DEVELOPMENTAL ASPECTS OF NMDA ANTAGONIST SENSITIVITY IN RATS-
AN ELECTROMYOGRAPHIC STUDY USING THE WITHDRAWAL REFLEX

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NMDA antagonists play an adjunctive role in many clinical pain management regimens including those in paediatric post-operative care. Progressive alterations in NMDA receptor structure and function during development have been well established. These changes may lead to significant changes in the antinociceptive effects of NMDA antagonists during this period. Unfortunately, pharmacodynamic studies of analgesic agents in human infants are fraught with methodological and ethical difficulties. Non-clinical means of investigating pain states and analgesic efficacy during early development are therefore particularly useful for defining appropriate paediatric dosing schedules.

The research project undertaken here is aimed at establishing an animal model for quantitative assessment of analgesic drug effects relevant to acute post-operative pain states. By studying an inflammatory state in rat pups at three ages, the study attempts to define age-related changes in the antinociceptive effects of NMDA antagonists.

The model is based on electromyographic recording of the hindlimb withdrawal reflex. Age related changes in the features of this reflex have been studied and described. The reflex has been used to characterise a carrageenan-induced inflammatory state at three developmental stages (Postnatal day 3, 10 and 21). The antinociceptive effects of epidurally delivered drugs on this state have then been assessed.

Ketamine, aminophosphonopentanoic acid (AP5) and morphine were injected epidurally and the effects on the EMG recordings determined. Dose-response curves have been created for each agent at the three developmental stages mentioned above. Age related changes in drug sensitivity have been defined by comparing ED50 values estimated from the dose-response curves generated.

At all three developmental stages tested, carrageenan inflammation significantly potentiated the withdrawal reflex. In carrageenan-inflamed pups the response was dose-dependently reduced by epidural ketamine, AP5 and morphine at doses that were ineffective if given systemically. NMDA antagonists (ketamine and AP5) did not have this effect in non-inflamed pups. The following ED50 values were estimated from dose-response curves.

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<th>DRUG</th>
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<th>P10</th>
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<tr>
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<td>0.001</td>
<td>0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>Ketamine</td>
<td>&lt;0.005</td>
<td>0.008</td>
<td>0.03</td>
</tr>
<tr>
<td>AP5</td>
<td>&lt;0.005</td>
<td>0.005</td>
<td>0.016</td>
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This animal model has successfully provided a means of assessing antinociceptive effects of drugs active at the level of the spinal cord during early development.
In acknowledgement of
those who teach and inspire,

Maria Fitzgerald
David Hatch

Dedicated to Joseph and Martha de Lima

In gratitude

to Ana Cristina
for her patience, understanding and support

also to
Tony Dickenson
The Portex Endowment
Children Nationwide Pain Research Group
The Great Ormond Street Hospital for Sick Children
(Department of Anaesthesia)
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Chapter 1 GENERAL INTRODUCTION

PAIN AND INFLAMMATION

1.1 Aims of this thesis

Clinical paediatric practice has, in recent years made large advances in the perioperative management of sick neonates and infants. Coupled with improvements in surgical and intensive care of these infants is an increasing awareness of pain and distress that may be imposed by modern care. Chapter 1 provides a background for the work presented in subsequent chapters. It outlines briefly the neurobiology of pain, the morphology and pharmacology of developing pain pathways and summarises current animal models in pain research.

This thesis set out to develop a reliable physiological measure of nociception in neonatal rats that can be correlated with pain behaviours. This was done by approaching the spinal withdrawal reflex as a model of nociceptive processing and is described in Chapter 2. Such models have been extensively used in adult rats to study acute nociception, inflammatory pain and neuropathic pain.

An interest in the clinical management of perioperative pain prompted the study of an inflammatory pain state. Carrageenan-induced inflammation was characterised using the model at three developmental stages. This is described in Chapter 3. A central premise of this chapter is that inflammation-induced changes in spinal reflex excitability are directly relevant to pain perception. It is these changes in excitability and their reversal by drug interventions that are then interpreted in terms of nociception and analgesia in later chapters.

The humane application of this model also required that the influence of anaesthetic agents be taken into account. A systematic study of the effect of anaesthetic agents on the model was therefore undertaken and described in Chapter 4.

The ability of the model to quantify and compare analgesic agents was tested by studying the actions of NMDA antagonists (ketamine and AP5) on the withdrawal reflex in carrageenan-inflamed pups. This allowed a final question to be addressed: whether or not the analgesic efficacy of NMDA antagonists is developmentally regulated in the postnatal period. This is described in Chapter 5.
1.2 Defining Pain

Pain is an unpleasant sensory or emotional experience associated with or expressed in terms of tissue damage. It is thus a subjective experience that assumes both consciousness and the capacity for expression and or action.

Pain has also been defined as the perceptual counterpart of the body’s response to stimuli that threaten the integrity of its tissues. It thus functions as a sensory warning system (Treede et al., 1992).

Pain is of course, not a unitary phenomenon and has been subject to an immense variety of classifications. These include-

a) first and second
b) acute and tonic
c) transient and pathological
d) procedural, inflammatory and neuropathic
e) thermal, mechanical and chemical
f) spinal and supraspinal
g) low and high intensity
h) deep and superficial
i) cutaneous and visceral
j) malignant and non-malignant

These taxonomies based on anatomy, duration or aetiology may simply reflect the large variety of inputs that can give rise to a common physiological substrate that is distinct from but may finally contribute to the perception of pain (Loeser and Melzak, 1999). Pain perception may then be better analysed using separate psychological dimensions. Price defines three such dimensions of pain in the attempt to find correlations with neural mechanisms: pain sensation, unpleasantness (emotions pertaining to the present and short term implications) and secondary affect (emotions directed toward long term implications) (Price, 2000). The latter two are accompanied by desires to terminate or escape the presence of pain.

In focusing largely on the first aspect/dimension above, Treede et al suggest that pain is little different to other sensory modalities such as vision and hearing (Treede et al., 2000). To support this purely sensory approach to pain, a search continues for features such as a strict anatomical pathway and projection that includes a cortical region specifically responsive to painful stimuli (Bushnell et al., 1999), localized sources of pain-evoked electrophysiological potentials (scalp or sub-dural recording) (Treede et al., 2000), single cell recordings from the cortex that show specific neuronal responses to painful stimuli (invasive sub-dural recording or intracerebral recording) and consistent cortical activation of pain related areas using functional scanning (fMRI or PET) (Casey, 1999).
Despite a degree of success in each of the above four endeavours, several features that distinguish the classical sensory modalities from pain suggest that pain is fundamentally different to other sensory modalities. Firstly, classical sensory modalities typically display high-fidelity between stimulus and perception. Response characteristics are relatively stable both within individuals (over time) and between individuals. Pain perception following a given stimulus on the other hand is notoriously subject to modulation by a large variety of factors e.g. attention, fear, anxiety, expectation, past events, culture and environmental cues. Secondly, classical sensory modalities are subserved by synaptic pathways that are notable for the strength of the connections formed, specificity of the modality transduced and for their precise topographic organization (somatotopy, tonotopy etc.). These features are well seen in the “secure nuclei” of the dorsal columns and thalamus. In contrast, the anterolateral system terminates at at least three levels in the central nervous system (CNS)- including the loosely organized reticular formation, the spinomesencephalic fibres in the superior colliculus/ peri-aqueductal grey matter (PAG) as well as the dorsal thalamus. Thirdly, primary afferent fibres of the classical sensory modalities generally synapse within well delineated nuclei in rostral parts of the CNS (cuneate/gracile/ lateral and medial geniculate nuclei etc.). In contrast, primary afferents subserving nociception terminate segmentally within superficial laminae of the spinal cord.

