skin, probably in the epidermis or very superficial dermis.
- (ii) **Mechano-heat neurons.** Some C fibre neurons with deeper receptive fields and all A fibre neurons with superficial or deeper receptive fields have polymodal-type response to noxious mechanical, thermal or chemical stimuli. These can be defined as mechano-heat neurons.
- (iii) **Thermoreceptive neurons.** These neurons initiate a considerable discharge for a small change in temperature with little or no response to non-noxious and noxious mechanical stimuli.
- (iv) **Nociceptors** respond to tissue damage and can cause pain sensation when activated. Nociceptors, first described by Charles Sherrington, convey sensory information, via electrical impulses, to the spinal cord from all areas of the body. They respond to multiple stimuli, such as high levels of pressure, high or low temperatures and chemicals but these types of stimuli only generate action potentials when they are of intensity sufficient to cause, or potentially cause, injury to the animal.

1.7.2.1.6 Ion channels

To transduce these stimuli into electrical activity, the sensory terminals contain a variety of specialized receptors that are activated by mechanical, thermal or chemical insults. Activation

of these receptors results in a conformational change in ion channels, allowing positively charged ions, such as sodium and calcium, to enter the cell, thus lowering the voltage across the membrane. The intensity of the stimulus is encoded in this way, with a larger stimulus resulting in a larger change in voltage. If the membrane voltage is lowered to the electrical threshold, voltage gated channels are activated, generating electrical impulses. The greater the intensity of the stimulation the greater the change in voltage at the terminal and the greater the discharge (Garell et al 1996). This is demonstrated in Figure 1-2 where mechanical stimuli (top panel) at different intensities have been applied to a sensory neuron. At low intensities, the stimulus has little impact, but at the higher level of mechanical stimulation the electrical threshold is surpassed and an action potential is induced.

Figure 1-2 The effect of varying stimulus intensity on fibre output

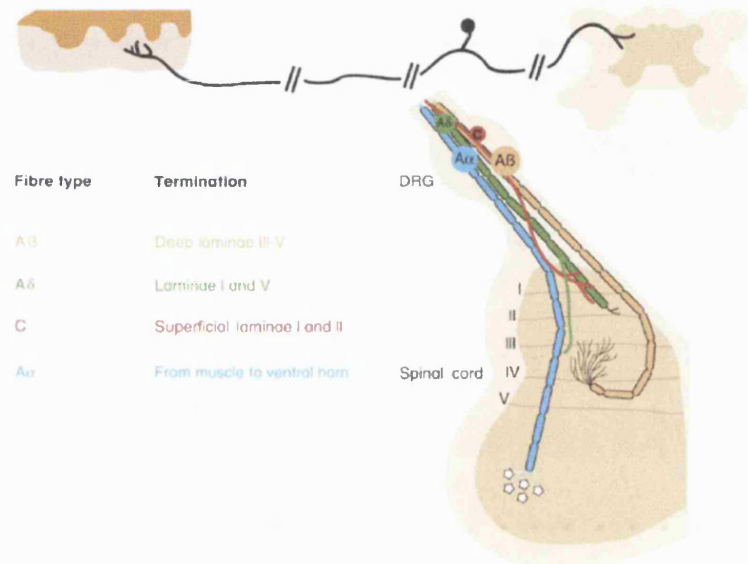


(From the Wellcome Trust)

1.7.2.1.7 Central terminal fields

A further distinguishing feature of the sensory neurons is their termination point within the spinal cord (Figure 1-3). Most nociceptive neurons (coloured green and red) terminate in laminae I and II of the dorsal horn of the spinal cord.

Figure 1-3 Sensory neurons in the dorsal horn



(From the Wellcome Trust)

1.7.2.2 Central Nociceptive pathways

A noxious stimulus is required to activate nociceptors but the production of a sensory response, and ultimately perception, will depend upon the ability to excite neurons within the central nervous system (CNS). The proliferation of research into adult pain mechanisms has demonstrated that pain transmission in the spinal cord is not through a simple circuit, but rather the arrival of noxious information from the periphery via primary afferent neurons initiates a cascade of events in the dorsal horn allowing modulation of the pain signal (Dickenson 1995; Woolf et al 1994).

Afferent fibres enter the spinal cord and travel in Lissauer's tract before sending processes into the dorsal horn. Aδ fibres synapse in laminae 1 and 5, C fibres synapse in laminae 1 and 2 (the substantia gelatinosa (SG)) with some visceral C fibres synapsing in lamina 10 and in the contralateral deep dorsal horn. Aβ fibres synapse in laminae 3, 4, 5 and 6. They then activate projection neurons, either directly or via interneurons, which will decussate within a few segments. Descending fibres projecting from brainstem nuclei modulate the incoming

information via Interneuronal networks, thus making the dorsal horn a significant site for the processing of incoming noxious information.

Nociceptive information is then conveyed up the spinal cord in white matter tracts traditionally thought to be in the anterior funiculus. These tracts ascend to the sensory cerebral cortex via the medulla, midbrain and thalamus. En route, projections are sent off to the medullary reticular formation, nucleus raphe magnus, midbrain reticular formation, periaqueductal grey matter, ventrobasal complex and the medial and lateral nuclei of the thalamus.

1.7.2.2.1 Pharmacology of central nociceptive transmission

The neurotransmitters involved in spinal nociceptive pathways have been the subject of intensive research (Dickenson 1995). The terminals of the primary afferents contain peptides, including SP, CGRP, somatostatin, cholecystokinin and vasoactive intestinal peptide and excitatory amino acids (EAAs) such as glutamate and aspartate. They are contained in separate vesicles within the same primary afferent, although location of these vesicles appears to be distinct to the individual fibre. A noxious stimulus causes their release with subsequent binding to their respective postsynaptic receptor (Yaksh and Malmberg 1994). EAAs produce a rapid, early depolarisation whereas peptides tend to provoke a delayed and prolonged depolarisation. The pattern of postsynaptic activity in the dorsal horn neurons will code the onset, duration, intensity and location of the stimulus. Fast excitatory synaptic transmission in the adult pain pathways is mediated by glutamate and aspartate acting on post synaptic amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainite ligand gated ion channels (Li et al 1999).

1.7.2.3 Central sensitisation

The secondary hyperalgesia and allodynia seen following tissue damage is the result of central synaptic rather than peripheral receptor alterations. Following inflammation, a state of hyperexcitability exists in sensory neurons in the dorsal horn which is termed central sensitisation (Woolf et al 1994). The simplest form of this sensitisation is known as 'wind-up' in which the activation of central cells by repetitive A δ and C fibres leads to an increase in the ratio of outgoing action potentials to incoming action potentials with each successive stimulus. Thus, normal inputs have an extended and exaggerated response (Woolf 1999b). The

resulting prolonged depolarisation allows previously ineffective inputs to activate the neuron and previously effective inputs to be even more so.

This is an example of plasticity in the CNS and current evidence shows that the N-methyl-D-aspartate (NMDA) glutamate receptor is primarily involved (Dickenson et al 1997; Woolf and Mannion 1999). AMPA and kainate are postsynaptic to primary afferents. Binding opens monovalent cation channels (Na^+ and K^+) to give depolarisation of the postsynaptic membrane, an effect that is short-lived. However, the NMDA receptor is located postsynaptically to interneurons and is only activated by the repeated activation of nociceptors associated with tissue injury. It thus requires previous membrane depolarisation by the activation of AMPA and kainate receptors which remove Mg^{2+} ions that normally block the channel of the NMDA receptor at resting membrane potential. Activation leads to the influx of Ca^{2+} as well as Na^+ ions. The increased intracellular calcium leads to increased synthesis of nitric oxide (NO) and prostaglandins (PGs) which act as secondary messengers and increase the sensitivity of primary afferent neurons and cause them to release more neurotransmitter in response to a fixed stimulus. NO and PGE_2 have also been postulated to diffuse out of cells and effect changes in neighbouring cells.

Other neurotransmitters also play a role in the activation of NMDA receptors. Neuropeptides, such as SP and CGRP and growth factors such as BDNF released by C fibres may potentiate the release of glutamate and its actions on the NMDA receptor. G-protein coupled receptors such as NK1 and glutamate receptors and receptor tyrosine kinases, such as trkB receptors, may also enhance NMDA currents via activation of protein kinase C (Woolf and Salter 2000). The rise in intracellular Ca^{2+} can also lead to longer term changes by the synthesis of novel genes. For example, after inflammation, A fibre neurons which do not normally express neuropeptides begin to express SP and BDNF and this may contribute to the allodynic response (Ji et al 1999). The effect of this enhanced neurotransmission and hyperexcitability includes the enlargement of receptive fields, increased spontaneous activity, greater discharges to mechanical, thermal and chemical stimuli and sometimes decreased thresholds (Ren and Dubner 1996). All this leads to increased neuronal activity transmitted to supraspinal sites and the onset of persistent pain.

1.7.2.4 Modulation of pain pathways

It is well established that descending pathways originating in higher centres can modulate the output of spinal nociceptive neurons. These pathways can act directly pre or postsynaptically or act on interneurons. Interneurons can also have an inhibitory as well as excitatory effect (Dubner and Ruda 1999).

Descending pathways release neurotransmitters such as 5HT, noradrenaline and endogenous opioids to exert inhibitory control. At spinal level there are several classes of local interneuron that contain one or more peptides such as enkephalins or inhibitory amino acids such as γ -amino-butyric acid (GABA) and glycine. Both these systems have pre and post synaptic action on primary afferents and function by decreasing the release of primary afferent neurotransmitter or decreasing postsynaptic excitability of second order neurons. Additional descending pathways release excitatory amino acids (EAAs) and can either excite the inhibitory interneurons, leading to a decrease in pre and postsynaptic activity, or excite the second order neuron, resulting in descending excitation. Inhibitory interneurons receive tonic excitation from A β fibres. Removal of inhibitory control leads to an increased discharge of the second order neuron following low-threshold input and may result in allodynia.

1.7.2.5 Higher brain systems

The activity of higher brain centres contributes to the experience of unpleasantness felt following a noxious stimulus. Sensory, autonomic, arousal and motor responses all contribute to the experience in both the meaning of the pain and in the context in which the pain presents (Price 2000). Several ascending pathways and brain regions are thought to be involved but there is little consensus as to the extent of involvement of these areas in adults (Davis et al 1998). Some pathways project directly from dorsal horn to the cortex via the brainstem and limbic system while others project to the cortex via multiple thalamic nuclei (Price 2000).

1.8 Pharmacology of spinal pain pathways

1.8.1 Overview of transmitter systems in the adult spinal cord

Several ligand-receptor systems have been implicated in physiological transmission of signals evoked by noxious stimuli. These include the excitatory amino acids glutamate and aspartate and the tachykinin family of peptides. The latter include SP, CGRP, somatostatin, vasoactive intestinal polypeptide (VIP), galanin, bombesin and neurotensin. Of these, somatostatin and galanin are thought to possess inhibitory actions. Glutamate and the peptide SP are of prime importance in excitatory transmission between primary afferent fibres and dorsal horn neurons in the spinal cord.

1.8.1.1 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS. Glutamate receptors are functionally either ionotropic or metabotropic. The ionotropic receptors by virtue of an integral transmembrane ion channel allow rapid alteration of the post-synaptic membrane potential. Metabotropic receptors are linked to an intracellular secondary messenger system. They belong to the family of G protein coupled receptors and can alter cell function and may then indirectly alter membrane responsiveness. The following table shows the classification of glutamatergic receptors (Gonzalez et al 1993).

Receptor type	Distribution and main characteristics
NMDA	Widely distributed in mammalian CNS, especially hippocampus and cortex Slow component in repetitive EPSP activity Important in synaptic plasticity
AMPA	Widespread distribution that parallels that of NMDA receptors Generation of fast component of EPSPs
Kainate	Concentrated in a few specific areas (e.g. hippocampus) Complimentary distribution pattern to NMDA/AMPA receptors (eg specific presence on some dorsal root C fibres and DRGs)
Metabotropic	Linked (positively) to inositol triphosphatase or (negatively) to cAMP Not antagonised by NMDA antagonists May be involved in developmental plasticity

Under normal synaptic conditions low frequency responses at glutamatergic synapses are mediated by AMPA and/or kainate receptors with very little contribution from NMDA receptors. NMDA receptors seem to play a crucial role in synaptic plasticity as is involved in many neurotoxic brain states.

1.8.1.2 Substance P

Substance P, containing 11 amino acids, acts on the G-protein coupled neurokinin 1 receptor (NK1). It has excitatory effects on both peripheral and central neurons and a variety of actions on non-neural tissues. SP belongs to the tachykinin family of peptides. It is synthesised by small diameter C sensory fibres and released into the dorsal horn of the spinal cord. Although only 10% of all lamina 1 neurons express the NK1 receptor, these neurons form a major component of the projection pathways rising to the parabrachial area, brainstem reticular formation, periaqueductal grey and thalamus (Hunt 2000). Studies with intrathecally administered SP and NK receptor agonists and neurokinin antagonists suggest that activation of peptide receptors is a requirement for nociceptive responses.

Neuropeptide receptors all appear to be over-expressed in early development. Despite the density of NK1 receptors being maximal at P11, levels of peptide are initially low and take 2 weeks to mature. A similar postnatal maturation in the expression of other peptides (CGRP, somatostatin and galanin) has also been documented (Marti et al 1987). The pattern of distribution of NK1 receptors undergoes considerable postnatal change- the concentration of receptors in the superficial dorsal horn does not occur until 2 weeks after birth (Charlton and Helke 1986).

1.8.1.3 BDNF

BDNF is now known to be synthesised and constitutively expressed by nociceptors expressing CGRP and trkA. It is released from the central terminals of these fibres in the superficial dorsal horn in an activity dependent way. Tissue trauma and conditions that increase NGF like inflammation result in the upregulation of BDNF receptors. BDNF is able to modulate spinal reflex excitability via a post synaptic mechanism, possibly by phosphorylation of NMDA receptors, making it an important neuromodulator of nociceptive neurons and a likely neurotransmitter in conditions of inflammatory pain (Thompson et al 1999).

1.8.1.4 Nitric Oxide

Nitric oxide is synthesised by neurons and glial cells expressing the enzyme nitric oxide synthase (NOS) and after diffusion into surrounding neurons is able to alter secondary messengers such as the guanylyl cyclase/ protein kinase system. DRG cells express NOS which appears to be co localised with SP and CGRP. Up regulation of the enzyme following noxious stimulation further suggests a role in spinal nociceptive processing (Robbins and Grisham 1997).

Three clinically relevant receptor-ligand systems appear to inhibit spinal nociceptive signalling. These are the ubiquitous gamma-butyric acid (GABA) system, the opioid system and the monoamines serotonin and noradrenaline.

1.8.1.5 GABA

In adults GABA is known to have inhibitory actions mediated via an increase in chloride conductance through post synaptic GABA_A and GABA_B receptors. In contrast, during early development GABAergic transmission is characterised by the transient over expression of GABA receptors (Vincent et al 1995) and by an excitatory rather than an inhibitory action. The depolarising action is a result of developmentally regulated changes in chloride homeostasis (Serafini et al 1995) and implies that the GABA receptor system may provide the excitatory input during early development that is later provided by the AMPA glutamatergic system (Leinekugel et al 1999).

1.8.1.6 Opioid system

The opioid system is discussed in more detail in chapter 2.

1.8.1.7 Monoamines

Of seven identified monoamine containing nuclei in the brainstem, four play a significant role in nociceptive processing- the raphe nuclei, peri-aqueductal grey, locus coeruleus and the rostromedial medulla (RVM). The pontine nuclei mostly secrete noradrenaline while the RVM projections to the spinal cord use serotonin (5HT) and neurotensin. Serotonin receptors comprise at least 16 subtypes most of which are G-protein linked. In the dorsal horn, 5HT₂ receptors have been implicated in anti-nociceptive actions via activation of phospholipase C (Coskun and Anand 2000).

1.8.2 Development and pharmacology of spinal pain pathways

EAA receptors undergo evolving patterns of change possibly reflecting developmental roles in the survival, migration and growth of neurites. Brain maturation is therefore characterised by a significant change in the pattern of excitatory synaptic transmission. Similarly the spinal cord is characterised by developmental regulation of expression of various receptors.

In the immature brain, primarily the hippocampus, synaptic transmission is weak and extremely plastic. A large proportion of it occurs via NMDA-type glutamatergic receptors (Durand et al 1996) although it is known if this is the case in the neonatal spinal cord. Later in life, transmission becomes stronger, less plastic and is then more usually mediated by AMPA-

type receptors. During development glutamatergic synapses that initially have small or no detectable AMPA currents display an increasing proportion of synapses with AMPA receptor currents together with a change in the kinetics of the NMDA receptor. These changes are possibly due to a developmental switch in the NMDA receptor subunit composition and a progressive insertion of AMPA receptors into the synaptic membrane. This latter may occur under the control of the NMDA receptors (Fox et al 1999).

Both uptake and binding of glutamate increases until P25. At the same time, the expression of mRNA for various subunits of the NMDA receptor reaches a peak at P20 (Monyer et al 1994). A general pattern of overproduction of synaptic terminals during development appears to be common to all EAA systems.

The early foetal cord and hippocampus have a widespread and high density of NMDA receptors. These receptors have a different subunit composition to that of their mature counterparts. This results in enhanced affinity for the ligand and a different channel open time. NMDA receptors are heteromeric ion channels composed of NR1 and NR2 subunits, with varying functional properties of the receptor being conferred by subtypes (A-D) of the NR2 subunit.

Less is known about the development of other amino acid receptors. AMPA receptors are thought to undergo a similar developmental process- an initial wide distribution progressing to a more restricted mature distribution. In the spinal cord, a developmentally regulated restriction of AMPA binding sites has also been documented. Following the transient high expression of these receptors in the ventral horn during early postnatal life, AMPA receptors become largely restricted to the SG in adults (Jakowec et al 1995). Dramatic changes in the pattern of expression occur during the first 3-4 postnatal weeks and with this changes in the subunit composition of the hetero-oligomeric receptor also occur (neonatal but not adult AMPA receptors are GluR1 positive).

Autoradiographic studies in human tissue confirm high level ligand binding throughout the spinal grey matter for all 3 ionotropic glutamatergic receptors. This transient expression diminishes in the early postnatal period in all regions but least so in the SG, until the adult pattern emerges (Kalb and Fox 1997). The duration of high level expression was found to be longest for kainate receptors and shorter for both NMDA and AMPA receptors. The

significance of this early high expression of excitatory receptors has been suggested to be in providing a necessary molecular component for activity dependent plasticity within a defined, critical period. Particularly dynamic changes in the relationship between NMDA receptors and those for AMPA and GABA suggest that these 3 receptor systems interact cooperatively in sequential developmental processes (Ben-Ari et al 1997).

GABA receptors are expressed in early embryonic stages, preceding the appearance of glutamatergic synapses (Durand et al 1996). Activation of the embryonic GABA_A channel results in depolarisation and excitatory currents in all CNS regions including the spinal cord (Wu et al 1992). The excitatory action is thought to be due to differences in chloride homeostasis in immature neuroblasts (Serafini et al 1995). As the post synaptic inhibitory effects of GABA_B receptor activation only mature later, GABA acts as an excitatory neurotransmitter in early development. The depolarising action of GABA is also associated with increases in intracellular calcium and provides an important excitatory input to immature neurons. At these early stages of development glutamatergic synapses lack functional AMPA receptors and are therefore quiescent at resting membrane potential. GABA receptor mediated excitatory inputs may well facilitate NMDA receptor currents in a pattern similar to their facilitation by AMPA receptors in adult neurons.

The importance of NMDA receptors in excitatory neurotransmission in early development is the result of:

- (a) the high density of these receptors
- (b) the slow decay of glutamate-induced currents
- (c) the synergistic action of GABA.

The latter undergoes an abrupt change as GABA_A receptors switch from an excitatory to an inhibitory synaptic role (Ben-Ari et al 1997).

1.9 Developmental issues

1.9.1 Development of the spinal cord

Spinal cord neurogenesis takes place in the ventro-dorsal direction beginning with motor neurons and ending with the local interneurons in the superficial dorsal horn or SG (Altman et al 1984). In the rat, both A and C fibres have grown into the spinal cord by birth but C fibre

terminals are immature, as many specific C fibre markers are not apparent in the spinal cord until the prenatal period (Jackman et al 2000). Synaptogenesis in the rat dorsal horn is at its maximum in the first postnatal weeks whereas in primates all types of primary afferent terminal and postsynaptic specialisation occurs early in the embryonic period (Knyihar-Csillik et al 1999).

1.9.1.1 A fibres terminate more superficially in the newborn dorsal horn

The growth of A and C fibres into the rat cord is somatotopically precise whereas laminar organisation is not (Fitzgerald 1985; Fitzgerald et al 1983). In the adult rat A β afferents are restricted to laminae 3 and 4. However, during the first three postnatal weeks of life, there is exuberant A-fibre termination in the superficial dorsal horn of the rat spinal cord (Fitzgerald et al 1994; Mirnics and Koerber 1995) such that both A- and C-fibres form synapses within substantia gelatinosa (SG) (Coggeshall et al 1996). Individual A-fibre terminals are seen to extend up to lamina I and II in the young spinal cord (Mirnics and Koerber 1997) although the physiological phenotype of these afferents is unclear (Woodbury et al 2001). A period of active synapse elimination over the first postnatal three weeks leads to A-fibre terminal withdrawal leaving only C-fibres terminating in the adult SG. Recent evidence shows that this refinement of afferent terminals is an activity-dependent process requiring NMDA receptor activation (Beggs et al 2002). Chronic, local exposure of the dorsal horn of the lumbar spinal cord to the NMDA antagonist MK801 from birth prevented the normal functional and structural reorganization of A-fibre connections. Dorsal horn cells in spinal MK801-treated animals, investigated at eight weeks of age by in vivo electrophysiological recording, had significantly larger cutaneous mechanoreceptive fields and greater A-fibre evoked responses than vehicle controls. C-fibre evoked responses were unaffected. Chronic MK801 also prevented the normal structural reorganization of A-fibre terminals in the spinal cord. The postnatal withdrawal of superficially projecting A-fibre primary afferents to deeper laminae did not occur in treated animals although C-fibre afferent terminals suggesting that their inputs are not regulated in the same way.

1.9.1.2 Evidence for the functional importance of A inputs in the newborn SG

An important piece of evidence supporting the functional importance of A inputs in the newborn SG is the EM study of labelled A β fibres in the neonate (Coggeshall et al 1996). Choleragenoid horseradish peroxidase (B-HRP) is a retrogradely transported marker that selectively labels large cutaneous myelinated primary afferent fibres. In this study, in adults, B-HRP labelled large afferent fibres were seen to enter laminae III-V, and to a lesser extent lamina I, whereas lamina II, which is the major termination site of unmyelinated primary afferents, remained unlabelled. In the neonate, however, there was extensive B-HRP label in lamina II. Importantly, this study showed that A β fibres in the neonate make numerous functional synaptic contacts in lamina II. This supports the idea that large primary afferent fibres in neonatal animals make synaptic contact with post-synaptic targets that presumably process nociceptive information.

Park et al (1999) demonstrated that neonatal SG neurons have more monosynaptic A β inputs (33%) than adults (7%) by recording synaptic responses evoked by large A β and fine A δ afferents from substantia gelatinosa (SG) neurons in slices obtained from immature and mature rats. These observations suggested that monosynaptic A β afferents were the predominant inputs to the SG neurons in the immature state and thereafter A δ afferents were substituted for the A β afferents to convey sensory information to the SG neurons.

Expression of c-fos in SG neurons can also be evoked by innocuous inputs and A fibre activation at this age, but is only seen with noxious A δ and C fibre inputs in adults (Jennings and Fitzgerald et al 1996). This suggests that in the neonatal spinal cord, low threshold A fibres are able to activate pathways in lamina I and II of the dorsal horn that in the adult are predominantly nociceptive.

The postsynaptic activity evoked in neonatal dorsal horn cells by cutaneous afferents differs considerably from that in adults. In vivo extracellular recordings from dorsal horn cells in both superficial and deep laminae in rats aged 3, 6, 10 and 21 days showed that repeated stimulation of cutaneous A fibres led to considerable sensitization in dorsal horn cells in the neonate. This sensitisation was unlike the classic C fibre-evoked 'wind-up' observed in adult dorsal horn. The direct A fibre-evoked activity did not increase, but the background activity increased during repetitive stimulation leading to a prolonged after-discharge beyond the

stimulation period. At P6, 33 % of cells were sensitized, at P10, only 6 % were sensitized, and by P21, sensitization was no longer observed (Jennings and Fitzgerald 1998).

1.9.1.3 Slow maturation of central C fibre evoked activity

Both A and C fibres enter the hindlimb of the rat embryo at E13-14. However, A fibres are the first to innervate the skin and dominate over small fibre innervation until later fetal life (Jackman et al 2000). All sensory terminals grow transiently to the skin surface before retracting subepidermally at late embryonic stages. The entry of A fibre terminals into the lumbar dorsal horn at E14 coincides with hindlimb skin innervation. In contrast, C fibres were not detected in the dorsal horn until E18, 4 days after peripheral innervation. In fetal skin, A fibre innervation dominates over C fibres. In addition, although peripheral and central innervation by A fibres coincide, this is not true for C fibres, suggesting that central target factors may control C fibre terminal growth within the dorsal horn.

Although C fibres enter the spinal cord before birth, functionally they are immature. While responses to A fibre input are enhanced in immature spinal cord, long-latency C fibre evoked spike responses are not seen in dorsal horn cells before the end of the second postnatal week (Nakatsuka et al 2000; Park et al 1999) and C fibre evoked spike activity is not seen before P8 (Torsney et al 2002). In addition, the C fibre irritant mustard oil fails to induce flexion reflexes or c-fos expression in dorsal horn neurons until the second postnatal week, despite its ability to excite C fibres in the newborn rat skin (Williams et al 1990).

1.9.1.4 Neonatal C fibre terminal evoked activity

The lack of C fibre evoked spike activity between P0 and P10 does not necessarily mean that no synaptic contacts have been formed. Little is known about the developmental profile of nociceptive fibre evoked EPSCs in the dorsal horn during the first 10 postnatal days. To study the postnatal development of nociceptive synaptic inputs in the superficial dorsal horn of the neonatal rat spinal cord Baccei et al (2003) examined the effect of capsaicin and menthol on mEPSCs in slices of spinal cord from P0 to P11 rats. They found that VR1-expressing nociceptive primary afferents form functional synaptic connections in the superficial dorsal horn from birth and that activation of the VR1 receptor increases spontaneous glutamate release via an undetermined mechanism. These results suggest that the failure of C fibre

electrical stimulation to evoke APs in superficial dorsal horn cells before P10 (Jennings and Fitzgerald 1998) may not necessarily indicate an absence of C fibre synapses during this period. This study suggests that the presence of synaptic contacts between VR1 expressing sensory neurons and dorsal horn neurons at P0 may mean C fibres begin forming synapses before birth. These synapses are capable of releasing glutamate and activating postsynaptic receptors on spinal neurons. But why are these C fibre inputs sub threshold in vivo until the second postnatal week? The number of C fibre synapses or their release probabilities may be too low to depolarise the postsynaptic neuron in the first postnatal days. Also, early on C fibre synapses seem to lack the ability synchronously release large numbers of presynaptic vesicles. Greater knowledge of the factors that govern the strengthening of C fibre inputs to the superficial dorsal horn has important implications for the understanding of infant pain processing.

1.9.1.5 Properties of neonatal dorsal horn cells in vivo

The receptive field properties and evoked activity of newborn dorsal horn cells to single repetitive and persistent innocuous and noxious inputs are developmentally regulated and reflect the maturation of excitatory transmission within the spinal cord. The size of dorsal horn cell peripheral cutaneous receptive field decreases with age (Fitzgerald 1985; Jennings and Fitzgerald 1999). In the neonate, therefore, receptive fields are not only dominated by low-threshold inputs but are also larger and will therefore overlap more than in the adult, increasing the chance of activation by peripheral skin stimulation.

In addition there is convergence of afferent inputs in the postnatal dorsal horn cells. Low-threshold inputs dominate the newborn dorsal horn when the responses to natural stimulation are examined. Background activity is generally absent when neonatal cells are initially isolated for extracellular recording, but strong responses can be evoked by mechanical stimulation of the skin of the receptive field. Some cells respond to both innocuous brushing and noxious pinching of the skin, but the convergence of input to dorsal horn cells changes over the postnatal period. The responses recorded from cells in the younger animals are elicited mainly by low-threshold mechanoreceptors, and there are few cells with convergent input in

the first week of life. This population gradually increases so that by P21 the percentage of neurons with convergent primary afferent input is similar to that seen in the adult.

1.9.1.6 Descending inhibition develops postnatally

Descending pathways to spinal cord originate from neurons in the upper cervical cord, brainstem nuclei, deep cerebellar, diencephalic and cortical neurons. Most of these are present at birth but many mature postnatally (Leong et al 1984). Although anatomical tract development appears almost complete at birth it is likely that synaptic maturity is achieved much later (Leong et al 1984).

Of particular interest are descending fibres from the brainstem travelling in the dorsolateral funiculus (DLF). These fibres originate within the rostral ventromedial medulla including the midline nucleus raphe magnus and adjacent reticular formation. This fibre tract is known to strongly modulate activity within the dorsal horn and this action is partly mediated by serotonin (5HT) (Marti et al 1987). Despite anatomical connection as early as P6, physiological maturity of the fibre tract is delayed until at least P10-12 (Fitzgerald and Koltzenburg 1986). Two possible explanations include a delay in the development of serotonergic transmission and the delay in the maturation of the local interneurons of the SG. No 5HT immunoreactivity is seen at E14, but by E18 5HT-immunostained axons in the white matter are evident throughout the cord and at birth diffuse light staining is seen also within the grey matter. The adult pattern and density of 5HT staining is only evident at P14 in the cervical cord and P21 in the lumbar cord (Bergman et al 1997)

1.9.2 Development of nociceptors

Knowledge of the developmental regulation of nociceptors and nociceptive signal transduction is still limited (Fitzgerald et al 2001; Alvares et al 1999). More is known about general anatomical and physiological development through work on animal models.

Sensory nerve fibres terminating in nociceptive or LTM endings grow out from the DRG prenatally and innervate the skin in an organised proximo-distal manner; by birth in the rat and the second trimester in man they have reached the most distal skin of the foot (Reynolds et al 1991). The large diameter A cells are born first and their nerve fibres reach the skin and

form the initial cutaneous nerve plexus before the C fibres which follow soon after (Jackman et al 2000). Two groups of C nociceptors have been distinguished. The first contain neuropeptides and express the neurotrophin receptor for NGF, trkA. The second do not but bind the lectin IB₄ and express the receptors for the neurotrophin GDNF. The functional differences between these two groups are not clear but in the rat the IB₄⁺ group only mature postnatally so that in early life the majority of C nociceptors express trkA (Bennett et al 1996). All the main functional cutaneous afferent types found in the adult rat hindlimb can be found at birth. C fibre polymodal nociceptors, responding to mechanical, thermal and chemical noxious stimuli, are fully mature in their thresholds, pattern and frequency of firing at birth. A δ mechanoreceptors, responding maximally to noxious mechanical rather than chemical and thermal stimulation can also be distinguished, but their peak firing frequencies are lower than in adults. Low threshold A β mechanoreceptors responding to touch or brush with brief, rapidly adjusting bursts of spikes are, relatively, the most immature at birth, with low frequencies of firing and amplitude of response.

Neurotrophins also play an important part in nociceptor development. The number of sensory afferents responding to a noxious stimulus, and their pattern and amplitude of response, will depend on the level of neurotrophic factors in the skin or other target tissues (McMahon 1996). There is a continuous cycle of neurons being born and innervating the skin alongside programmed cell death and the survival of neurons is dependent upon access to neurotrophic factors (Coggeshall et al 1994). Neurotrophic factors also play an important role in the development of normal receptor properties independent of their effect on survival. NGF and neurotrophin 3 (NT3) regulate the differentiation of myelinated and C fibre nociceptors and their mechanical thresholds and BDNF regulates mechanoreceptor properties (Koltzenburg 1999).

1.9.3 Development of higher centres

Little is known of the development and maturation of pain pathways to the higher brain centres or on the extent and degree of activation of these centres in neonates and infants. In the rat, spinothalamic afferents reach the thalamus at E19 and thalamic axons also reach the cortical plate at E19. By P0 there is an organised plexus in the cortical plate and some axons have reached the marginal zone (Erzurumlu et al 1990). At P2-5 non-functional AMPA

receptors ('silent synapses') are found which are converted to being functional at P8-9 (Isaac et al 1999). By P7 cortical cells are organised in columns but have larger receptive fields than in the adult which suggests a lack of inhibition similar to that seen in the spinal cord. The rat cortex remains immature for up to 6 weeks after birth. The human cortex takes many years to develop fully. Thalamocortical axons are first seen at 22-34 weeks gestation but synaptogenesis continues for many years.

2 Opioids and development

Opioids remain the most powerful analgesics available. It is now accepted that opioid analgesia can be used safely in neonates, infants and children although the thresholds for treatment and response are not yet defined, particularly in the youngest patients.

Developmental factors are likely to be very important in the regulation of opioid analgesia in the neonate and infant and may explain the variability and unpredictability of opioid action observed in clinical practice.

The neonatal central nervous system is both structurally and functionally immature, and significant changes in opioid analgesic mechanisms occur before and after birth. In this chapter I will discuss the current understanding of the developmental neurobiology of opioids. I will review opioid actions in adults before focussing on opioid actions in neonates.

2.1 Opioid receptor function

2.1.1 Introduction

Preparations of the opium poppy *papaver somniferum* (Figure 2-1) have been used for many hundreds of years to relieve pain. In 1803, Sertürner isolated a crystalline sample of the main constituent alkaloid, morphine, which was later shown to be almost entirely responsible for the analgesic activity of crude opium. The rigid structural and stereochemical requirements essential for the analgesic actions of morphine and related opioids led to the theory that they produce their effects by interacting with a specific receptor. The concept that there is more than one type of opioid receptor arose to explain the dual actions of the synthetic opioid nalorphine, which antagonises the analgesic effect of morphine in man but also acts as an analgesic in its own right. Martin (1967) concluded that the analgesic action of nalorphine is mediated by a receptor, later called the κ -opioid receptor, which is different from the morphine receptor. Evidence for multiple receptors, μ , κ and σ , came from the demonstration of different profiles of pharmacological activity in the chronic spinal dog with the prototype

agonists morphine, ketazocine and N-allylnormetazocine (SKF 10047) (Martin et al 1976).

The existence of the δ -receptor was subsequently proposed to explain the profile of activity *in vitro* of the enkephalins (the first endogenous opioid peptides), and on the basis of the relative potency of the non-selective opioid antagonist naloxone to reverse endogenous opioid peptide inhibition of the nerve-evoked contractions of the mouse vas deferens (Lord et al 1977). Its existence was further confirmed by radioligand binding studies using rat brain homogenates.

It is now clear from work carried out over the last 20 years that there are 3 well-defined or "classical" types of opioid receptor μ , δ and κ which are located on neuronal cells in the brain, spinal cord, myenteric plexus, peripheral nociceptors and many other cell types, including lymphocytes, monocytes, skeletal and cardiac muscle.. Genes encoding for these receptors have been cloned (Kieffer et al 1992). More recently, cDNA encoding an "orphan" receptor was identified which has a high degree of homology to the "classical" opioid receptors. On structural grounds this receptor is an opioid receptor and has been named ORL₁ (opioid receptor-like) (Mollereau et al 1994).

2.1.2 Opioid receptors and subtypes

Pharmacological studies have attempted to further subdivide opioid receptors in each of the three major subgroups. Although suggestive, pharmacologically defined subclasses of μ , δ , and κ receptors are not well established. The cloning of each of the three major opioid receptors has done little to support further expansion of opioid receptor classification (Raynor et al 1994). There are reports of alternate splice variants of the μ , δ , and κ receptors which share ~60% sequence homology. However, it is not clear at what level they are expressed or if they can be distinguished pharmacologically (Gaveriaux-Ruff et al 1997; Pan et al 1999). The lack of molecular evidence for more than four opioid receptor subtypes indicates that further sub classification of receptors may result from mechanisms that might include post translational regulation, receptor dimerisation, or even interactions with accessory proteins. The use of ligands with differing efficacy in tissues having varying receptor reserve is one potential confounding problem in the pharmacological classification of multiple opioid receptors. The description of the epsilon opioid receptor in the rat vas deferens is one such example. In this preparation β endorphin decreased the muscle contraction evoked by

electrically stimulating transmitter release from the nerves. Morphine was ineffective in this preparation. From this observation the β -endorphin selective epsilon receptor was characterized (Garzon et al 1985). Subsequent work showed that the receptor reserve of μ -receptors in this preparation was low enough that a partial agonist, such as morphine, acted as a pure antagonist (Sheehan et al 1988). The characterisation of multiple receptors based on results obtained in more complex tissues using indirect assays are subject to the same difficulties in interpretation.

Many G protein-linked receptors exist as dimers. The most dramatic demonstration of dimerisation of G protein-linked receptors is with the GABA_B receptor, where heterodimerisation with two subtypes of the receptor are required for functional expression (Jones et al 1998). Both κ and δ opioid receptors have been reported to form homodimers. Heterodimers of κ and δ opioid receptors have been expressed in Chinese hamster ovary (CHO), HEK 293, and COS cells (Jordan and Devi 1999). The pharmacological profile of heterodimers was not completely characterized but differed from the homodimers of both δ and κ receptors. Heterodimerization of receptors in vivo could account for complex pharmacology even if there is only a single gene for each receptor.

Analyses of opioid receptor 'knock out' mice (which are deficient in a receptor or part of a receptor) have clearly shown that MOR plays a central role in opioid-induced analgesia (Matthes et al 1996). MOR-KO mice show reduced analgesia after administration of morphine, an MOR agonist, but also after administration of DOR (Sora et al 1999) and KOR agonists. KOR-agonist-induced analgesia is also reduced in CXBK mice that have an abnormal MOR gene (Ikeda et al 1999). The formation of opioid receptor heterodimers (Jordan and Devi 1999) might be one of the mechanisms underlying this cross-communication between different opioid signals. DOR-KO mice show retained supraspinal delta-like analgesia and intact spinal analgesia (Zhu et al 1999). KOR-KO mice show no analgesia after administration of KOR agonists, whereas the analgesic effects of morphine are intact (Simonin et al 1998).

2.1.3 Opioid peptides

There are more than 20 endogenous opioid peptides, and most of them are derived from three precursor proteins: pro-opiomelanocortin, prepro-enkephalin (PPE), and prepro-dynorphin (PPD) (Vaccarino and Kastin 2001). While dynorphin is relatively selective for kappa-opioid receptors (KORs), β -endorphin and enkephalins activate μ - and δ -opioid receptors (MORs and DORs, respectively). It has been demonstrated that β -endorphin knockout (KO) mice show enhanced stress-induced analgesia (Rubinstein et al 1996) and that supraspinal responses to pain are increased in PPE-KO mice (Konig et al 1996). Although dynorphin induces analgesia, PPD-KO mice do not show any differences in opioid-induced analgesia (Sharifi et al 2001). Endomorphins, selective MOR agonists, are amidated tetrapeptides whose genes have not yet been identified (Zadina et al 1997). There are other gene-unidentified opioid peptides, such as the peptides in the MIF-1 (melanocyte-stimulating hormone release inhibiting factor 1) family (Erchegyi et al 1992) and morphiceptin (Chang et al 1981). Although nociceptin/orphanin FQ structurally resembles dynorphin, the peptide is not classified into the opioid peptide family (Meunier et al 1995).

2.1.4 Biological effects of receptor activation

Opioid agonist binding and subsequent receptor activation initiate a cascade of events that result in a varied array of biological effects. These include analgesia, miosis, bradycardia, general sedation, hypothermia, insensitivity and depression of flexor reflexes. An important site of opioid analgesic action is the spinal cord where opioids inhibit neurotransmitter release from dorsal root ganglion projections in the dorsal horn of the spinal cord (Macdonald and Nelson 1978; Yaksh 1993). The diversified repertoire of opioid actions is possible because all of the cloned opioid receptors belong to the G protein coupled receptor family. Their signals are transduced through interaction with guanine nucleotide-binding proteins. They all possess the same general structure of an extracellular N-terminal region, seven transmembrane domains and intracellular C-terminal tail structure (Figure 2-2 and Figure 2-3). They form a 4 member gene subfamily together with the orphanin FQ/nociceptin receptor discovered later (Darland and Grandy 1998). There are four major families of G proteins: Gi, Gs, Gq, and G₁₂ (Linder and Gilman 1992). Opioid receptors are coupled with six members of Gi family proteins, Gi₁₋₃, Go_{1,2} and pertussis-toxin (PTX)-insensitive Gz (Jeong and Ikeda 1998).

2.1.5 Second messengers/ Effectors

Activation of any of the three opioid receptor subtypes produces common cellular actions through coupling G proteins. The profile of coupling of the three opioid receptors to the spectrum of G proteins is similar, although subtle differences have been identified (Connor and Christie 1999). The most commonly reported actions include

- (i) inhibition of adenylyl cyclase,
- (ii) activation of a potassium conductance,
- (iii) inhibition of calcium conductance;
- (iv) inhibition of transmitter release (Figure 2-3).

More recent observations have extended the actions of opioids to include the

- (i) activation of protein kinase C (PKC),
- (ii) the release of calcium from extracellular stores,
- (iii) the activation of the mitogen-activated protein kinase (MAPK) cascade, and
- (iv) the realisation that receptor trafficking plays an important role in receptor function.

2.1.5.1 Inhibition of adenylyl cyclase

Two physiological consequences of the acute inhibition of adenylyl cyclase by opioids have been identified: one is mediated by the modulation of a voltage-dependent current (I_h), which is also termed the pacemaker current (Ingram and Williams 1994). This cation nonselective current is activated at hyperpolarized potentials to cause an inward current that depolarizes the membrane potential toward threshold. The voltage dependence of this current is regulated by cAMP, being activated at less negative potentials when cAMP levels are elevated (Ingram and Williams 1996). Opioids shift the voltage dependence to more negative potentials by decreasing intracellular cAMP. This inhibition was most easily observed after the activation of adenylyl cyclase with forskolin or PGE₂ (Ingram and Williams 1994). The consequence of this action of opioids was to decrease the amplitude of the inward current that drives spontaneous activity and thus decreases excitability. A family of these cation channels has now been cloned, some of which show the same cAMP-dependent changes in voltage dependence. A similar effect of opioids has also been observed on tetrodotoxin-insensitive, cAMP-sensitive sodium current in cultured sensory neurons (Gold and Levine 1996). Activation of adenylyl cyclase with prostaglandin E increased a sodium current that was depressed by [D-Ala²,N-

Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO). This effect, similar to the decrease in I_h , would be expected to reduce excitation caused by agents that are thought to mediate hyperalgesia.

The second consequence of the inhibition of adenylyl cyclase was an inhibition of transmitter release that was dependent on the activation of adenylyl cyclase (Ingram et al 1998).

Previously there was no indication that the inhibition of adenylyl cyclase affected transmitter release. Under conditions where adenylyl cyclase was activated and caused an increase in transmitter release through activation of cAMP-dependent protein kinase (PKA), opioids decreased transmitter release via a PKA-dependent mechanism. This action of opioids was not observed at all opioid-sensitive synapses, which may suggest differential distribution of adenylyl cyclase isoforms at individual synapses.

Activation of adenylyl cyclase by opioids has also been reported in both primary afferent neurons (Cruciani et al 1993) and the olfactory bulb (Onali and Olinas 1991). Studies in the olfactory bulb suggest that this response was mediated through activation of δ -opioid receptors (Onali and Olinas 1991). The increase in adenylyl cyclase activity was not affected by pretreatment with cholera toxin and was blocked with pertussis toxin. More recently, the same group has found that the increase in adenylyl cyclase activity was mediated by the release of β/γ -subunits from pertussis toxin-sensitive G proteins (Olinas and Onali 1999). A similar mechanism for the opioid activation of adenylyl cyclase was proposed in a study using a membrane preparation of longitudinal muscle-myenteric plexus from guinea pigs chronically treated with morphine (Chakrabarti et al 1998). Thus it appears that the opioid regulation of adenylyl cyclase is dependent on the isoform under study and the absence or presence of coactivated G_{α} . In the olfactory bulb it appears that the conditions are such that acute administration of opioids can activate the cyclase, whereas in other tissues, this response is observed only after adaptations induced by chronic morphine treatment.

2.1.5.2 Activation of potassium conductance

G protein-activated inwardly rectifying potassium (GIRK) channels (also known as Kir3 channels) are activated by various GPCRs, such as MOR, DOR, KOR, and nociceptin/orphanin FQ, M_2 muscarinic, α_2 adrenergic, and D_2 dopaminergic receptors (Ikeda et al 1997). GIRK channel opening is triggered by the direct action of $G\beta\gamma$ released from PTX-

sensitive G proteins, including G_i and G_o . Activation of GIRK channels induces membrane hyperpolarisation of the neurons via efflux of potassium ions and ultimately reduces neural excitability and heart rate (Wickman et al 1998). With regard to nociception, this inhibitory hyperpolarisation results in a decrease in nociceptive induced excitation and pain. GIRK channels are members of a family of inwardly rectifying potassium (IRK) channels which include seven subfamilies (Reimann and Ashcroft 1999).

The cDNAs for four GIRK channel subunits have been cloned from mammalian tissues (Reimann and Ashcroft 1999). Neuronal GIRK channels in most CNS regions are predominant heteromultimers composed of GIRK1 and GIRK2 subunits (Liao et al 1996), whereas atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky et al 1995). GIRK₁, GIRK₂, and GIRK₃ subunits are widely and distinctively expressed in the CNS (Liao et al 1996), indicating their possible involvement in various CNS functions such as cognition, memory, emotion, and motor coordination. GIRK channels coexist with opioid receptors in various neurons. It is most likely that the immediate analgesic effects of opioids are mediated by rapid signal transduction similar to the direct activation of GIRK channels by G proteins.

2.1.5.3 Inhibition of calcium conductance

There are many examples of the inhibition of calcium currents by activation of all opioid receptor subtypes. The inhibition of high-threshold calcium currents by opioids, in common with other receptors linked to pertussis toxin-sensitive G proteins:

- (i) is mediated by the β/γ -subunits of G proteins,
- (ii) decreased the rate of current activation such that the inhibition was greater immediately after the voltage step, and
- (iii) showed relief of inhibition following a depolarization to positive potentials (Wilding et al 1995).

Inhibition of calcium currents by opioid peptides results in a decrease in transmitter release and reduced Ca^{2+} entry on depolarisation. This in turn results in a reduction in second messenger effects.

2.1.5.4 Inhibition of transmitter release

The opioid inhibition of acetylcholine release in the guinea pig ileum and ATP release in the vas deferens has been used as pharmacological assays for many decades. In various peripheral preparations from different species, the activation of all three receptor subtypes has been found to cause inhibition of transmitter release (Kosterlitz et al 1986). The activation of potassium conductance and/or the inhibition of calcium conductance and not the inhibition of adenylyl cyclase have been argued to account for this action (Bhoola and Pay 1986), although recent work suggests that under some conditions the inhibition of adenylyl cyclase can also account for some of the decrease in transmitter release. Direct inhibition of the release machinery, independent of potassium and calcium conductances, has also been reported (Capogna et al 1993).

Depending on the site, opioids inhibit release of excitatory and/or inhibitory transmitters. Opioid inhibition of GABA release in local circuits, first observed in the hippocampus, has become a common observation that accounts for indirect excitatory, or disinhibitory, effects of opioids (Nicoll et al 1980). Opioids caused direct hyperpolarisation of interneurons, thus decreasing excitability of these cells (Madison and Nicoll 1988). In addition, spontaneous quantal release of GABA from terminals was decreased by opioids, suggesting that opioids also acted directly on axon terminals to decrease the probability of GABA release (Cohen et al 1992). A similar disinhibitory mechanism mediated by opioids acting on local circuits has now been described in brain regions where the local circuitry is not as well defined, such as the raphe magnus, ventral tegmental area, periaqueductal gray (PAG) and dorsal raphe.

Of equal significance is the fact that transmitter release is the result of a complex series of events with numerous protein-protein interactions such that there are multiple sites of potential regulation. Opioid receptors are one of a vast number of G protein-linked receptors that modify transmitter release. Given the potential interactions between these receptors and the effects of prior activity in any given terminal, the effects of opioids may vary considerably.

2.1.5.5 Activation of protein kinase C

The activation of PKC by opioids appears to result from the activation of phospholipase C and/or phospholipase A₂, which is thought to result from an interaction of β/γ -subunits of pertussis toxin-sensitive G protein and may require coactivation with the α -subunits of pertussis toxin-insensitive G proteins (Smart et al 1997). The results suggest that in order for opioids to have a robust effect, co activation with G_q α subtype G proteins is required. A similar pathway is also thought to mediate the release calcium from inositol 1,4,5-trisphosphate (IP₃)-sensitive stores.

2.1.5.6 Other effectors

Opioid receptors are also coupled with PLC β via Gi/o and Gq proteins. Because PLC β -KO mice exhibit enhanced morphine-induced analgesia (Xie et al 1999), it is unlikely that PLC β is the main mediator of the analgesic effects of morphine, although PLC β certainly plays a role in the nociceptive responses.

Mitogen-activated protein kinases (MAP-kinases), the major effectors for growth-factor receptors, are activated by opioid receptors via G $\beta\gamma$. The role of MAP-kinases in analgesic effects of opioids remains unknown.

The best-characterised pathways of effector activation of opioids are illustrated in Figure 2-4 and the wide array of cellular responses produced by opioid receptor activation are summarised in Table 2-1 below.

Table 2-1 Cellular responses produced by opioid receptor activation

Direct G-protein binding or subunit-mediated effects

- activation of an inwardly rectifying potassium channel
- inhibition of voltage operated calcium channels (N, P, Q and R type)
- inhibition of adenylyl cyclase

Responses of unknown intermediate mechanism

- activation of PLA₂
- activation of PLC b (possibly direct G protein bg subunit activation)
- activation of MAPKinase
- activation of large conductance calcium-activated potassium channels
- activation of L type voltage operated calcium channels
- inhibition of T type voltage operated calcium channels
- direct inhibition of transmitter exocytosis

Responses which are a consequence of opioid-evoked changes in other effector pathways

- activation of voltage-sensitive potassium channels (activation of PLA₂)
- inhibition of M channels (activation of PLA₂)
- inhibition of the hyperpolarisation-activated cation channel (I_h) (reduction in cAMP levels following inhibition of adenylyl cyclase)
- elevation of intracellular free calcium levels (activation of PLCb, activation of L type voltage operated calcium conductance)
- potentiation of NMDA currents (activation of protein kinase C)
- inhibition of transmitter release (inhibition of adenylyl cyclase, activation of potassium channels and inhibition of voltage operated calcium channels)
- decreases in neuronal excitability (activation of potassium channels)
- increases in neuronal firing rate (inhibition of inhibitory transmitter release - disinhibition)
- changes in gene expression (long-term changes in adenylyl cyclase activity, elevation of intracellular calcium levels, activation of cAMP response element binding protein (CREB))

2.1.6 Spinal actions

There is ample evidence that the dorsal spinal cord is an important site for the mediation of the antinociceptive effect of morphine and opioids (Yaksh 1981, Akil et al 1984 and Basbaum and Fields 1984). It is well documented that in the formalin-induced flinching test intrathecal (i.t.) administration of morphine as well as other opioid drugs into rats over a dose range of 1.0–20 nmol produces a reliable antinociceptive effect by interacting with opioid receptors in the spinal cord.

The highest levels of opioid receptors are around the C fibre terminal zones in lamina I and the SG with lower levels found in deeper layers. In the rat MOR predominate in the spinal cord comprising 70% of opioid receptors, with DOR and KOR comprising less than 33% of the total. The spinal effects of opioids are mediated via calcium channels, GIRK channels and the other effectors discussed in the previous section.

50-70% of the total number of opioid receptors in the superficial dorsal horn is found presynaptically on the central terminals of primary afferents (Besse et al 1990). Dorsal root section produces marked ipsilateral depletions in opioid receptor binding sites in the superficial laminae (Ninkovic et al 1981; Stevens and Seybold 1995), the major site of termination of fine diameter A and C-fibres. Similarly, destruction of sensory C-fibres by neonatal capsaicin treatment depletes opioid receptor binding sites in the dorsal horn (Nagy et al 1980). The presynaptic action of opioids has been demonstrated for MOR and DOR agonists but not KOR on the basis of a reduced release of the primary afferent transmitters present in C fibres. Evidence for a presynaptic action of opioids comes from studies of opioid inhibition of C fibre evoked release of transmitters (SP and glutamate) and from in vitro and in vivo studies (Hirota et al 1985; Aimone and Yaksh 1989; Hori et al 1992). Capsaicin can elicit the release of these transmitters, so at least a proportion of the release must be from C fibre afferents. The association of opioid receptors with C fibre but not large A fibre terminals (in adults at least) explains the observed selective effects of spinal opioids on noxious evoked activity (Duggan and North 1983).

However, opioids can have facilitatory effects on C fibre transmission at low doses, probably due to release of excitatory transmitters from the afferent terminals. A characteristic of MOR agonists is that the lowest doses of all intrathecal μ opioids enhance C fibre activity. However, inhibitions occur and then predominate as the dose is increased (Wiesenfeld-Hallin et al 1990). This mild increase in C fibre activity elicited by low doses could explain the itching around the extremities of the spinal morphine injection that occurs in humans. These effects may be the one exception to the primary inhibitory actions of opioids (Crain and Shen 1990).

The postsynaptic actions of opioids are more difficult to interpret because any direct hyperpolarisation of a cell soma would inhibit all responses of the cell. Responses of the substantia gelatinosa cells are enhanced by opioids following various routes of administration. This enhancement is via GABA_A mediated disinhibition of enkephalin interneurons or vice versa (Magnuson and Dickenson 1991). If these disinhibited cells fed onto the output cells, excitatory interneurons, or dendrites, they would be an additional source of inhibition of nociceptive transmission. Comparative studies in animals with and without the presynaptic sites provide electrophysiological evidence that the post synaptic actions of opioids require higher doses of morphine than do the presynaptic effects (Lombard and Besson 1989).

2.1.7 Supraspinal actions

Opioids also act supraspinally to produce analgesia. They reduce nociceptive input to the brain by altering ascending and descending control systems. This can be demonstrated by the analgesic efficacy of the injection of systemically inactive doses of opioids intraventricularly and intracerebrally (Lazorthes 1988).

In the very first experiments to localise opioid receptors within the CNS Pert and Snyder observed major regional variations in [³H] naloxone binding, with negligible binding in the cerebellum and highest levels in the corpus striatum (caudate–putamen). The levels in the caudate–putamen were more than four times the levels found in the cerebral cortex and midbrain, and seven times the levels found in the brain stem (Pert and Snyder 1973). The very high levels of opioid receptors that were observed in the caudate–putamen enabled the imaging of receptors in human subjects by positron emission tomography (PET) scanning

(Frost 2001). In a detailed dissection of monkey brain, variations that could explain some of the pharmacological actions of opioids were described (Kuhar et al 1973). Receptors were greatly enriched in the periaqueductal gray, where electrical stimulation produces analgesia that is blocked by naloxone. The medial thalamus, which conveys to the cerebral cortex information about 'emotional' components of pain, had almost four times the density of receptors than the lateral thalamus, which handles touch sensation and the 'pin prick' type of pain that is not influenced by opiates. Limbic regions of the brain, such as the amygdala, which regulate emotional behavior, also possessed a high opioid receptor density.

The most important advance in localising receptors came with the development by Kuhar and associates of autoradiographic techniques for localizing receptors after first administering the radiolabelled ligand to the intact animal and subsequently, and more importantly, following incubation of the radioligand with brain slices (Atweh and Kuhar 1977). Autoradiography revealed extremely dense concentrations of opioid receptors in nuclei such as: the locus coeruleus, the source of the major noradrenaline-containing cell bodies in the brain; the substantia gelatinosa of the spinal cord and brain stem, the first site for integrating sensory information within the CNS; and vagal nuclei such as the nucleus ambiguus and nucleus solitarius. These 'hot spots' for opioid receptors correlated very closely with the localization of enkephalin-containing neurons (Mansour et al 1995) and provided some of the first robust evidence that the enkephalins are endogenous ligands for the opioid receptor.

In summary, in rats, opioid receptors are widely distributed throughout the CNS being most dense in the brainstem, thalamus, amygdala, hippocampus and cortex (Mansour et al 1993). MOR are found in the majority of these areas, whereas DOR are more restricted, found mostly in forebrain regions with poor binding in the midbrain and brainstem. KOR are widely distributed throughout the forebrain, midbrain and brainstem comprising only 10% of the receptors in the rat brain but up to 33% in humans (Mansour et al 1993).

2.1.8 Peripheral actions

Opioids have long been thought to act exclusively within the central nervous system. An increasing number of studies recently reported the existence of opioid receptors outside the central nervous system and therefore suggested that opioids are also able to produce

analgesic effects in the periphery. Such effects are particularly prominent under painful inflammatory conditions, both in animals and in humans (Brack et al 2004). During inflammatory processes, opioid receptors are transported from dorsal root ganglia towards the peripheral sensory nerve endings. At the same time, immune cells containing endogenous opioid peptides accumulate within the inflamed tissue. Environmental stimuli (e.g. stress) as well as releasing agents (e.g. corticotropin releasing factor, cytokines) can liberate these opioid peptides to interact with the neuronal opioid receptors and elicit local analgesia. The inflammation-induced activation of opioid production and the release of endogenous opioids from immune cells may lead to novel approaches for the development of peripherally acting analgesics. Clinical investigation now focuses on the development of new peripheral opioid agonists as well as on ways to stimulate the endogenous analgesic system in order to induce effective peripheral analgesia with reduced central side effects typically associated with opioids (for review see Janson and Stein 2003).

2.2 Opioid efficacy and age

2.2.1 Clinical studies

Historically neonates were considered to be relatively insensitive to pain. This belief was based on misconceptions such as small infants have an immature nervous system and are unable to feel pain. There was a feeling that infants do not remember pain, they do not experience pain in the same way as adults do and so they do not require the same level of pain relief as adults. With increased research and knowledge of the developing pain pathways these beliefs have been clearly shown to be incorrect (Anand et al 1987; Anand and Hickey 1992). These studies demonstrate the need for opioid-based post operative analgesia which result in clinical benefits to the neonate.

As well as uncertainty about the need for pain relief in young patients, reluctance to prescribe opioids has occurred as a result of concerns about an apparent increased susceptibility of neonates to opioid-related side effects. Early reports have suggested that newborns are more prone to the respiratory depressant effects of morphine than adults (Way et al 1965) as well as the CNS depressant effects of opioids. However, this is not supported by animal studies

(Abbott and Guy 1995). Early studies indicating that neonatal rats are more susceptible to the convulsant effects of morphine have not been confirmed and high dose morphine has shown to produce no behavioural convulsions (van Praag and Frenk 1992).

There have been no studies in humans using a constant and readily quantifiable measure of pain at different ages that allow accurate comparisons of the potency of commonly used opioids at differing ages. Direct comparisons between drugs and across ages are often not feasible due to lack of standard assessment techniques, dependent measures, and ethical concerns over the use of human subjects. The few studies that have addressed the issue of morphine efficacy have used subjective criteria such as nursing observations. Scott et al (1999) attempted to correlate morphine serum concentrations with analgesia in premature neonates by using a recognised objective validated scale. However steady-state morphine concentrations varied greatly so no correlation was found. Other studies in older children and adults also determined that there is not a simple relationship between serum concentration and analgesic efficacy (Dahlstrom et al 1982; Lynn et al 1984; Olkkola et al 1988).

Hansen et al (1996) investigated whether small children require less morphine for post operative analgesia than older children and adolescents. They compared the morphine consumption of 28 consecutive children using PCAs after major surgery. The median age-specific morphine requirements between 2 comparable groups of children aged 4-8 years and 9-15 years were compared. The children aged 4-8 years had significantly higher total postoperative morphine requirements compared to children aged 9-15 years (11.6mcg/kg/h and 7.5mcg/kg/h respectively).

More recent studies have highlighted age as the most important factor affecting morphine requirements in neonates and infants postoperatively (Bouwmeester et al 2003). This group has investigated age-related differences in morphine requirements and metabolism intensively in a cohort of neonates treated on their neonatal intensive care unit following noncardiac surgery. After surgery, patients were randomly assigned to continuous morphine or intermittent morphine. Additional morphine was administered on guidance of pain scores measured using visual analogue scales and the Comfort behavioural scale. Younger neonates were found to have significantly lower morphine requirement (median 10 vs. 10.8

µg/kg per hour) than older neonates. Importantly, morphine plasma concentrations were not correlated with analgesia or respiratory depression. They concluded that neonates aged 7 days or younger require significantly less morphine postoperatively than older neonates.

2.2.2 Laboratory studies

Paediatric clinical trials have many drawbacks. They generally involve the administration of drugs to patient populations for whom the drug has potential benefits. Phase I studies for the introduction of new drugs to human beings usually involve healthy volunteers to investigate potential adverse effects of the drug under investigation and there is no potential benefit to the participants. This is ethically impossible in infants and children. Sample size in paediatric studies is restricted by ethical, financial and logistical reasons. For these reasons it has become desirable to perform drug studies in developing animals. Animal studies have various powerful advantages e.g. genetic methods have been used to produce particular strains of animal and in mice specific gene manipulation has produced various knock-outs that allow investigation of the effects of specific genes; animal models allow the study of mechanisms by using various physiological, histological or biochemical methods which would not be possible in a human study.

Adult rats are commonly used in studies of nociception and infant rats are used for developmental studies. As well as offering the opportunity to study the ontogeny of nociceptive functions the rat model also has potential pitfalls. It is important to be aware of these when interpreting data and extrapolating the results to human subjects.

At birth maturation of peripheral and spinal somatosensory functions in rat pups is roughly equivalent to a 24-week premature neonate. A 7-day-old infant rat is equivalent to a term neonate. Rats are weaned at about 15 days and at 21 days can be considered to be roughly analogous to school aged humans.

Conflicting reports exist about the effectiveness of opioids in producing analgesia in young animals. These differences may reflect the varying dose regimens, age ranges and behavioural tests studied.

The types of nociceptive stimuli used in various models include electrical, thermal, mechanical or chemical. None is ideal although chemical stimuli probably most closely mimic

acute clinical pain. Behavioural models have been developed based on these nociceptive stimuli. The tests using short-duration stimuli or phasic pain are the most common and, in general, involve measuring thresholds and the response time to a stimulus of increasing intensity with the assumption that this reaction time is related to the threshold.

On the whole, current literature suggests that opioids produce a significant dose-related antinociception young in rats. Windh and Kuhn (1995) reported that neonatal rat pups (P10) are more sensitive to morphine antinociception than are weanlings (P27) using the hot plate test. This test consists of introducing the rat into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated. The heated plate produces 2 behavioural components that can be measured in terms of their reaction times, namely paw-licking and jumping. Rats can demonstrate more complex behaviour, however, which makes assessment of reaction times difficult and they are able to learn about the noxious nature of the hot plate, which results in a progressive shortening of the reaction time. This test consists of stimulating the four limbs and even the tail of the animal simultaneously. Such heterotopic stimuli involving large body areas must trigger inhibitory controls that are likely to affect the observed responses. Although these factors make this test a difficult one to use, it remains a valuable tool in the investigation of nociceptive processes in animals. McLaughlin and Dewey (1994) found that opioids were effective antinociceptive agents in both neonates and adults with three different types of nociceptive tests; tonic (formalin-induced inflammation) and phasic (tail flick and hot plate) and that the relative potencies of these agents appeared to be similar in neonates and adults. In general the pups were more sensitive to the antinociceptive agents when tested in the phasic tests. The tail-flick test involves the application of thermal radiation to the tail of the animal, which provokes the withdrawal of the tail by a brief vigorous movement. It is the reaction time of this movement that is recorded. Although the tail flick test can be a useful nociceptive test it does have various limitations. The rodent tail has a very specific function – it is an essential organ of thermoregulation and balance. How representative is it of other peripheral nociception? Also reaction time varies with the part of the tail that is stimulated – it decreases when the stimulus is applied to increasingly distal parts of the tail (Ness et al 1987). There is consensus; however, that this test is truly efficient

only for revealing the activity of opioid analgesics and it is adequate for predicting their analgesic effect in humans.

Marsh (1999) has also shown that P3 animals demonstrate a greater response to morphine as well as the delta (DPDPE) and kappa (U69593) opioid receptor agonists administered both systemically and epidurally than P21 animals using the cutaneous flexion withdrawal reflex elicited using von Frey hairs. This test is based on the use of mechanical stimuli. A pressure of increasing intensity is applied to a punctiform area on the hind paw. The measured parameter is the threshold for the appearance of a given behaviour that is the reflex withdrawal of the paw. In heat tests the pattern differed. The efficacy of the κ agonist decreased with postnatal age, morphine efficacy increased over the same period and the effects of the δ agonist remained relatively unchanged. Marsh concluded that whereas heat pain is particularly sensitive to spinal κ opioids in neonates, mechanical sensory thresholds are generally sensitive to all spinal opioids in the newborn.

Possible explanations for the increased sensitivity of neonatal rats to morphine compared to their older counterparts include a poorly developed blood brain barrier, immature pharmacokinetic handling of opioids and developmental changes in receptor number and/or function.

These studies stand in contrast to previous reports that indicated marked insensitivity to the antinociceptive properties of morphine in P2 rat pups (Zhang and Pasternak 1980 and 1981). The hot plate test was used in these studies and there are important methodological differences between the studies.

Morphine analgesia to thermal nociception was shown to increase with age in neonatal rat pups, progressing to a 40 fold analgesic potency at P14 compared to P3 (Giordano and Barr 1987).

As a result of widely varying behavioural tests, age ranges, dose ranges and even routes of administration used it is difficult to compare these studies and draw conclusions from the conflicting results.

2.3 Developmental issues

2.3.1 Pharmacokinetics

Dose requirements of opioids and responses to them change markedly in premature and term neonates, infants and children. Several factors contribute to these differences, one of which is the alteration in pharmacokinetic handling of opioid agonists.

Many pharmacokinetic studies have been undertaken to evaluate the handling of opioids in neonates and infants. Most of these have investigated morphine. There is a large degree of variability between these studies as a result of differences in study design (e.g. duration of measurement, timing of sampling, assay method), indication for morphine usage (e.g. intraoperative anaesthesia, postoperative analgesia, sedation for mechanical ventilation), analytical procedures and pharmacokinetic assumptions and calculations.

In a meta analysis performed by Kart et al the volume of distribution of morphine was found to be 2.8 ± 2.6 l/kg irrespective of the age of the neonates and children studied. Standard deviations are very large as a result of great individual variation. Protein binding is reduced in preterm and term neonates (18-22%) when compared with adults (20-35%). However, the degree of protein binding is relatively low in both populations and therefore leads to little discrepancy in free morphine concentration (Kart et al 1997). These parameters would suggest that the same bolus dose per kg of morphine would give rise to similar plasma concentrations at differing ages. This is not the case. Animal studies have found higher initial plasma and brain concentrations in younger animals after single doses. This is due to a delayed redistribution phase – 150 minutes in P10 rat pups compared with 26 minutes in P27 rat weanlings (Windh and Kuhn 1995). The duration of action of a drug is affected by its clearance and elimination half-life and it is these parameters that differ most significantly with age. All studies have shown that clearance is affected by postnatal age with adult clearances being reached by 2 months of age in some studies (Lynn and Slattery 1987; Pokela et al 1993) or as late as six months of age (Olkola et al 1988). Hepatic glucuronidation is the main metabolic route for the elimination of morphine with morphine 3 glucuronide (M3G) and morphine 6 glucuronide (M6G) being the main metabolites. The kidneys excrete the

glucuronides and impaired renal function leads to the accumulation of M3G and M6G. M6G has significant analgesic properties. The majority of preterm neonates are capable of conjugating morphine to either M3G or M6G or both but this metabolic capacity is reduced contributing to the slow clearance of the drug. Birth weight may be correlated with the glucuronidation capability, but other factors such as postnatal age are also important. Thus there is a tendency for half-life to decrease with increasing age although there are diverging reports concerning the influence of gestational and postnatal age.

In addition to inter patient variability there are reports of great intra patient variability. Concurrent illness is extremely important in morphine kinetics in infants. Lynn et al (1993) demonstrated that infants who underwent cardiac surgery showed consistently lower morphine clearance compared with their age –matched peers undergoing noncardiac surgery. Pharmacokinetic data is summarised in Table 2-2 below.

Table 2-2 Pharmacokinetics of opioids in neonates, infants and children

	Half life (hours)	Clearance (ml/kg/min)
Preterm neonates	9.0 +/- 3.4	2.2 +/- 0.7
Term neonates	6.5 +/- 2.8	8.1 +/- 3.2
-after cardiac surgery		5.5 (3.4 – 13.8)*
-critically ill		2.1**
Infants and children	2.0 +/- 1.8	23.6 +/- 8.5

From Kart et al 97 (meta analysis)

*Lynn et al 93

**Pokela et al 93

Opioids are now often administered as continuous infusions e.g. postoperatively and in intensive care. Lower doses than adults will maintain similar serum concentrations in neonates and infants as a result of reduced clearance. Regimens producing steady-state plasma concentrations of 25-20ng/ml have been described (Lynn et al 1998). However these doses have not been suggested to achieve analgesic efficacy but rather to minimise the

incidence of respiratory depression. In fact, an effective minimum analgesic plasma concentration for morphine has not been widely studied in infants and children. In a study comparing morphine by infusion or intermittent bolus dosing improved pain scores were reported in the infusion group, however this group also received a higher total dose of morphine. Interestingly no correlation was found between steady state morphine concentrations and pain scores (Lynn et al 2000).

Establishing pharmacokinetic differences at differing ages has been very important. It has enabled dosage guidelines to be established especially for continuous infusion regimens where excessive accumulation of the drug is a problem. However, for the management of pain in infants in a clinical setting the knowledge of pharmacokinetic parameters alone clearly has its limitations. Firstly, there is huge inter patient variability in kinetics as described above. Secondly, there is huge intra patient variability. Clearance is significantly lower in critically ill infants. And thirdly, there is little data relating plasma concentration and analgesic efficacy.

Pharmacokinetic handling of opioid agonists is only one factor that affects the changes in response to opioids with age. Pharmacodynamic differences due to developmentally regulated changes in opioid receptor expression, distribution and function are also important.

In addition to pharmacokinetics, pharmacogenetics plays a role in opioid efficacy during development.

The liver is the primary site of biotransformation of most drugs, including opioid drugs. Metabolism reactions can be divided into one of two broad groups: Phase I (conjugation reactions, usually with glucuronic acid) or Phase II reactions (both N- and O-dealkylations catalysed by various isoforms of the predominantly hepatic cytochrome P450). There are families (>40% amino acid sequence homology) and subfamilies (>55% sequence homology) of 35 human cytochrome P450s, of which 18 subfamilies are involved in the metabolism of drugs and other xenobiotics. The inter patient expression of various isoforms of cytochrome P450 may well be an important factor in explaining variability in the pharmacokinetics of opioids by influencing the rate and extent of metabolism. Cytochrome P450 3A4 is the major isoforms in the liver and the small intestine, where it constitutes 30% and 70% of total

cytochrome P450 protein; Thus cytochrome P450 3A4 is the major isoforms responsible for the metabolism of drugs. Its activity can be manipulated by environmental factors; e.g. hepatic levels of this cytochrome can be induced and its activity inhibited by concomitantly administered drugs. However, the large inter individual variation of cytochrome P450 3A4 activity is of major importance and is presumed to be under genetic control (Thummel and Wilkinson 1998).

Cytochrome P450 2D6 is involved in the metabolism of codeine (and its derivatives) to morphine (and corresponding derivatives) by O-demethylation. There is a polymorphic distribution of this isoforms in the Caucasians such that 8 to 10% of the population lack the capacity to perform this conversion. This variation results in the subdivision of patients into two groups, either extensive or poor metabolisers. Thus, poor metabolisers will not experience analgesia after codeine administration. Essentially similar metabolic considerations apply to oxycodone, hydrocodone and dihydrocodeine.

The pharmacodynamic effects of this bimodal distribution of cytochrome P450 2D6 activity for codeine and its various derivatives in humans depend on the relative binding (to μ and possibly other receptors), intrinsic activity, and pharmacological effects of the parent compound relative to its O-demethylated metabolite.

2.3.2 Developmental Pharmacology

Endogenous opioids are developmentally regulated as are the densities and distribution of their receptors, both in the brain and the spinal cord.

Opioid receptors are present from early foetal life in the rat and human, in the brain and the spinal cord, and display pre- and postnatal maturational changes (Kirby 1982; Attali et al 1990; Xia and Haddad 1991; Kar and Quirion 1995) before achieving the final adult distribution.

Many studies have described the development of opioid receptors using tissue homogenate binding methods providing information relating to the overall receptor density for the tissue, but they do not resolve localisation within the tissue (Attali et al 1990). In addition, the use of different ligands, concentrations and methods make comparison of data difficult.

Most in vitro studies on the cellular effects of opioids are performed using cells from very young animal tissue so it can be presumed that opioid receptors are functional at early stages (Sivilotti et al 1995; Faber et al 1996). As the antinociceptive effects of opioid receptor agonists are not necessarily entirely predictable from the density of receptor binding sites, a number of receptors may not be functional. Immunohistochemical studies with antibodies to MOR and DOR have reported MOR and DOR like immunoreactivity at intracellular sites, such as on the vesicular membrane (Arvidsson et al 1995; Zhang et al 1998), as have studies where μ and δ opioid receptor binding in membranes from endoplasmic reticulum and Golgi complexes were investigated. These studies concluded that opioid receptors in adult microsomes were not functionally coupled to G proteins, whereas receptors on synaptic plasma membranes were functionally coupled (Szucs and Coscia 1992). Thus G protein coupling and effector mechanisms can be developmentally regulated. Alternatively, G protein coupling activity has shown to be much greater in adulthood than in neonatal life (Szucs and Coscia 1990). The levels of G_i and G_o have been reported to be present in 100-1000 fold excess over the amount of MOR and DOR in the brain of neonatal and adult rats.