Finally and possibly most importantly, while classical sensory modalities transduce signals from the external environment, pain pathways can be rapidly modulated to transduce signals from within the interior milieu. This process of modulation- an example of plasticity within nociceptive pathways- is manifest as sensitization in the face of tissue injury or inflammation. Moreover, through the peripheral release of neuropeptides and growth factors into innervated tissues, nociceptor afferents may significantly alter the very milieu that generates the original signals.

In contrast to the purely sensory view of pain, and perhaps focusing on the unpleasantness and secondary affect of pain perception, Wall proposes that pain is best seen as a “need state” much like hunger or thirst during which attention, orientation, analysis and cognitive planning are geared toward motor responses that abolish the pain producing stimulus (Wall, 2000). It has also been expressed as a fundamental and essential “organism-generated provocation to action” (Sullivan, 1999). By expressing pain perception as a need-state or even as a motivational drive, Wall’s proposal allows separate consideration of pain perception from the processing of nociceptive inputs. The latter may be thought of as an intermediate physiological substrate with a distributed dynamic nature that can be objectively studied and modulated (Coghill,
This substrate may well include reflex responsiveness, autonomic activity and the hypothalamic-pituitary axis balance. Each of these are known to maintain a dynamic equilibrium which can be shifted by a variety of inputs. The overall pattern or sum of these individual equilibria may then be the substrate for pain perception.

This schema for conceptualising pain and nociception may also allow for the consideration of non-neural inputs to the substrate mentioned above. Non-neural inputs include a constellation of endocrine and immune-to-brain communications e.g. pro-inflammatory cytokines and spinal cord glial reactions (Watkins and Maier, 1999; Salzet et al., 2000).

Extension of this model might allow a classification of pain states according to the primary mechanism by which the substrate is altered. Broadly then,

a) Pain that is generated by purely neural signals and during which the immune system not activated. e.g. pin prick, acute thermal stimuli. This may be equivalent to acute, physiological or procedural pain.

b) Pain generated in situations where the peripheral immune system is activated e.g. abscess, arthritis, burn. This essentially describes inflammatory pain states which may be labelled for convenience under “mast cell activation”. It does not exclude states in which immune competent cells are secondarily activated within the CNS, say by acute phase reactants. (Wall, 1995)

c) Pain states in which CNS immune mechanisms are primarily activated. That is, wherever neural tissue is damaged and microglia are activated e.g. amputation, nerve injury, multiple sclerosis. These are currently described as neuropathic pain states (Bennett, 1999).

This presupposes a role for the immune system in all aspects of pathological pain. An attempt to represent this schema diagramatically is presented in Diagram 1.

The above division may also allow some compromise between the opposing views of pain as a classical sensory modality and pain as a motivational drive. Acute (physiological or procedural) pain may in fact share many more features with classical sensory modalities than either inflammatory pain or neuropathic pain states. The latter two can be considered to comprise both a pure sensory aspect as well as motivational drives.
Pain processing and the immune system

Interactions between the immune and nervous system may occur at several levels. Both tissue and nerve damage activate immune competent cells triggering an inflammatory reaction which can alter neuronal function. Acute phase reactants (inflammatory cytokines) produced in the periphery may cross the blood-brain barrier in another possible interaction. The result of these processes is a sensitisation (either peripheral, central or both) that can be detected as an increase in the responsiveness of the withdrawal reflex.

Activation of sensory nerves is known to cause the peripheral release of several pro-inflammatory neuropeptides (Steinhoff 2000). These and other neuro-endocrine mediators (enkephalins, glucocorticoids, adrenaline and noradrenaline) released via the autonomic nervous system and the hypothalamo-adrenal axis are able to modulate the function of the immune system. Abbreviations are explained overleaf.
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>BK-1</td>
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<tr>
<td>CB-1</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
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<td>Substance P</td>
</tr>
<tr>
<td>TTXr-Na</td>
<td>Tetrodotoxin-resistant sodium channel</td>
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<td>VR-1</td>
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1.3 Pain in Infancy - a special case

Advances in developmental anatomy and neurobiology have been responsible for several major changes in the approaches of paediatric pain research and practice. These include firstly, the realisation that infants are not just scaled down adults - pain in infancy is a special case. Secondly, early development is not a state of simple immaturity - in the sense of "a lack of or incomplete function", but that the developing organism is fully functional and extremely well adapted to its primary role i.e. the process of growth and development.

The implication of this last premise is that many important neurobiological systems such as neuronal fibre tracts as well as neurotransmitter and trophic signalling systems are in a more dynamic state than the same systems in adult organisms. This state of extended functions and evolving anatomy is apparent not only in the central and peripheral nervous systems but also in other systems (e.g. the skin and immune systems) that are crucial in the responses to noxious stimuli (Longaker and Adzick, 1991). Examples of this dynamic state include the changing pattern of reflex activity in human infants, changes in the pattern of primary afferent terminations in the dorsal horn of developing organisms and developmental changes in the distribution and subunit composition of many receptor systems including the glutamatergic NMDA receptor. These are described further in Chapters 2 and 5. While these developmental sequences are adapted to the process of growth and development, in general infants are not well adapted to direct self-protection. Instead, protection against threatened tissue damage is provided through complex interactions that result in maternal care. It is tempting to argue teleologically that the infants' sensory warning/pain system is adapted to this effector mechanism rather than direct self-protection, explaining the infant's raised sensitivity and exaggerated responses to environmental stimuli and low specificity for noxious stimuli. (A mammalian neonate serves itself well by responding immediately and vigorously (even if non-specifically) to any minor perturbation in its environment.)

While significant progress has been made in the research and management of paediatric pain, several constraints beyond those encountered in adult practice remain.

Firstly, the issue of specificity of pain responses and behaviours in infancy: Pain in adults can be defined in terms of its subjective report which is generally very specific even if of variable sensitivity (i.e. severe pain is usually clearly announced but many daily mild-moderate pains go unreported). Communication from neonates and infants on the other hand is limited. While often quite sensitive to minimal disturbance, infants lack specificity in their behaviours. Pain management and research then depends on a range of devised pain assessment tools that act as proxies for the self report of pain (Stevens et al., 2000). Pain assessment tools are currently based on a variety of
physiological, metabolic and behavioural variables. Many of these variables can unfortunately be confounded by alterations in other systems e.g. cardio-respiratory and endocrine and by pathologies not related to the pain being measured e.g. sepsis. In many animal models of pain the cutaneous withdrawal reflex is used as a proxy for nociception, on the basis that the stimulus threshold of this physiological variable, at least in adult humans, is closely related to the pain perception threshold (Willer 1977). Further the reflex is relatively unaffected by alterations in other systems.

A second important constraint on the study and management of paediatric pain is that it must necessarily make assumptions or indirect conclusions regarding pain perception and memory that cannot be directly tested. Various historical beliefs have included that pain in neonates is not perceived because of cognitive immaturity or not remembered and therefore of no consequence. The issues become even more complex if attempts are made to define and postulate neonatal suffering (Walco and Cassidy, 2000).

A third constraint is that there appears to be no major economic imperative to treat paediatric pain. Coupled with this is the continuing debate regarding the long term consequences of untreated pain. Should these be clearly defined, perhaps economic arguments for aggressive pain management in infancy may become more powerful (Grunau, 2000).

Fourthly, pain management interventions in neonates and infants may still be limited by adverse reactions and side effects - whether real or imagined. The difficulty of accurately profiling the toxicology of analgesic agents in young patients represents an example of yet another constraint on paediatric pain research i.e. the ethical considerations regarding the enrollment of paediatric subjects into scientific and clinical trials. The difficulties involved limit the rate at which evidence can be gathered regarding drug safety and long-term consequences of analgesic therapy (Olkola and Hamunen, 2000; Ohlson et al., 2000).