2.3.2.1 Ontogeny of spinal opioid receptors and ligands

The onset of spinal opioid receptor expression is unclear as few studies have investigated the postnatal ontogeny of opioid receptor binding sites in the spinal cord (Kirby 1981; Attali 1990; Kar and Quirion 1995). The findings from the early Kirby study, using the non-selective opioid antagonist [3 H] diprenorphine, described fluctuations in the development of opioid receptor number in the spinal cord. Opioid receptors were present in the cord from embryonic day 16 (E16) and subsequently a massive linear increase in receptor density is seen up to E22. A significant drop in receptor density was seen at P1, after which a further rapid increase in receptor numbers was observed, peaking at P6. The biphasic development of receptor number is likely to relate to the differential development of the various opioid receptors in the cord. A more recent study (Attali et al 1990) investigated the pre and post natal development of μ , δ and κ opioid receptor binding in spinal cord homogenates. No opioid receptor binding was detected before E15. Both μ and κ receptor binding sites displayed a rapid increase in density from E18. κ receptor number peaked at E20 followed by a 20% drop in receptor density between P1 and P3. MOR binding peaked a little later at P1 and the levels of MOR

binding remained relatively stable from P1 to adulthood. In contrast to the pattern seen for MORs a second peak of KOR binding was seen at P7, with receptor density declining to mature levels by P15. DOR binding was detected later than that of MOR and KORs at about P1. Little change in the density of DOR was observed from first appearance to adulthood. The use of non-selective ligands which cross-react with more than one opioid receptor, such as ethylketocyclazocine and diprenorphine, in these two studies has led to controversy as to when the δ opioid receptor is first expressed, and as to the relative proportions of μ , δ and κ receptors at different postnatal ages in the spinal cord. An interesting study by Rahman et al investigated the postnatal ontogeny of μ , δ and κ opioid receptor binding sites in the spinal cord of rat pups at various postnatal days using in vitro autoradiographical methods and selective ligands. In addition, the functional effect of spinal morphine was assessed using in vivo electrophysiological methods in rats at P14, P21 and adults (P56). Both μ and κ opioid receptor binding-sites were found to be present from P0 and spread relatively diffusely throughout the spinal cord. Overall binding peaked at P7 and subsequently decreased to adult levels with the μ opioid receptor binding sites regressing to become denser in the superficial dorsal horn. δ opioid receptor binding were first seen at P7, and no distinction between superficial and deeper laminae was seen. C-fibre evoked dorsal horn neuronal responses recorded from anaesthetized rat pups were found to be highly sensitive to spinal morphine at P21, (EC_{50} 0.005 μ g), compared to the adult (EC_{50} 0.9 μ g). However, the EC_{50} (0.2 μ g) at P14 was greater than at P21 despite the fact that μ receptor binding was greater at P14. They concluded that the number of μ receptor binding sites appears not to be the only determinant of functional sensitivity to spinal morphine. Other factors, such as coupling of the receptors are likely to be important.

In the adult, the endogenous opioid peptides methionine and leucine-enkephalin, the endogenous ligands for δ and μ receptors, and dynorphin A for κ receptors, are present within local dorsal horn neurons. The pre and post natal distribution and development of met-enkephalin-arg6-gly7-leu8 (Enk-8) containing neurons in the sensory ganglia of the rat has been investigated. Enk-8 like immunoreactivity first appeared in neurons of the DRG at E21, similar to the appearance of leu-enkephalin. The number of immunoreactive neurons in these

sensory ganglia peaked at P5-7. Subsequently levels of Enk-8-like immunoreactivity in the sensory ganglia decreased to barely detectable levels in adult animals (Senba et al 1989).

2.3.2.2 Ontogeny of supraspinal opioid receptors and ligands

Endogenous opioid peptides appear in the rat CNS from early foetal ages, before their receptors. This excludes the cerebellum, where enkephalin-like immunoreactivity in neuronal cells is not even detected at P1 (Osborne et al 1993). β -endorphin, met-enkephalin and dynorphin are all found at E11.5, followed by μ receptor binding at E12.5, κ receptor binding at E14.5 and δ receptor binding at P0 (Rius et al 1991). Enkephalin-like immunoreactivity has been detected in the rat brain between E 16 and 18 (Pickel et al 1982). Leucine enkephalin (LE) positive cells were detected in the grey matter at E20-21, with content peaking at P1-3 followed by a decline to adult levels (Senba et al 1982).

Studies which have investigated both the development of endogenous opioids and their receptors, report some similarity in the patterns of elevation and decline in the amounts of opioid binding sites and in the level of opioid peptides (Tsang et al 1982).

The region-specific appearance of messenger RNA for the three opioid receptors has been described in both the nervous system and peripheral tissues of the embryonic and fetal mouse (Zhu et al., 1998). Although the prenatal appearance of opioid receptor binding in whole brain homogenates has been detected (Rius et al 1991), an anatomical comparison of opioid receptor proteins with the RNA distribution during prenatal development is still lacking.

Conclusions drawn from gene expression or ligand binding studies examining opioid system development are limited to the extent that neither provide functional assessment of receptor activity. Nitsche and Pintar have used GTP γ 35S binding assays (Nitsche and Pintar 2003) and its adaptation to autoradiography, to determine the anatomical pattern of opioid receptor coupling to G-proteins in histological sections. These techniques were used to extend previous gene expression and ligand binding studies and ascertain at what stage and in which fetal brain regions the mu opioid receptor (MOR) and delta opioid receptor (DOR) become active and how the onset of this functional parameter of receptor activity is related to gene activation.

MOR activation was first detected in the caudate-putamen (CPU) at embryonic day 12.5 (E12.5), and by E15.5, activity had not only increased in this region but also expanded to include the midbrain, medial habenula, hypothalamus, pons, and medulla. DOR activity first appeared at E17.5 in the hypothalamus, pons, medial habenula, and medulla and at P1 in the CPU at levels noticeably less than those of the MOR. In general, MOR and DOR activation lagged only slightly behind the appearance of MOR-1 and DOR-1 mRNA but delayed activation was particularly pronounced in the trigeminal ganglia, where MOR-1 gene expression was first detected at E13.5, but MOR activity was not observed even at birth. Thus, the data demonstrate temporal and often region-specific differences in the appearance and magnitude of functional activity in cell groups expressing either the MOR-1 or DOR-1 genes, suggesting that interaction between the opioid receptors, G-proteins, and other signalling cofactors is developmentally regulated.

2.3.2.3 What is the role of the opioid system in development?

A role for the opioid system in the development of both nervous and somatic structures has been suggested based on studies showing many detrimental effects of perinatal exposure to both morphine and methadone, including lowered survival rates (Hutchings et al 1992), decreased body weight (Kunko et al 1996), impeded brain growth delayed appearance of neuromuscular re-flexes and behaviours, restricted neuronal proliferation, and physical dependence (Zagon 1987). Chronic administration of the general opioid antagonist naltrexone to newborn pups or pregnant mice was initially shown to produce several interesting phenotypes, including increased weight, enlarged brains, increased cerebellar volume, an acceleration in the appearance of sensorimotor behaviours, and increased Purkinje cell dendritic arborisation. More recent studies have shown that continuous opioid receptor blockade for the duration of gestation results in even greater increases in body, brain, and organ growth (McLaughlin et al 1997), accelerates the appearance of physical characteristics, reflexes, and spontaneous motor behaviours (McLaughlin et al 1997), and produces a decrease in MOR binding density and decreased sensitivity to the analgesic effects of morphine (Zagon et al 1998).

In fact opioid peptides are known to act as growth factors in neural and non-neural cells and tissues, in addition to their role in neurotransmission/neuromodulation in the nervous system

(for review see Zagon et al 2002). One of the endogenous opioid peptides, [Met5]-enkephalin, is a tonic inhibitory peptide that plays a role in cell proliferation and tissue organization during development, cancer, cellular renewal, wound healing, and angiogenesis. To distinguish the role of [Met5]-enkephalin as a growth factor in neural and non neural cells and tissues, and in prokaryotes and eukaryotes, this peptide has been termed opioid growth factor (OGF). OGF action is mediated by a receptor mechanism. Studies show that the receptor for OGF (OGFr) is an integral membrane protein associated with the nucleus. Using antibodies generated to a binding fragment of OGFr, this receptor has been cloned and sequenced in human, rat, and mouse. The interaction of OGF with OGFr obeys the pharmacological principles of the definition of an opioid receptor, yet unexpectedly the OGF receptor has no homology to the opioid receptors in terms of nucleotides or amino acids. Co-localization studies have detected OGFr on the outer nuclear envelope where it interacts with OGF. The peptide–receptor complex associates with karyopherin, translocate through the nuclear pore, and can be observed in the inner nuclear matrix and at the periphery of heterochromatin of the nucleus. Signal transduction for modulation of DNA activity is dependent on the presence of an appropriate conformation of peptide and receptor. It is interesting that the endogenous opioid peptide, [Met5]-enkephalin, can function as a neurotransmitter/neuromodulator by way of classical opioid receptors, and it can also function in growth—and does so by way of a totally different receptor—OGFr.

It seems that alteration of the balance in one or both of peptide and receptor could have a profound effect on growth. The effect of disrupting the equilibrium on developing pain systems is not known.

2.4 Other opioid systems

2.4.1 Orphanin/FQ

The classic three opioid receptors have been defined by their affinity with selective agonists and antagonists. In 1992, the amino acid sequence of the δ opioid receptor was determined and, subsequently, the amino acid sequences of the μ and the κ opioid receptors were determined (for review see Satoh and Minami 1995). The cDNAs of the μ , κ and δ opioid

receptors encode highly homologous proteins which have a primary structure typical of G protein coupled membrane receptors and inhibit adenylyl cyclase. Further attempts to clone the novel opioid receptor types were made and a cDNA encoding a novel receptor protein, the orphan opioid receptor, has been identified in human, mouse and rat. The human orphan opioid receptor was called ORL1 (opioid receptor like1) (Mollereau et al 1994)

ORL1 receptor is a G protein-coupled receptor and mediates inhibition of adenylyl cyclase. The sequence of the ORL1 receptor is closely related to that of the classical opioid receptors μ , δ and κ . Classical opioid agonists and antagonists, such as morphine and naloxone, are not active on the ORL1 receptor. In 1995, the endogenous agonist for ORL1 receptor was identified by two groups (Meunier et al 1995; Reinscheid et al 1995) and the agonist was named nociceptin (Meunier et al 1995) or orphanin FQ (Reinscheid et al 1995).

Nociceptin/orphanin FQ is a 17 amino acid peptide and has a sequence that is similar to dynorphin A. Nociceptin/orphanin FQ is not active at the classical opioid receptors. As well as the role of nociceptin/orphanin FQ in nociceptive information transmission this system has numerous other functions, including effects on feeding, learning, locomotion, circulation and autonomic nervous system effects.

The precise role of nociceptin/orphanin FQ on nociceptive transmission is still unclear.

Localisation of nociceptin/orphanin FQ and ORL1 receptor strongly suggests that nociceptin/orphanin FQ and ORL1 receptor play an important role in the processing of nociceptive information. The effect of intracerebroventricular (i.c.v) injection of nociceptin/orphanin FQ on the nociceptive transmission is completely different from those of intrathecal injection of nociceptin/orphanin FQ. Although some researchers reported that i.c.v. injection of nociceptin/orphanin FQ had no effect on the nociceptive transmission, most of the studies demonstrated that i.c.v. injection of nociceptin/orphanin FQ produced hyperalgesia or allodynia and the effect of i.c.v. injection of nociceptin/orphanin FQ was not antagonised by naloxone. On the other hand, intrathecally administered nociceptin/orphanin FQ produces complex effects on the spinal nociceptive transmission. In the hot plate test, no analgesic effect was seen after the intrathecal injection of high dose nociceptin/orphanin FQ, and, in the

tail flick test, intrathecal injection of high dose nociceptin/orphanin FQ produced analgesic effects. On the other hand, a low dose of nociceptin/orphanin FQ produced allodynia. Moreover, i.c.v. injection of nociceptin/orphanin FQ antagonised the morphine analgesia but nociceptin/orphanin FQ did not act as an anti-opioid peptide in the spinal cord.

N/OFQ and its NOP receptor display a widespread localization in the central nervous system and seem to participate in many distinct neuronal circuits, thereby exerting a multitude of diverse actions. Although structural similarities to opioid receptors led to the discovery of NOP, this receptor shows low-affinity binding to selective opioid agonists or antagonists. Conversely, N/OFQ does not activate classical opioid receptors. At the cellular level, signaling events appear very similar to those elicited by opioid peptides, whereas the behavioral effects of N/OFQ proved to be unique. Physiological roles intensively studied since their discoveries comprise opioid-like as well as anti-opioid or opioid-opposite actions. Recently, potent and highly selective antagonists and non peptide agonists have been developed, the behavioral effects of which are currently being investigated in a large number of studies. This will help to clarify some disputed issues about the physiological role of the N/OFQ system.

Some believe that the spinal ORL1 receptor may be the next receptor which should be targeted by drugs designed for the treatment of intractable pain. Further investigations are needed to clarify the role of ORL1 receptor on the nociceptive transmission.

The role and effects of this system during development remain unclear. Knowledge of the distribution of the orphanin system during development, in human as well as the rodent, is crucial in understanding what role, if any, orphanin may play in the evolution of stress and pain circuitry. Using in situ hybridization techniques, a study by Neal et al (2001) was undertaken to determine the normal pattern of expression of ORL1 mRNA in the human and rat brain at various developmental stages. In situ hybridization was performed on rat embryos, postnatal rat brains and postmortem human brains using riboprobes generated from cDNA containing representative human and rat ORL1 and OFQ sequences. Both ORL1 and OFQ mRNA is detected as early as E12 in the cortical plate, basal forebrain, brainstem and spinal

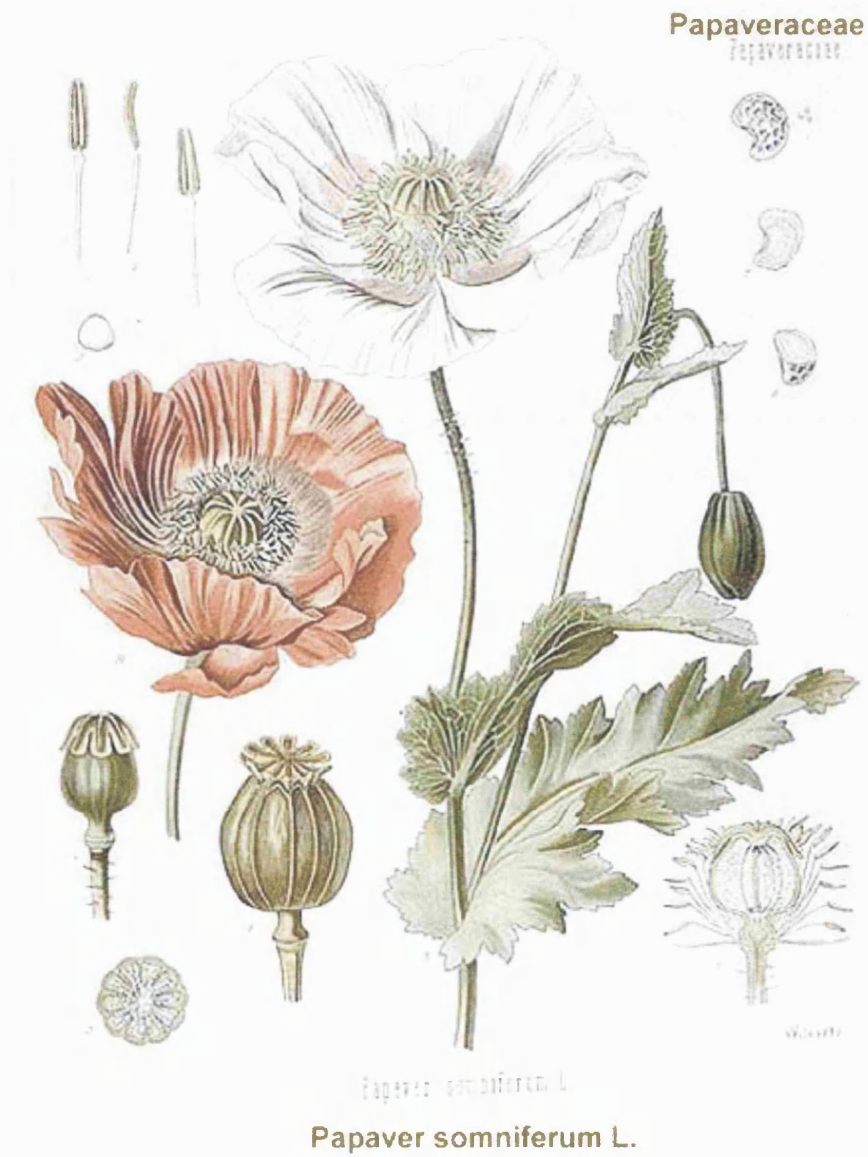
cord. Expression for both ORL1 and OFQ is strongest during the early postnatal period, remaining strong in the spinal cord, brainstem, ventral forebrain, and neocortex into the adult. Human ORL1 and OFQ expression is observed at 16 weeks gestation, remaining relatively unchanged up to 36 weeks. The influence of early orphanin expression on maturation of stress and pain circuitry in the developing brain remains unknown.

The ORL1 receptor and its endogenous ligand OFQ have been implicated in numerous behavioral and physiologic processes, including pain perception and stress. Studies have demonstrated that mRNAs for prepro orphanin and the ORL1 receptor are expressed early in rat and human gestation (Neal et al 2001). Although no evidence for significant transient expression of OFQ or ORL1 has been observed, the presence of elements of the orphanin system on numerous populations of neurons soon after their differentiation, in the human and rat, suggests possible participation in early developmental events.

In addition to the stress of critical illness, premature neonates are routinely exposed to many stressors during their stay on neonatal intensive care units, including pain, cold, light, noise and handling. As part of their management, most of these infants are frequently exposed to opioids. This exposure to a powerful exogenous neuroactive agent occurs during a critical phase of development in the nervous system.

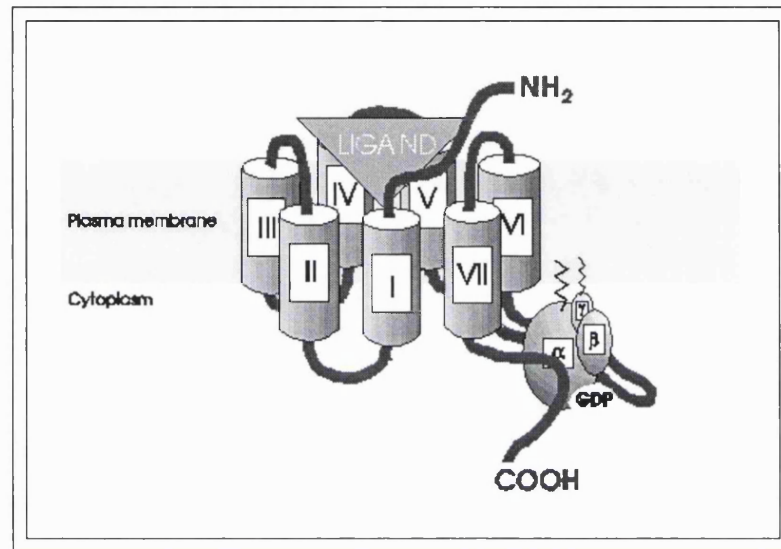
The mechanisms of action and consequences of early opioid administration are in need of greater robust investigation.

Figure 2-1 The opium poppy



(from www.opioids.com, artist unknown))

Figure 2-2 A three dimensional representation of a G protein coupled receptor



(from <http://3D> presentation of the 7 helices transmembrane structure of a GPCR)

Figure 2-3 The opioid receptors

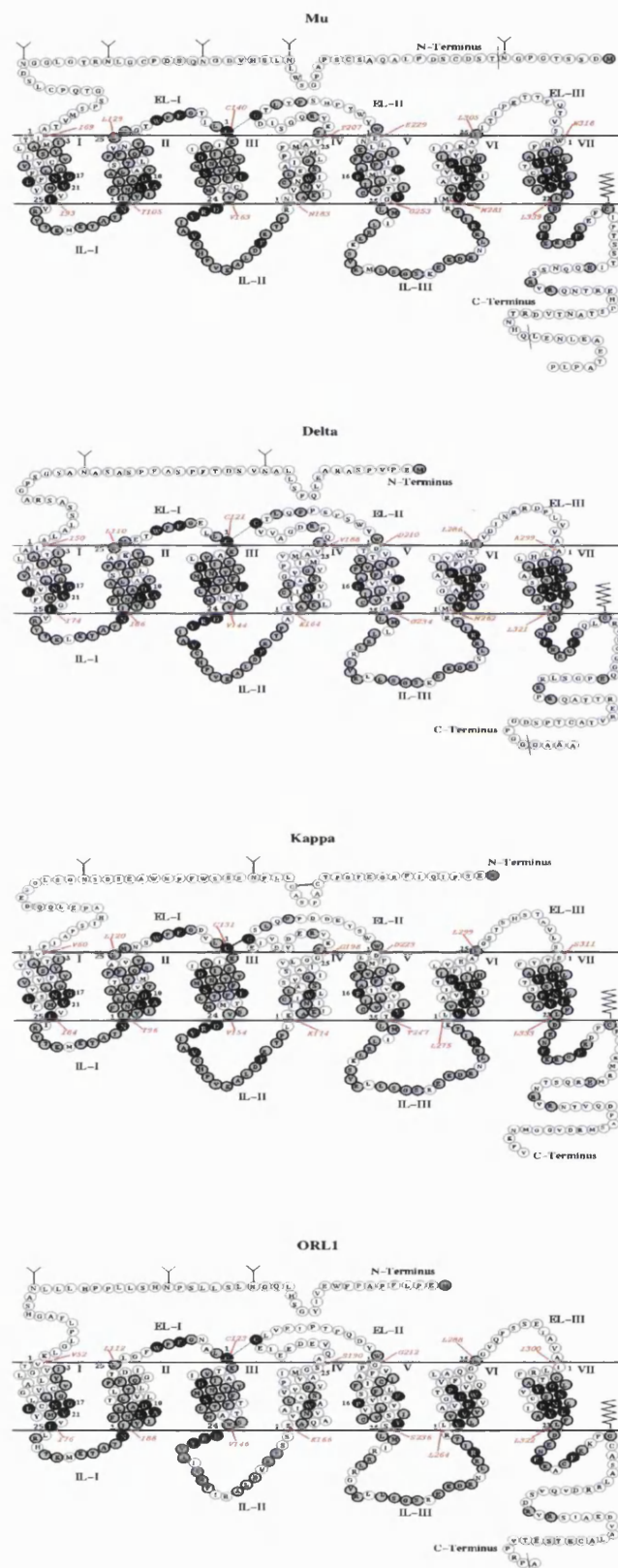
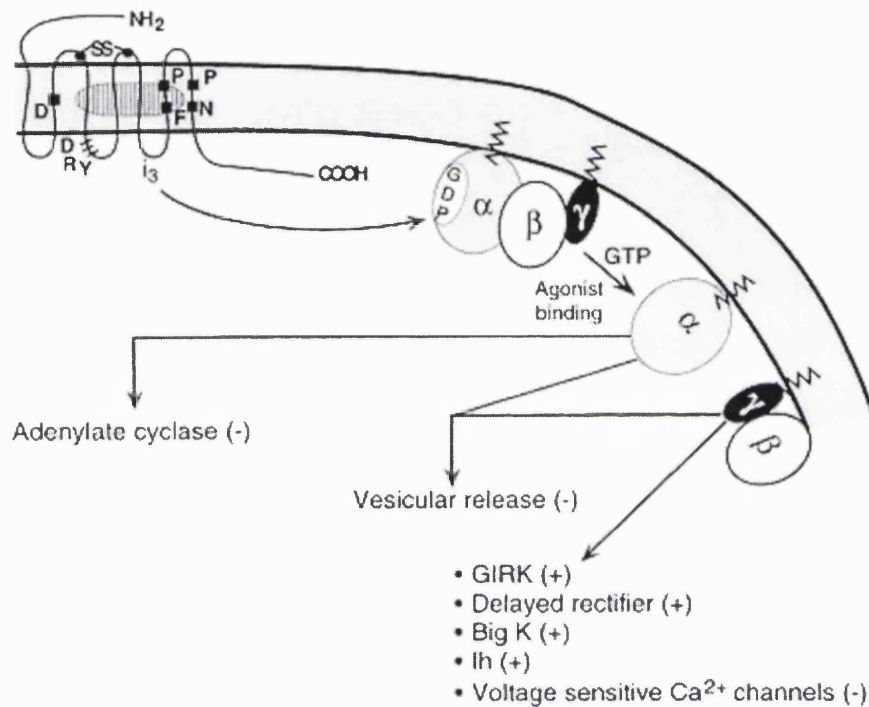


Figure 2-4 The best-characterised pathways of effector activation of opioids



An illustration of the best-characterized pathway of effector activation of opioids. Three primary classes of effectors include the inhibition of adenylyl cyclase, inhibition of vesicular release, and interactions with a number of ion channels. These effectors are affected by both the GTP-bound form of the α-subunit as well as free β/γ-subunits of pertussis toxin-sensitive G proteins. GIRK, G protein inwardly rectifying conductance.

(from [http:// secondary messengers](http://secondarymessengers))

3 MOR expression in primary sensory neurons during postnatal development

3.1 Introduction

Evidence from animal and human studies suggest that opioids have different analgesic efficacy in the immature compared to the mature nervous system (Bouwmeester et al 2003; Thornton et al 1998; Rahman and Dickenson 1999). Opioids also exert an effect on non-nociceptive sensory processing in the developing nervous system (Faber et al 1997; Marsh et al 1999). One of the possible explanations for this difference in opioid effect may be a postnatal regulation in the expression of opioid receptors, particularly in the spinal cord which is an important site of opioid action.

Opioid agonists are known to exert their most powerful analgesia after spinal administration in animals and humans (Stevens and Yaksh 1988). Spinal opioid receptors have been shown to contribute to the analgesia produced by morphine regardless of the route of administration i.e. spinal, supraspinal or systemic. In the superficial dorsal horn of the spinal cord, the majority of opioid receptors are located presynaptically on the central terminals of primary afferent neurons (Lamotte et al 1976; Besse et al 1990). This presynaptic location for opioid receptors has been inferred from in vivo (Yaksh et al 1980; Kuraishi et al 1983) and in vitro studies (Macdonald and Nelson 1978; Mudge et al 1979) demonstrating an opioid inhibition of neurotransmitter release from the central terminals of primary afferent fibres. Further evidence for the presynaptic location of opioid receptors is provided by labelling studies which demonstrate a dramatic decrease of opioid binding sites in the superficial laminae of the dorsal horn after deafferentation by dorsal rhizotomy (Lamotte et al 1976; Jessell et al 1979; Ninkovic et al 1981; Daval et al 1987; Zajac et al 1989; Besse et al 1990; Hohmann et al 1999). Besse et al found that up to 75% of mu and 65% of delta opioid receptor binding in the dorsal horn is primary afferent derived after lesioning seven consecutive dorsal cervical roots. More recently Abbadie has shown that approx 50% of MOR in the cervical cord derives from small diameter primary afferents. Her study uses immunocytochemical analysis and may

have identified a greater percentage of postsynaptic MOR by using antisera that immunostains both cytoplasmic and membrane receptors (Abbadie et al 2002). Destruction of sensory C fibres by neonatal capsaicin treatment depletes opioid receptor binding sites in the dorsal horn (Nagy et al 1980) due to their predominantly presynaptic location. Opioids act presynaptically on MOR and DOR on sensory nerve terminals to inhibit voltage-dependent calcium currents and suppress the neurotransmitter release including substance P and glutamate. Opioids also act on postsynaptic sites and depolarise dorsal horn neurons. (Dickenson 1995).

There are few studies investigating the postnatal ontogeny of opioid receptors in the spinal cord (Kirby 1981; Attali et al 1990; Kar and Quirion 1995). Results can be difficult to interpret especially when non-selective ligands which cross react with more than one opioid receptor are used (Kirby 1981; Attali et al 1990). Rahman et al have used autoradiographical techniques and selective ligands to investigate the distribution and postnatal ontogeny of μ , δ and κ opioid receptor binding sites within the lumbar region of the spinal cord in the rat (Rahman et al 1998) and found considerable MOR and KOR binding at birth. DOR develops more slowly and is first seen at P7. MOR was the predominant opioid receptor in the spinal cord throughout development and in adulthood which is consistent with previous reports (Spain et al 1985). At P0, MOR binding sites were diffusely distributed over the dorsal horn of the spinal cord although there was already some localisation in the superficial lamina. The number of binding sites increased over the first postnatal week and peaked at P7. After this, the number of binding sites decreased until P21. As development progressed MOR binding sites were more dense in the superficial dorsal horn with relatively little binding in the deeper laminae by adulthood.

There is some information about opioid receptor ontogeny in DRG neurons from opioid receptor mRNA expression studies. In embryonic mice DRG DOR mRNA is detected first at embryonic day (E) 12.5 followed by MOR at E 13.5. Expression of KOR mRNA is seen much later at E 17.5 (Zhu et al 1998). MOR binding was also recently reported in P1 rat DRG (Ray and Wadhwa 1999).

Despite the importance of presynaptic opioid receptors in the dorsal horn of the spinal cord there is limited information on the development of opioid receptor expression in primary afferent neurons (Zhu et al 1998). Beland et al have investigated the postnatal development of MOR and DOR expression in dorsal root ganglia of neonatal and adolescent rats (Beland and Fitzgerald 2001). They found that a greater proportion of cells were immunoreactive for MOR and DOR in neonatal rat DRG section at P0, P3 and P7 compared to P21. During the first postnatal week both opioid receptors were expressed in cells across the whole diameter range but by three weeks of age, expression was restricted to small and medium diameter cells. There was a down regulation of opioid receptors in the largest diameter cells, determined by measurement of cell diameter. However, the largest diameter cells at P3 are considerably smaller than at P21. To demonstrate a genuine postnatal downregulation of opioid receptors on large diameter cells coexpression of MOR with NF200, a marker for large diameter cells, was also investigated. It was found that in P0 pups a significantly higher proportion of NF200 positive neurons co expressed MOR. This study provided the first information about the postnatal developmental regulation of OR in large and small to medium populations of DRG cells. Physiological studies have shown that the majority of large diameter, NF200+ve neurons in DRG are the cell bodies of non-nociceptive A β mechanoreceptors and proprioceptors, while nociceptors are generally NF200 –ve (Lawson and Waddell 1991). Similar, selective neurofilament staining has also been shown in human DRG cells (Holford et al 1994). The results suggested, therefore, that opioid actions in the newborn could affect both low threshold and nociceptive neurons and therefore be less selective than in the adult.

The aim of this study was to test the functional significance of this difference in MOR expression in neonatal DRG compared to adults. To do this we needed to establish dissociated lumbar DRG cell culture as a suitable model for the investigation of MOR expression in the DRG of newborn and adult rats and establish that the same developmental pattern of MOR expression occurs in neonatal culture as in the adult. The advantages of cell culture are that the cellular environment can be manipulated and many different cellular phenomena can be investigated e.g. the DRG cell culture model has been used to display the

effects of repeated morphine exposure on levels of CGRP and SP in DRG cultured neurons (Belanger et al 2002). This group has demonstrated μ -, δ - and κ -opioid receptor-like IR in post-natal cultured DRG neurons and have found that opioid receptors are in an active configuration or state in the DRG culture model. The advantage of using cell culture for my study is that having described the distribution of MOR on cultured DRG neurons in rats of different postnatal ages the functional development of the MOR in culture can be investigated using calcium microfluoroscopy as discussed in chapter 4.

Mu opioid receptor (MOR) expression was studied using immunohistochemistry in neonatal and adult rat dissociated dorsal root ganglia (DRG). To differentiate between small and large classes of DRG cells, immunostaining for a high molecular weight neurofilament marker, NF 200 was also used (Lawson and Waddell 1991). The coexpression of MOR and NF200 provided a picture of the distribution of MOR across two identified subpopulations of DRG, small NF200-ve and large, NF200 +ve sensory neurons in cultures from neonatal and mature rats

3.2 Materials and methods

Sprague-Dawley rats of both sexes were obtained from UCL Biological Services. All experiments were performed under personal and project licences in accordance with the UK Animals (Scientific Procedures) Act, 1986.

3.2.1 Tissue culture in the neonatal DRG

Lumbar dorsal root ganglia, L1 to L5, (DRG) were dissected from freshly killed newborn rats and enzymatic degradation was commenced by treatment with 0.125% collagenase (Worthington Biochemical Corporation, Berkshire) in 2ml of Neurobasal culture medium (Gibco, Invitrogen Corporation, USA) at 37°C for 30 minutes. 10ml of Neurobasal was then added, the tube inverted twice gently and spun at 400g for 1 minute to settle the tissue. The supernatant was discarded and 2ml Neurobasal with 0.25% trypsin added to the pellet for the second phase of enzymatic dissociation. The pellet was resuspended and then placed in an incubator at 37°C for 40 minutes. After this the tube was spun again at 800g for 1 minute and

the supernatant discarded from the digested tissue. 2ml of Neurobasal with 4% USG and 20% DNase Trypsin Inhibitor (made up with 20mg DNase I (deoxyribonuclease I) (SIGMA) was added.

Following enzymatic degradation the ganglia were triturated to mechanically liberate the cells from the loose network of connective tissue that remains following enzymatic treatment. A fine flamed Pasteur pipette was used to triturate the solution 10-15 times. This was repeated using gradually smaller Pasteur pipettes until the DRGs were fully dispersed and the solution had become cloudy.

Differential sedimentation was then used to separate the neurons from their investing axonal and cellular debris. The solution was carefully pipetted onto a column of 5% BSA (Sigma, St. Louis, USA) in Neurobasal. The tube was spun at 1000g for 8 minutes. Debris remains at the interface of the Neurobasal and BSA and the dissociated neurons form a pellet at the bottom of the tube. The supernatant was carefully aspirated and the pellet resuspended in an appropriate volume of Neurobasal with USG ready for plating.

200mcl of cells were plated onto 2% poly-DL-ornithine (hydrobromide) (SIGMA, 1:50 diluted in sterile distilled water) pre-treated, washed 2% laminin (SIGMA 1:50 diluted in sterile PBS) coated coverslips, with laminin removed just before plating. Cells were then placed in a 37°C incubator to adhere for 30 to 60 minutes. Neurobasal with the nutrient 10% fetal calf serum was then added. All neurons were left to grow overnight in a 5% CO₂ buffered air incubator at 37°C.

Neurons from 4 neonatal rats were cultured overnight, fixed for 20 minutes in 2% paraformaldehyde and double immunostained for MOR and NF200 as described below.

3.2.2 Immunohistochemistry

Fixed DRG neurons were washed and treated with 10% normal goat serum (Vector, Peterborough, UK) for 30 minutes. This serum contains a spectrum of antibodies that block non-specific binding. This reduces background staining, allowing clear identification of cells that specifically bind the antibody. Primary antibodies in phosphate buffered saline (PBS) containing 2% normal goat serum and 0.4% triton (Sigma, St. Louis, USA) were applied for 24 hours at room temperature. After several washes, the secondary antibody was applied for 2 hours.

To investigate co expression of MOR and NF200, DRG were double immunostained with anti-MOR in combination with anti-NF200. The antibody specific to MOR is raised in rabbits against a peptide corresponding to the predicted carboxy terminus of cloned rat MOR1 (Arvidsson et al 1995b) (polyclonal rabbit Mu Opioid Receptor RA10104, Neuromics, Minneapolis, USA). The antibody specific to NF200 is raised in mice and binds to phosphorylated and unphosphorylated high molecular weight neurofilament (monoclonal mouse anti-neurofilament 200, phosphorylated and non-phosphorylated Clone N52, Sigma, St. Louis, USA). It can be used to label large cells in neonatal and adult rats. The primary antibody solution contained both anti-MOR at 1:10,000 and NF200 at 1:1000. The secondary antibodies were red emitting Alexa fluor 594 conjugated anti-rabbit and green emitting Alexa fluor 488 conjugated anti mouse (Molecular Probes, Eugene, OR) both at 1:200. Slides were cover slipped using Vectashield and air-dried.

3.2.3 Tissue culture in the adult rat DRG

There were only a few differences in the procedure for tissue culture in the adult rat DRG cells. Preliminary incubation of dissected DRGs in collagenase was for 3 hours compared with 30 mins for the neonatal DRG cells. DNase Trypsin Inhibitor was not required before trituration of the cells. Apart from this all processes were as described above. Neurons from 4 adult rats were cultured overnight, fixed for 20 minutes in 2% paraformaldehyde and double immunostained for MOR and NF200 as described above.

3.2.4 Analysis

In order to quantify MOR and NF200 coexpression, fluorescent double-stained cells in dissociated DRG culture were manually counted. NF 200 positive cells, stained with green emitting Alexa fluor 488, were visualised with a G-2A filter on a fluorescence microscope while MOR positive cells, stained with red emitting Alexa fluor 594, were visualised with a B-2A filter. By switching between the two filters it was possible to count, in the same field, the cells that were green emitting and therefore NF200 positive, cells that were red emitting and therefore MOR positive and cells that were both red and green emitting and therefore MOR and NF 200 positive.

Lumbar DRGs from 4 animals at both age groups were cultured. Cells from each animal were plated onto 4 coverslips. 10 fields of cells per coverslip were counted in a single sweep across each coverslip. Each field had approximately 50 cells so at least 500 cells were counted per coverslip and 2000 cells per animal. As there were 4 animals at each age 8000 cells were counted per age group. Proportions of labelled cells from the two age groups were analysed using non parametric statistics.

3.3 Results

Neuronal cell cultures from both newborn and adult DRGs contained large, medium and small cells with abundant neurite outgrowth after overnight culture. There was less clear distinction in sizes in the newborn and the overall sizes were smaller. Cell densities were comparable in both age groups with approximately 50 cells per visualised field.

3.3.1 NF200 staining

NF 200 was highly expressed in both the neonatal and adult DRG cell culture with strong immunoreactivity in the cell body cytoplasm as well as in the neurites. The positive and negative cells were easily distinguishable. Figure 3-1 and Figure 3-2 show examples of NF 200 positive cells in neonatal and adult culture respectively. It is clear that at both ages the NF200 staining is restricted to the large/medium cell population.

3.3.2 MOR staining

Moderate MOR immunoreactivity was detected in DRG cell bodies of both neonatal and adult rats. Positive and negative cells could be distinguished reliably. The pattern of immunostaining was quite different from NF200 being primarily confined to the plasma membrane of cell bodies. Little immunoreactivity for MOR was detected in the neurites at either age (Figure 3-3).