These constraints and various difficulties have each contributed to sub-optimal pain management in infants and children in the past and may continue to do so now. Some of the issues await the results of clinical and pre-clinical trials or improvements in clinical and research tools (e.g pain scoring tools). Other issues will by their nature remain conjectural (e.g. infants perception of pain). Many issues can be addressed through the use of appropriate animal models including the careful definition and measurement of physiological parameters within these models. All the issues strongly benefit from a greater understanding of the developmental neurobiology of pain processing.
1.4 The Neurobiology of Pain

1.4.1 Physiological and Pathological Pain

A common classification of pain in both clinical and research literature comprises a division into physiological and pathological pain.

Physiological pain is the sensation experienced in response to stimuli that threaten to damage tissue or cause localized injury insufficient to provoke an extensive 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. These are:

i) The pain may occur in the absence of any apparent stimulus (spontaneous pain).

ii) The response to suprathreshold stimuli may be exaggerated in either amplitude or duration (hyperalgesia/ hyperpathia) and commonly outlasts the period of stimulation or damage.

iii) Usually innocuous stimuli may elicit pain as a consequence of a reduced pain threshold (allodynia).

iv) The sensation of pain may spread from the site of injury to an uninjured or unaffected side (referred pain).

v) Pathological interactions may occur between the sympathetic and somatosensory systems (sympathetic dystrophy).

Viewed in a negative light, 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 (“adequate stimulus”) and prolonged and excessive responses (Woolf, 1989). 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, in fact 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 psychophysical 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 (Lewis 1942, Raja 1984).

The anatomical site (central or peripheral) of the mechanisms responsible for hyperalgesia have been the subject of intense investigation (Campbell 1992). The important role of central mechanisms was confirmed by Woolf in 1983 using a spinal-decerebrate rat model (Woolf, 1983). In the newly described model, he showed that a localized thermal injury induced an increase in the excitability of the flexion reflex (measured electrophysiologically from α-motor neurons) and that this change persisted despite complete sensory block (local anaesthesia) of the injured tissue. Extensive study of the phenomenon since then has documented significant changes in both central (Cook 1987) and peripheral (Kocher 1987) nociceptive systems. All suggest that the pattern of hyperalgesia is dependent on the type of injury, the stimulus used to test it and the proximity of the test site to the injured tissue (La Motte et al., 1992; Cervero 1993; Koltzenburg et al., 1992).

1.4.2 Mechanisms of Pathological Pain

While physiological pain is a sensation that is very closely correlated with peripheral noxious stimuli, pathological pain is a sensation that arises from changes within the nervous system that result in altered sensory processing of both noxious and innocuous stimuli. Documented changes in this form of somatosensory processing provides much evidence for the more recent view of the brain and spinal cord as a modifiable or plastic system.

There are four categories of change to the nervous system that can result in pathological pain: i) peripheral sensitisation ii) central sensitisation of dorsal horn neurons iii) abnormal properties in central circuits and iv) permanent change in the nervous system.

Peripheral sensitisation.

Following tissue injury the stimulus threshold for eliciting pain decreases substantially within the area of the injury (primary hyperalgesia). This is true of both thermal and mechanical stimuli. In the area surrounding the injury, thresholds for eliciting pain with mechanical stimuli are also reduced (secondary hyperalgesia) (Raja et al., 1984).
Part of this change in sensitivity is due to alterations in the nociceptors themselves. Evidence for this includes a decrease in threshold, an augmented response to supramaximal stimuli and spontaneous activity measured in thermal nociceptors within the area of injury (Besson, 1987). Mechanical hyperalgesia on the other hand, can be evoked by pressure stimuli in the area of injury (primary or static hyperalgesia)- and by brush (allodynia) in a halo surrounding the area of injury (secondary hyperalgesia). The latter, also termed dynamic hyperalgesia appears to be mediated by large myelinated afferents via altered central processing while the former is thought to be mediated by sensitisation of peripheral nociceptors and unmyelinated afferents (Koltzenburg et al., 1992).

Studies of the mechanism of peripheral sensitisation highlight the extensive cellular interactions that occur between the immune and nervous systems in peripheral tissues. Alterations of peripheral nociceptors may involve mediators from several sources such as i) plasma and cell membrane derived inflammatory mediators e.g. bradykinin, leukotrienes and prostaglandins, ii) endothelial cell derived mediators e.g. nitric oxide, iii) mast cell derived mediators e.g. histamine, Nerve growth factor (NGF) and iii) neuropeptides (e.g. substance P and calcitonin-gene related peptide) released by somatic cutaneous and post-ganglionic sympathetic efferents.

The molecular basis of plasticity within peripheral nociceptive signalling pathways includes several hypotheses including a) bradykinin- induced sensitisation (Calixto et al., 2000), b) vanilloid receptor (VR-1) mediated sensitisation (Cesare et al., 1999) c) prostaglandin-induced changes in TTX resistant sodium channels (England et al., 1996)(Gold, 1999), d) cannabinoid receptor (CB-1) mediated modulation of primary afferent activity (Richardson et al., 1998) e) cytokine (interleukins and tumour necrosis factor)-induced alterations in nociceptor sensitivity (Opree and Kress, 2000) and f) purinergic receptor (P2X-3) mediated sensitisation (Souslova et al., 2000).

Interestingly, all bar the last mentioned mechanism have in common, an interaction with the neurotrophin signalling system - NGF/trkA (Lindsay, 1996; Winston, 2001). Although Nerve Growth Factor (NGF) is primarily a survival factor for developing sensory neurons it has also been shown to induce profound behavioural hyperalgesia (Lewin et al., 1993 and 1994a). The interaction between NGF and the mechanisms listed above assume greater relevance given that injury and inflammation result in an elevation of NGF levels in skin. NGF is produced by a large range of skin cells in response to a variety of cytokines (Woolf et al., 1994) and can interact with trkA receptors on unmyelinated peptidergic afferents (McMahon et al., 1994), postganglionic sympathetic neurons (Smye et al., 1994) and many immune cells (Lomen-Hoerth and Shooter, 1995). A close association between immune cells and
cutaneous nerve fibres further emphasises neuro-immune communications (Misery, 1997). With regards to f) above, tissue injury results in platelet degranulation with consequent release of purines.

While the role of peripheral inflammation in modulating the activity of peripheral sensory nerves is established, the converse, that peripheral sensory nerves play an important part in modulating tissue inflammatory processes is becoming increasingly recognised (Felten, 2000; Brain, 2000). Activation of sensory nerves has been shown to cause the release of several pro-inflammatory neuropeptides such as substance P (sP) and calcitonin-gene-related peptide (CGRP) from nerve terminals (Levine et al., 1993). The process involves the activation of "proteinase-activated receptors" (PAR2) on sensory nerves (Steinhoff et al., 2000).

Substance P is an active neutrophil chemotactic agent (Helme et al., 1987) and can stimulate tissue fibroblast proliferation (Nilsson et al., 1985) while CGRP is a potent vasodilator. These functions suggest that the capacity to mount an inflammatory response to injury may be somewhat dependent on these neurally derived peptides at the site of injury. A novel source of these peptides, the intrinsic innervation of nerves (nervi nervorum) may play a role in pathological processes affecting nerves (Sauer et al., 1999).

Central sensitisation

Investigating the expansion of receptive fields following brief conditioning stimuli, Wall and Woolf et al realised the changes in excitability could not be ascribed entirely to either afferent fibres or motor neurons and therefore focussed attention on central (dorsal horn) neurons (Cook et al., 1987). Both injury-induced re-organization of receptive fields and the phenomenon of neuronal "windup" first described by Mendell in 1966, then led to the formulation of the concept of central sensitization.