3.3.3 Coexpression of MOR and NF200 – quantitative analysis

Table 3.1 shows data from individual animals. Figure 3-3 shows that MOR and NF200 expression was detected in both newborn and adult cultured DRG neuronal cell bodies. Quantitative analysis of the number of cells that co expressed MOR and NF200 showed that

in newborn rat pups a significantly higher proportion ($p < 0.05$) of DRG cells co expressed MOR and NF200, with $32.8 \pm 2.0\%$ (mean \pm SEM, $n=4$) in newborn pups compared to $21 \pm 1.1\%$ (mean \pm SEM, $n=4$) for the adult rats. These results are displayed as a bar chart in Figure 3-4.

3.4 Discussion

Immunohistochemistry was used in dissociated DRG neuronal cell culture to investigate expression of MOR across two different subpopulations of primary sensory neurons, large NF200 +ve cells and smaller NF200 -ve cells.

We found that in newborn rat pups, a significantly higher proportion of DRG neurons co express MOR and NF200 - $32.8 \pm 2.0\%$ compared to $21 \pm 1.1\%$ in adult rats. The abundant neonatal MOR expressed on large NF200 +ve neurons are downregulated postnatally. Information about MOR expression in DRG cell culture, either developmentally or in the adult, is limited. Previous studies have demonstrated μ -, δ - and κ -opioid receptors-like immunoreactivity in post-natal cultured DRG neurons and found that the opioid receptors are in an active state (Belangera et al 2002). However, proportions of DRG neurons expressing MOR were not reported. Information regarding MOR expression in the DRG is mainly from immunoreactivity studies on DRG tissue sections and investigation of MOR mRNA expression. Our findings are reasonably consistent with those of Beland et al who reported, in DRG sections, that at P0 $26.3 \pm 0.6\%$ of MOR positive cells expressed NF200 compared with $14.3 \pm 1.5\%$ at P21. They also found that in immature pups at age P3 and P7 the proportion of MOR +ve cells was significantly higher ($39.5 \pm 1.0\%$) compared to mature P21 rats ($30.1 \pm 1.7\%$). Others have reported MOR immunoreactivity in section to be 17% (Zhang et al 1998) and 21% (Ji et al 1995) of total adult rat DRG cells. The larger values found here may be accounted for by the effect of dissociation on the DRG cells and the process of immunostaining in culture which may stain more cells. Counting methods were also different and generally the proportions of subpopulations can only be estimates. Higher levels of MOR mRNA expression, from 40 - 55% of all DRG neurons, have been reported (Minami et al 1995; Wang and Wessendorf 2001) suggesting that cells are capable of protein expression

which is not always detectable. While both small and large DRG neurons express MOR mRNA the majority were small (less than 700µm).

There is an increase in mean cell size of rat DRG neurons during embryonic and postnatal development (Lawson et al 1974). This is why it was important to study MOR and in combination with an A fibre marker (NF200) to confirm downregulation of MOR in large DRG neurons. In this study the presence or absence of NF200 immunostaining was used to identify 2 main subdivisions of DRG neurons. Neurofilament subunits in rat dorsal root ganglion (DRG) neurons have been examined using antibodies such as RT97 and NF200 that recognise the 200 kDa subunit of neurofilament (Perry et al 1991). NF200 recognises the phosphorylated form of neurofilament whereas RT97 is specific for the phosphorylated and non-phosphorylated forms. The antibodies distinguish between the two populations of neurons corresponding to the 'large light (L) cell' and 'small dark (SD) cell' types as previously described from classical histological methods, showing that L and SD neurons contain different levels of neurofilament and that it is the presence of the phosphorylated form of neurofilament that provides the basis for discrimination between the two cell types. Observations made from unfixed DRGs indicate that all neurons contain some neurofilament and neurofilament rich and neurofilament poor populations are also apparent. L neurons contain more neurofilament than SD neurons and both cell types contain non-phosphorylated NF200, but the L neurons also contain a much greater amount of the phosphorylated form.

Rapid growth in diameter of the whole population of rat DRG neurons occurs between P3 and P20 followed by a plateau in cell growth. In DRG sections, DRG cell bodies immunoreactive to NF200 had a significantly larger diameter than the NF200 –ve population (unpublished data) at P0, 3, 7 and 21. At all ages the NF200 +ve population included the largest cells in the DRG. Two populations, NF200 +ve and –ve, were found to be normally distributed (Beland and Fitzgerald 2001).

NF200 is expressed in A cells from before birth and the NF200+ve population remains stable between birth and adulthood (Jackman and Fitzgerald 2000). Beland and Fitzgerald have

found that the proportion of NF200 +ve cells in DRG section remained constant between P3 ($46 \pm 1.4\%$) and P21 (44.2 ± 1.1). Jackman and Fitzgerald found that, using RT97 immunohistochemistry and cell diameter analysis these fibres arise from the large population of DRG cells at E18. These RT97 positive cell bodies are in the first wave of DRG cells to be born beginning at E12 (Kitao et al 1996). This is important because it means immunostaining for A fibres by RT97 and NF200 marks the same subpopulation of neurons in neonates and adults and that the postnatal change in MOR expression in this group of A cells is not a result of postnatal alterations in RT97/NF 200 expression.

This method of differentiating DRG cell type has been used here because physiological studies have shown that the majority of large diameter, L NF200+ve neurons in DRG are the cell bodies of non-nociceptive A β mechanoreceptors and proprioceptors, while nociceptors are generally NF200 –ve (Lawson and Waddell 1991). Similar, selective neurofilament staining has also been shown in human DRG cells (Holford et al 1994). Some nociceptive DRG neurons, however, have fibres that conduct in the A α / β fibre conduction velocity (CV) range as well as the C and A δ range but C fibre nociceptive neurons remain the most numerous of the nociceptive fibres. The properties of nociceptive A fibres have been very well characterised and the existence of myelinated nociceptive afferent fibres that have CVs in the range of the faster conducting A α / β cutaneous low threshold mechanoreceptors was clearly shown some time ago in the cat (Burgess and Perl 1967; Burgess et al 1968). That a proportion of A-fibre nociceptive neurons have fibres that conduct in the A α / β range has been confirmed with intracellular recordings in DRG neurons in vivo in several species. The proportions of A fibre nociceptive neurons with A β -fibre CVs has been found to be 23% in the cat (Koerber et al 1988), 65-67% in the rat (Ritter and Mendell 1992) and 42% in the guinea pig (Djouhri et al 1998). These proportions do not provide an indication of the real proportions, however, since they may vary according to many factors, including sampling bias, species and the rostrocaudal level of the DRG. Interestingly Koerber et al suggest that this is the group that terminates in the substantia gelatinosa in the neonate. Although a proportion of A-fibre nociceptive neurons do conduct in the A α / β range, the peak of the distribution of CVs of A-fibre nociceptors is in the A δ range (Burgess and Perl 1967; Burgess et al 1968). Also there

are a very large number of low threshold mechanoreceptor neurons that conduct in the A α / β -fibre range, dominating the wave of the compound action potential. It is clear that a small proportion of NF200 +ve neurons will in fact be nociceptive A-fibres. While it is not possible to determine whether the down regulation of MOR in the adult is from nociceptive or non-nociceptive A neurons this does not alter the finding that there is significantly greater expression of MOR on A fibres in the neonate.

The model of primary dissociated cell culture for the investigation of MOR distribution and ultimately function is a very useful one. The cellular environment can be manipulated and many different cellular phenomena can be investigated e.g. Gavazzi et al have investigated the growth responses of different subpopulations of adult sensory neurons to neurotrophic factors using this model (Gavazzi et al 1999). They found that cultured DRGs neurons retained the heterogeneity described in vivo when grown as dissociated primary cultures and that the different subpopulations are present in approximately the same proportions in vivo and in vitro. However there are also some problems with this model. Neurons in dissociated DRG cultures are in an axotomised state, since the surgical manipulations associated with removing the DRGs include axotomy of both the peripheral and central branch close to the cell bodies. In addition, the injured, dissociated neurons are grown in culture for 12 hours in an artificial extracellular environment that may not reflect the in vivo situation.

Axotomy is known to markedly change expression of peptides and peptide receptors in DRG neurons (Hokfelt et al 1994). Results from earlier analyses of such cultures have shown increased levels of vasoactive intestinal polypeptide (VIP) (Mulderry and Lindsay 1990) and galanin (Kerekes et al 1997), changes that have been reported to occur in DRG neurons after peripheral axotomy in vivo (Villar et al 1989). Nóra Kerekes et al have demonstrated that removal of DRGs and subsequent culturing result in appearance of many neuropeptide tyrosine (NPY) positive neurones which cannot be detected in DRG in vivo under normal conditions (Gibson et al 1984). After peripheral nerve transection there is a marked upregulation of this peptide. Importantly, there is a reduction in the expression of opioid receptors after nerve injury (Besse et al 1992). Peripheral axotomy has been shown to cause a reduction in the number and intensity of mu-opioid receptor-positive neurons in the rat and

monkey dorsal root ganglia, and of mu-opioid receptor-like immunoreactivity in the dorsal horn of the spinal cord (Zhang et al 1998). However, this down regulation was first detected two days after axotomy. Such changes may also explain the differences in MOR expression in vitro compared with in vivo although our neurons were investigated after 12 hours of culture only. The culture medium used Neurobasal and fetal calf serum will have a wide range of neurotrophic and other growth factors necessary for survival and will ameliorate the effects of axotomy. Importantly, the neonatal and adult DRGs have been cultured in the same way so that the effect of axotomy and maintenance in culture for 12 hours is likely to be similar in both groups. Cultures from both age groups looked equally healthy after 12 hours with comparable, abundant neurite outgrowth. Our main interest is the effect of postnatal age on MOR expression rather than absolute percentages.

The developing sensory nervous system undergoes considerable structural and functional reorganisation in the neonatal period (Fitzgerald and Jennings 1999). Sensory neurons possess a huge array of molecules which contribute to their structural and functional characteristics and many of these are developmentally regulated. The pattern of early OR over expression followed by postnatal downregulation is seen in many other transmitter systems (Attali et al 1990; Gonzalez et al 1993; Kar and Quirion 1995). There are greater numbers of excitatory receptor systems at early ages which after peaking early in life, decline towards adult levels. E.g. NMDA receptor binding has been shown to peak between P6 and P10 in the mouse lumbar cord (Gonzalez et al 1993) and substance P receptor density is maximal in the first two postnatal weeks with the adult spinal cord containing only one sixth of the binding sites present at P11 (Kar and Quirion 1995). Calcitonin gene related peptide receptors are also over-expressed in the immature spinal cord (Kar and Quirion 1995). Similarly, in the DRG there are many receptor systems that undergo postnatal downregulation. Nerve growth factor (NGF), a member of the neurotrophin family, has functional roles dependent upon the stage of development under investigation. NGF binds to the tyrosine kinase receptor Trk A. Trk receptor protein is observed in early embryonic DRG neurons but not in neural crest or neuronal precursors (Farinas et al 1998). Between E10 and E13 TrkA expression in DRG neurons rises from 20 to 80% (Farinas et al 1998). By E13 the

majority of DRG cells are labelled with TrkA mRNA in the rat. The level of TrkA mRNA in the DRG decreases two fold during the postnatal period (Ehrhard and Otten 1992). From postnatal day 0 to 14 the percentage of DRG cells expressing the TrkA receptor decreases from 70 to 45%.

The abundance of opioid receptors in the early postnatal period may be required for the maturation of synapses and fine tuning of spinal circuitry as there is increasing evidence that the endogenous opioid system has a trophic role in the neonatal period (Zagon and McLaughlin 1984; Hauser et al 1989; Steine-Martin et al 1991). Continuous opioid receptor blockade for the duration of gestation results in huge increases in body, brain and organ growth (McLaughlin 97b), accelerates the appearance of physical characteristics, reflexes and spontaneous motor behaviours (McLaughlin et al 1997a), and produces a decrease in MOR binding density and decreased sensitivity to the analgesic effects of morphine (Zagon et al 1998).

The underlying mechanisms and functional significance of the decline of the downregulation of MOR postnatally are not known. Large scale cell death occurs within the CNS during development but this is unlikely to be the cause of the decline in MOR number since no significant cell death was seen in postnatal DRGs (Coggeshall et al 1994). Considerable amounts of opioid receptors are presynaptic and are synthesised in the DRG (Besse et al 1991), but most cell death in the DRG occurs at much earlier stages (Coggeshall et al 1994). It is possible that dorsal horn neuronal death occurs later which may lead to loss of postsynaptic receptors.

Functional C fibre maturity occurs mostly from the second postnatal week (Fitzgerald 1988) and peak synaptogenesis occurs in the first two postnatal weeks. Since it is around this time that the opioid receptor numbers start to decline, it is possible that increased synaptic activity from maturing fibre afferents leads to greater activity dependent release of endogenous opioid peptides which may ultimately regulate the level of receptors. This developmental pattern of opioid receptor number can vary depending on the area of the CNS. In most areas of the brainstem, the density of binding sites for MOR and DOR peaks between P10 and P21 and then either remained constant or decreased to adult levels, but in some nuclei no peak

binding was seen and MOR and DOR binding sites were seen to continue increasing to adulthood (Xia and Haddad 1991).

The functional nature of the over expressed neonatal DRG MOR is investigated in chapter 4.

Figure 3-1 Neonatal DRG neurons immunostained for NF200.

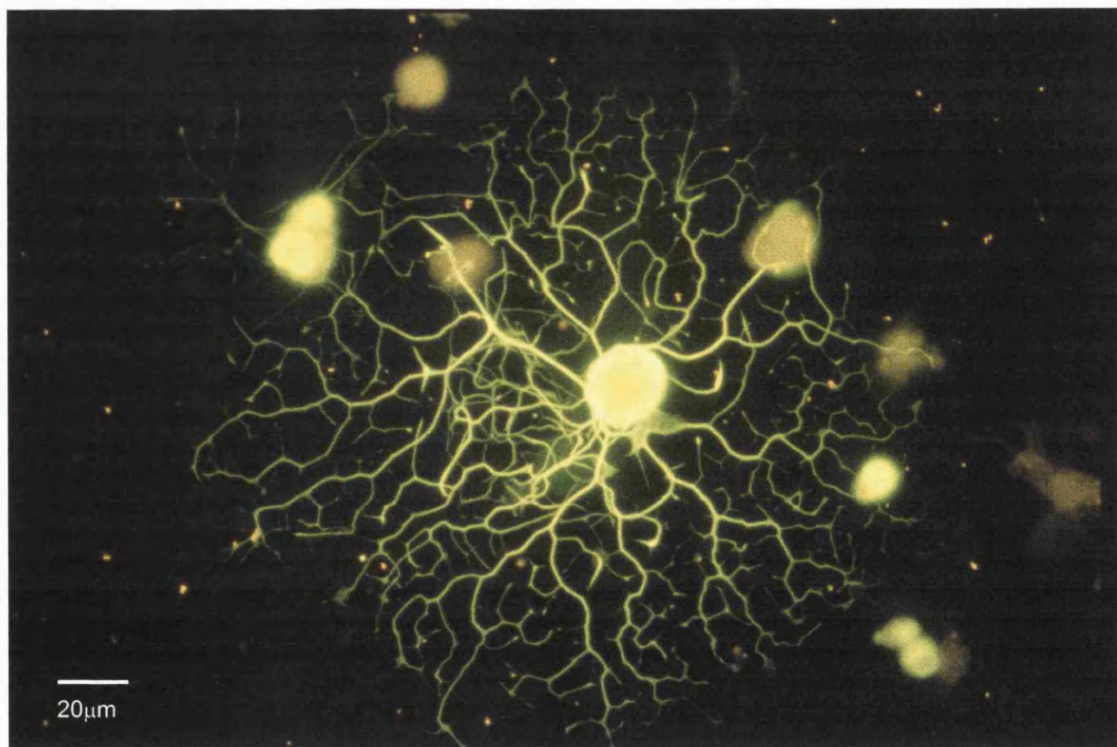
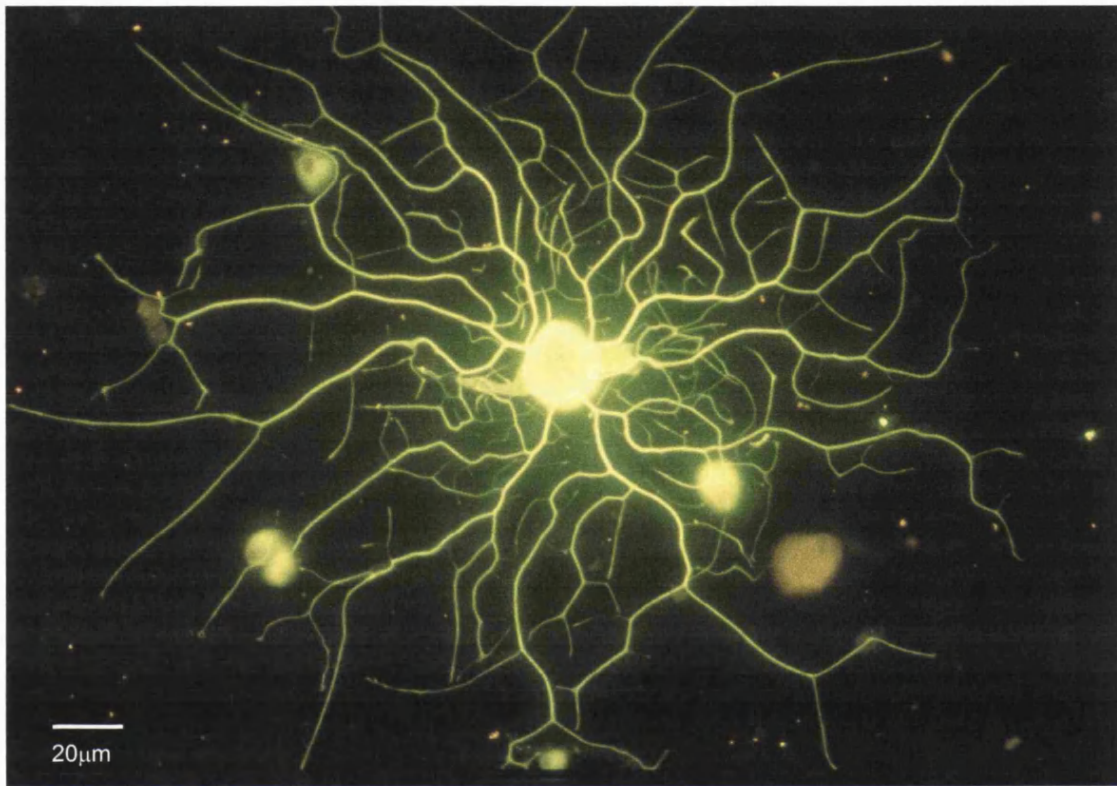


Figure 3-2 **Adult DRG neurons immunostained for NF200**

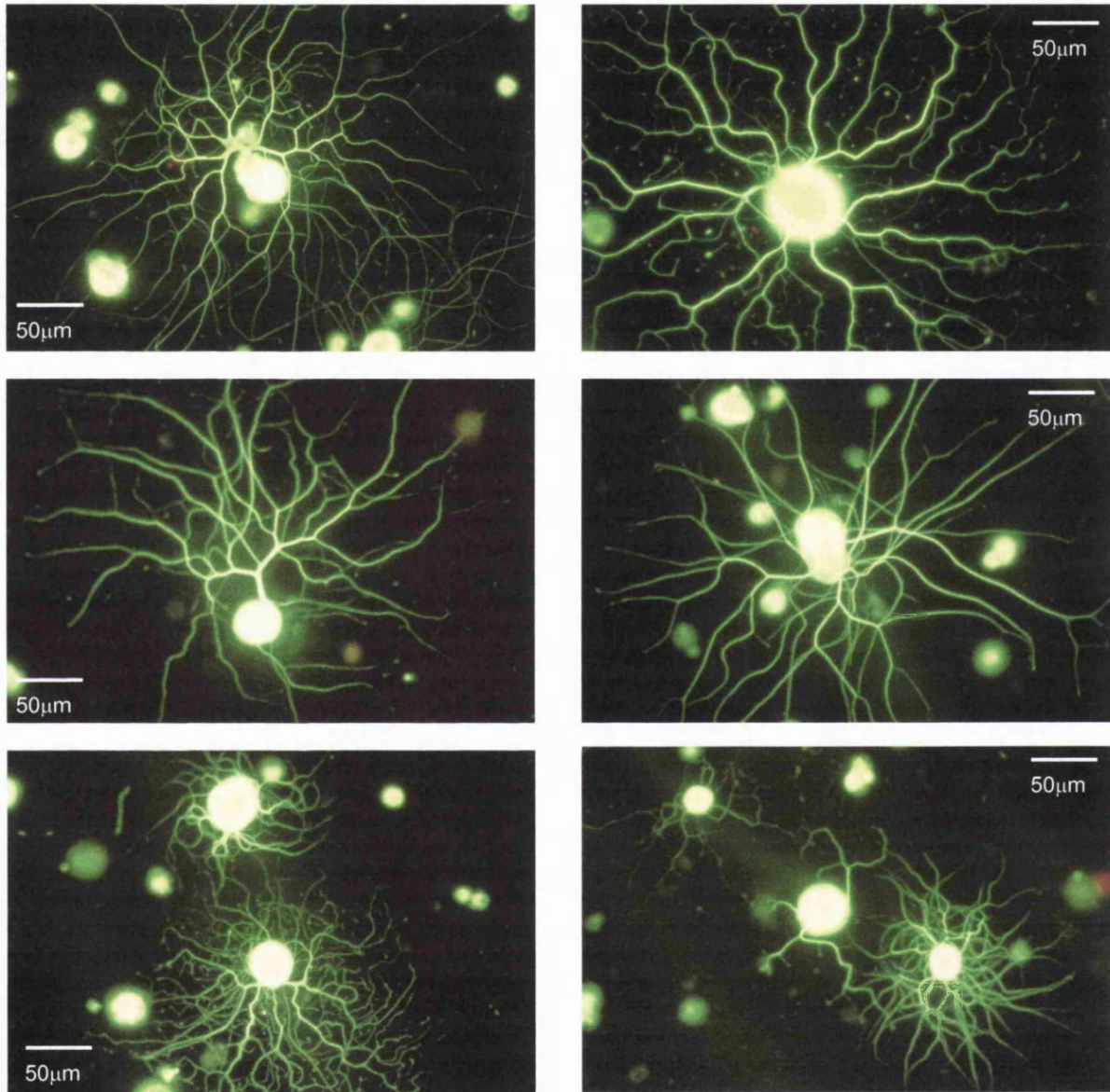
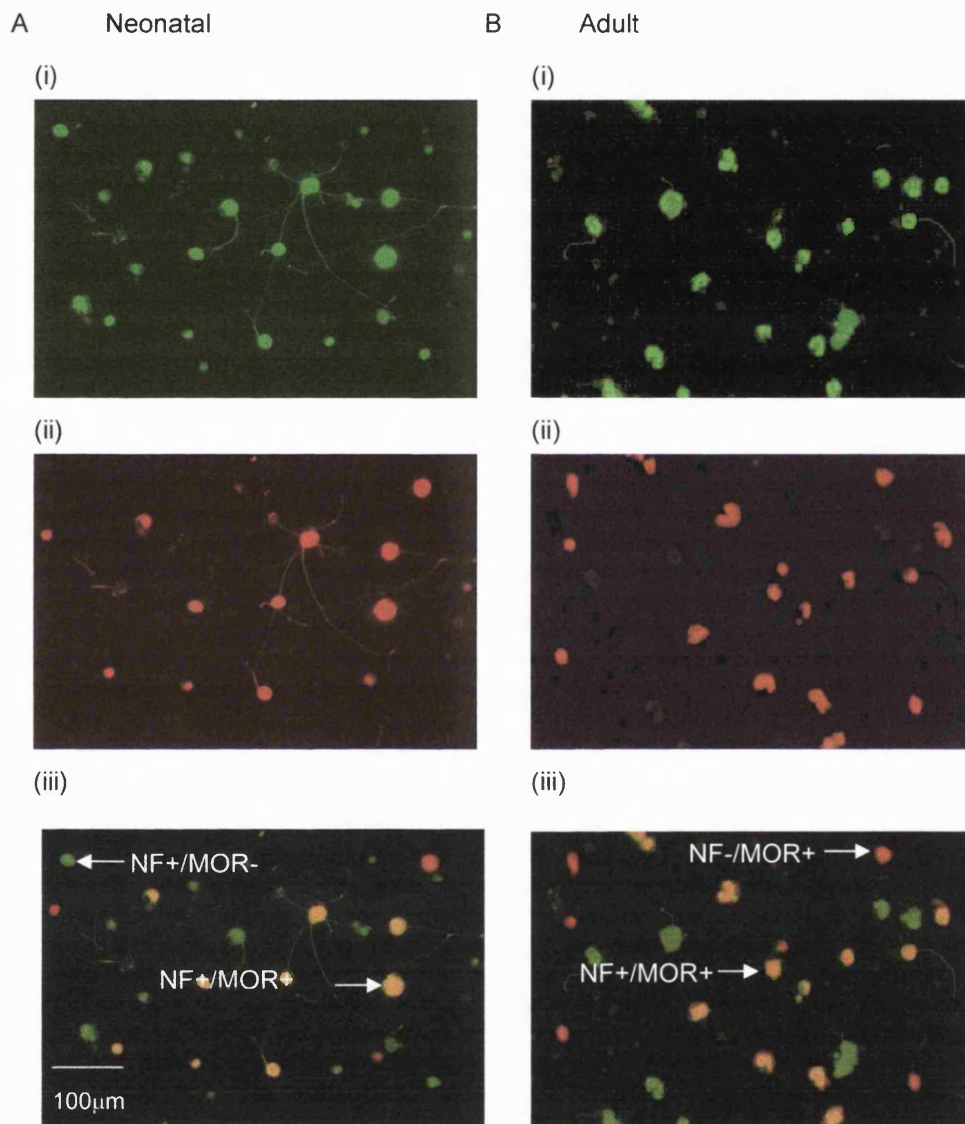


Figure 3-3 Fluorescence images of typical fields of double-labelled neuronal DRG cell cultures from (A) neonatal rat pups and (B) adult rats.



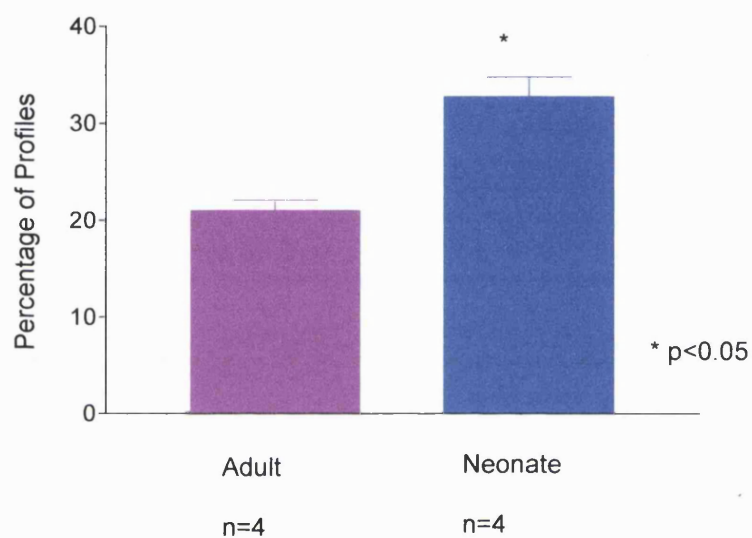
(i) NF200 positive cells. These represent the large, non-nociceptive A fibres and are labelled green.

(ii) MOR positive cells. The same cells have been immunostained for MOR. Cells that express MOR are labelled red.

(iii) Superimposed image of NF200 and MOR labelled cells. By overlaying the image of the green NF200 positive cells onto the red MOR positive cells the double-labelled cells appear orange, becoming more readily identifiable. Examples of NF200 positive/MOR negative (NF+/MOR-), NF200 negative/MOR positive (NF-/MOR+) and NF200 positive/MOR positive (NF+/MOR+) are illustrated.

Significantly more large, NF200 positive neonatal neurons co express MOR than adult neurons.

Figure 3-4 Percentage of DRG neurons co expressing NF200 and MOR in neonatal and adult rat DRG neuronal culture.



There is significantly greater co-expression of MOR and NF 200 in neuronal DRG cells of neonatal compared with adult rats (n=4, mean \pm SEM, $p<0.05$).

4 The functional development of MOR in primary sensory neurons

4.1 Introduction

In the previous chapter I have shown that the distribution of MOR in DRG neurons in the neonate differs from MOR expression in the adult. Using immunohistochemistry, a greater proportion of large, neonatal DRG A cells were found to express MOR. These widespread neonatal MOR are then subject to postnatal down regulation with the majority of MOR in the adult expressed on smaller, non-A cells.

Are these widespread neonatal MOR functional?

Opioids produce many effects on the nervous system by interacting with a widely distributed G-protein–coupled receptor system, especially μ , δ and κ classes, as discussed in chapter 2. Opioid receptors in the spinal cord are a critical site in the production of analgesia. This is clearly demonstrated by the profound analgesic effects of opioids administered in the subarachnoid and epidural spaces. The spinal analgesic action of opioids results from a reduction in neurotransmitter release from primary afferent fibres (Jessell and Iversen 1977), an effect attributed to presynaptic suppression of Ca^{2+} channel current/conductance ($I_{\text{Ca}}/g_{\text{Ca}}$) in sensory nerve terminals (Hori Y et al 1992). This hypothesis is supported by the observation that opioids suppress high-voltage-activated (HVA) I_{Ca} or Ca^{2+} -dependent action potentials in the cell bodies of dorsal root ganglion (DRG) neurons (Werz and MacDonald 1982; Werz et al 1987; Moises et al 1994a, b; Taddese et al 1995; Womack and McCleskey 1995). Activation of opioid receptors also activates an inwardly rectifying K^+ conductance (g_{KIR}) in spinal cord, brain, or DRG neurons (Faber et al 1996; Vaughan and Christie 1996, 1997; Abdulla and Smith 1997; Vaughan et al 1997). Such effects are indistinguishable from those of morphine (Jessell and Iversen 1977; Williams et al 1988; Surprenant et al 1990; Schroeder et al 1991; Hori et al 1992; Moises et al 1994a, b; Taddese et al 1995; Womack and McCleskey, 1995).

A particularly interesting study by Xu et al introduced a recombinant adeno-associated viral (rAAV) vector containing the cDNA for the μ -opioid receptor (μ OR) into primary afferent neurons in dorsal root ganglia (DRGs) of rats causing a long-lasting (>6 months) increase in MOR expression in DRG neurons. The increase greatly potentiated the antinociceptive effects of morphine in rAAV-MOR-infected rats with and without inflammation. Perforated patch recordings indicated that the efficacy and potency of opioid inhibition of voltage-dependent Ca^{2+} channels were enhanced in infected neurons, which was the underlying factor in the increase in opioid efficacy (Xu et al 2003).

Opioid receptor function can be investigated using various techniques, including:

(i) Neurophysiological techniques

Voltage-clamp recording can be used to study the effects of opioids on the modulation of ionic conductances. This technique involves controlling the membrane voltage and measuring the current required to maintain the voltage. Step voltage changes lead to instantaneous current changes. This is ideal for analysing channel activity because, by controlling voltage, there is control over the key variable that controls the opening and closing of many ion channels.

Also, because the net driving force for ions across the channel is constant, any changes in current can be interpreted as ion channels opening or closing, not as changes in driving force of ions across the channel. Nevertheless, the patch-clamp technique suffers some limitations. The channel of interest must be physically accessible to the patch pipette; seal formation may disrupt the local cytoskeletal architecture; independent measurements cannot be obtained from multiple channels; and little information is provided regarding the spatial distribution of channels.

In vivo electrophysiology can also be used in anaesthetised animals. Recordings can be made from single neurons in the presence of morphine or other opioid agonists and their effect on the activity of sensory neurons measured.

(ii) Measurement of neurotransmitter release using microdialysis techniques.

The first paper involving microdialysis was applied to the study of dopamine neurotransmission (Ungerstedt and Pycock 1974). Microdialysis is a widely accepted

technique for sampling, allowing measurement of endogenous and exogenous substances in the extracellular fluid surrounding the microdialysis probe. A microdialysis probe containing a dialysis membrane is implanted into blood vessel, tissue, organ or culture dish and perfused with a suitable perfusate at a constant flow rate. By means of diffusion down their concentration-gradient, molecules small enough to cross the semi permeable membrane of the probe then enter the probe lumen, and are taken with the perfusion flow. The resulting dialysate can be analyzed by an appropriate analytical technique. E.g. neuropeptides can be measured by radioimmunoassay. Using this method in cell culture, it is possible to measure neurotransmitter release and the effect of opioid agonists on neurotransmitter release can be studied. Examples of the use of microdialysis in cell culture include the measurement of intracellular catecholamines in PC-12 cell and the direct measurement of pyruvate and lactate in primary liver cell culture medium under hypoxia and reperfusion.

(iii) Measurement of Ca^{2+} entry into cells in response to depolarisation using optical imaging.

This technique was utilised to investigate MOR function in vitro in this study. Fura-2 based calcium microfluoroscopy is a well established technique known to provide a sensitive measure of MOR function. It has been used to investigate opioid receptor function in various areas of the nervous system. Rhim and Miller (1994) used calcium microfluoroscopy to demonstrate the opioid receptor modulation of diverse types of calcium channels in the nucleus tractus solitarius of the rat. Ortiz-Miranda (2003) measured intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) using Fura-2 calcium imaging to investigate the MOR modulation of peptide release from rat neurohypophysial terminals. Using this technique they were able to demonstrate DAMGO mediated, MOR antagonist reversible inhibition of calcium entry in nerve terminals of the hypothalamic-neurohypophysial system

Depolarisation of cultured neurons with 50 mM KCl results in Ca^{2+} influx into the cell via voltage-gated channels. Application of a MOR agonist such as DAMGO onto opioid sensitive cells has been shown to result in suppression of Ca^{2+} influx by opioid receptor-mediated inhibition of Ca^{2+} channels (Shen and Surprenant 1991; Taussig et al 1992) and it is this mode of action of opioids that is being exploited in the study OR function using calcium imaging.

Recently attempts have been made to use optical techniques for monitoring the kinetic activity of multiple single channels. It provides an alternative, less invasive approach to patch-clamping. The use of highly sensitive fluorescent Ca^{2+} indicators has permitted imaging of Ca^{2+} flux through clusters containing a few Ca^{2+} -permeable channels, and even through individual channels (Zou et al 1999; Wang et al 2001). However, until recently, this approach did not provide sufficient resolution to study channel kinetics, and single-channel imaging was restricted to channels with unusually high Ca^{2+} permeability and long open times, or to channels whose lifetime was pharmacologically prolonged. The use of high-resolution confocal microscopy has now been used to image Ca^{2+} flux through individual N-type voltage-gated Ca^{2+} channels expressed in *Xenopus* oocytes under physiological conditions, and demonstrate the utility of this methodology as a practicable, non-invasive tool to measure the functional kinetics and spatial localization of Ca^{2+} -permeable channels (Demuro and Parker 2003).

4.2 Calcium microfluoroscopy

Calcium imaging is the production of a picture of a cell or group of cells in which the brightness at any point relates not to the superficial structure of the cell but to the local value of ionised calcium concentration ($[\text{Ca}^{2+}]$). A series of such images, relating to the calcium distribution at different times, makes it possible to trace the evolution of the calcium signal. 'Real – time' calcium imaging is the appearance of these images in video form as a constant stream throughout the experiment. The requirements for such imaging are (i) a chemical indicator which can be introduced into the cells and whose optical properties change measurably with $[\text{Ca}^{2+}]$; (ii) a microscope and suitable light source; (iii) a sensitive video camera and (iv) a computer controlled device known as an 'image processor' which digitises and processes the video information to produce the calcium image. If the processing is fast enough a real time image can be produced.

4.2.1 Optical indicators for intracellular calcium

Measurement of intracellular calcium is possible using (i) ion-selective micro – electrodes; (ii) bioluminescent proteins (e.g. aequorin) which emit light upon binding to calcium; (iii) azo –

dyes e.g. arsenazo III and antipyrilazo III whose absorption of red light changes in the presence of calcium; (iv) fluorescent dyes e.g. quin – 2, fura – 2 and indo -1.

4.2.2 Fluorescent Calcium indicators

Fluorescent calcium indicators currently in use belong to a series developed by R. Y. Tsien et al (Grynkiewicz et al 1985). They have made a major contribution to advances in the understanding of the role of calcium in cellular regulation and of agents that affect intracellular calcium flux. Fura-2 and indo-1 have been cited in thousands of papers that describe their application in a wide variety of cells. They are based on a tetra – carboxylic acid calcium chelator, to which are added side – chains whose fluorescence is altered by the electron – withdrawing effect of a bound calcium ion. The first to appear was the dye quin – 2, but this has been surpassed in performance by indo – 1 and fura – 2. Fura – 2 has a calcium affinity such that it is useful at $[Ca^{2+}]$ from 50 nM to 2 μ M, and its superior UV absorption and quantum efficiency mean that, at a given concentration, and with a given UV dose, it can provide about 30 times as much fluorescence as quin – 2. Indo – 1 has similar efficiency and calcium-affinity but a different optical spectrum. Fura – 2 is currently the most widely used fluorescent calcium indicator for intracellular measurements. Figure 4-3 shows the structure of fura – 2.

4.2.3 Cell loading

Fura – 2 is available as a membrane permeable acetoxymethyl ester. This is hydrolysed by non-specific esterases present in most animal cells producing the free acid which is hydrophilic and not membrane permeable and is thus trapped within the cell. In this way cells can be loaded quickly, without disruption of the plasma membrane and to a final concentration many times higher than that of the extracellular suspension of fura ester.