Central sensitisation is a pathophysiological process that contributes to pathological pain. It can be defined as an increase in the excitability of central neurons in response to sustained nociceptive input, typically that associated with tissue damage and inflammation. It is characterised by a decrease in response thresholds, an increased response to suprathreshold stimuli and an increase in the neuronal receptive field size (Woolf and King, 1990).

The perceptual correlates of these electrophysiological phenomena may include 1) allodynia — defined as the perception of usually innocuous stimuli as painful. This is often thought of as pain generated by low threshold Aβ mechanoreceptors, 2) hyperalgesia: the perception of greater pain than would be normally expected for a given noxious stimulus. This may occur both at the site of injury (primary
hyperalgesia) and/or distant to it (secondary hyperalgesia) and 3) spontaneous and referred pain.

In the intact laboratory animal, behavioural manifestations include reduced nociceptive thresholds (allodynia), exaggerated responses to noxious stimuli and spontaneous pain behaviours. In spinalised, anaesthetised and in-vitro laboratory preparations, sustained nociceptive input (typically C fibre activity) appears to be the primary factor in the generation of central sensitisation in tissue injury states (Woolf and Wall, 1986).

In both intact animals and the preparations described above, central sensitisation can be clearly observed as a facilitation of the withdrawal reflex. This facilitation is heterosynaptic (i.e. conditioning nociceptive inputs in one group of afferents increases the response to other groups of afferents) but not entirely generalized (i.e. remains lateralized) reflecting a discrete spatial organisation. Nociceptive inputs from muscle produce longer lasting excitability changes than cutaneous inputs (Wall and Woolf, 1984). Other factors suggested to affect the degree and duration of this sensitisation include:

a) the pattern and duration of conditioning stimuli, in particular electrical stimulation activates both A and C fibres synchronously whereas chemical inflammatory agents may not.
b) afferent inhibition of the reflex which may vary on a segmental basis
c) varying densities of innervation in skin, muscle and joint
d) variations in the type of synaptic contact made by C fibre afferents
e) differences in the central terminations of C afferents from skin, muscle and joint.

Long lasting facilitation of the reflex resulting from temporal summation of nociceptive inputs like those described above have been documented in intact halothane anaesthetized rats (Gozariu et al., 1997; Weisenfeld-Hallin 1985, 1990). Data from either obex transected or spinal anaesthetized rats, suggest that the mechanism of C-fibre induced hyperexcitability has a spinal origin but that a supraspinally mediated inhibition of reflex excitability is able to counteract it.

The exact mechanism, anatomical site and molecular basis of central sensitisation has been the subject of considerable investigation (Woolf and Salter, 2000). “Wind-up” is thought to be one manifestation of central sensitisation and has provided some insight into the mechanisms underlying the process (Woolf and King, 1990).
Activity dependent changes- wind-up and long term potentiation

Windup is an electrophysiological phenomenon defined as a facilitation of neuronal activity in response to repetitive primary afferent stimulation (Mendell, 1966). It becomes apparent only after C-fibres are activated and when the input from these fibres arrive at frequencies greater than 0.3 Hz. The resulting facilitation can be recorded as an increase in the magnitude of neuronal response and the development of significant “after-discharges”. In short, it is the pattern of neuronal firing that results from the temporal summation of C-fibre evoked, slow synaptic potentials. It has become a useful tool in the study of neural mechanism of plasticity and sensitisation because it is reproducible, occurs within a relatively convenient time scale and is easy to manipulate (Li et al., 1999).

Typically, 10-16 electrical stimuli at a frequency of 1 Hz (range 0.5-2Hz) and at strengths above those required to activate C fibres, are used to elicit neuronal excitatory postsynaptic potentials. The evoked depolarizations are then counted, comparing the number of spikes elicited by the first stimulus in the train with the number elicited by the last.

Wind up has been documented in a variety of neural circuits including both dorsal and ventral horns of the spinal cord (Mendell 1965), trigeminal nuclei and the thalamus (Kawakita 1993) and in both mammalian and non-mammalian species (Clatworthy 1993; Russo 1994).

The presence of this phenomenon in neurons subserving the withdrawal reflex arc is crucial to linking the electrophysiological data to behavioural data obtained from subjects in a pathological (e.g. inflammatory) pain state. A particular class of dorsal horn neurons - suggested to be intercalated within the polysynaptic pathway of the withdrawal reflex (class 2D-multireceptive neurons in deeper layers see Chapter 2 Introduction) and therefore likely to drive the reflex, differ from other classes in the degree of wind-up elicited. These neurons display wind-up to a greater extent than other classes (2S, I and III) of neurons. Further, in a large proportion of these 2D neurons, an increase in the A-fibre evoked discharge is also noted. The frequency potentiation in these particular neurons may be pivotal in the process of “late” or “second” pain (Schouenborg and Sjolund, 1983).

Interestingly, though researchers admit that wind-up is typically not stable and the variability is usually large, the reduction in the response of neurons when more than 16 stimuli are applied (i.e. “wind-down”) is rarely reported (Svendsen et al., 1999).

Though windup and central sensitisation are distinct phenomena, they most probably share a common mechanism as both require tonic activity of C-fibres and can be blocked by both substance P and NMDA receptor antagonists (Woolf and
Thompson, 1991; Dickenson and Sullivan, 1987; Price 1994). Furthermore, neurons displaying windup also display an increase in the size of their receptive fields (Li et al., 1999). It is likely that windup is a contributing mechanism of central sensitisation.

Wind-up is distinct from long term potentiation which is a more prolonged phenomenon that requires a short, high frequency conditioning input (Woolf, 1996). Both windup and LTP may contribute to central sensitisation. Other possible mechanisms of central sensitisation include, i) repetitive stimuli at rates below those necessary for windup but sufficient to lead to an increase in intracellular calcium levels, ii) activation of metabotropic receptors of either the excitatory amino acid (EAA) or peptidergic type which similarly increase intracellular calcium but do not cause direct current flows.

Correlates of windup in awake human have been described. Single peripheral C-fibre strength electrical shocks in experimental subjects produce only a poorly localized non-painful sensation. Repetitive stimulation for several seconds at least 0.3 Hz is required before pain is reported (Richards 1972). Clinically, the slightly delayed but excruciating pain produced by light touch in cases of herpetic neuralgia may also be a manifestation of windup in central neurons (Wu et al., 2000).

Molecular basis of central sensitisation

Though not fully elucidated, the molecular mechanisms involved in the augmented responsiveness of central nociceptive neurons in pathological pain states almost certainly involve activation of N-methyl-D-Aspartate (NMDA) and substance P (sP) receptors. Activation of these receptor systems and subsequent neuronal depolarization leads to intracellular molecular cascades that must finally alter the responsiveness of the neuron. Several likely intracellular mechanisms have been investigated including arachidonic acid metabolism, kinase and phosphorylase activity as well as nitric oxide synthesis.