4.2.4 Quantification

Fura – 2 responds to calcium by a change in the UV excitation spectrum. Figure 4-4 shows a family of excitation spectra for fura – 2 as a function of calcium activity. Binding to calcium causes not only an increase in fluorescence but also a shift in the UV wavelength at which it

is maximally excited, from about 365 nm to 340 nm. This shift in wavelength is essential since fluorescence intensity is also dependent on many other poorly quantified or variable factors such as illumination intensity, emission collection efficiency, dye concentration and effective cell thickness in the optical beam. The ratio of the fluorescence at two suitably chosen wavelengths then signals calcium while cancelling out most or all of the possible variability due to instrument efficiency and content of effective dye.

There is an isobestic point at 361 nm where fluorescence of fura-2 is independent of calcium. For quantitative calcium assay 2 wavelengths are chosen equally spaced around the isobestic point and the ratio between fluorescence intensities, excited at each wavelength, measured. As fura – 2 binds calcium, exposure to 340 nm wavelengths will produce an increase in emission intensity whereas exposure to 380 nm wavelengths will cause a decrease in emission intensity. Emission is collected at 510 nm and registered once every second. Changes in calcium level are expressed as the ratio between the emission intensity at both wavelengths (340: 380). This ratiometric principle acts to calibrate fluorescence changes so that any variation in fura -2 loading is not mistaken as a variation of the intracellular calcium level.

In this study, MOR function was measured as the effect of DRG cell depolarisation, in the presence and absence of an opioid agonist, on intracellular calcium levels, using ratiometric calcium imaging with Fura-2. The opioid agonist used was D-Ala²,N-MePhe⁴,Gly⁵-ol-enkephalin (DAMGO), a pure MOR agonist. Application of DAMGO onto MOR responsive neurons causes suppression of Ca²⁺ influx in response to a depolarising K⁺ stimulus by inhibition of voltage-operated Ca²⁺ conductance through MOR activity. This method was used to identify MOR sensitive neurons in newborn and adult rat DRG neurons, the numbers of responsive cells at each age quantified and cell areas measured so that the distribution of MOR sensitive cells across different cell sizes could be evaluated. Cultured cells were then fixed, immunostained for the large DRG cell marker, NF200 and the fields investigated with Ca²⁺ imaging relocated using a confocal microscope. Immunostaining with NF200 made it possible to compare the distribution of MOR responsive neurons across two different

subpopulations of sensory neurons, large non-nociceptive, NF200 positive neurons and small nociceptive, NF200 negative neurons, in newborn and mature rats.

4.3 Materials and Methods

4.3.1 Cell culture

Dissociated neonatal and adult lumbar DRG neurons were grown in culture for 12 hours according to the protocol described in detail in chapter 3.

4.3.2 Calcium imaging

A solution of 5 mM fura – 2 ester (Molecular Probes reference F-1221) was prepared in pure, dry dimethyl sulphoxide (DMSO). This was added to external solution to produce a final concentration of 2 μ M Fura – 2AM. Dispersion was assisted by the addition of the emulsifying agent 0.01% pluronic F127 (Molecular probes, Eugene, OR). The dissociated lumbar DRG neurons were loaded with this membrane permeable acetoxymethyl–ester form of the Ca^{2+} sensitive dye for 40 minutes at room temperature. After loading, cells were rinsed in isotonic external solution, ECF, (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, pH 7.4 with NaOH) and then allowed 20 minutes to de-esterify the dye.

The coverslips plated with Fura-2-loaded neurons were glued with petroleum jelly to the recording chamber of an inverted microscope. A manifold with inputs from the solution reservoirs and a single outflow pipe was lowered into place over the coverslip, allowing free perfusion of solution over the coverslip. This arrangement allows rapid and complete exchange of the solution that flows across the field of cells immediately below the flow pipe.

The cells were exposed to a constant flow of ECF. They were then bathed in solutions according to 3 experimental protocols given in Table 4-1, Table 4-2 and Table 4-3.

Flow of solutions was regulated by a computer controlled and gravity-driven perfusion system. As the computer-controlled valves open in turn, solution passes over the cells and is vacuum-aspirated when the level surpasses a critical maximum. This enables complete exchange of solution and rapid wash-out thereby minimising cross-contamination of stimuli. Great care was taken to ensure that the cells were only exposed to the intended stimuli. Figure 4-5,

Figure 4-6 and Figure 4-7 show a schematic drawing and photographs of the experimental apparatus.

Table 4-1 Experimental protocols of calcium imaging studies investigating MOR function in neonatal and adult rat DRG neuronal cultures.

The control experiment – testing the stability and reliability of the measurements

Control	Application	Effect
Expt	Time (s)	
External	10	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline

Table 4-2 Experimental protocols of calcium imaging studies investigating MOR function in neonatal and adult rat DRG neuronal cultures.

The agonist experiment – testing the effect of MOR function

DAMGO	Application	Effect
Expt	Time (s)	
External	10	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	40	Baseline
DAMGO	20	Block with MOR agonist
DAMGO/High K ⁺	5	Depolarisation
External	40	Baseline
DAMGO	20	Block with MOR agonist
DAMGO/High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline

Table 4-3 Experimental protocols of calcium imaging studies investigating MOR function in neonatal and adult rat DRG neuronal cultures.

The agonist/antagonist experiment – testing the selectivity of the MOR function

Antagonist	Application	Effect
Expt	Time (s)	
External	10	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	40	Baseline
DAMGO	20	Block with MOR agonist
DAMGO/High K ⁺	5	Depolarisation
CTAP	40	Inhibit MOR agonist block
CTAP/DAMGO	20	Inhibit MOR agonist block
CTAP/DAMGO/High K ⁺	5	Depolarisation
External	60	Baseline
High K ⁺	5	Depolarisation
External	60	Baseline

4.3.3 The 3 experimental protocols

In the control experiment, neurons were stimulated 7 times for 5 seconds with a 50mM K⁺ solution in ECF with stimulus interval of 60 seconds. Large, consistent transient increases in intracellular Ca²⁺ levels, reflecting neuronal excitability, were produced.

In the agonist experiment, to identify MOR sensitive neurons, DAMGO was co administered with the 4th and 5th K⁺ stimuli. The effect of DAMGO on the Ca²⁺ transient amplitude was measured in all cells that were depolarised by the K⁺ stimuli.

In the agonist/antagonist experiment the specific MOR antagonist CTAP (D-Phe-Cys-Tyr-D-Tryp-Lys-Thr-Pen-Thr-NH₂) was applied with the second DAMGO application to demonstrate the selective effects on MOR. There were 2 K⁺ stimuli after DAMGO or DAMGO/CTAP application to demonstrate recovery.

Both the agonist and agonist/antagonist protocols were preceded with a control experiment of repeated K stimulation to ensure that there was a consistent response to the standard depolarising stimulus and that the cultures were healthy. This control response was analysed in at least 20 cells per group.

The DAMGO protocol was undertaken in 8 animals and the antagonist protocol in 4 animals per age group. Two coverslips comprising 50 cells per field per animal were used for the DAMGO protocol and 1 per animal for the antagonist protocol, therefore 800 neurons per age group were treated with DAMGO and 200 neurons per age group were treated with DAMGO/CTAP.

4.3.4 Observation of neurons

During the experiments individual neurons were observed on an inverted microscope (Zeiss Axiovert 200) with a 20x objective. Neurons were exposed to epi-fluorescence illumination using a Polychrome IV system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) with a 150 W xenon lamp with light of alternating excitation wavelengths (340 and 380nm). Emission fluorescence (F) was led to an IMAGO CCD camera through a dichroic filter (DCLP410) and then a 440 nm longpass filter. Fluorescent image intensities were expressed as the ratio F340/F380 to allow quantitative estimates of changes in intracellular calcium concentrations. F340 and F380 represent the fluorescence intensity elicited by 340nm emission light (for Fura

– Ca^{2+}) and 380nm emission light (for Fura without Ca^{2+}) respectively. Intracellular calcium concentrations were measured using TILLvisION Imaging System v4.01 (T.I.L.L. Photonics GmbH, Germany, Gräfelfing, Germany). All experiments were performed at room temperature (23-25°C).

4.3.5 Immunostaining of cells for NF200

At the end of each experiment coverslips were marked to enable relocation of the fields and fixed in 2% paraformaldehyde for 20 minutes. The cells were then immunostained for NF200 to investigate the distribution of MOR responsive cells across 2 subpopulations of sensory neurons: the large NF200 positive A neurons and smaller NF200 negative neurons.

The protocol for immunostaining for NF200 is as discussed in chapter 3 and will be only be briefly described here.

Fixed DRG neurons were washed and treated with 10% normal goat serum (Vector, Peterborough, UK) for 30 minutes. The primary antibody in phosphate buffered saline (PBS) containing 2% normal goat serum and 0.4% triton (Sigma, St. Louis, USA) was applied for 24 hours at room temperature. After several washes, the secondary antibody was applied for 2 hours. The antibody specific to NF200 is raised in mice and binds to phosphorylated and unphosphorylated high molecular weight neurofilament (monoclonal mouse anti-neurofilament 200, phosphorylated and non-phosphorylated Clone N52, Sigma, St. Louis, USA). It can be used to label large cells in neonatal and adult rats. The primary antibody solution contained NF200 at 1:1000. The secondary antibody was green emitting Alexa fluor 488 conjugated anti mouse (Molecular Probes, Eugene, OR) both at 1:200. Slides were cover slipped using Vectashield and air-dried.

4.3.6 Analysis of Ca^{2+} imaging

Ratiometric fluorescence levels were recorded every second during the course of each Ca^{2+} imaging experiment. This information was entered onto Microsoft Excel spreadsheets and line graphs were generated from this data as shown in Figure 4-8. All the Ca^{2+} peaks produced by depolarisation with 50mM K^{+} were averaged to remove any effects of gradual run down as a result of repeated stimulation leading to calcium depletion. The Ca^{2+} transients produced after

application of DAMGO were expressed as a percentage of the average peak produced by 50 mM K⁺ alone. Following analysis of variability between runs, suppression in the peak of greater than 15% was considered to be a positive response to DAMGO and the neuronal cell was categorised as MOR sensitive.

4.3.7 Analysis of DRG cell size and distribution across NF200 +ve cells

The cross-sectional areas of all the cells were measured using SCION software. The circumference of all cells in a studied field were marked using this software and the area of the cell was automatically calculated.

Following immunostaining for NF200, fields were relocated using a confocal scanning microscope to enable correlation of NF 200 expression with MOR sensitivity.

4.4 Results

To test the function of MOR in neonatal DRG fura-2-based Ca²⁺ microfluorimetry was used on the DRG cultures. This is based on the reduction of intracellular Ca²⁺ flux evoked by depolarisation through MOR agonist mediated inhibition of voltage dependent Ca²⁺ channels.

4.4.1 A greater proportion of neonatal DRG cells express functional MOR than adult DRG cells

Figure 4-8 shows typical fields of cell investigated with calcium imaging in (A) adult DRG neurons and (B) neonatal DRG neurons. Ratiometric changes in fluorescence as a result of Ca²⁺ transients from cultured DRG cells evoked by exposure to 50 mM K⁺ are shown in Figure 4-9. In control experiments, depolarisation using a 5 second exposure to 50 mM K⁺ increased the fluorescence ratio 3 to 5 fold in both neonatal and adult cells. Fig 4.7A demonstrates the effect of K depolarisation on fluorescence in a typical cell. The 340/380 fluorescence ratio went from a baseline of 0.15 to 0.43. Seven constant transient increases in [Ca²⁺]_i were elicited as a result of seven repeated depolarising K⁺ stimuli (figure 4.7A). The peak ratios are consistently around 0.45 with return to baseline of 0.15 between each depolarisation. This consistency was observed in both neonatal and adult cells. No differences were observed in these responses between neonatal and adult cells. Similarly, in the control runs preceding the agonist and agonist/antagonist experiments the 20-30 cells analysed per group demonstrated

minimal run down. Control data showed that peak ratios were consistently around 0.53 with return to baseline of 0.10 in the agonist experiments and approximately 0.47 with return to baseline of 0.19 in the agonist/antagonist experiments.

All the Ca^{2+} peaks produced by depolarisation were averaged and the Ca^{2+} transients produced after application of DAMGO were expressed as a percentage of the average peak. Suppression in the peak of greater than 15% was considered to be a positive response to DAMGO and the neuronal cell was categorised as MOR sensitive.

The MOR selective agonist DAMGO (1 μM) suppressed the 50 mM K^+ - induced increase in $[\text{Ca}^{2+}]_i$ in a proportion of cells in both neonatal and adult preparations (figure 4.7B).

The DAMGO mediated inhibition in cells from both age groups was completely reversed by addition of the highly selective MOR antagonist CTAP (1 μM). Application of CTAP after DAMGO resulted in peak fluorescence recovering to pre DAMGO levels (figure 4.7C – baseline ratio 340/380 of 0.22 to peak of 0.49 after K^+ depolarisation. DAMGO mediated a suppression of the ratio to 0.41. CTAP reversed this suppression and peak ratios were 0.49 after its application).

Similar proportions of DRG cells were depolarised in adult and neonatal culture. A greater proportion of DRG cells were found to be MOR sensitive in neonatal compared to adult DRG neurons (Figure 4-10). In neonatal culture $56.5 \pm 3.4\%$ (mean \pm SEM, $n=8$) of all neurons that were depolarised by K^+ application responded to DAMGO compared to $39.9 \pm 1.5\%$ in the adult neuronal culture. This difference is highly significant ($p<0.001$).

4.4.2 The increased number of functional MOR expressing cells in neonatal DRG are large diameter NF200+ve cells

To test whether the increased number of opioid sensitive cells obtained in the Ca imaging studies were distributed across all subpopulations of DRG cells, two further analyses were performed.

Firstly the distribution of MOR responsive cells across neuronal cell sizes was investigated. Lumbar DRGs were dissected from 8 rats at age group. Cells were grown on 2 coverslips per rat and one field per coverslip was investigated with Ca imaging. There were approximately

100 cells per field so I studied 200 cells per animal, overall, over 1600 cells per age. Figure 4-11 shows cell cross-sectional areas of MOR responsive and MOR unresponsive cells in neonatal and adult DRG neuronal culture. MOR responsive cells are much more widely distributed across neuronal cell sizes in the neonate compared to the adult, where they are confined to smaller diameter cells.

Secondly, neurons were immunostained for NF200 and opioid responsive cells were relocated using confocal microscopy to determine their distribution across the two different subpopulations of sensory afferents. It was only possible to relocate fields from 2 animals per age and approximately 100 cells per rat, at each age were studied. Figure 4-13A shows typical fields from neonatal cultures and illustrates how fields were relocated for analysis. Figure 4-12A and B show that the proportion of NF200 positive neurons, was unaffected by age (45.3% in neonatal, compared with 40.8% in adult rat DRG neurons) and that the percentage of NF 200 negative MOR responsive neurons is also not significantly altered with age. However, there was a very clear difference in the expression of NF 200 in opioid sensitive cells between neonatal and adult neurons; $33.1 \pm 2.6\%$ of neonatal neuronal NF200 positive cells were found to be opioid responsive compared with only $11.3 \pm 1.9\%$ in the adult ($p < 0.001$).

4.5 Discussion

Ratiometric calcium imaging with Fura-2 was used to quantify MOR sensitive neurons in newborn and adult rat dissociated DRG neuronal culture. The distribution of MOR sensitive cells across different cell sizes was evaluated and immunostaining for NF200 was used to compare distribution of MOR responsive neurons across two different subpopulations of sensory neurons, large NF200 positive neurons and small NF200 negative neurons.

We found that, in the neonatal DRG, the MOR that are over-expressed compared to the adult, are functional. This is important because it was possible that all the widespread MOR observed on small and large cells in newborn animals might not have been biologically active. Using calcium imaging, we have shown that significantly more neonatal DRG cells respond to

the MOR agonist DAMGO than adult cells - $56.5 \pm 3.4\%$ and $39.9 \pm 1.5\%$ respectively. By correlating cell size with MOR function, we found that functional MOR expression in the adult rat was predominantly in small and medium sized neurons, the cell bodies of C and A δ fibres consistent with previous reports (Silbert et al 2003). In the neonate, a considerably higher proportion of large diameter A cells also express functional MOR. Combined calcium imaging with neurofilament NF200 immunostaining confirmed that the additional opioid sensitive cells are not spread across all DRG cell populations but are restricted to large diameter A cells - $33.1 \pm 2.6\%$ of neonatal neuronal NF200 positive cells were found to be opioid responsive compared with only $11.3 \pm 1.9\%$ in the adult. Importantly, the proportions of NF200 positive cells were not affected by age ($45.3 \pm 1.6\%$ in the neonate compared with $41.1 \pm 2.1\%$ in the adult). The proportions of NF200 positive cells are consistent with previous reports (Beland and Fitzgerald 2001). Also, the proportion of small C and A δ , nociceptive cells that are MOR sensitive in neonates is not significantly different from older animals.

The change in MOR expression with postnatal development observed in lumbar DRG tissue sections (Beland and Fitzgerald 2001) and in postnatal dissociated neuronal cell culture in earlier chapters of this thesis has now been shown to have direct functional consequences on the actions of opioids on neonatal sensory neurons.

4.5.1 DRG MOR function in adult sensory neurons

Previous studies have shown that, in sensory neurons from the mature nervous system, responsiveness to MOR agonists is quite variable. In random populations of acutely isolated sensory neurons, between 44 and 90% of cells studied are opioid sensitive (Schroeder et al 1991; Schroeder and McCleskey 1993; Moises et al 1994a, b; Liu et al 1995; Abdulla and Smith 1998;). We found that $39.9 \pm 1.5\%$ of all mature neurons cultured were MOR sensitive. Some of this variability in opioid sensitivity among cells might be explained by differences in the sensory modality transmitted by individual neurons. In the adult, nociceptive neurons are likely to be sensitive to the effects of opioids, whereas other neurons that transmit innocuous mechanical and thermal sensations should be relatively insensitive to opioids (Brennum et al 1993). Few studies have attempted to characterise the opioid responsiveness of different subpopulations of sensory neurons. Schroeder and McCleskey (1993) tested the effect of DAMGO on a group of putative nociceptive neurons identified by using antibodies against a

specific oligosaccharide present only on cells that project to laminae I and II in the dorsal horn of the spinal cord, the site of most nociceptive input. They found that the average DAMGO mediated inhibition of I_{Ca} in the labelled neurons (25%) was actually less than that observed in random samples of neurons (38%). This result may be partly explained by the fact that while primary nociceptive neurons do terminate extensively in laminae I and II in the adult, they also project to other areas of the dorsal horn (Light and Perl 1979). Another limitation of this labelling technique is that some non-nociceptive neurons also project to laminae I and II, which could explain their finding that not all of the labelled neurons were sensitive to DAMGO. Using a different approach Taddese et al (Taddese et al 1995) studied the opioid responsiveness of neurons from the trigeminal ganglion that were labelled when they took up a fluorescent indicator placed in the tooth pulp. They reasoned that neurons innervating tooth pulp are a pure population of nociceptors based on the assumption that the only sensory modality transmitted from the tooth is pain. They found that 79% of small nociceptors were inhibited by DAMGO, and the response rate for nociceptors of all sizes was only 49%. They also found that smaller nociceptors were much more likely to be opioid sensitive than larger nociceptors. A study by conducted by McDowell investigated whether opioids decrease Ca^{2+} currents (I_{Ca}) in primary nociceptive neurons, identified by their response to the algogenic agent capsaicin (McDowell 2003). They identified nociceptive neurons by their sensitivity to capsaicin. Capsaicin is a vanilloid compound isolated from hot peppers that produces burning pain when injected intradermally or arterially or applied to mucous membranes. It is an agonist for the vanilloid receptor TrpV1, a membrane protein involved in the transduction of noxious heat (Tominaga et al 1998; Caterina et al 2000). TrpV1 is expressed primarily by small- to medium-sized neurons of sensory ganglia (Tominaga et al 1998). Single-unit recordings from peripheral nerves innervating the skin demonstrate that capsaicin selectively activates heat-sensitive nociceptors. The majority of heat-sensitive nociceptive neurons are C-fibre polymodal nociceptors, which respond to noxious heat, noxious mechanical stimuli, and noxious chemical stimuli. Some C- and A δ neurons respond to noxious heat and noxious mechanical stimuli (mechano-heat nociceptors), while purely mechanical nociceptors, which do not respond to noxious heat, are insensitive to capsaicin. Nerve fibres transmitting non-noxious stimuli, such as light touch and hair sensors, are rarely found to be capsaicin

responsive (Seno and Dray 1993). Capsaicin responsiveness, therefore, is a sensitive and specific test for identifying heat-sensitive nociceptors. I_{Ca} was recorded from acutely isolated rat dorsal root ganglion neurons using the whole cell patch clamp technique before, during, and after application of the μ -opioid agonist fentanyl (0.01–1 μ m). Capsaicin was applied to each cell at the end of the experiment. Approximately 80% of all the cells were found to respond to capsaicin. This is consistent with other studies that have reported the capsaicin sensitivity of small sensory neurons from young rats, (Vyklícký et al 1998; Stucky et al 1998) though in older rats, the percentage of capsaicin-sensitive neurons is less, ranging from about 50 to 65% (Cardenas et al 1995; Gold et al 1996; Del Mar and Scroggs 1996). Fentanyl reduced I_{Ca} in a greater proportion of capsaicin-responsive cells (62 of 106, 58%) than capsaicin-unresponsive cells (2 of 15, 13%; $p < 0.05$). However, in contrast to the Taddese study, 58% of all nociceptors were opioid sensitive and fentanyl-sensitive cells were larger, on average, than the fentanyl-insensitive cells. There were also a few extremely large cells that were opioid sensitive. Little is known about opioid-insensitive nociceptors that have been identified by this and other studies. Although these studies have attempted to investigate MOR expression and function in nociceptive neurons there is no information about the developmental regulation of MOR in these fibres. Also little is known about the proportions of largely non-nociceptive A fibres that we found to express MOR and how this might change with CNS development.

4.5.2 The use of calcium microfluoroscopy to measure MOR function

The technique of fura-2 based calcium imaging is known to provide a sensitive measure of MOR function (Rhim and Miller 94). This optical imaging technique has some advantages over other methods of investigating MOR function. It is inherently less invasive than patch-clamp recording, as there is no need for physical access of a patch pipette, and mechanical disturbance to the membrane and cytoskeleton associated with seal formation, which may adversely affect calcium channel activity, are avoided. It also provides higher temporal and spatial resolution than other conventional methods and, particularly using a cell culture model, allows measurements from sites inaccessible using conventional methods. It allows recording in real time and has recently even been used for monitoring the kinetic activity of multiple single channels (Demuro and Parker 2003). For the purposes of this study it was a very

effective technique, allowing, in total, the examination of over 3000 cells for MOR responsiveness. While the issues discussed in chapter 3 regarding the use of cell culture as a model for the investigation of functional MOR also apply here, MOR are clearly functional in cell culture and an unequivocal difference in the proportions of MOR responsive cells was found at the two ages studied.

In this study a cell was considered to be MOR responsive if the application of DAMGO with the depolarising stimulus suppressed peak fluorescence by a minimum of 15%. This figure was arbitrary. High K^+ -induced depolarisation evoked large and reversible increases in peak fluorescence that could be reliably repeated multiple times without any significant rundown. Based on this a 15% reduction in fluorescence was thought to be significant and considered to have occurred as a result of the modulation of intracellular calcium flux. It seems unlikely that such a reduction in peak fluorescence would have occurred in a cell that was not MOR sensitive in response to DAMGO. Also, these reductions were completely reversed by the application of the MOR antagonist CTAP. Conversely, the criterion used here may have been too stringent leading to an underestimation of the number of MOR sensitive cells. But the cut off was applied to both age groups so if MOR responsive cells were overlooked this was the case at both ages. And, based on this criterion we found a significant difference in the number of MOR responsive cells between the adult and the neonate.

After immunostaining of cells for NF200 it was only possible to relocate 4 fields that had been studied with calcium imaging because cells had become displaced during the process of immunostaining. This meant that correlation of MOR responsiveness and NF200 staining was possible in about 200 cells per age. Although numbers were smaller than examined overall, immunostaining for large diameter A cells confirmed that the additional opioid sensitive cells are not spread across all DRG cell populations.

4.5.3 The role of calcium channels in MOR function and analgesia

It is well established that a block of Ca^{2+} channels at the central presynaptic terminals of DRG cells profoundly affects synaptic transmission in the spinal cord (Kohno et al 1999; Iwasaki et al 2000). Acute opioid administration leads to reductions in intracellular Ca^{2+} levels due to the inhibition of voltage dependent calcium channel activity (Werz and MacDonald 1984) and

decreases in Ca^{2+} binding to synaptic membranes and synaptic vesicles (Yamamoto et al 1978). This in turn results in a reduction in the release of neurotransmitters that are involved in nociceptive transmission.

Calcium ions play an important role in the regulation of pain sensitivity and considerable evidence indicates a close relationship between opioid antinociception and Ca^{2+} levels within the central nervous system (Miranda and Paeile 1990). Agents that increase cytosolic Ca^{2+} level in neurons block opioid antinociception (Smith and Stevens 1995). Conversely, Ca^{2+} chelators (Kakunaga et al 1966) or Ca^{2+} channel antagonists potentiate opioid antinociception (Del Pozo et al 1987; Contreras et al 1988; Cahill et al 1993). Agents such as opioids that modulate Ca^{2+} can exert important effects on the ability of neurons to interpret extracellular signals since Ca^{2+} plays a critical role in neurotransmission. Calcium levels outside cells are 10 000 times higher than free intracellular Ca^{2+} . However, free $[\text{Ca}^{2+}]_i$ is the physiologically active form of calcium. The level of free intracellular calcium ($[\text{Ca}^{2+}]_i$) is regulated and maintained as low (~100 nM) through the action of a number of binding proteins and ion exchange mechanisms. Each cell has a unique set of Ca^{2+} signals to control its function. Ca^{2+} signal transduction is based on rises in free cytosolic Ca^{2+} concentration and Ca^{2+} can flow from the extracellular space or be released from intracellular stores. The endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Ca^{2+} is accumulated into intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5-trisphosphate (IP_3) via IP_3 receptors and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (Clapham 1995a, b; Putney 1999). Extracellular Ca^{2+} enters the cell through various types of plasma-membrane Ca^{2+} channels. Membrane-intrinsic transporting proteins are important for buffering cell Ca^{2+} and soluble proteins, such as calmodulin, also contribute. Ca^{2+} is transported across the plasma membrane and the membranes of organelles via channels, pumps and $\text{Na}^+/\text{Ca}^{2+}$ exchangers. External signals arriving at the cell engage plasma membrane receptors to initiate cell signaling pathways. One of the end results is increased intracellular calcium concentration. On stimulation, this level can rise globally to in excess of 1 M. This increase can be generated from sources both within and outside the cell. The formation of IP_3 is the focal point for two major pathways, one initiated by a family of G

protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly. These separate receptor mechanisms are coupled to energy-requiring transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-bisphosphate to generate both DAG and IP₃. The latter then binds to an IP₃ receptor (IP₃R) to mobilize stored calcium and to promote an influx of external calcium. This is summarized in the figure below.

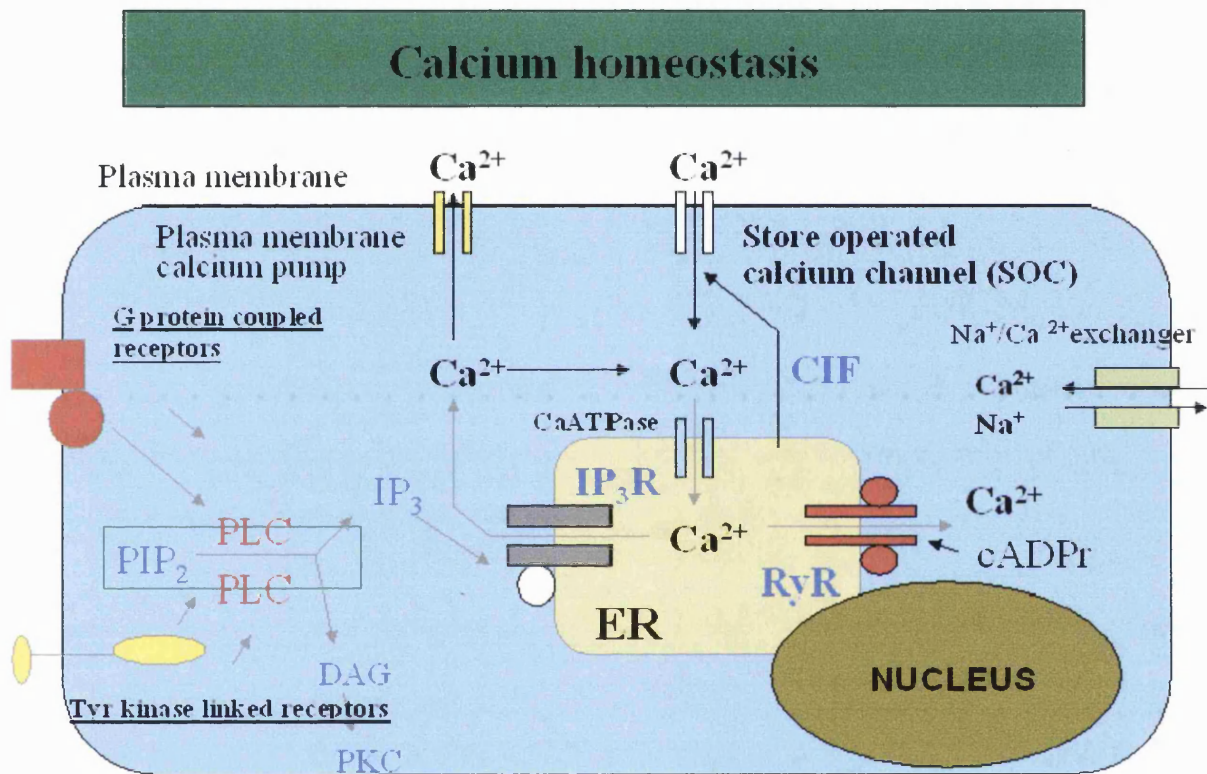


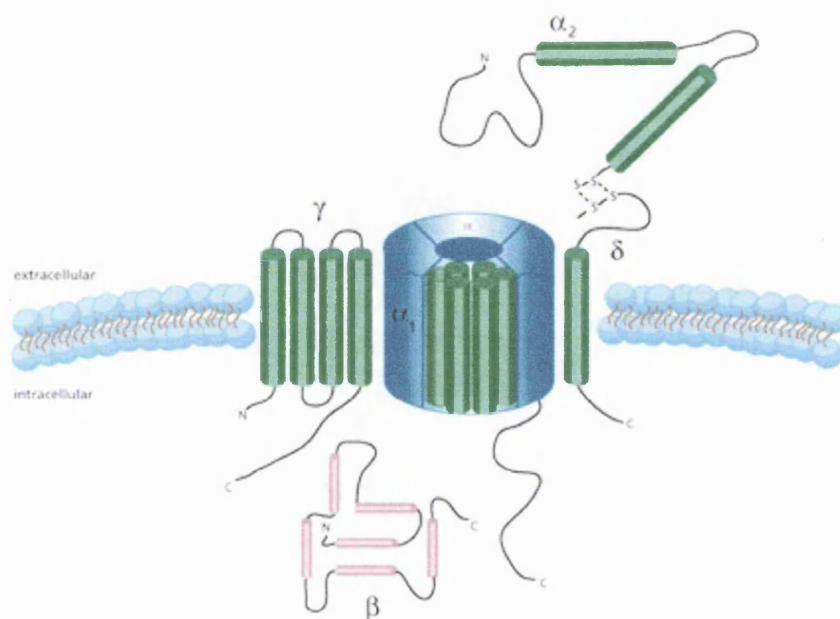
Figure 4-1 Representation of calcium homeostasis in a single cell.

Extracellular Ca^{2+} enters the cell through plasma membrane Ca^{2+} channels and leaves the cell using Ca^{2+} pumps and $\text{Na}^+/\text{Ca}^{2+}$ exchangers. Endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Ca^{2+} is accumulated in intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5- trisphosphate (IP₃) via IP₃ receptors (IP₃R) and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (RyR).

The main route of extracellular calcium influx to the cells is through voltage dependent Ca^{2+} channel (VDCCs). Functionally, Ca^{2+} channels have been classed into 'high' and 'low' threshold on the basis of the voltage range at which they are activated. Low threshold Ca^{2+}

channels have also become known as T-type and exhibit a unique pharmacological profile (Herrington and Lingle 1992). High threshold Ca^{2+} channels can be grouped into various classes according to their sensitivity to dihydropyridines (L-type), ω -conotoxin-GIVA (N-type), ω -agatoxin-IVA (P- or Q-type) or none of these, for example the R-type currents of cerebellar granule cells. Voltage-gated calcium channels are heteromultimers composed of a α_1 subunit and three auxiliary subunits, α_2 - δ , β and γ . The α_1 subunit forms the ion pore and possesses gating functions and, in some cases, drug binding sites. Ten α_1 subunits have been identified, which, in turn, are associated with the activities of the six classes of calcium channels. L-type channels have α_{1c} (cardiac), α_{1d} (neuronal/endocrine), α_{1s} (skeletal muscle), and α_{1f} (retinal) subunits; N-type channels have α_{1B} subunits; P- and Q-type channels have α_{1A} subunits, and T-type channels have α_{1G} , α_{1H} , and α_{1I} subunits. The α_1 subunits each have four homologous domains (I-IV) that are composed of six transmembrane helices. The fourth transmembrane helix of each domain contains the voltage-sensing function. The four α_1 domains cluster in the membrane to form the ion pore. The β -subunit is localized intracellularly and is involved in the membrane trafficking of α_1 subunits. The γ -subunit is a glycoprotein having four transmembrane segments. The α_2 subunit is a highly glycosylated extracellular protein that is attached to the membrane-spanning δ -subunit by means of disulfide bonds. The α_2 -domain provides structural support required for channel stimulation, while the δ domain modulates the voltage-dependent activation and steady-state inactivation of the channel (Jones 1998).

Figure 4-2 Schematic of a voltage-dependent calcium channel



Although the physiological roles of these different types of calcium channels are not fully understood, neurotransmitter secretion seems to be preferentially coupled to one or more of these channel types under different circumstances (Luebke et al 1993; Takahashi and Momiyama 1993; Toth et al 1993). L-type and N-type VDCC have been implicated in the release of neurotransmitters / neuromodulators from sensory neurons in the spinal cord (Perney et al 1986; Anwyl 1991) and L-, N- and T-type VDCC have been found to coexist in sensory neurons of the dorsal root ganglion (Nowycky et al 1985). T-type channels are thought to be responsible for neuronal excitability and oscillatory activity (Bertolino and Llinas 1992).

Opioids inhibit a variety of neurotransmitters and neuromodulators in a variety of locations in the peripheral and central nervous system (Mudge et al 1979; Yaksh et al 1980; Duggan and Morton 1983). μ , δ and κ agonist-induced inhibition of calcium currents (Werz and MacDonald 1985; Werz et al 1987; Su et al 1998) is thought to mediate opioid reduction of calcium-dependent neurotransmitter release from presynaptic terminals (Werz and MacDonald 1985). It is possible that the activation of μ receptors blocks L-type and especially T-type VDCC reducing neurotransmitter release and producing analgesia. It has been shown previously that the activation of μ receptors inhibits N-, P- and Q-type VDCC in rodent sensory neurons, but

not L-type and T-type VDCC (Rusin and Moises 1995). However, in contrast to these studies, there are reports that activation of μ receptors inhibits T-type VDCC (Schroeder et al 1991) and L-type VDCC (Piros et al 1996). δ receptor agonists do not modulate VDCC in DRG neurons (Liu et al 1995; Rusin and Moises 1995) and L-type and T-type VDCC are not regulated by κ receptors in DRG cells (Gross and Macdonald 1987; Wiley et al 1997). Interestingly, there are also reports showing that opioids can increase the release of some neurotransmitters and neuromodulators (Cahill et al 1993; Cesselin 1995; Devillers et al 1995; Gustafsson et al 1999) perhaps due to increased Ca^{2+} uptake into synaptosomes (Jin et al 1992).

Differentially regulated patterns of Ca^{2+} channel expression have been proposed from electrophysiological studies of development in dorsal root ganglion neurons. Hilaire et al investigated non-N, non-L Ba^{2+} through Ca^{2+} channels in freshly dissociated large diameter embryonic mouse DRG neurons using the whole-cell patch-clamp technique. A P-type current present at embryonic day 13 was found to disappear by day 15 whereas the Q-type current increased during the same embryonic period. In contrast, the contribution of the non-L, non-N, ω -agatoxin IVA resistant current (R-type) was constant during this developmental span. These results showed that P-type and Q-type Ca^{2+} currents are differentially expressed during ontogenesis in large diameter DRG neurons (Hilaire et al 1996)

Opioid receptors couple to PTX-sensitive G proteins, thereby activating inwardly rectifying K^{+} channels and inhibiting voltage-sensitive Ca^{2+} channels and adenylyl cyclases (Murthy and Makhoulf 1996; Piros et al 1996). However, G_i/G_o -coupled opioid receptors also activate phospholipase C and phosphatidyl inositol turnover (Murthy and Makhoulf 1996) and elevate intracellular Ca^{2+} levels in neuronal and non-neuronal tissues (Connor and Henderson 1996; Hauser et al 1996). This occurs either by stimulating influx of extracellular $[\text{Ca}^{2+}]_e$ or release of intracellular $[\text{Ca}^{2+}]_i$ stores via soluble second messengers (inositol trisphosphates), or both. Whereas MOR agonists predominantly inhibit neuronal activity by modulating potassium channels and voltage-dependent Ca^{2+} channels, excitatory opioid effects have also been noted. For example, opioid enhancement of evoked enkephalin release in guinea pig

myenteric plexus was found to involve elevation of intracellular Ca^{2+} levels (Xu and Gintzler 1992). In the rat locus coeruleus, morphine did not simply decrease firing rates of LC neurons, but it also induced persistent oscillatory discharges (Zhu and Zhou 2001). Opioid-dependent Ca^{2+} influx pathways could contribute to these effects. In this study the issue of increased $[\text{Ca}^{2+}]_i$ as a result of MOR mediated release of intracellular Ca^{2+} stores was not addressed. It may be possible to conduct the calcium imaging experiments after pre-treatment of cells with caffeine to deplete intracellular stores of Ca^{2+} .