Increases in intracellular calcium following neuronal depolarization leads to the activation of phopholipase A2. Cyclooxygenase (COX) and lipoxygense products that result include the prostaglandins PGE2 and PGF2a. (COX2 appears to be a constitutive enzyme within the spinal cord.) The released prostaglandins can then interact with specific receptors (EP2/3) on primary afferent fibres or dorsal horn neurons to alter transmitter release or post-synaptic responsiveness (Malmberg and Yaksh, 1992). Activation of these receptors can then lead to a further increase in calcium flux through voltage-sensitive Ca channels and enhanced peptide release from primary afferents (Yaksh et al., 1999).
Neurokinin-1 (NK-1 or sP) and NMDA receptor activation may also increase intracellular calcium via the inositol triphosphate pathway. This leads to the activation of a series of phosphorylating enzymes (kinases) within dorsal horn cells including cAMP-dependent kinases, CAM-Kinase II and protein kinase A and C (PKA, PKC). The latter, consisting of a large family of isoenzymes appear to play a particularly large role in the superficial dorsal horn. Judging from the anti-hyperalgesic effects of spinally applied PKA/PKC inhibitors, phosphorylation of NMDA receptors and or other downstream mediators appears to be central to the development of spinal hyperalgesic states (Lu et al., 1999; Yaksh et al., 1999). Among the various protein kinases (serine, threonine and tyrosine) a particular tyrosine kinase (Src) has been recently identified as an endogenous and possibly integral component of the NMDA receptor complex that is able to regulate the receptors function (Yu and Slater, 1999).

Intracellular calcium may also be the trigger for another secondary messenger—nitric oxide (NO) (Robbins and Grisham, 1997). This volatile product of the enzyme NO synthase, is considered a likely candidate to act on presynaptic terminals and alter neural transmission within the dorsal horn (Malmberg and Yaksh, 1993). Competitive inhibitors of NO synthase, if delivered intrathecally have been shown to reduce hyperalgesia (Yaksh et al., 1999).

The proteins and enzymes involved in the several signalling cascades outlined above, together form a post-synaptic complex intimately associated with the NMDA receptor (Sheng and Lee, 2000). This is described further in Chapter 5. The macromolecular assembly of these signalling modules gives the NMDA receptor a variety of effector pathways (e.g. Ras-ERK/MAP cascade and Ras-P13 kinase). These are able to alter neuronal excitability through: i) phosphorylating the NMDA receptor itself and thereby altering synaptic efficacy (Zou et al., 2000) ii) altering the trafficking, insertion or phosphorylation of AMPA receptors (Zhu et al., 2000). The activity-dependent insertion and removal of AMPA receptors into otherwise silent NMDAR-only synapses is a popular hypothesis for the mechanism of long term potentiation and depression (Carroll and Malenka, 2000) iii) modifying the cytoskeletal structure of the dendritic spine iv) regulating local protein translation and/or v) inducing gene transcription.

Several issues may cloud the interpretation of experiments investigating the molecular mechanisms of central sensitisation. Firstly, although wind-up appears to be particularly sensitive to NMDA antagonists, in fact a wide range of neurotransmitter receptors can modulate wind-up also. (Baranauskas and Nistri 1998) (Svendsen et al., 1999). Secondly, inadvertent sensitisation in control in-vivo experiments has been
suggested to be a confounding factor that is often un-recognised (Svendsen et al., 1999).

Resolving these difficulties requires both careful experimental design and consideration of mechanisms of central sensitisation that are not directly downstream of NMDA receptor activation. Two such mechanisms include descending facilitatory pathways and the action of the neuromodulators/neurotransmitters, such as brain-derived neurotrophic factor (BDNF) and the tachykinin, substance P (sP).

While much experimental evidence exists implicating spinal neurons in the process of central sensitisation, the involvement of higher neural centres is receiving increasing attention. Animal models utilising either pharmacological manipulation or lesions of brain stem centres demonstrate the importance of the rostral ventromedial medulla and nucleus tractus solitarius in modulating the development and maintenance of hyperalgesia (Urban and Gebhart, 1999).

Closely related to NMDA receptor dependent sensitisation is the role of BDNF and sP (Urban 1994). Conditions inducing central sensitisation result in the peripheral production of NGF which in turn is able to up regulate the expression of both sP and BDNF in the central terminals of nociceptors. Post-synaptic actions of BDNF via trk-B receptors may play a role in central sensitisation by phosphorylation of post-synaptic NMDA receptors (Thompson et al., 1999).

1.4.3 The development of pain behaviours in rat pups

Current evidence supports two fundamental points in relation to the development of pain behaviour. The first is that in early development spinal responses to nociceptive inputs are often greater than those in the mature organism.

The second is that in early development tissue injury leads to consistent changes that parallel but are not identical with the ‘algesic’ or “pain state” described in the mature organism. The magnitude of the injury-induced changes tends to be much smaller in younger organism but this must be interpreted in light of the fact that baseline evoked responses are often much greater.

The first three post natal weeks in rats are characterised by a complex pattern of evolving behavioural responses. Wriggling and hyper-reactivity are elicited in rat pups by punctate or nociceptive stimuli from birth. This response is replaced by a simpler, directed response by 6-8 days of age (Stelzner, 1971). Together with this, sensory thresholds- as measured by the mechanical and thermal stimuli required to elicit the withdrawal reflex are much lower than in adults (Collier and Bolles, 1980; Fitzgerald, 1985; Falcon et al., 1996).
Pups respond vigorously to s.c. injections of formalin from shortly after birth. Persistent limb flexion is more prominent over squirming, kicking and whole body convulsive jerking. The latter behaviours are not specific to noxious stimulation but can also be observed in response to non-noxious handling. The pattern of behaviour changes with age- by 10 days of age shaking and licking of the injected paw become prominent and specific indicators of formalin injection (Teng and Abbott, 1998). In general the duration of the response is longer in younger pups and the typical biphasic pattern seen in mature rats is not evident until pups reach 15 days of age (Guy and Abbott, 1992).

The difficulties in separating specific nociceptive behaviours from a wide repertoire of non-specific behaviours in neonatal rat pups is complicated by the difficulties in selecting age/size-appropriate doses of inflammatory/algesic substances. Either the concentration (Teng and Abbott, 1998) or the volume (Marsh et al., 1999) (or both) of algesic agents must be adjusted for age and size.

Clear reductions in mechanical thresholds following s.c. injection of 2% carrageenan have been documented in rat pups as young as 3 postnatal days, although this reduction is less marked than that seen in older (postnatal day 21) pups. Similarly, topical capsaicin results in a significant decrease in response latency to thermal stimuli across all ages in this range (Marsh et al., 1999).

A state of hyperalgesia has also been produced using Complete Freunds Adjuvant (CFA) in 10 day old rat pups (Ren et al., 1997). The state is characterised by reduced paw withdrawal latencies to thermal stimuli and somatotopically appropriate Fos protein expression in the dorsal horn of inflamed pups.

Mustard oil (MO)- induced sensitisation has been studied using EMG recordings in spinal transected rats as well as tail flick latencies in intact rats. In both paradigms augmentation of the response due to MO application has been documented in rat pups as young as 2 postnatal days although, once again this effect is less marked than the effect in older animals (Jiang and Gebhart, 1998).

1.4.4 Developmental correlations between rats and humans

Estimating equivalent neurodevelopmental stages in rats and humans is based on both morphological detail and the patterns of reflex responses. The first is described in section 1.5 while the latter is described in section 2.5. For obvious reasons, more detail is available for rat development. The available data suggest that the final trimester of human foetal development is comparable to the first 7-10 days of rat post natal life (Fitzgerald 1988). By three weeks of age (weanling stage) rat neural development may be thought to be roughly equivalent to that of human adolescence.
1.5 Morphological development of spinal pain pathways

1.5.1 Birth and organization of spinal cord neurons

Since the original work of Ramon y Cajal (1911) (who used a silver impregnation technique) various aspects of neural development have been studied using light microscopy, electron microscopy and histochemical techniques in various mammalian species. In 1984, Altman and Bayer (Altman and Bayer, 1984) presented a detailed and comprehensive study of the development of the rat spinal cord using histological and thymidine radiographic preparations. Times of origins of major neuronal classes as well as sites of origin, migratory movements and settling patterns were examined from the early neural tube stage i.e. embryonic days 11 and 12 (E11-12). The onset of cell differentiation within the cervical cord begins with ventral horn motor neurons (E11-13) and is followed by intermediate grey neurons (E12-15) and finally by neurons of the substantia gelatinosa (E14-16). This developmental gradient was noted to occur in a ventral to dorsal, rostral to caudal and lateral to medial fashion.

The bulk of skeletal motor neurons are born over a two day period, peaking at E12 in the cervical cord and E13 in the thoracic and lumbar cord. Motor fibres reach the mid-portion of the myotomes as early as E13 but penetrate into the limb bud in a slower proximo-distal process. Contralateral projection neurons develop before ipsilateral ones.

The functional significance of this ventral to dorsal developmental sequence may lie in the observation that endogenous ("spontaneous") movements become apparent relatively early as they require function of ventrally placed motor neurons only. These movements precede exogenous (reflex evoked) movements which require the function of dorsally placed, sensory neurons also (Altman and Bayer, 1984).

The developmental dorso-ventral gradients observed by Altman and Bayer support Rexed's classical subdivisions only within the head region of the dorsal horn and suggested a more nuclear (rather than laminar) structure for the neck of the dorsal horn and ventral horn. A difference between Rexed’s lamination scheme and the physiological laminar organization of the dorsal horn had also been pointed out by Fitzgerald and Wall following a study of dorsal horn cells that respond to C fibre stimulation in cats (Fitzgerald and Wall, 1980). These observations may have implications for understanding spinal processing. The laminar patterns of the dorsal horn support a somatotopic (surface-to-surface projection) for afferent processes while the nuclear pattern of the more ventral regions support a more modular organization for efferent activity. This is discussed further in Chapter 2.
Rostro-caudal developmental patterning while definitely observed, is less convincing than the strictly unilinear cephalocaudal gradient in development of the vertebrate body. The cervical spinal cord in the rat appears to lead the maturation process (in front of the even more rostrally located brainstem motor nuclei). This may represent functional adaptation, in that it would allow early maturation of control mechanisms for the snout, neck and forelimb—all relevant to suckling behaviour (Altman and Bayer, 1984).

Defining the exact morphology of substantia gelatinosa cells has been problematic because of their small size and lack of Nissl substance. Early descriptions (Cajal 1890) of three cell types—limiting, central and transverse assumed these to be projection/propriospinal neurons. Two further cell types (stalk and islet cells) have also been described though their exact nature remained elusive until recently. Using toluidine blue and golgi stains, two neuronal populations can be differentiated on the basis of axonal projection, dendritic geometry and developmental sequence. Projection/ propriospinal neurons including central, limiting and transverse cells are discernible from embryonic day 15 (E15) using these stains. Extensive dendritic arbors can be discerned by E19. Intrinsic neurons appear to mature later—axonal and dendritic development of these neurons occurring just before birth. These cells (interneurons) develop from small SG cells in a star-like pattern eventually taking the form of stalk and islet cells (Bicknell and Beal, 1984).

The neurogenesis of spinal cord neurons with supraspinal projections have been more recently studied using a combination of fluorescent axonal tracers and thymidine autoradiography. Supraspinally projecting neurons are born first in the intermediate zone and ventral horn at E13 but a day later, the birth rate of neurons in the dorsal horn outstrips that of the ventral horn, the superficial dorsal horn being most prolific at this stage. These studies also demonstrate a consistent projection-distance gradient whereby neurons with supraspinal projections (long projections) are generated prior to those with propriospinal (short) projections. This pattern of neurogenesis is consistent with similar projection-distance gradients in many other parts of the CNS.

Neurons projecting to both the dorsal thalamus (spinothalamic neurons) and to the cerebellum (spinocerebellar neurons) are born between E13-15. While the birth of neurons in some projection tracts appears to also follow a ventral-to-dorsal gradient (e.g. spinocerebellar tract), the birth rate of spinothalamic tract neurons does not follow the same topographical order in which it is initiated. Subpopulations of spinothalamic neurons in each area of the spinal grey are generated at different rates (Beal and Bice, 1994).

Apart from well recognised projections to the dorsal thalamus (Burstein et al., 1990), superficial dorsal horn neurons have been shown to project also to the
hypothalamus (Burstein et al., 1987), midbrain areas (superior colliculus, periaqueductal grey and parabrachial area) (Hylden et al., 1985; Kitamura et al., 1993) and to nuclei of the medulla (lateral and dorsal reticular nuclei)(Lima et al., 1991). By these three principal routes (the spinoreticular, the spinomesencephalic and the spinothalamic tracts) nociceptive signals are thought to mediate a) general arousal, b) autonomic and affective aspects and c) discriminative aspects of pain respectively. A greater proportion of these projection fibres from the rat lumbar cord have neurons situated within lamina I than in lamina II and in general, the projections from the superficial layers are characterised by numerous collaterals to multiple centres along the neuraxis (Hylden et al., 1989). Approximately half the projections from the superficial laminae are spinothalamic with collaterals to the midbrain while only 3% form a purely spinomedullary system. The remaining proportion (45%) project to both areas i.e. are spinomesencephalic with collaterals to the medullary reticular formation. (Bice and Beal, 1997). In keeping with a projection-distance gradient, neurons projecting to the thalamus and /or midbrain complete neurogenesis prior to those projecting only to the medulla.

The neurogenesis of spinal projection neurons from the superficial dorsal horn parallels the generation of ventrally located motor neurons and precedes that of neurons with intraspinal projections, the maturation of descending fibre tracts and the in-growth of primary afferent fibres. Complementing the thymidine/fluorescent tracer studies of Altman and Beyer (1984) and Bicknell and Beal (1994), immunostaining with the growth associated protein GAP-43 provides information regarding the temporal and spatial pattern of axonal elongation following neuronal birth. The protein marker is synthesized in developing neurons, transported to axonal growth cones and incorporated into the membrane. These studies show that axonal growth in the spinal cord commences at E12 in dorsal root ganglia, motor neurons and laterally placed (both ipsi and contralateral) projection neurons. Following the birth of projection neurons, their axons express GAP-43 as they project into the dorsolateral funiculus. This occurs together with the growth of axons entering the grey matter from white matter tracts at around E17-P2 in the lumbar cord. This represents the newly extending ascending and descending tracts. Axonal growth within the substantia gelatinosa is most prominent between E19 and P2. In keeping with the delayed anatomical development of the corticospinal tract, GAP-43 expression remains apparent in these fibres to as late as P29 (Fitzgerald et al., 1991).

Primary afferent fibres are dealt with in the following section.
1.5.2 Birth of DRG cells

The birth and differentiation of primary sensory neurons do not fit into the general ventral-to-dorsal developmental sequence described for the spinal cord neurons. Dorsal root ganglion cells along the entire body axis are born between embryonic day 12 (E12) and 15, i.e. relatively early, together with the ventrally placed motor neurons. A size-sequence similar to that seen within the spinal cord is apparent though, with larger ganglion cells preceding smaller ones.

Two cell types can be distinguished- large light and small dark neurons. These two cell groups differ also in birthdays- the former being born slightly earlier. Centrally directed processes from these cells with large and small diameters respectively, can be observed within the spinal roots by E13. Fibres appear to be segregated according to diameter- small fibres travel more laterally (Lawson et al., 1974).

The entry of dorsal root afferents into the cord begins at E13 (cervical region) with bifurcating intrasegmental collaterals forming dorsolaterally (E15) before dorsomedial intersegmental collaterals. By E17 the growth of ascending suprasegmental collaterals has commenced. The first afferents to penetrate the dorsal horn are thought to reach the motor neuron pool in the ventral horn and are followed by low threshold mechanoreceptive fibres which terminate in the deep dorsal horn on E16.5-E17.5. Small diameter afferents enter the dorsal horn at or around birth (Fitzgerald, 1987b).