4.5.4 The postnatal regulation of MOR function

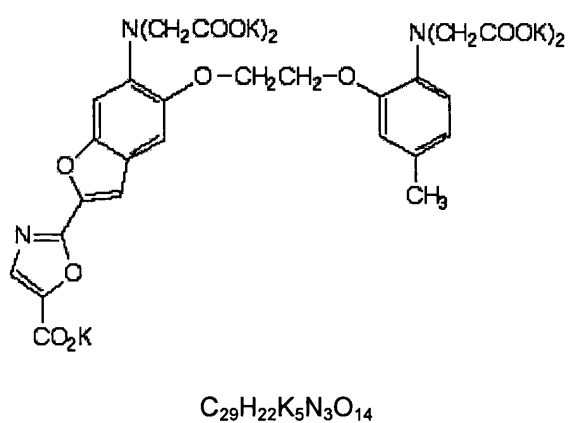
There is limited information of the function of postnatally regulated MOR in DRG neurons. A study by Hamra et al compared opioid modulation of calcium currents in neonatal and adult cultured nodose sensory neurons (Hamra et al 1999). DAMGO-mediated inhibition of I_{Ca} was present in essentially all neonates (95%). In the general population of neurons from juvenile or adult animals, the response to DAMGO was less frequent (60-65%). DAMGO was found to act via only the N-type channel in neonates but modulated I_{Ca} through multiple channel types in older animals. This is consistent with studies in adult dorsal root ganglia neurons (Rusin and Moises 1995). The difference in the response at the two ages may reflect differences in the relative expression of the specific Ca^{2+} channels or age-dependent changes in the coupling of the receptor to the Ca^{2+} channels and G protein. In this study, the T-type current in neonatal or juvenile neurons appeared not to be altered by DAMGO. I am unaware of studies comparing the relative age-dependent expression of Ca^{2+} channels in DRG neurons.

What do these results tell us about the regulation of DRG cells by opioids? Receptor modulation of Ca influx into the cell soma has many effects including the regulation of a variety of Ca-dependent ion channels. Effects on gene expression and metabolism are possible. The effects of opioids are likely to occur on the terminals of the same neurons. This would represent a mechanism by which opioids could regulate NT release from these cells.

The differing magnitude and pattern of opioid responsiveness in neonatal sensory neurons will directly affect the actions of morphine on neonatal central sensory and pain pathways. In the adult the functional expression of MOR in DRG cells parallels the actions of opioid

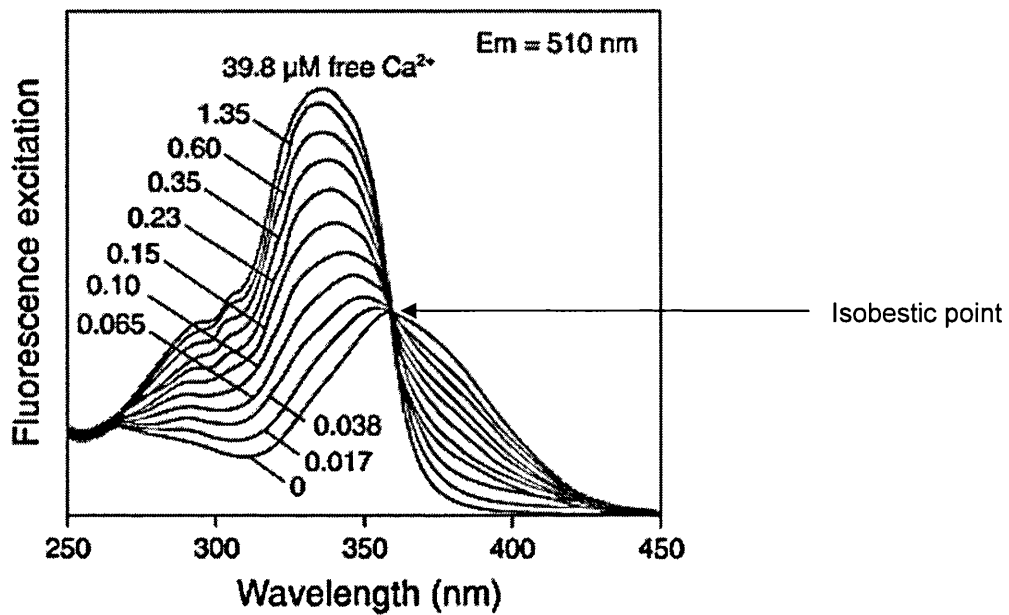
agonists on nociceptive transmission in the spinal cord. Opioid agonists inhibit membrane currents in small but not large adult neurons (Taddese et al 1995; Wilding et al 1995) and selectively depress nociceptive C and A δ evoked activity in the dorsal horn of the spinal cord (Sivilotti et al 1995; Rahman and Dickenson 1999). The presynaptic site of action of opioids on nociceptive terminals in the spinal cord is a key mechanism underlying opioid analgesia in the whole animal, leading to reduced neurotransmitter release and consequently reduced postsynaptic excitation in central nociceptive pathways. In the neonatal rat, however, studies in vivo and in vitro suggest that opioids depress non-nociceptive mediated activity (Faber et al 1996; Marsh et al 1999) as well as C and A δ -mediated nociceptive inputs. This wider, non-selective effect in the immature neonatal rat spinal cord is consistent with widespread MOR on A cells.

Figure 4-3 The structure of Fura-2



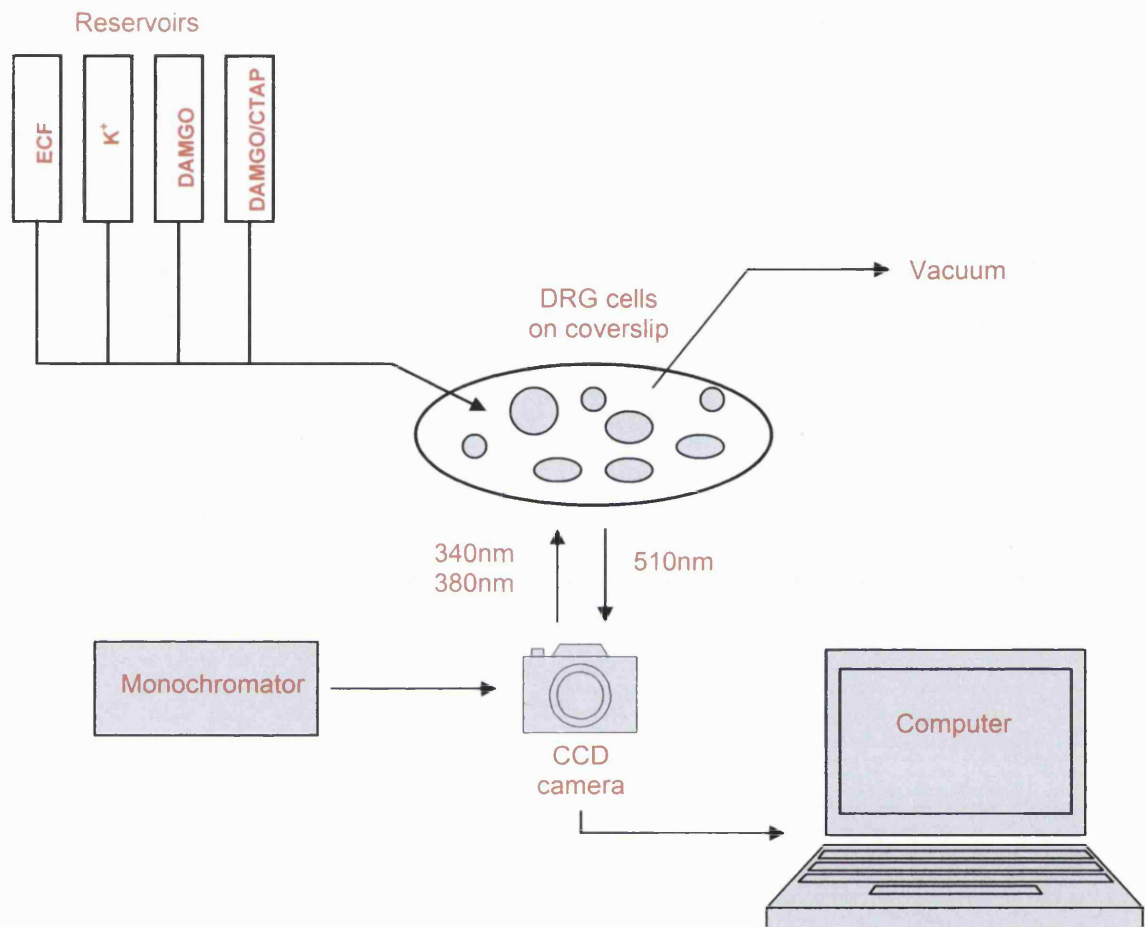
1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]- 2-(2-amino-5-methylphenoxy)
ethane-N,N,N',N'-tetraacetic acid, pentapotassium salt
[11364-64-7]

Figure 4-4 Fluorescence excitation spectra for Fura-2



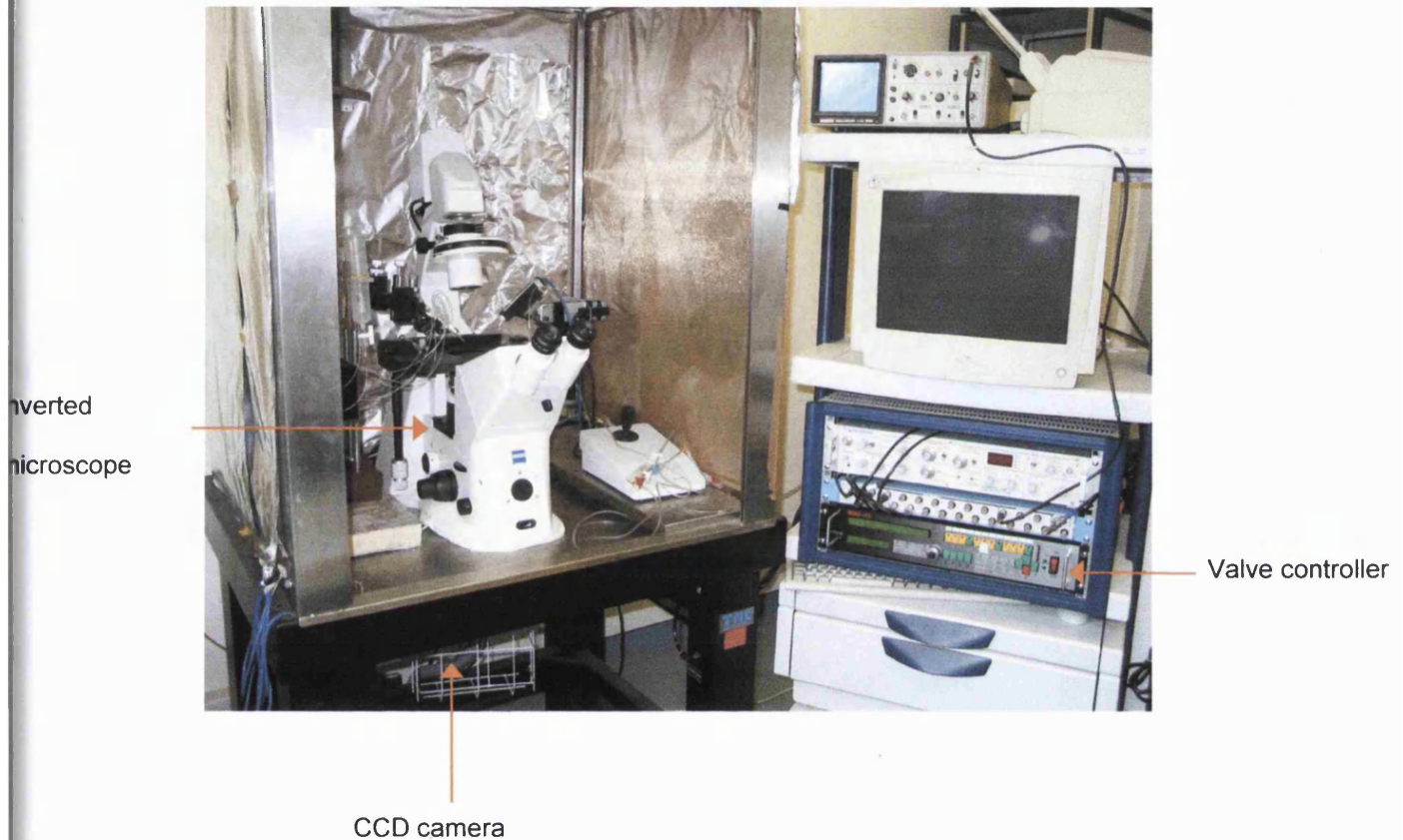
This set of fluorescence excitation spectra for fura-2 is in calibration buffers containing 0–39.8 μM free Ca^{2+} . Emission is collected at 510 nm.

Figure 4-5 Schematic drawing of calcium imaging apparatus.



Solution reservoirs contain the extracellular fluid and experimental solutions applied to the cells. A monochromator controls the excitation wavelength delivered to the cells and a CCD camera records the emission of fluorescent light from the preparation. The output from the camera is recorded on the computer

Figure 4-6 The calcium imaging apparatus



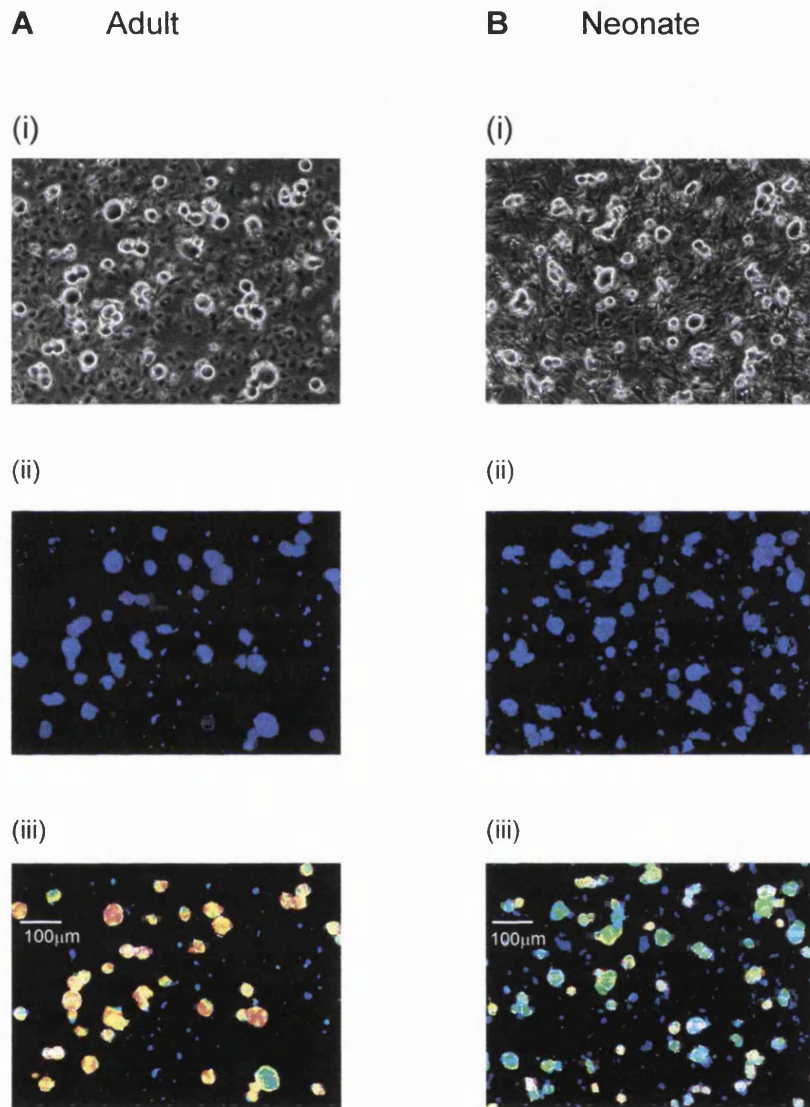
The valve controller is used to program the protocols for solution application. The CCD camera can just be seen mounted below the microscope. The microscope station is covered in tin foil so that all experiments can be conducted in complete darkness.

Figure 4-7 The calcium imaging apparatus – detail



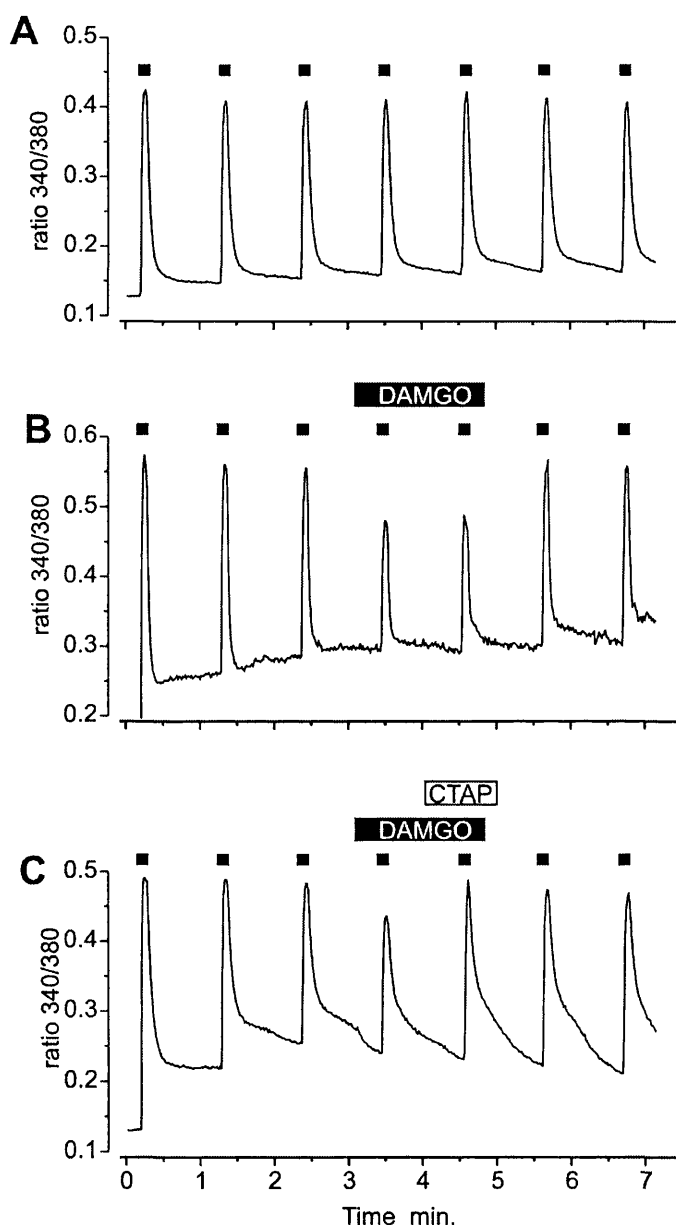
There are 6 reservoir chambers each of which contains a different experimental solution. All lines from these reservoirs converge into the white Teflon manifold which has minimal dead space allowing precise and rapid control of the solutions passing over the cells. The coverslip on which the healthy dissociated DRG neuronal cells are plated can be seen mounted on a bridge on the stage of the inverted microscope.

Figure 4-8 **Calcium imaging in (A) adult and (B) neonatal rat lumbar DRG neurons.**



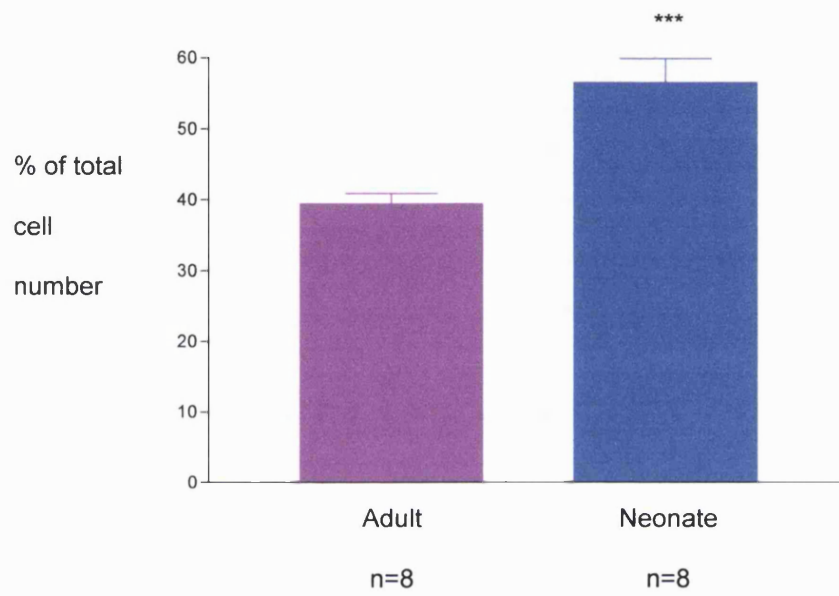
- (i) Bright field image of cultured DRG cells to be investigated with calcium imaging.
- (ii) Fluorescence images of cells loaded with calcium sensitive Fura-2, before depolarisation. These healthy cells are mainly dark blue as a result of baseline fluorescence.
- (iii) Fluorescence images of cells following depolarisation with K^+ . Most cells fluoresce more brightly following depolarisation with K^+ which results in Ca^{2+} flux into the cell.

Figure 4-9 Changes in fluorescence in neonatal DRG neuronal cells produced by intracellular Ca^{2+} flux.



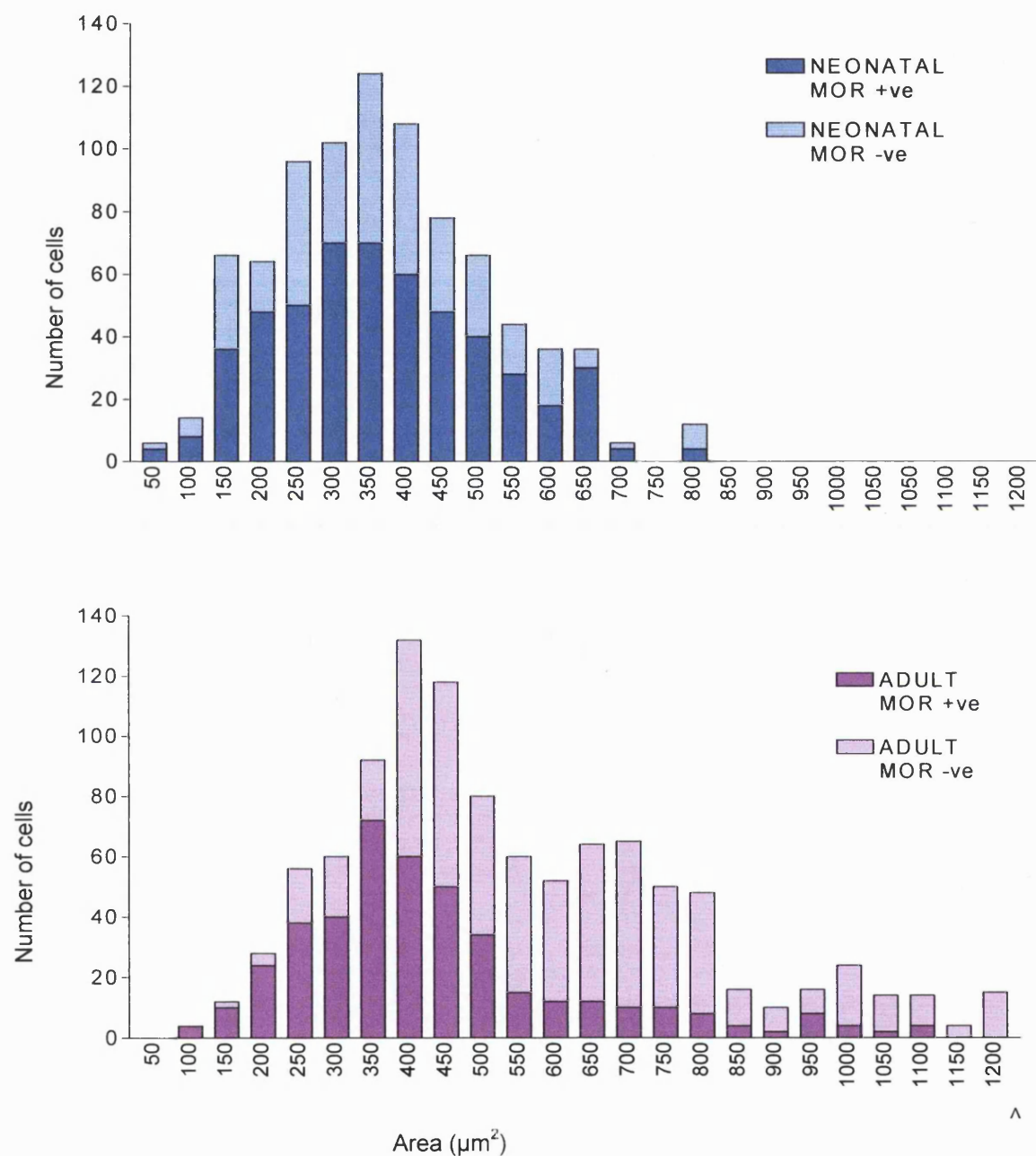
- A. Control Experiment. Several consistent transient increases in $[\text{Ca}^{2+}]_i$ were elicited by repeated depolarising K^+ stimuli in neonatal DRG neuronal cells.
- B. Application of DAMGO causes suppression of depolarisation induced increase in $[\text{Ca}^{2+}]_i$ in an opioid responsive neonatal DRG neuronal cell
- C. DAMGO mediated reduction in $[\text{Ca}^{2+}]_i$ following depolarisation is completely reversed by simultaneous treatment with the MOR antagonist CTAP.

Figure 4-10 Proportions of opioid responsive cells in neonatal and adult rat DRG neuronal cells using calcium imaging.



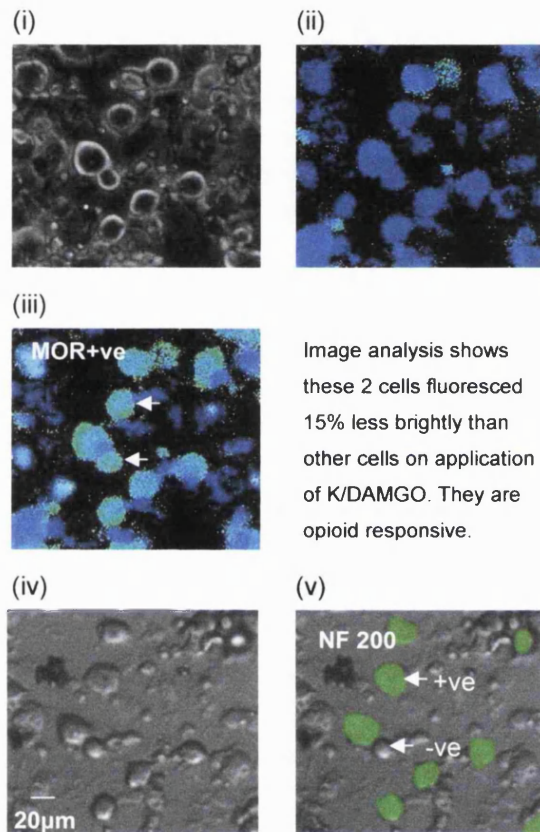
There are significantly greater numbers of opioid responsive cells in the neonate compared with the adult (n=8, mean \pm SEM, ***p<0.001).

Figure 4-11 The distribution of MOR positive and negative cells according to cell diameter in neonatal rat DRG neurons (n=8) and adult rat DRG neurons (n=8).



MOR are expressed evenly across the full range of small to large diameter cells in the neonatal rat DRG neurons whereas MOR are expressed predominantly in medium and small cells in the adult rat DRG neurons.

Figure 4-12 Calcium imaging and relocation of fields following immunostaining for NF 200 in neonatal rat lumbar DRG neurons.



- (i) Bright field image of cultured DRG cells investigated with calcium imaging
- (ii) Fluorescence images of cells loaded with calcium sensitive Fura-2, before depolarisation. These healthy cells are mainly dark blue as a result of baseline fluorescence. There are a few cells that are brighter due to higher levels of baseline fluorescence. They are non-functional and did not respond to K^+ depolarisation
- (iii) Fluorescence images of cells following depolarisation with K^+ . Most cells fluoresce more brightly following depolarisation with K^+ which results in Ca^{2+} flux into the cell. Opioid sensitive cells are detected by their response to the application of DAMGO. Cells that demonstrate a suppression of at least 15% in peak fluorescence, measured by CCD camera,

as a result of K with DAMGO application were considered opioid sensitive. This 15% drop is not detectable in these photographs therefore opioid sensitive cells are represented diagrammatically here. In this field 2 opioid sensitive cells were detected after analysis of the fluorescence ratios.

(iv) Cells relocated using confocal microscopy.

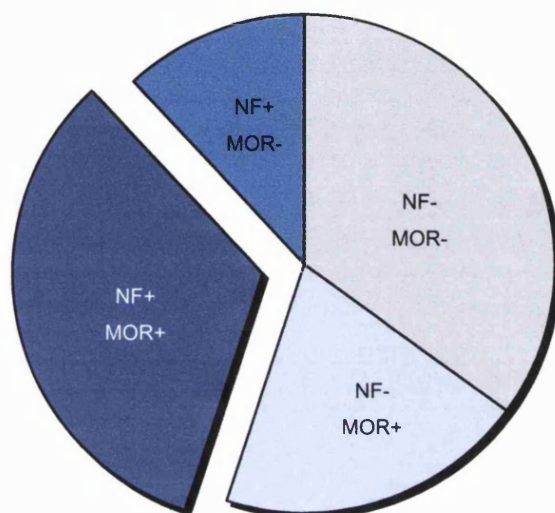
After calcium fluoroscopy cells were fixed and immunostained for NF200. The field of cells investigated with calcium fluoroscopy were then relocated using a confocal microscope. This is a confocal image of the relocated cells.

(v) Confocal fluorescence image of NF200 positive cells in relocated field.

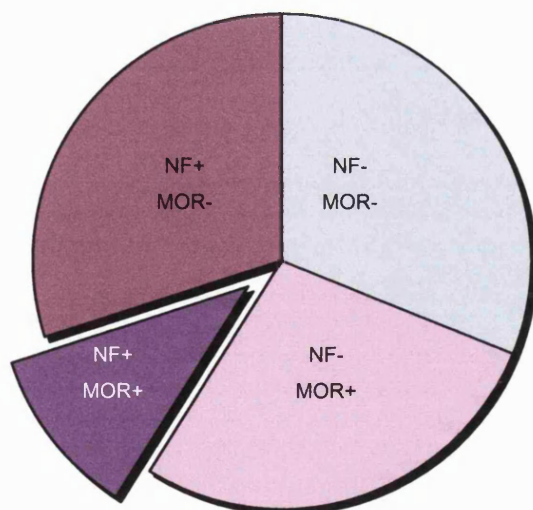
Following immunostaining, NF200 positive cells are seen as fluorescent. Since calcium fluoroscopy had revealed the opioid responsive cells, cells that co-express NF200 and are opioid responsive can be identified using this image.

Figure 4-13 The proportions of NF+/- and MOR+/- cells with postnatal development

(A) Neonate



(B) Adult



Pie charts showing the percentage of cell expressing NF200 and MOR in

A. neonatal and B. adult neuronal DRG culture from combined immunohistochemistry and calcium imaging.

In the neonatal rat there was a greater percentage of MOR responsive cells (53% compared with 39% in the adult). The increased expression of MOR in the neonate was found to be mainly on the large NF200 positive A cells (33% compared with only 11% in the adult). Importantly the proportions of NF 200 positive cells were found to be similar at both ages (45% in the neonate and 41% in the adult).

5 Morphine selectivity in different sensory modalities in neonatal and older rats

5.1 Introduction

We have established that the functional effects of morphine in neonatal dorsal root ganglia differ substantially from that in adults using calcium imaging of morphine sensitivity in identified subpopulations of small and large sensory neurons. Functional MOR expression was found to be greater in large A cells in the neonate than in older rats. To test the implications of this in the whole animal, in this chapter we directly compared the selectivity and sensitivity of morphine on different sensory modalities in behavioural tests in neonatal and older rats. Reflex hind limb withdrawals to mechanical and thermal stimuli were used to investigate the effects of postnatal age on the efficacy of morphine in rats. These behavioural techniques are a well-established method of quantifying nociceptive and non-nociceptive spinal sensory responses in both adults and neonates (Fitzgerald M 1999; Le Bars et al 2001).

Although research in human infants and in animals has focussed on various biochemical indicators for pain (e.g. catecholamines, corticoids, and opioids), physical signs of pain remain the most reliable since biochemical markers can be non-specific (Stevens and Franck 2001). This can also be true for other methods such as electrophysiological parameters e.g. electroencephalograms, evoked potentials (Ichinose et al 1999). The study of behavioural reactions in animals provides the best indicator of the perceived unpleasant sensation resulting from a stimulus that would be painful in humans. Behavioural techniques allow the assessment of analgesic interventions and careful extrapolation of these to human subjects. An increase in nociceptive threshold from control values is typically interpreted as an analgesic effect. The rationale for this assumption derives from psychophysical experiments in human volunteers that show a close parallel between self reported pain and the thresholds for activation of the withdrawal reflex (Willer 1977). There is no doubt that animal models of

acute pain are extremely useful although they suffer from some shortcomings and the data they generate must be interpreted with care.

5.2 The withdrawal reflex

5.2.1 Definition

A withdrawal reflex is a response to a painful cutaneous stimulus e.g. electrical, thermal, mechanical or chemical, that results in the removal of the limb from the source of potential tissue damage. The size and strength of the reflex muscle contraction is graded according to the intensity of the stimulus.

Sherrington, in 1910, observed that painful electrical stimulation of the limb in an experimental animal causes an ipsilateral hip, knee, and ankle reflex withdrawal that he termed the nociceptive flexion reflex (NFR). The NFR is a physiological, polysynaptic reflex allowing for painful stimuli to activate an appropriate withdrawal response. The method became an established clinical research tool for pain as a result of evidence that the reflex cannot be elicited without the activation of nociceptive fibres. Kugelberg published the first human study using NFR in 1960. Standard NFR testing includes transcutaneous electrical stimulation of the sural nerve in the retromalleolar space and recording of the impulse from the surface of the short head of the biceps femoris muscle, ipsilateral to the stimulated side (Arendt-Nielsen et al 1994). The minimal intensity of the stimulus that is sufficient to elicit a reflex at a well-defined latency, known as the reflex threshold, usually corresponds to the minimal stimulus intensity that elicits a perception of pain (Willer 1977; Chan and Dallaire 1989). A voluntary knee flexion can be excluded when the reflex latency (i.e. the interval between application of the stimulus and muscle contraction) lies below 150 ms (Willer 1984; Arendt-Nielsen et al 1994). Therefore, this method can be used as an electrophysiological parameter for quantifying the excitability of spinal neurons (Petersen-Felix et al 1996). A number of studies suggest a linear correlation between NFR threshold and the subjective perception of pain in humans. The threshold for maximal reflex response is similar to that for intolerable pain. (Willer 1977). Wall advocated caution, however, in the interpretation of these experiments that suggest such a linear relationship between the intensity of the stimuli that reaches the pain

threshold to that which activates the flexion withdrawal reflex. He was uncomfortable with the artificial nature of the experiments that could suffer from systematic errors in design. He also felt that the generalisation from sensory thresholds to pain perception may be a gross oversimplification. This is supported by studies of thermal nociception. In these, withdrawal magnitude, which is measured electromyographically, and pain intensity are both proportional to stimulus intensity. However, the magnitude of withdrawal does not always reflect the intensity of pain sensation. This is particularly true at higher temperatures. The threshold for withdrawal from radiant heat far exceeds the pain threshold (Campbell et al 1991). This is in contrast to studies of mechanical and electrical stimuli.

The withdrawal reflex can be considered a reasonable model for the study of spinal nociceptive processing but not necessarily of pain perception. Cutaneous reflexes provide information about the sensitivity and selectivity of the nervous system to nociceptive stimuli. As such it is a very useful tool in animal experimental work and both experimental and clinical work with non-verbal (paediatric and developmentally delayed) humans.

The withdrawal reflex arc consists of several components:

- (i) Primary afferent nociceptors in skin and deeper tissues;
- (ii) Synaptic transmission between primary afferents and dorsal horn neurons;
- (iii) Transmission through somatotopically and musculotopically organised interneurons within the dorsal horn of the spinal cord which project either intraspinally or supraspinally;
- (iv) Activation of motor neurons and muscle groups that act synergistically to produce limb withdrawal.

In the rat, the nociceptive withdrawal reflex system has a modular organization. Each reflex module controls a single muscle or a few synergistic muscles, and its cutaneous receptive field corresponds to the skin area withdrawn upon contraction of the effector muscle(s) when the limb is in the standing position. Thus, the nociceptive withdrawal movement is a compound reflex linked to a number of reflex pathways acting on single muscles or small groups of synergistic muscles. The withdrawal reflex is therefore an integrated series of

parallel reflex pathways each sub serving different muscles (Schouenborg and Sjolund 1983; Schouenborg and Kalliomaki 1990; Schouenborg et al 1995).

The components of cutaneous primary afferent input involved in spinally-organized NFR circuitry, namely flexion reflex afferents, have been generally believed to involve primary A and C afferent fibres (Schomburg 1990). It was also further demonstrated that A fibres are involved in the early response component, while C fibres in the late response component of the NFR by using tetrodotoxin (TTX) with a concentration capable of isolating TTX-resistant C-fibres and TTX-sensitive A-fibres (Schomburg et al 2000). Spinal deep DH neurons were also found to receive convergent input directly from mATP-sensitive/capsaicin-insensitive (A) primary afferent inputs and indirect capsaicin-sensitive (C) primary afferent inputs mediated via SG neurons of the spinal cord by using a spinal slice patch-clamp preparation (Nakatsuka et al 2002). The data suggest that primary C fibre afferent mediated EMG activity of NFR is organized by a longer polysynaptic transmission pathway while A fibre mediated EMG activity of NFR is organized by a shorter polysynaptic transmission pathway.

Electrophysiological studies have revealed that peripheral cutaneous or deep tissue injury and inflammation can result in hyperexcitability or central sensitization in spinal DH nociceptive neurons (Chen et al 1998) as well as a temporal or spatial facilitation of the NFR (Woolf 1983; Woolf and Wall 1986; Herrero and Cervero 1996; You et al 2002) in both human and animal experimental models of persistent pain and hypersensitivity.

Tissue damage and inflammation produce a variety of local biochemical events (Rang et al 1991) that sensitize the peripheral receptors (Treede et al 1992) and may activate normally inactive nociceptors (Schmidt et al 1995). Peripheral inflammation induces a gene expression in the dorsal root ganglion resulting in an increased synthesis of peripheral receptors (Michael and Priestley 1999). These events mediate primary hyperalgesia, whereby a reduced threshold for eliciting pain within the injured area can be detected. In animal preparations, tissue damage induces profound plasticity changes in the spinal cord that result in increased responsiveness to peripheral stimulation (Woolf and Salter 2000). An expansion of receptive fields of individual dorsal horn neurons is documented (McMahon and Wall 1984). As a result,

a peripheral stimulus activates a higher number of dorsal horn neurons and hyperalgesia may also be evoked in healthy areas surrounding the injured region.

The withdrawal reflex has been confirmed as a powerful tool in pain research e.g. in the demonstration that activity-dependent changes form the central component of post-injury pain hypersensitivity (Woolf 1983, 2000). In chronic decerebrate rats, tissue inflammation or brief high-threshold afferent inputs were shown to change the receptive field properties of flexor motor neurons. In addition, the reflex has been used to test antinociceptive agents. Increase in reflex withdrawal threshold can be attributed to the analgesic action of the agent. However, some forms of innocuous stimulation can evoke a withdrawal reflex. The responses are weaker though and it is clearly still nociceptors that provide the major input from the skin to the reflex pathways. Skin inflammation and tissue injury can significantly increase the magnitude of a response to a normally innocuous stimulus and repeated thermal stimulation has been shown to reduce the threshold of nociceptors to thermal stimuli. The strength of the association between reflex responses and subjective perception is critical to the interpretation of animal experimental work and both experimental and clinical work with non-verbal humans.

5.2.2 Development of the cutaneous flexion withdrawal reflex

Nociceptive reflexes demonstrate a clear developmental pattern both in humans and rats. The properties of the reflex are the same in neonatal rats and humans and are a reflection of sensory neuron activity in the developing dorsal horn. In newborn rat pups and humans cutaneous flexion reflexes can be elicited with much lower, often non noxious, mechanical stimuli than adults and show more persistent and synchronised responses. In rats the reflex is sensitised by repetitive stimulation up to P8 and it is only after this time that it shows the more mature pattern of habituation (Fitzgerald 1988). Some studies have shown that thermal thresholds are lower too in neonatal rat pups compared to adults (e.g. Marsh et al 1999). Responses to noxious mechanical and electrical stimuli are characterised by prolonged durations that outlast the stimulus duration. Furthermore, activation of low-threshold A fibre afferents in rats leads to an increase in central excitation normally associated exclusively with high threshold C fibre afferents in the adult (Jennings and Fitzgerald 1996). Expression of c-fos, the early onset gene, in the spinal cord dorsal horn again only occurs after C fibre

activation in adults, but in neonates it can also be activated by low threshold A fibres (Jennings and Fitzgerald 1996).

Cutaneous reflexes to mechanical stimulation can be clearly elicited in human neonates using von Frey hairs (vFh) from as early as 23 weeks post conceptual age (PCA). These neonates typically display low mechanical thresholds which increase strikingly with increasing age (Fitzgerald 1988). As in the rat, the withdrawal reflex in human neonates is characterised by low thresholds resulting in responses to non-noxious stimuli, prolonged responses to stimuli and often large receptive fields (Andrews and Fitzgerald 1994, 1999, 2000). Withdrawal responses early on demonstrate sensitisation in response to repeated stimulation at 5 second intervals (Fitzgerald 1988). As infants reach 29-35 weeks PCA this pattern of sensitisation to repeated stimuli is replaced by habituation. As well as having a flexor reflex response to a mechanical stimulus that would be non-noxious in the adult, neonatal withdrawal is much less specific. Limb withdrawal in the neonate the ankle dorsi-flexors and tibialis anterior as well as the knee flexors.

The increased efficacy of A fibres in the neonate may be due to:

(i) Changes in central afferent terminals. During development, thickly myelinated A β fibres are the first to penetrate the dorsal horn at E15, whereas the central terminals of C fibres penetrate the dorsal horn at E19, and synaptogenesis with neurons in the substantia gelatinosa is noted by P5 in a somatotopically precise manner (Fitzgerald 1987; Pignatelli et al 1989). In the adult spinal cord, A β fibres form connections with deep neurons in laminae III and IV of the dorsal horn, but in the developing cord, their collateral terminals extend up to superficial neurons in the substantia gelatinosa (Fitzgerald 1994). After synaptogenesis between C-fibre terminals and substantia gelatinosa neurons is completed, these unique superficial terminals from the A β fibres disappear (Fitzgerald 1994). Thus, in the neonatal rat it appears that the same high velocity A β axons transmit tactile and pain sensations, explaining the similar patterns of neuronal activation noted by c-fos expression following innocuous and noxious stimuli (Yi and Barr 1995; Jennings and Fitzgerald 1996). Acute pain or inflammation produces robust and specific pain behaviours, neuronal c-Fos expression in

the dorsal horn (Yi and Barr 1995), leading to A β fibre mediated sensitisation and C-fibre-mediated temporal summation, or wind up. Because of developing synapses with the dorsal horn, pure C fibre stimulation remains sub threshold until the second postnatal week (Jennings and Fitzgerald 1998).

(ii) weak intrinsic or descending inhibitory mechanisms in the neonatal spinal cord (Jiang and Gebhart 1998; Fitzgerald and Koltzenburg 1986). Descending axons grow down from the PAG, pontine reticular nuclei, locus coeruleus and other foci in the brainstem into the spinal cord in early fetal life, but they do not extend collateral branches into the dorsal horn until around birth (Fitzgerald and Koltzenburg 1986). Boucher et al demonstrated that diffuse noxious inhibitory controls (DNICs) were functionally mature by P21 but not effective at P12 (Boucher et al 1998). The development of DNICs follows a rostrocaudal pattern, leading to higher pain thresholds in the forelimbs compared to the hindlimbs in P10 rats. This delayed maturation of descending inhibition may be due to a deficiency of neurotransmitter in the axon terminals or lack of specific receptors in their spinal targets. Alternatively, a delayed maturation of interneurons (Bicknell and Beal 1984) or the excitatory of neurotransmitters such as GABA and glycine in the dorsal horn may contribute to the delayed maturation of inhibitory mechanisms in the spinal cord. While GABA and glycine are inhibitory neurotransmitters in the adult, in the neonatal dorsal horn they mediate increased calcium influx into immature neurons and enhance the action potential duration from these neurons (Wang et al 1994).

(iii) Differences in distribution and sensitivity of a variety of neurotransmitter/receptor systems (for review please see Pattinson and Fitzgerald 2004).

With increasing age, thresholds to noxious stimuli increase. Intensity discrimination and the range of behavioural responses develop gradually over the first two weeks of postnatal life (Collier and Bolles 1980).

Local tissue injury in the neonate results in significant reductions in reflex thresholds indicating that even at this early age hypersensitivity states can develop. Repeated heel lance in human premature neonates has been shown to result in measurable allodynia (Fitzgerald

1989). A similar finding has been documented following ischaemic leg injury in infants (Andrews and Fitzgerald 1999). The abdominal skin reflex in neonates also shows significant changes with age and becomes sensitised following tissue injury which is clinically relevant (Andrews et al 2002).

In our experiments, stimulus response curves were constructed for mechanical stimulation using vFhs in rats of different ages. The effect of the administration of varying doses of morphine on this stimulus response curve was recorded. The effect morphine on thermal withdrawal latencies in rats at P3, P10 and P21 was also investigated.

5.3 Materials and Methods

Sprague-Dawley rats of both sexes were used for these experiments. They are bred in-house and kept at a constant temperature of 20-22⁰ C, humidity of 50-55% and a light cycle of 12 hours full light separated by 1 hour half light from a 10 hour dark phase.

All rats were kept fed, watered and with their mother until the experiments were started.

Litters of rats at 3 days (P3), 10 days (P10) and 21 days of age (P21) were used to investigate the changes in mechanical and thermal sensory threshold following systemic injection of varying doses of morphine. The mean weight for each group was 10g at P3, 25g at P10 and 50g at P21.

5.3.1 Morphine Injection

5.3.1.1 Mechanical sensory thresholds

Morphine was administered via the intraperitoneal route in all animals. The injections were administered with the animals awake but carefully restrained. It was felt that a general anaesthetic would affect experimental results. All drugs were diluted using sterile normal saline to give a volume of 10ml/kg. Based on this P3 rats received 0.1ml, P10 rats 0.25ml and P21 rats 0.5ml. The injections were administered into the lower left quadrant of the abdomen using a 0.5ml insulin syringe or a 1ml syringe with a 30-gauge needle. A timeline for the experimental protocol is illustrated in Figure 5-1.

4 rats at each age group were given ip morphine doses of 0.1, 0.3, 0.5, 1 and 2mg/kg as described above. In addition 4 rats at each age group were given the equivalent volume of sterile normal saline ip.

5.3.1.2 Thermal thresholds

Baseline thermal thresholds were measured in 8 animals per age group. Four rats at each age were given 2mg/kg ip morphine or normal saline to test the effect of morphine on thermal thresholds at these postnatal ages .

5.3.2 Blinding

For both mechanical sensory threshold and thermal withdrawal latency experiments all solutions were made up and labelled according to concentration by the experimenter. They were then relabelled with an alphabetical code known only to an independent worker. In this way the experimenter was unaware of the dose being administered and experimenter bias was removed. A randomisation table was also generated so that each consecutive rat received a randomly allocated dose. The code was broken only when all the experiments had been completed.

5.3.3 Behavioural Testing

5.3.3.1 Mechanical sensory thresholds

The different age groups of rats required different degrees of handling before the experiments were undertaken. P3 pups required minimal handling and were tested almost immediately without any restraint. P10 rats were habituated for 1 hour prior to testing in the environment they were to be tested in and required minimal restraint when testing. P21 rats required greater habituation and were habituated for one hour daily for two days before testing as well as 1 hour immediately prior to testing. They needed gentle restraint during testing.

Baseline mechanical sensory thresholds were recorded in all animals prior to any ip injection.

The mechanical threshold of the cutaneous flexion withdrawal reflex was determined using calibrated von Frey hairs (vFh). Von Frey hairs are single nylon monofilaments of graded diameter (0.08-1.0mm) attached at right angles to a Perspex handle. They are made according to a logarithmic scale of the weight that they apply. Each was calibrated using a

balance accurate to 1mg. Each hair was pressed onto the balance, in the way it would be during an experiment, and a value ascribed according to the weight produced. Table 5-1 shows the weights and natural logarithmic values of the numbered vFhs when calibrated in our laboratory. The absolute values for their weights lie on an exponential scale. The natural logarithm of each weight is calculated and assigned a number from 1 to 20. This converts the thresholds to a linear scale, and gives results in units that are directly interpretable as fractions. The advantage of using a natural log scale is that, multiplied by 100, natural log differences are equivalent to symmetric percentage differences i.e. percentage differences calculated with the mean of the two numbers as a denominator. The percentage difference of ordinary numbers is not symmetric e.g. an average woman at the age of 20 is 7.7% shorter than an average man whereas the same man is 8.4% taller than the woman. Thus conversion to a natural log scale allows changes in threshold to be compared even if the original baselines are not the same e.g. a change from vFh number 9 to 10 is equivalent to a change from vFh number 19 to 20. An increase of 1 in the vFh number corresponds to a 66% increase in the applied force. In addition using the natural log scale for vFhs is preferable to a log scale with a different base because differences in this can be interpreted directly, without back transformation to the original scale (Cole 2000). Natural logs, as differences, are easy to interpret. The difference between the natural logs of 2 numbers is the fractional difference between the numbers.

Von Frey hairs were applied perpendicular to the surface of the skin of the hindpaw of the rat with a pressure just sufficient to bend the hair. The dorsal surface of the hind paw was tested while the rat was in a normal weight-bearing position. Each hair was applied 5 times at approximately 2s intervals. If the hair did not produce 5 flexion withdrawals of the limb with the 5 stimuli administered the process was repeated using the next stiffer vFh in the scale. The lowest calibrated hair that elicited 5 clear withdrawals for each of the 5 stimuli was said to produce a 100% response. All responses elicited by vFhs before the mechanical threshold was reached were also recorded so that 1 out of 5 withdrawals was considered a 20% response, 2 out of 5 40% and so on. The vFh number that elicited a 50% response, derived from the stimulus response curves, was taken as the 'ER₅₀', and defined as the threshold.

Mechanical thresholds were repeated in this way every 10 minutes after ip injection for a total of one hour. A dose response curve 30 minutes after morphine administration was obtained for all the doses of morphine given.

Table 5-1 **Calibrated weights and loge of each numbered vFh when applied to a calibrated balance.**

vFh number	Weight	Loge
1	0.015	-4.12
2	0.04	-3.22
3	0.06	-2.81
4	0.12	-2.12
5	0.17	-1.77
6	0.36	-1.02
7	0.55	-0.59
8	0.92	-0.08
9	1.37	0.31
10	2.2	0.79
11	3.4	1.22
12	5.5	1.71
13	10.5	2.35
14	17.1	2.84
15	25.8	3.25
16	37	3.61
17	63.5	4.15
18	105	4.65
19	157	5.06
20	248	5.51

5.3.3.2 Thermal thresholds

Hind-limb withdrawal latencies to a noxious thermal stimulus were measured using the Hargreaves method. The animals were placed in a clear plastic box on a glass surface maintained at constant temperature. A calibrated infra red radiant heat source (intensity 50%), activated with a timer, was focused on the hind paw. Paw withdrawal exposed a photocell that stopped both lamp and timer, giving the latency to withdrawal, which is the heat threshold. A 30 second maximum exposure was used to avoid tissue injury.

Each animal had a baseline thermal threshold recorded immediately before ip injection. The threshold was measured again 30 minutes post ip morphine so that each animal had one injection and one test. Withdrawal latency was measured 5 times in each foot in the middle of the footpad and the 5 observations were averaged for each animal. The thermal thresholds experiment was conducted in the same way in different rats.

5.3.4 Termination

At the end of each experiment all the animals were sacrificed by ip injection of pentobarbitone (University College Hospital Pharmacy) in a dose suitable to produce terminal analgesia.

5.3.5 Analysis

Data from these experiments were analysed electronically using Graphpad Prism 3.0.

Comparisons between continuous variables were made using analysis of variance (ANOVA) with Bonferroni post test corrections. For all comparisons $p < 0.05$ was considered significant.

5.4 Results

The analgesic effects of varying doses of systemic morphine at different postnatal ages were investigated using the cutaneous flexion withdrawal reflex elicited with vFhs and noxious radiant thermal stimulation.

5.4.1 Changes in baseline thresholds with age

5.4.1.1 Mechanical thresholds

Figure 5-2 shows the mean baseline mechanical thresholds of the flexion withdrawal reflex at each age group. Each bar at the age indicated represents the mean ER_{50} with standard errors of the mean. It consists of data pooled from the 24 animals tested per age group.

It demonstrates that the baseline mechanical withdrawal threshold increases with increasing age. The baseline threshold is vFh number 9.6 in P3 rat pups compared with 11.8 for P10 and 14.8 for P21 rats. There is a significant difference between the baseline values at all age groups ($p < 0.05$).

5.4.1.2 Thermal withdrawal latencies

Figure 5-3 shows that baseline withdrawal latency to thermal stimulation did not change significantly with age, although there was a trend towards decreasing thermal threshold between P10 and P21. Baseline withdrawal latencies were $6.4 \pm 1.2s$, $7.8 \pm 1.4s$ and $6.3 \pm 0.9s$ at P3, P10 and P21 respectively.

5.4.2 Effect of morphine on baseline mechanical thresholds

The mechanical threshold was measured in the hindpaw of rats of varying ages after the administration of different doses of systemic morphine over one hour. 4 animals were tested for each dose and at each age and the experimenter was blinded to the dose being administered. Stimulus response curves were constructed from this data i.e. % responses versus vFh number at the different doses of morphine.

5.4.3 Stimulus response curves

Figure 5-4, Figure 5-5 and Figure 5-6 demonstrate the stimulus response curves of the P3, P10 and P21 rats respectively. The baseline dose response curve is clearly shifted to the right with increased postnatal age. There is a clear dose related effect of morphine at P3 and P10 with rightward shift of the curves with the sequentially increasing doses of morphine. At P21 this is less clear, there is less rightward shift even at the higher doses of morphine and there is little separation at the lower doses. This illustrates the reduced efficacy of morphine at this age.

5.4.4 Comparison of ER₅₀s

From the stimulus response curves in Figure 5-4, Figure 5-5 and Figure 5-6 it is clear that administration of morphine to the animals resulted in a displacement of the curve to the right in a more or less parallel fashion. The threshold was defined as the vFh number that produces a 50% withdrawal response after the administration of morphine and the vFh number required to produce this 50% response was called the ER₅₀. ER₅₀ was determined from the stimulus response curves every 10 minutes for 60 minutes after morphine administration in all rats. This data showed that the maximal response to morphine at all doses at all age groups occurred consistently 30 minutes after ip injection. The ER₅₀ was therefore determined for each dose of systemic morphine at each age at the 30 minute time point. The mean change in ER₅₀ was plotted to demonstrate the effects of increasing doses of systemic morphine at different postnatal ages of rats (Figure 5-7).

5.4.5 Effect of age on morphine efficacy

5.4.5.1 Mechanical thresholds

Figure 5-7 shows that morphine efficacy on mechanical sensory thresholds markedly decrease with age. 2mg/kg of morphine caused a significantly greater increase in mechanical sensory threshold (ER₅₀) at P3 and P10 than P21. There was a statistically significant difference between the P3 and P21 animals at 0.5 and 1.0mg/kg of morphine. There were no statistically significant differences between the ages at doses below 0.5mg/kg or between the P10 rats and the other age groups below 2mg/kg.

5.4.5.2 Thermal withdrawal latencies

Figure 5-8 shows that 2mg/kg ip morphine significantly increased thermal latencies at all ages. Morphine was significantly more effective at P21 than at P3 and P10, increasing withdrawal latency from a baseline of 5.9 ± 0.9 s to 13.1 ± 1.4 s at P21 compared to 8.1 ± 1.3 s to 13.8 ± 1.0 s and 9.4 ± 1.8 s to 13.9 ± 1.4 s at P3 and P10 respectively. This is in direct contrast to the effect of morphine on mechanical thresholds. The administration of ip saline had no significant effect on thermal thresholds at any age. Baseline data differs from that shown in figure 5.3 as tests were conducted in a different subject group.

5.5 Discussion

Reflex hind limb withdrawal to mechanical and thermal stimuli were used to directly compare the selectivity and sensitivity of morphine in neonatal and older rats.

Our experiments show that baseline mechanical thresholds increase with age. These findings, using Von Frey testing with a range of stimulus strengths, are in keeping with previous reports (Andrews and Fitzgerald 1994; Falcon et al 1996; Marsh et al 1999). We found that the analgesic potency of morphine in mechanical sensory tests is significantly greater in neonatal compared to older animals on investigation of the morphine sensitivity of these mechanical responses across the postnatal period. In contrast, the enhanced neonatal morphine analgesic potency seen in mechanical tests was not observed in thermal tests. Although 2mg/kg systemic morphine significantly increased thermal withdrawal latencies at all ages, there was no difference in efficacy with increasing age.

This is the first time that such studies have been conducted blinded. Blinding eliminates experimenter bias, removing the link from treatment to experimenter effect and assures that observed effects are a result of a direct link between the independent and dependent variables. In addition, doses administered were randomised, i.e. not sequential, removing unsuspected sources of bias.

5.5.1 The flexion reflex during development

Developmental data from peripheral nociceptors and primary afferent fibres shows that these elements of the withdrawal reflex arc are unlikely to show major changes in function over the age range being studied here (P3 to P21) (Fitzgerald 1987). The motor limb of the reflex arc, however, does undergo significant postnatal maturation. In particular the pattern of inputs to ventral horn motor neurons may result in a changing order of recruitment of motor units with increasing age (Ben Ari et al 1997). The neuromuscular junction also shows maturational changes. The greatest changes occur prior to birth in the rodent (e.g. metabolic turnover increases 10 fold). More subtle changes continue over 2-3 weeks and include a subunit

switch in the acetylcholine receptor (Hall and Sanes 1993) as well as the elimination of polyneuronal innervation of myofibrils.

A greater reflex excitability in early development is consistent with findings from several other experimental models (Stelzner 1971). Immature rats are hyper responsive to various noxious stimuli including mechanical (Stelzner 1971) and chemical (Guy and Abbott 1992) stimuli.

Most of this data has been obtained from behavioural tests and measurements of reflex thresholds e.g. mechanical thresholds in human neonates (Fitzgerald 1987).

Electrophysiological studies of dorsal horn cell activity adds further support to the idea of a largely uninhibited spinal cord in early development. Consistent with this are larger receptive field sizes and longer after discharges in the dorsal horn neurons of the newborn rat compared to older pups (Fitzgerald 1985).

Changes in sensory threshold with age have been well documented in both human and animal models (Fitzgerald 1988; Andrews and Fitzgerald 1999; Marsh et al 1999; Howard et al 2001; Andrews et al 2002; Ririe et al 2003). The flexion reflex in neonates has some particular characteristics that are not seen in the adult:

- (i) The amplitude of the reflex is much larger in the neonate. Both human and animal studies of neonatal withdrawal responses to noxious stimuli have found them to be exaggerated compared to the adult (Andrews and Fitzgerald 1994).
- (ii) The neonatal cutaneous withdrawal reflex shows sensitisation in the response to repeated mechanical stimulation whereas in the adult, repeated stimulation is more likely to cause habituation of a reflex (Dimitrijevic and Nathan 1970).
- (iii) The threshold for the reflex is very low in neonates with a gradual increase in mechanical threshold with age commencing from a post conceptual age (PCA) of 29.5 weeks. This maturational process was correlated with PCA rather than postnatal age indicating its genetic basis. The same researchers demonstrated a similar developmental pattern in rat pups over the first 4 postnatal weeks with much of the increase occurring in the second postnatal week (Fitzgerald 1988).

(iv) The receptive field for the reflex is considerably larger in neonates and decreases with age

Hypersensitivity can be clearly demonstrated in neonates following tissue injury.

The consequences of the increased excitability and A fibre activation of nociceptive systems in the newborn is that the neonatal spinal cord lacks selectivity and spatial discrimination in its pain pathways.

Several maturational processes within the first 3 weeks of rat neonatal life are likely to play a significant role in the finding that reflex responsiveness declines during this period. Three issues can be considered:

- (i) the anatomical arrangements of the central terminals of neurons in the withdrawal reflex arc
- (ii) the pattern and strengths of synaptic connections as well as the intrinsic membrane properties of the neurons in this arc
- (iii) the role of modulatory influences on the arc

An evolving pattern of laminar organisation in the spinal cord has been well described in the rat. The central terminals of primary afferent fibres enter the dorsal horn and make synaptic connections in a sequential pattern in which initial connections are made between A β fibres and substantia gelatinosa (SG) cells. During the first 2 postnatal weeks these undergo gradual restriction to deeper laminae (Fitzgerald 1994; Coggeshall et al 1996). Prior to P5, superficial spinal laminae are dominated by larger afferent fibres carrying signals from low threshold stimuli. The withdrawal reflex in early development is therefore likely to be elicited by weaker stimuli. As A δ and C afferents come to predominate in the SG inputs the reflex is more likely to be driven by nociceptive afferents (Nakatsuka et al 2000).

In early development, synaptogenesis and synaptic efficiency undergo marked age related changes. Histological studies show that a large proportion of synaptogenesis occurs postnatally. The maturation of these synapses is a slow process (Stelzner 1971). The gradual

maturation of C fibre synapses within the dorsal horn commences only from P5 (Pignatelli et al 1989; Cabalka et al 1990).

In younger rat pups, despite weak excitatory synaptic transmission, natural cutaneous stimulation can evoke long lasting excitation which may in part be due to larger NMDA evoked calcium currents than those in the adult (Hori and Kanda 1994). NMDA receptors in early life are uniformly and densely distributed through the dorsal horn before becoming restricted. Substance P (sP) receptors too display a dense distribution in the newborn spinal cord. Despite low levels of the peptide, long lasting ventral root potentials induced by C fibre stimulation can be blocked by sP antagonists, confirming the ability of these peptidergic synapses to contribute to reflex excitability (Akagi et al 1985).

Extrinsic modulatory influences on the withdrawal reflex arc include both segmental and descending inhibitory inputs. The functional effects of these inputs include alterations in receptive field sizes and the pattern of convergence (proportion of cells responding to both innocuous and noxious stimuli). Receptive field sizes of dorsal horn neurons have been shown to undergo striking age related changes. Cutaneous receptive fields rapidly reduce during the first postnatal week (Fitzgerald and Jennings 1999). These studies also demonstrated a changing pattern of convergence of inputs to dorsal horn cells with development. With increasing age a greater degree of convergence is seen (Fitzgerald 1985). Supraspinal centres and descending pathways to the spinal cord also mature postnatally. Functional maturity of the dorsolateral funiculus, a particularly important inhibitory influence on dorsal horn cells, occurs around P8 to P10 (Fitzgerald and Koltzenburg 1986). Studies of spinal cord lesions during development suggest that descending tracts become a major influence on spinal cord activity from about 2 weeks after birth (Weber and Stelzner 1977). Stimulus induced analgesia, a phenomenon that is dependent on functional descending tracts, is only detectable after 3 postnatal weeks (van Praag and Frenk 1991).

5.5.2 Thermal latencies and development

The response to noxious heat has also been frequently used in pain research and in assessment of antinociceptive agents. In this study thermal thresholds were found to be unaffected by postnatal age. This is in contrast to the results with mechanical thresholds.

Only a few animal studies analyzing age-related changes in thermal nociception exist, and these conflict over whether nociceptive responses in animals change with development. Although it might be anticipated that the younger animals would have a shorter latency to the thermal stimulus, thermal latency has been reported to vary widely and may depend on not only age but also the type of thermal test employed as well as heat source, distance from the heat source, location of the thermal stimulus, and the heat absorption of the tissue itself (Blass et al 1993; Conway et al 1998). Studies in this area use a wide variety of thermal tests and intensities on rat paws or tails and test rats of greatly varying ages. These significant differences make the studies hard to compare. Some studies have shown thermal hypersensitivity to noxious thermal stimuli in juvenile rats e.g. Marsh et al 1999; Hu et al 1997; Falcon et al 1996. In the study by Falcon et al, hypersensitivity to noxious thermal stimulation was seen in developing rats. Rats, at postnatal days 3, 6, 9, 12, 15, 21 and 90 were tested for reflex responsiveness to noxious heat, using tail withdrawal from hot water as the assay. Thermal nociceptive thresholds were considerably lowered, relative to adults, up to postnatal day 12. From P21, sensitivity to noxious stimuli started to decrease with increasing age. Conway et al studied the escape latencies of immature (5-to 25-day-old) and adult (3-to 4-month-old) albino rats following tail exposure to different intensities of radiant heat (650-W halogen lamp placed 10-30 mm from the tail) or conductive heat (35-50⁰ C water). The tail escape responses of rats exposed to noxious thermal stimuli varied with age and stimulus intensity, regardless of whether radiant or conductive heat was used. Rats less than P15 exhibited shorter latencies than older animals when their tails were exposed to moderately intense radiant or conductive heat. Others have reported a decrease in thermal nociceptive threshold with age (Hammond and Ruda 1991; Ririe et al 2003) although the youngest rats tested in these studies were 10 days old. There is also a report of minimal change in thermal threshold with age using both the radiant heat technique and the hot plate test (Hiura et al 1999). The findings of these studies along with tests and ages of animals used are summarised in the table below.

Thermal latency is primarily a measure of C-fibre function (Ossipov et al 1999). Because of this the finding that thermal thresholds tend to decrease over the first three postnatal weeks in

rats may be consistent with the C-fibre input not developing fully until the second postnatal week, as previously suggested (Fitzgerald 1985).

In our study, the same intensity of thermal stimulus was used in the animals of different ages, such that the baseline withdrawal latency is different. We felt that it was important to use the same intensity of thermal stimulus across the age groups and not change the thermal stimulus to achieve a fixed withdrawal threshold across the age ranges.

5.5.3 The effect of morphine on flexion reflexes

The effect of morphine on electrophysiological recordings of the nociceptive flexion has been investigated both in animals and healthy human volunteers. This reflex is a polysynaptic spinal reflex elicited by electrical stimulation of a cutaneous sensory nerve and recorded from a flexor muscle on the ipsilateral limb. The threshold and amplitude of this reflex response are closely related to those of the concomitant painful sensation evoked by electrical stimuli (Willer 1977). This reflex has been used in numerous pharmacological studies in humans, most notably to demonstrate the direct spinal effects of morphine (Willer and Busnel 1980; Willer 1985). The nociceptive flexion reflex and the corresponding subjective pain scores elicited by sural nerve stimulation were studied in healthy volunteers by Willer in 1985. The reflex and the pain thresholds were found to be almost identical and the threshold of the maximal reflex response was very close to that of intolerable pain. These four parameters were studied before and after intravenous administration of morphine (0.05, 0.1, 0.2 and 0.3 mg/kg) and subsequent administration of naloxone hydrochloride. While 0.05 mg/kg morphine remained without any effect, higher doses produced an increase in the four thresholds. Morphine also depressed, in a dose-dependent fashion, the nociceptive reflexes elicited by constant stimulation intensity. All these effects were immediately reversed by subsequent naloxone. In addition, variations in the reflex and pain thresholds as well as in the threshold of the maximal reflex response were found to be very significantly linearly related, indicating a close relationship between the effects of morphine on the nociceptive reflex and on the related pain sensation. These results suggest that the mechanisms of morphine-induced analgesia can be explained by a depressive effect on the nociceptive transmission directly at a spinal level.

The hypothesis that an important site of action of morphine is located directly at spinal level is supported by a study of the effects of low doses of epidural morphine on the nociceptive flexion reflex of the lower limb and on postoperative pain in volunteer patients after orthopaedic surgery on one knee (Willer et al 1985). It was found that, 40-50 min after injection, morphine produced an increase of 87% and 83% of the reflex threshold and of the threshold of maximal reflex response, respectively, as well as a 80-90% depression of the nociceptive responses when elicited by a constant level of stimulation.

This C-fibre reflex, elicited by electrical stimulation within the territory of the sural nerve has also been recorded from the ipsilateral biceps femoris muscle in anesthetized rats. High doses (4-10 mg/kg) of intravenous morphine were found to induce a depression of the C-fibre reflex in a dose-dependent manner (Guirimand et al 1995). Intravenous naloxone (0.4 mg/kg) completely antagonised the depression induced by high dose morphine.

Morphine is known to act at both spinal and supraspinal levels, as discussed in chapter 2. The spinal action is well documented (Yaksh 1997). It is generally agreed that opioids reduce the spinal transmission of nociceptive signals predominantly at presynaptic sites (i.e. reduction of transmitter release from C-fibre afferents), although a postsynaptic inhibition of spinal dorsal horn nociceptive neurons has also been demonstrated (i.e. hyperpolarisation due to an increase of potassium currents).

5.5.4 Postnatal development of morphine efficacy on flexion reflexes. Morphine is less selective in the neonate.

Our results clearly demonstrate that the analgesic potency of morphine in mechanical sensory tests is significantly greater in the neonatal compared to older animals. This was not due to generalized sedation since there was a clear dose response. Comparison of the sedative and analgesic effects of systemic morphine and pentobarbital in infant rats has shown that morphine produces analgesia to an intra-plantar injection of formalin at all ages, which is qualitatively different from the sedative effects of pentobarbitone 10 mg/kg (Abbott and Guy, 1995).

Importantly, this enhanced neonatal morphine analgesic potency in mechanical tests was not observed in thermal tests. This is in agreement with earlier studies showing that the analgesic effect of systemic morphine to a thermal noxious stimulus increases with age in neonatal rats (Giordano and Barr 1987; Marsh et al 1999). The heat evoked flexion reflex is likely to be mediated by A δ and C fibres (Lynn 1975; Fitzgerald 1987) and the low efficacy of morphine in these tests may reflect the immaturity of C fibres in the newborn (Fitzgerald 1987).

It could be argued that differences in maturity and function of the blood-brain barrier (BBB) explain the increased effects of systemic morphine on the mechanical thresholds in younger animals. However the BBB has been shown to be intact both histologically and physiologically at birth in the neonatal rat (Butt et al 1990). After subcutaneous administration of morphine-6-glucuronide (M6G), a potent analgesic, to neonatal guinea pigs, the proportion of M6G 'transferred' into the brain did not alter with either dose or age, suggesting a functionally intact BBB at birth (Murphey and Olsen 1994). This is supported by the low levels of [3 H] morphine binding in the spinal cord at all ages following systemic administration. The general regulation of metabolism or drug access cannot explain the increased efficacy of morphine in neonatal mechanical tests since morphine demonstrated differential potency in mechanical versus thermal tests. Pharmacokinetic, pharmacodynamic and BBB factors would be expected to affect both tests. Rather, our findings suggest that opioid sensitivity and selectivity in neonatal rats differ from those of older animals as a result of developmental regulation of MOR pharmacology.

5.5.5 Behavioural responses

Changes in neonatal rat behaviour follow the stereotypical responses observed in adult animals (Abbott et al 1995) in classical tests of nociception, such as formalin (McLaughlin et al 1990), hot plate (Anand et al 1999) or stimulation with von Frey filaments (Fitzgerald 1995). Following formalin injection into the hind limb, a pattern of acute response, followed by paw-lifting, paw-licking and other recuperative behaviours occurs in rats at P3, 7 and 10 (McLaughlin et al 1990; Guy and Abbott 1992; Barr 1998). All studies have noted a uniphasic response to formalin in neonatal rats (P0 to P14) which lasts up to 60 minutes in the younger rats and 30 minutes in the older rats (Guy and Abbott 1992; Barr 1998). The combination of paw flexion, shaking and licking as well as kicking movements were specifically related to

increases in formalin concentration (Teng and Abbott 1998); specific pain behaviours increase and non-specific behaviours decrease with advancing neonatal age (Guy and Abbott 1992).

5.5.6 Comparison with other reports

Conflicting reports exist about the analgesic efficacy of opioids in young animals. These differences may reflect the varying dose regimens, age ranges and behavioural tests studied. On the whole, current literature suggests that opioids produce a significant dose-related antinociception in rats. Windh and Kuhn (1995) reported that neonatal rat pups (P10) are more sensitive to morphine antinociception than are weanlings (P27) using the hot plate test. ED50 values for morphine and sufentanil induced antinociception were determined in the paw-lift assay on days 10 and 27. The ED50 for morphine analgesia in 10-day-old pups was 0.35 mg/kg and increased with age to 5.3 mg/kg on day 27. Similarly, sufentanil was more potent in pups than in weanlings, the ED50 increasing from 1.7 to 7.6 micrograms/kg. Serum and brain morphine levels after 5 mg/kg of morphine were higher in neonates (P10) than weanlings (P27), largely due to a more rapid redistribution phase in weanlings than in pups. Additionally, a substantial (70%) antinociceptive response was achieved in neonates at brain morphine levels that were one-half those producing an equal effect in weanlings. McLaughlin and Dewey (1994) found that opioids were effective antinociceptive agents in both neonates and adults with three different types of nociceptive tests; tonic (formalin-induced inflammation) and phasic (tail flick and hot plate) and that the relative potencies of these agents appeared to be similar in neonates and adults. In general, pups were more sensitive to the antinociceptive agents when tested in the phasic tests. Marsh et al (1999) have also shown that P3 animals demonstrate a greater response to morphine (as well as the delta (DPDPE) and kappa (U69593) opioid receptor agonists) administered both systemically and epidurally than P21 animals using the cutaneous flexion withdrawal reflex elicited with von Frey hairs. In contrast to the effects on mechanical thresholds, morphine was less effective on nociceptive heat thresholds in younger animals and increased its analgesic potency with age in these tests. A study by Thornton SR et al (1998) showed that morphine elicited dose-dependent antinociception and was completely efficacious in P3, 6, 9, 14, 17 and 21 rats using the tail-flick test. Morphine was the least potent in P3 rats, with potency reaching a peak at P9 and

remaining essentially the same to P21. They found it difficult to account for the lower potency of morphine in P3 and P6 rats. These studies stand in contrast to previous reports that indicated marked insensitivity to the antinociceptive properties of morphine in P2 rat pups (Zhang and Pasternak 1980, 1981). Morphine analgesia to thermal nociception was shown to increase with age in neonatal rat pups, progressing to a 40 fold analgesic potency at P14 compared to P3 (Giordano and Barr 1987). The hot plate test was used in these studies and there are important methodological differences between the studies. As a result of widely varying behavioural tests, age ranges, dose ranges and even routes of administration used it is difficult to compare these studies and make sense of their often conflicting results. In addition, none of these studies used blinding and are therefore likely to be subject to some experimenter bias. In our blinded study this cannot be a confounding variable. Data from these behavioural studies are tabulated below in Table 5-2.