Immunostaining with the growth associated protein GAP-43 (demonstrating axonal growth) provides a more detailed developmental picture. Although axonal growth from DRG cells can be discerned from E12, invasion of the spinal grey occurs after a delay (E15), during which stained DRG axons form the rostrocaudal bundle of His. At E14-15 the pattern progresses to intense expression in ventral roots and motor nerves. At the same time, the grey matter- previously unstained, begins to express the protein. The ingrowth of these collaterals is coincident with the closure of the ventral root reflex arc as well as the innervation of peripheral skin target innervation by primary afferents. Later still (E17-P2) axons projecting into the dorsolateral funiculus from dorsal horn neurons and axons entering the grey matter from white matter tracts express the marker (Fitzgerald et al., 1991).

1.5.3 Dorsal root ganglion cell phenotype

Dorsal root ganglion (DRG) cells are divided into two main categories. Large (L) cells give rise to large myelinated axons while small dark (SD) cells give rise to unmyelinated (C) and thinly myelinated (Aδ) axons. The traditional classification of
DRG cells into L and SD cells has given way to more sophisticated classification based on molecular contents. The persistence of the general classification of primary afferents into large diameter A fibres and unmyelinated C fibres possibly reflects the well established anatomical correlates of the division. Unmyelinated C fibres terminate in laminae I and II of the dorsal horn, thin myelinated (mechano-nociceptive) fibres terminate in laminae I and V while large diameter myelinated afferents terminate in lamina III (Willis and Coggeshall 1991). Correlations between fibre size, sensory modality and laminar distribution remain the basis of the principle of modality segregation (Hendry; 1999). A good deal of correlation exists between the fibre diameter, myelination and conduction velocity of DRG neurons and the pattern of expression of peptide neurotransmitters within these primary afferents. Neurons giving rise to fine unmyelinated fibres form two distinct biochemical classes based on their expression of peptide neurotransmitters and surface receptors. The first: peptidergic neurons can be labelled for the preprotachykinin product, substance P (sP) and co-express calcitonin-gene-related peptide (CGRP) as well as the trkA receptor. This class of cells makes up approximately 40% of all DRG neurons. The second class of fine unmyelinated neurons - non-peptidergic neurons, express an α-galactosyl epitope which can be stained with the plant lectin, IB4. These cells comprise about 30% of DRG neurons and are also noted to express fluoride resistant acid phosphatase and immunostain for the purinergic (P2X3) receptor (Bradbury et al., 1998). Both populations of unmyelinated neurons (85% of peptidergic and 60-80% of non-peptidergic neurons) express the vanilloid receptor (VR-1) (Tominaga et al., 1998). Most of the remaining 30% of DRG cells extend myelinated Aδ and Aβ fibres, can be stained for neurofilament (NF200) and about 10% of these express the VR-1 receptor. These patterns are represented in diagram 2.

Diagram 2 Classification of dorsal root ganglion neurons

Almost three quarters of all dorsal root ganglion (DRG) cells transduce high threshold stimuli. Most of these are unmyelinated C-fibres. A proportion of nociceptive fibres are thinly myelinated Aδ fibres. Immunostaining for calcitonin-gene-related -peptide (CGRP), the purinergic receptor -P2X3 and for neurofilament 200 identifies nearly all DRG cells. Unmyelinated C fibres are divided into two categories peptidergic (which co-express CGRP and substance P (sP) as well as the trkA receptor) and a non-peptidergic group.
The terminations of peptidergic C fibres tend to concentrate in lamina I and outer lamina II of the superficial cord and target projection neurons that express the NK-1 receptor. Non-peptidergic terminations target neurons in the deeper part of lamina II and often innervate interneurons (Basbaum, 1999).

1.5.4 Survival, differentiation and maintenance of DRG neurons- the neurotrophins

The survival, differentiation and maintenance of both central and peripheral neurons are dependent on a family of polypeptide hormones termed neurotrophins. At least two broad groups of ligand-receptor pairs appear to have co-evolved. The neurotrophin group includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and NT3 and 4 while the glial-cell line derived neurotrophic factor (GDNF) group includes GDNF, neuturin (NTN) and persephin (PSP). The former family of ligands interact with two receptor types, the p75 neurotrophin receptor (low affinity) and the Trk family of receptor tyrosine kinases (high affinity) and can elicit biological actions through each of these two receptor systems independently. The Trk family of receptors includes TrkA, TrkB and TrkC receptors which are expressed on 45%, 5% and 18% of adult DRG neurons respectively (Snider 1996). TrkA expressing neurons have small and medium sized soma, are generally unmyelinated and co-express the peptide CGRP.

On the other hand the GDNF family of ligands interact with a multicomponent receptor system in which ligand binding and downstream signalling are segregated. In the case of GDNF, ligand is bound by GFRα-1 and subsequent signalling occurs via the receptor tyrosine kinase (RET) (Ibanez 1998). Ret expressing DRG neurons include a population of unmyelinated cells that can be labelled with the lectin IB4.

NGF acts as a target derived trophic factor regulating the survival and growth of dorsal root ganglion cells and sympathetic neurons. Disruption of the NGF-TrkA signalling system results in the loss of 70-80% of DRG cells (Smeyne 1994). NGF produced by keratinocytes, fibroblasts and Merkel cells, plays a role in determining the pattern and density of neuronal arborization in the skin as well as the physiological phenotype of sensory neurones (Constantinou 1994). The maturation of high threshold mechanoreceptors and C-mechanoheat receptors appears to be dependent on NGF (Ritter 1991; Lewin 1994). Importantly, NGF production can be modulated by tissue injury and inflammation and plays an important role in the sensory alterations that accompany inflammatory pain (Woolf 1996b).

The dependence of DRG neurons on neurotrophins is developmentally regulated and correlated with the expression of particular receptors on neuronal
subpopulations. In the rat post natal period, a subgroup of DRG cells appear to down regulate expression of the TrkA receptor and begin to express the GDNF receptor, Ret. This division of DRG neurones (particularly of unmyelinated neurones) according to neurotrophin receptor expression coincides with several other distinguishing characteristics of the two main subpopulations of unmyelinated DRG cells. These are summarised below:

<table>
<thead>
<tr>
<th>TrkA expressing</th>
<th>Ret expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidergic: contain sP and CGRP</td>
<td>Non-peptidergic</td>
</tr>
<tr>
<td>Can be labelled with lectin IB4</td>
<td>Sensitive to GDNF-GRFα1-Ret interaction</td>
</tr>
<tr>
<td>Survival and function regulated by NGF-TrkA interaction</td>
<td></td>
</tr>
<tr>
<td>Predominant form of visceral afferent</td>
<td>Central terminals tend to synapse in lamina II</td>
</tr>
<tr>
<td>Central terminals tend to synapse in lamina I and IIo</td>
<td>Central terminals tend to synapse on projection neurones - 50% of projections travelling within the spinothalamic tract and sending collaterals to the midbrain</td>
</tr>
<tr>
<td>Central terminals tend to synapse on interneurones</td>
<td></td>
</tr>
</tbody>
</table>

1.5.5 Peripheral growth of afferent fibres

Studies using carbocyanine dyes also show that the first fibers to exit the lumbar ventral horn and DRGs do so at E12. At E13 major nerve trunks (e.g. femoral and sciatic) are visible as they exit the plexus region. By E14 afferent fibers are present in the epidermis of the proximal hindlimb, and the major nerve trunks extend into the leg. From the lumbar region, fibres reached the paw by E14.5-E15, and the epidermis of the most distal toes is innervated by E16-E16.5. By this stage the dermatomes resemble mature forms with substantial overlap only between adjacent ones. While spinal nerves are mixed, little interfascicular mixing of fibres occurs within plexuses and major nerves, confirming that the adult pattern of spatial relationships between cutaneous afferent fibers in the periphery is established early in development (Mirmics and Koerber, 1995a).