Table 5-2 Response to opioids in behavioural studies

Authors	Age	Test - THERMAL	Response to opioid
Ririe et al 2003	P14-112	Hind paw withdrawal -IR heat	↓ sensitivity at P14
Marsh et al 1999	P3-21	Hind paw withdrawal -hot water bath	↑ sensitivity at P3 & P10
Hiura et al 1999	P12-20	Hind paw withdrawal -IR heat & hot plate	No change in thermal threshold with age
Conway et al 1998	P5-25	Tail withdrawal -IR heat & hot water bath	↑ sensitivity up to P15
Thornton 1998	P3-21	Tail flick test -IR heat	↑ sensitivity up to P9
Hu et al 1997	P1-14	Hind paw withdrawal -hot plate	↑ sensitivity up to P14
Falcon et al 1996	P3-21	Tail withdrawal -hot water bath	↑ sensitivity up to P12
Windh and Kuhn 1995	P10-27	Hind paw withdrawal -hot plate	↑ sensitivity at P10 compared with P27
McLaughlin and Dewey 1994	P3, adult	Tail flick test -IR heat & hind paw withdrawal -hot plate	↑ sensitivity at P3
Hammond and Ruda 1991	P10-84	Hind paw withdrawal -hot plate	↓ sensitivity up to P42 and then ↑ sensitivity
Giordano and Barr 1987	P3, P14	Hind paw withdrawal -hot plate	↓↓ sensitivity at P3 compared to P14
Zhang and Pasternak 1981	P2-14	Hind paw withdrawal -hot plate	↓ sensitivity at P2 and P7
Test - MECHANICAL			
Williams et al 2004	P3-21	Flexion withdrawal reflex to stimulation with vFh	No change with postnatal age
Marsh et al 1999	P3-21	Flexion withdrawal reflex to stimulation with vFh	↑ sensitivity at P3 and P10

5.5.7 Possible difficulties

Although behavioural testing in animal models are an essential tool in the study of nociception and analgesics various issues must be considered when interpreting studies employing these methodologies. Behavioural models of nociception in an animal should demonstrate as many of the following characteristics as possible:

- (i) **Specificity.** The stimulus must be nociceptive. The appearance of a flexion reflex does not inevitably mean that the stimulus is nociceptive or that it is a nociceptive flexion reflex. If a flexion reflex is not triggered exclusively by nociceptive stimuli this can lead to misinterpretations.
- (ii) **Sensitivity.** It must be possible to quantify the response and to correlate this variable with the stimulus intensity within a reasonable range (from the pain threshold to the pain tolerance threshold). In other words, the quantified response must be appropriate for a given type of stimulus and monotonically related to its intensity.
- (iii) **The model must be sensitive to manipulations and notably pharmacological ones,** which would reduce the nociceptive behavior in a specific fashion. A sensitive test must be able to show effects for the different classes of antinociceptive agents at doses comparable to those used for analgesics in humans.
- (iv) **Validity.** The model must allow the differentiation of nonspecific behavioral changes (e.g. in motility and attention) from those triggered by the nociceptive stimulus itself. The response being monitored should not be affected by simultaneous disturbances related to other functions. The degree to which the test actually measures what it claims to measure, is one of the most difficult problems to resolve.
- (v) **Reliability.** Consistency of scores must be obtained when animals are retested with an identical test or equivalent form of the test. The repeated application of the stimulus must not produce lesions.
- (vi) **Reproducibility.** Results obtained with a test must be reproducible not only within the same laboratory but also between different laboratories.

Clearly, there is no perfect behavioral model of acute pain in animals so choosing a particular model inevitably involves a compromise. The stimuli used to trigger a nociceptive reaction are

not always specific and can be difficult to control. It can be extremely complex to produce an effective stimulus that activates the peripheral nociceptors as this is dependent on the physiological state of the target tissues. There are many different sources of variability or plasticity in the biological responses evoked by stimuli, even if they are constant, because of changes brought about by concomitant physiological factors. The validity of behavioural models can be affected by many factors that are very difficult to control. Pharmacokinetics can be very different in humans and different species of animals for a variety of reasons, notably the bioavailability, the tissue distribution, the metabolism, and the rate of elimination. Environmental factors are also important. Tests are often made on restrained animals and animals that are exposed to new surroundings having been reared in the carefully controlled environment of laboratory animal houses - the effects of morphine on the tail-flick test are greatly facilitated by the restraint and/or the novelty of the environment (Kelly and Franklin 1984a, b). Certain physiological factors pose an almost insoluble problem. Thermoregulation can effect nociceptive testing. In the rat the main peripheral organ of thermoregulation is the tail and also, although to a lesser extent, the parts of the paws not covered by fur. These are the areas used for testing thermal thresholds and consequently greatly affected by ambient temperature. The fact that fluctuations in room temperature are an important source of variations in thermal threshold has been known for a long time. Vasomotor tone, systemic arterial blood pressure and circadian rhythm can all effect nociceptive reflexes in rodents.

Another weakness of these models lies in the threshold of a motor reaction. In most animal models of pain, the only measurement is of a nociceptive threshold. However, clinical pain is rarely limited to threshold intensities (0-1 on a visual analogue scale of 1-10). This restriction to measurements of threshold in classic tests is very limiting. If a stimulus-response curve is constructed and an analgesic administered resulting in the parallel displacement of the curve to the right, then measuring the threshold will provide evidence of the action of the substance. If, on the other hand, the slope of the stimulus-response curve is reduced without any overall shift, then the measurement of the threshold alone will not permit any conclusion to be drawn about the substance. Measuring a threshold does not permit an evaluation of changes in the

gain of a system although it is well known that the nociceptive systems that generate pain can show changes in gain.

Mechanical thresholds can be determined in humans and animals by applying probes of different surface areas with varying force. The most common method was published by von Frey in his work on pressure sensitivity (von Frey 1896). Originally, the filaments were made by using human and horse hair. Today nylon is the most common material. These materials are hygroscopic, and the bending forces of the filaments are significantly affected by temperature and humidity (Andrews 1993) which may result in discrepancies in withdrawal thresholds. In addition, the material can be deformed by extensive use and these factors call for frequent calibration.

Usually, to get one threshold value, the filaments are applied repeatedly. Investigators use one or any number of filaments in different ways, starting with the finest or with one in the middle of the chosen series. This is time-consuming, and may lead to sensitization of the investigated tissue. Furthermore, for each application the investigator has to decide whether there is an adequate response, leading to subjectivity and possibly different interpretations. Thresholds are computed in several ways, but not all possible values can be generated due to the non-continuous bending forces of the filaments and the ways they are used. Moreover, probe size can affect the pain and pressure thresholds. As a consequence, results from different laboratories and investigators are difficult to compare. Moller K et al (1998) have suggested using an electronic algometer to overcome some of these problems. The pressure algometer they used consists of a hand-held force transducer with a stable nylon probe resembling a von Frey filament of defined diameter, connected to a computerized data collection system. The algometer probe is applied manually, while the applied force as well as a digital signal processing (DSP) function of the force is displayed. They found that using a stable probe of fixed diameter provided direct measurements with improved objectivity of the withdrawal responses.

Thermal testing using the Hargreave's method uses an infrared radiant heat source. The weak caloric power of the stimulators that are generally used (radiant lamps or contact thermodes) can be a limitation of this method. If the speed of cutaneous heating induced in this way is slow ($<10^{\circ}\text{C/s}$), an asynchronous activation of peripheral and central neurons results and it becomes difficult to study neural phenomena e.g., reflexes, evoked potentials, and reaction times for which a synchronous excitation of fibres is required. The thermal radiation needed to increase the temperature to the pain threshold depends on several parameters:

- (i) the radiation properties of the skin, namely reflectance, transmittance, and absorbance, which depend on the electromagnetic spectrum emitted by the source of radiation, which itself varies with the intensity of the electrical current through incandescent bulbs
- (ii) the conduction properties of the skin
- (iii) the initial temperature of the skin
- (iv) the amount of caloric energy delivered to a given surface area of skin, which in turn depends on both the power spectral density of the bulb and the duration of exposure.

Clearly (i) to (ii) are likely to be affected by postnatal age since the plantar surface skin in a P3 pup is considerably thinner and more delicate than that of an older animal. Despite these factors and the use of the same IR intensity to produce the noxious thermal stimulus at all ages in our study there was little change in thermal withdrawal latency with age.

Our results could be explained by a difference in the density, distribution and/or pharmacology of opioid receptors during development. The increased efficacy of morphine on neonatal mechanical withdrawal thresholds is entirely consistent with the high levels of functional MOR expression in largely non-nociceptive neonatal NF200 positive A cells, identified using calcium imaging. The down regulation of A fibre MOR is likely to contribute to the decreasing efficacy of morphine on mechanical thresholds with increasing age. Thermal nociceptive latency, however, is mainly a measure of C fibre activity (Ossipov et al 1999) so the finding that morphine is more efficacious in prolonging withdrawal latencies at P21 than

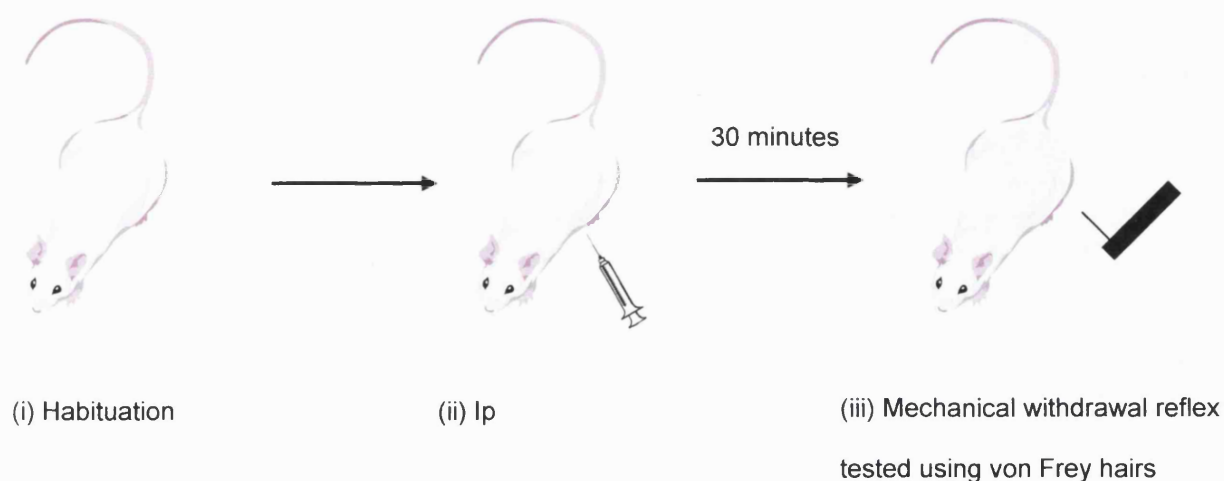
P3 is consistent with our previous finding that the proportion of small C and A δ MOR sensitive neurons does not alter with age.

Another factor to consider regarding age-dependent MOR activity include MOR coupling to second messenger G proteins. Although the presence of GTPase activity and both Go and Gi subunits in postnatal rats has been demonstrated (Szucs and Coscia 1992; Windh and Kuhn, 1995), it remains unclear what percentage of the MOR population are efficiently coupled to the G proteins during the postnatal period. The presence of G protein activity and the Go and Gi protein in postnatal rats implies appropriate coupling of MOR and G proteins and resulting in signal transduction. However, researchers have reported the presence of a large amount of uncoupled MOR in the endoplasmic reticulum of postnatal rats compared to adult rats (Bem et al 1991). Since receptor ligand studies rarely examine binding in subcellular components, postnatal MOR density likely reflects both coupled and uncoupled receptors. However, since a greater proportion of sensory neurons were found to express MOR which were found to be functional using calcium imaging it seems unlikely that this is a significant factor in determining the changing selectivity and sensitivity of morphine with increasing postnatal age.

The changing morphine sensitivity in the postnatal period may be part of a general reorganisation in the pattern of central synaptic connectivity that occurs in the sensory nervous system over this time. The neonatal dorsal horn undergoes considerable changes in the structure and function of primary afferent synapses, neurotransmitter/receptor expression and function and excitatory and inhibitory modulation from higher brain centres (Alvares and Fitzgerald 1999; Fitzgerald and Beggs 2001; Pattinson and Fitzgerald 2004). Neonatal spinal sensory processing is characterised by lack of selectivity and exaggerated cutaneous responses (Fitzgerald et al 1988). Both high intensity C fibre stimuli and low intensity A fibre stimuli can activate neonatal spinal pathways that are purely nociceptive in the adult (Jennings and Fitzgerald 1996). In the adult, *c-fos* expression and other activity dependent excitability changes in dorsal horn neurones are evoked by A δ and C inputs only, but in the neonate, low intensity mechanical stimulation and A β activation can also evoke *c-fos* expression and sensitization of responses (Jennings and Fitzgerald 1996; Jennings and

Fitzgerald 1998). Opioid receptor activity may therefore be appropriate to the particular characteristics of cutaneous transmission in the neonate.

Figure 5-1 **Timeline for mechanical sensory testing**

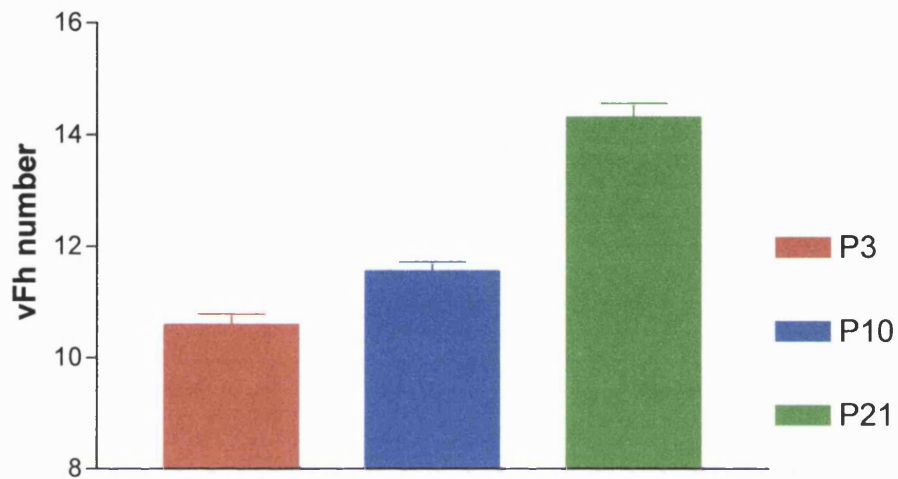


(i) P21 rats were handled and habituated for 3 days prior to testing, P10 for 3 hours prior to testing and P3 rats did not require habituation.

(ii) Rats were given ip injections of varying doses of morphine as described. Each rat was given one ip injection only. The investigator was blinded to the solution administered.

(iii) 30 minutes after ip injection the mechanical withdrawal reflex was tested using calibrated von Frey hairs. Each rat given an ip injection was tested once only.

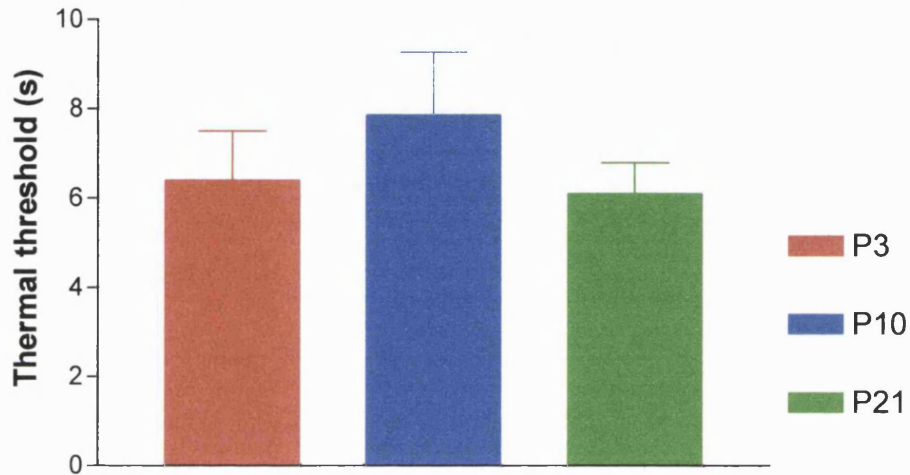
Figure 5-2 The effect of postnatal age on mechanical ER₅₀s in rats



This bar chart demonstrates the change in ER₅₀ of mechanical stimulation with vFhs in rats age 3, 10 and 21 days. Each bar represents the mean threshold with standard errors of the mean. It consists of data pooled from 24 animals in each age group.

There is a clear increase in baseline mechanical threshold with age.

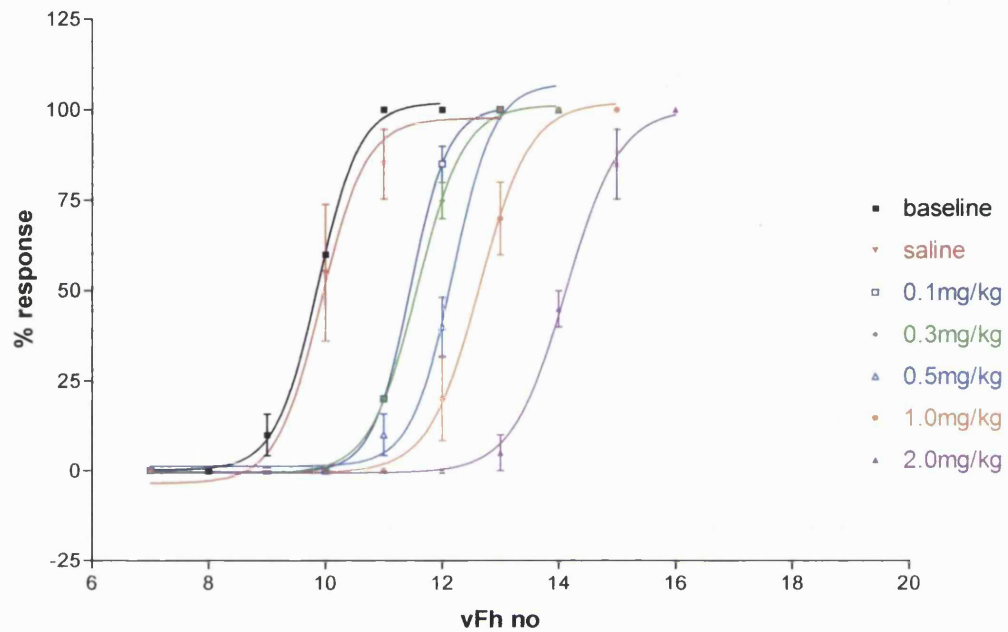
Figure 5-3 The effect of postnatal age on thermal withdrawal latencies



This bar chart demonstrates the change thermal withdrawal latencies in rats age 3, 10 and 21 days. Each bar represents the mean threshold with standard errors of the mean. It consists of data pooled from 8 animals in each age group.

Heat thresholds are defined as the latency of withdrawal from an infrared heat source (Hargreave's method) applied to the hindpaw. Unlike mechanical thresholds no age related change in thermal thresholds was detected using this method.

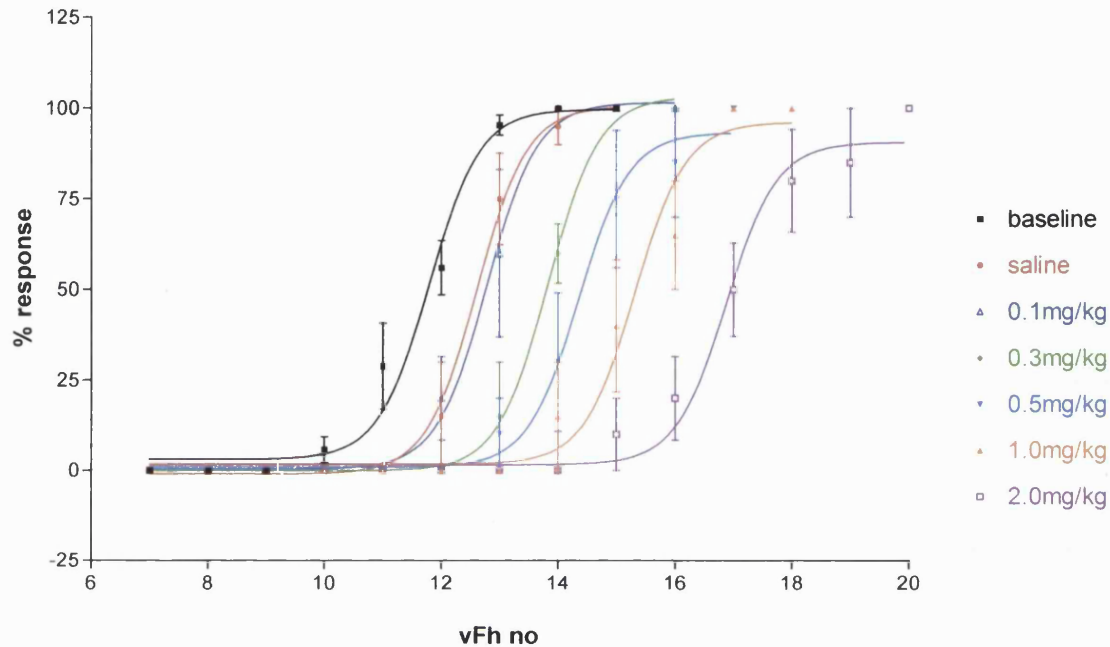
Figure 5-4 Morphine 'stimulus response' curves in P3 rats



The effect of morphine dose upon mechanical stimulus response curves in P3 rats.

Hind limb withdrawal responses are plotted against stimulus intensity of von Frey hair applied to the hindpaw after 0.1, 0.3, 0.5, 1 or 2mg/kg morphine i.p. or saline. The curves demonstrate a clear dose related effect of morphine at P3 leading to a rightward shift of the curves with increasing doses of morphine.

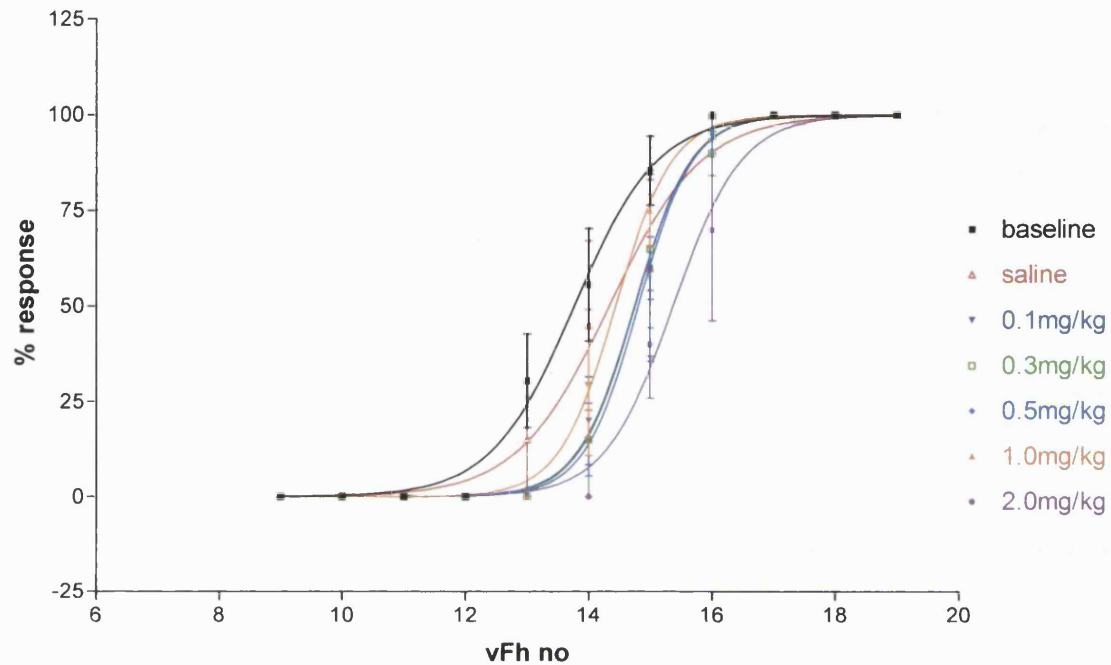
Figure 5-5 Morphine 'stimulus response' curves in P10 rats



The effect of morphine dose upon mechanical stimulus response curves in P10 rats.

Hind limb withdrawal responses are plotted against stimulus intensity of von Frey hair applied to the hindpaw after 0.1, 0.3, 0.5, 1 or 2mg/kg morphine i.p. or saline. The curves demonstrate a clear dose related effect of morphine at P10 leading to a rightward shift of the curves with increasing doses of morphine.

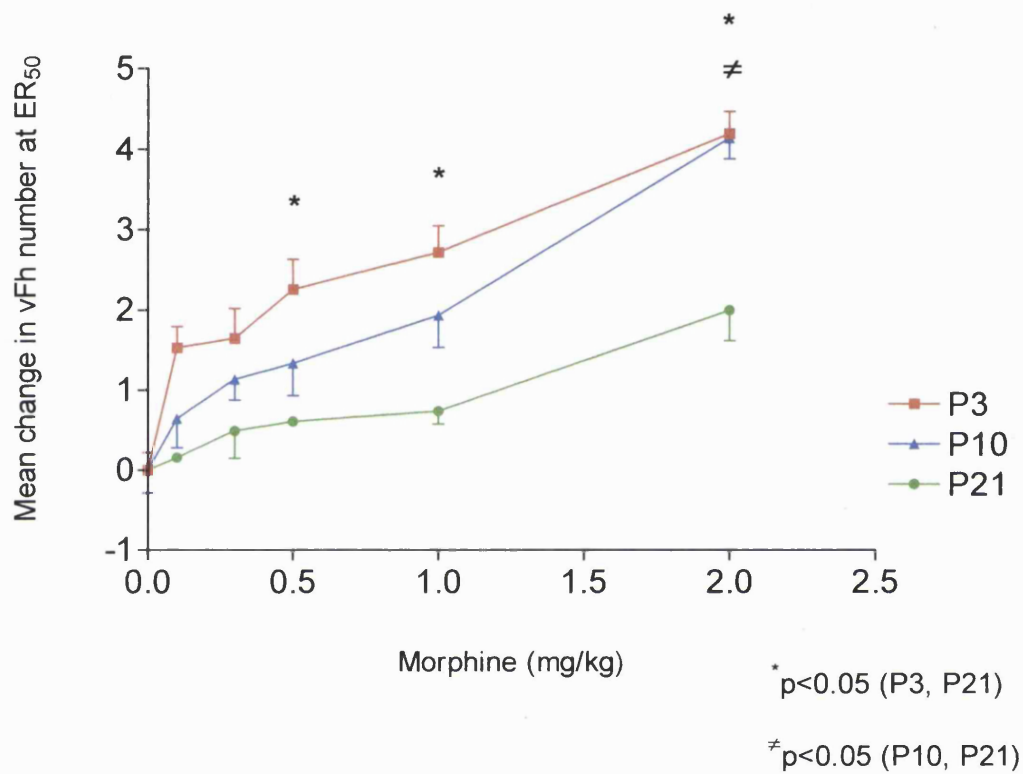
Figure 5-6 **Morphine 'stimulus response' curves in P21 rats**



The effect of morphine dose upon mechanical stimulus response curves in P21 rats.

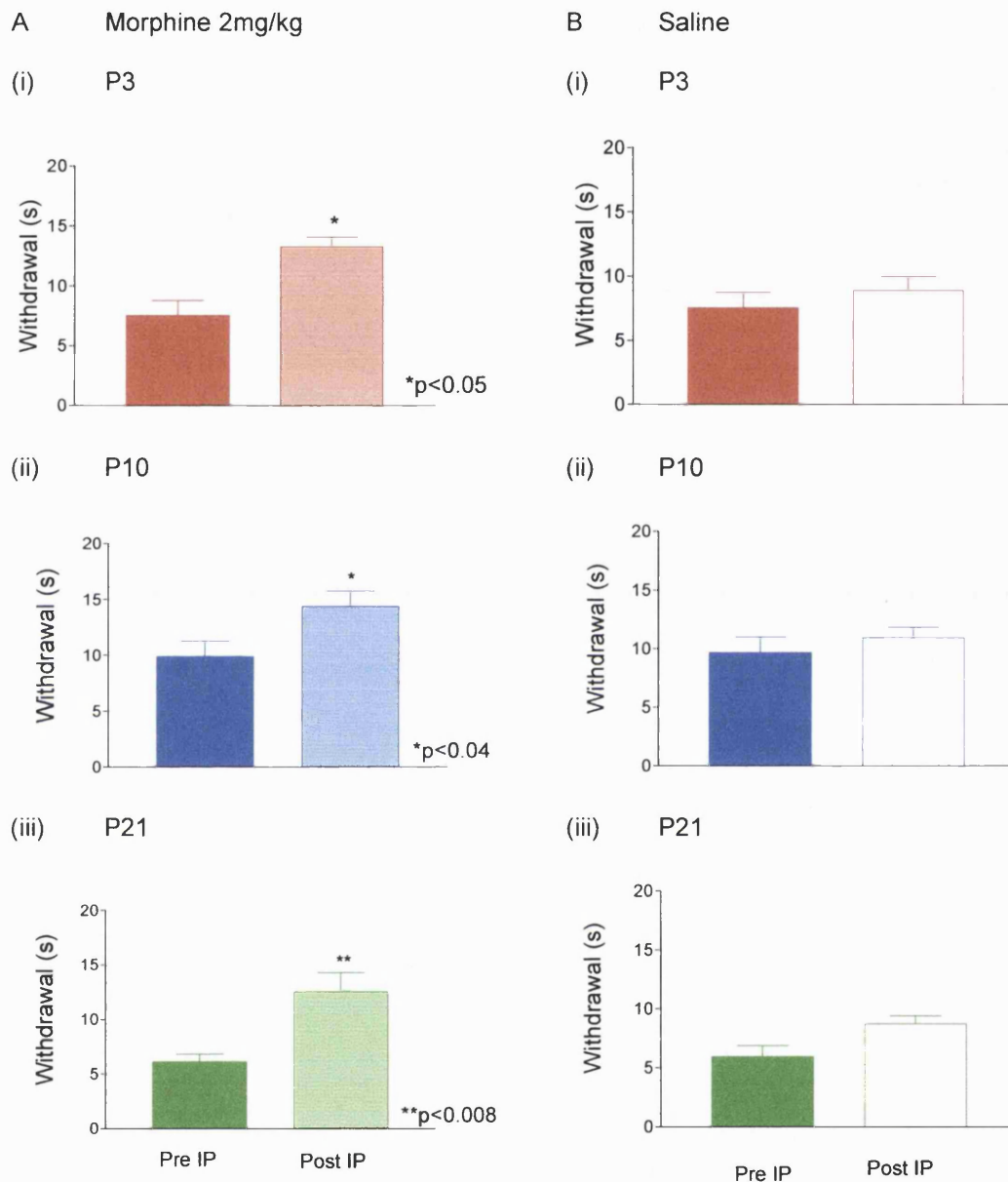
Hind limb withdrawal responses are plotted against stimulus intensity of von Frey hair applied to the hindpaw after 0.1, 0.3, 0.5, 1 or 2mg/kg morphine i.p. or saline. At this age although the curves demonstrate a dose related effect of morphine leading to a rightward shift of the curves with increasing doses of morphine this trend is much less pronounced than at P3 or P21.

Figure 5-7 Stimulus response curves of systemic morphine on the mean change in vFh threshold from predrug baseline in P3, P10 and P21 rats.



Threshold (ER₅₀) is defined here as the intensity which caused hind limb withdrawal in 50% of tests. The curves show that that morphine efficacy on mechanical sensory thresholds markedly decreases with age.

Figure 5-8 The effect of ip (A) morphine and (B) saline on heat thresholds in P3, P10 and P21 rats.



The effect of 2mg/kg ip morphine or saline on the thermal withdrawal latencies in P3, P10 and P21 rats. Each bar represents the mean threshold with standard errors of the mean. It consists of data pooled from 4 animals in each age group. Baseline data differs from that shown in figure 5.3 as tests were conducted in a different subject group.

Morphine significantly increased thermal withdrawal thresholds at all ages. In contrast to mechanical thresholds, morphine was significantly more effective at P21 than at P3 and P10.

6 Concluding Remarks

The study of the neurophysiology of neonatal pain has been limited and many important questions about basic function remain unanswered. In this MD thesis I have attempted to move the field forward by investigating neonatal sensitivity to opioids and proposing a possible mechanism for the increased responsiveness to opioids in the immature nervous system that I have observed.

The results presented here confirm that mu opioid receptor (MOR) expression is more abundant and widespread in newborn sensory neurons in the dorsal root ganglion (DRG) compared to the adult (Beland 2001). Importantly, it has been shown here for the first time that these widely expressed receptors are functional. Using calcium imaging, we have shown that significantly more neonatal DRG cells respond to morphine than adult cells.

Furthermore, combined calcium imaging with neurofilament, NF200 immunostaining demonstrated that the additional opioid sensitive cells are not spread across all DRG cell populations but are restricted to large diameter, non nociceptive A cells.

Non-nociceptive and nociceptive neurons were identified both by cell diameter and by the presence or absence of NF200 immunostaining. NF200 is expressed in A cells from before birth (Jackman 2000) and we have shown here that the NF200+ve population stays stable between birth and adulthood. This is important because it means that the postnatal change in MOR expression in this group of A cells is not a result of postnatal alterations in NF 200 expression. Consistent with previous reports (Silbert et al 2003), we found that MOR expression in the adult rat was found predominantly in small and medium sized neurons, the cell bodies of C and A δ fibres, while in the neonate, a considerably higher proportion of large diameter A cells also express MOR.

The differing magnitude and pattern of opioid responsiveness in neonatal sensory neurons will directly affect the actions of morphine on neonatal central sensory and pain pathways. In the adult opioid agonists inhibit membrane currents in small but not large adult neurons (Taddese et al 1995; Wilding et al 1995) and selectively depress nociceptive C and A δ

evoked activity in the dorsal horn of the spinal cord (Dickenson et al 1987; Sivilotti et al 1995; Rahman 1999). In the neonatal rat, however, studies in vivo and in vitro suggest that opioids depress non-nociceptive mediated activity (Faber et al 1997; Marsh et al 1999) as well as C and A δ -mediated nociceptive inputs. This wider, non-selective effect in the immature neonatal rat spinal cord is consistent with widespread MOR on A cells.

Using behavioural studies, we compared the selectivity and sensitivity of morphine on different sensory modalities in neonatal and older rats. Reflex hind limb withdrawal to a range of mechanical stimuli using Von Frey hairs confirmed that baseline mechanical thresholds increase with age (Andrews 1994; Falcon et al 1996, Marsh et al 1999). However, for the first time, we have performed blinded studies of morphine sensitivity on these mechanical responses across the postnatal period. Morphine dose response curves clearly demonstrate that the analgesic potency of morphine in mechanical sensory tests is significantly greater in the neonatal compared to older animals. This result is entirely consistent with high levels of functional MOR expression in neonatal non-nociceptive A cells. The down regulation of A fibre MOR is likely to contribute to the decreasing efficacy of morphine on mechanical thresholds with increasing age.

Importantly, this enhanced neonatal morphine analgesic potency in mechanical tests was not observed in thermal tests. Our results show that morphine was actually less effective on thermal nociceptive thresholds in younger animals and increased its analgesic potency at P21. Since thermal nociceptive latency is primarily a measure of C fibre activity (Ossipov et al 1999) this result is consistent with the finding that the proportion of small C and A δ MOR sensitive neurons does not alter with age. The differential morphine potency in neonatal mechanical versus thermal tests also suggests developmental regulation of specific receptors rather than a general regulation of metabolism or drug access, which might be expected to affect both tests.

What are the implications of these alterations in neonatal MOR expression and function for the human neonate? Many factors, including immature pharmacokinetics and metabolism will determine the clinical efficacy of opioids in the human neonate (Lynn et al 2000). In addition morphine acts on multiple receptor sites within the brain that will also be subject to

developmental regulation and, since morphine was administered systemically in this study, we cannot know its precise site of action. Nevertheless, the functional expression of MOR in human neonatal primary sensory neurons in the DRG is likely to be critical to morphine analgesic actions in the newborn.

The change in functional MOR expression identified here is one example of the many structural and functional alterations that occur in the peripheral and central nervous systems during extensive postnatal reorganisation. Ion channels, neurotransmitters, regulatory factors such as neurotrophins, and intracellular regulatory proteins are further examples of elements that are postnatally regulated and therefore affect the nature of nociceptive signalling at different stages of development. A good example of the fundamental differences in pain processing in neonates is their great hypersensitivity to sensory stimuli compared to adults. Thresholds to mechanical stimuli are reduced and further sensitisation can occur with sustained or repetitive inputs in the noxious and non-noxious range. Postnatal maturation of primary sensory neuron function, modulation of inputs by spinal and descending mechanisms and the processes of central and peripheral sensitisation contribute to these striking differences.

Now, just over 25 years after the first reports of the inadequate treatment of children's pain, the far reaching consequences of pain, such as the stress response to pain and the effects of pain on the immune system, are known to be of great importance at all ages. As a result, children are benefiting from substantial improvements in the treatment of pain. The study of the underlying mechanisms of pain and analgesia in development, which are particularly important and exciting fields of neurobiology at the moment, has enabled this huge advance in our clinical practice. However, there are still many children whose pain is underestimated or poorly understood, resulting in inadequate treatment. Therefore, continued research and intensive study in this area is essential for further effective analgesic intervention and the discovery of new targets for therapy.

7 Reference List

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