At birth, both myelinated and unmyelinated fibres are present in a dermal plexus in hindlimb skin. The maturation of skin sensory end-organs though occurs over a prolonged postnatal period. This is supported by the continuing expression of the growth associated protein GAP-43 and its mRNA in sensory neurons for some weeks.
postnatally (Fitzgerald et al., 1991). The maturation of various sensory end organs is dependent on the type of receptor. Polymodal nociceptors (C fibres) appear to be fully mature in terms of threshold and firing pattern at birth. High threshold mechano receptors (Aδ fibres) can be distinguished although their firing patterns are not completely mature while low threshold mechanoreceptors (giving rise to Aβ fibres) are the least mature with low firing frequencies and response amplitudes (Fitzgerald, 2000).

Immunostaining markers of subpopulations of primary afferent neurons has recently allowed Jackman and Fitzgerald (2000) to provide further detail of both peripheral and central innervation by dorsal root ganglion cells. These studies confirm the entry of fibres into the rat hindlimb at E13-14 and show that A fibres do so ahead of C fibres, reaching the distal hindpaw at E17. Exuberant innervation of skin (terminal arborisations reaching the skin surface) is seen peaking at E17, with subsequent retraction to a subepidermal plexus prior to birth. Interestingly, peptide (CGRP) expression in unmyelinated fibres appears at E19 in a pattern that suggests a phenotypic switch in existing fibres (perhaps related to target connection) rather than the birth and growth of a new population. The late (postnatal) appearance of lectin (IB4) binding on small diameter fibres is also consistent with a developmentally regulated change in C fibre phenotype (Jackman and Fitzgerald, 2000).

1.5.6 Central growth of afferent terminals

The topographical arrangement of primary afferent terminals within the spinal cord is critical to the coding of both the modality and location of sensory inputs. In the adult spinal cord collaterals of large diameter fibres (A fibres) coursing ventrally in the medial aspect of the dorsal horn recurve dorsally and terminate with “flame shaped” arbors in the nucleus proprius (lamina III). These are thought to innervate hair follicles. Other collaterals terminate within the intermediate grey (lamina IV, V) or complete a monosynaptic arc with motor neurons. Small diameter primary afferents (C fibres) on the other hand enter the dorsal surface of the horn and via Lissauer’s tract, terminate mainly within the marginal layer and substantia gelatinsa (Altman and Bayer, 1984). These fibres have been implicated in nociceptive and thermal sensory processing and appear to constitute the central processes of free nerve endings.

Detailed morphological analysis obtained from horseradish peroxidase (HRP) injections of single afferent fibres show specific patterns of terminal arborizations depending on the type of peripheral receptor being innervated (Woolf, 1987). These studies confirm also that somatotopy is based on the mediolateral positioning of terminal arborizations while modality is coded by laminar arrangement.
The initial fiber penetration of the lumbar spinal grey matter begins at embryonic day 15 (E15) and is restricted to the segments of entry. Subsequent growth of fibers into grey matter of adjacent segments begins after a delay, approximately one day later. Small diameter afferents begin a second wave of in-growth at E19. Somatotopic organization is evident from E18 and has a mature pattern that does not change significantly throughout embryonic development. No particular proximo-distal sequence is seen in fiber entry which occurs before peripheral innervation is complete. This suggests that in-growth may be independent of the establishment of specific peripheral connections (Mimics and Koerber, 1995b).

The first sensory fibers to grow into the dorsal roots are large diameter myelinated ones. In the lumbar cord this begins at E12. These afferent fibers then send collaterals into the dorsal grey at E15 (Fitzgerald et al., 1991). Some of these, presumed Ia muscle afferents, reach the ventral horn to synapse within the motor neuron pool while other cutaneous afferents (Aδ) remain within the dorsal horn.

Transganglionic labelling of afferent fibers with wheat germ agglutinin-horseradish peroxidase (WGA-HRP), allows the study of fine diameter (Aδ and C) primary afferents. These fibers are found to grow into the lumbar dorsal roots at E19 (i.e. later than the larger A fibers) These fine fibers only penetrate the dorsal horn some 12 hours later with increasingly dense terminal labelling, progressively reaching lamina II by birth (E21.5) (Fitzgerald, 1987a; Fitzgerald and Gibson, 1984).

Immunostaining subpopulations of DRG cells confirms the above pattern of dorsal horn innervation. In-growth of A fibers (stained with RT97) commences at E14 with Ia afferents projecting ventromedially and putative Aδ fibers coursing ventrolaterally. These fibers also are present in lamina II for a period of time. Only later (E17) do unmyelinated fibers (trkA stained) appear in the lateral region of the superficial dorsal horn. Interestingly, coincident innervation of peripheral skin and dorsal horn is observed for A fibers only which may imply a causal relationship between the two events for these fibers only. C fibers on the other hand, enter the dorsal horn some time after innervating the skin suggesting other factors determine central innervation (Jackman and Fitzgerald, 2000). Further insights into factors determining timing of innervation and “waiting periods” (brief delays in the axonal invasion of a tissue being innervated) have been obtained from elegant experiments in the chick. In these experiments, the “waiting periods” of both motor and sensory axons are determined by maturation of the target tissue and do not appear to influence the accuracy of peripheral innervation nor determine the timing of central projections (Wang and Scott, 2000).

Both A and C fibers grow into the spinal cord in strict somatotopic order. The pattern of termination of individual peripheral nerves is laid down from the outset in a
pattern that preserves a topographic representation of the body surface throughout development (Fitzgerald and Swett, 1983). Laminar organization on the other hand does undergo some developmental alteration and has been studied by selective labelling (of A fibres) with HRP conjugated with the B subunit of choleratoxin (B-HRP). Early in development, A fibres have terminations throughout laminae I to V, including lamina II (Subst. gelat.). This distribution, wider than that in the adult persists for up to three weeks postnatally. A gradual post natal rearrangement involving a restriction of Aβ terminations to deeper laminae (III-V) occurs up to 23 days after birth. The adult pattern of terminations (restricted to laminae III-V) is noted from P30 onwards. For a significant post-natal period then, the superficial layers of the spinal cord (substantia gelatinosa) are shared by both C fibre afferents and the larger Aβ and Aδ fibres (Fitzgerald et al., 1994)(Mimics and Koerber, 1995b). It must be noted that the selectivity of B-HRP for A fibres in the face of axotomy/neuronal death has been questioned (Tong 1999) and the selectivity of the staining technique in neonatal tissue is not known. Several lines of evidence though continue to support the conclusions reached above, notably: B-HRP labelled profiles are predominantly those of large DRG soma irrespective of postnatal age (Fitzgerald 1994), the pattern of terminal labelling is unlike that of C fibres (Torsney 2000) and results obtained from intradermal B-HRP injection (avoiding nerve damage) do not differ significantly form those obtained from nerve injections (Fitzgerald and Shortland 1994). Finally and more importantly, recent electrophysiological evidence confirms that 51% of immature substantia gelatinosa neurons receive Aβ synaptic inputs and that this proportion reduces to 9% in adult life (Nakatsuka 2000).

By staining and examining neurons positive for fluoride resistant acid phosphatase (FRAP) Coimbra et al also demonstrated significant post natal (up to P5) laminar changes that appeared to reflect the development of dendritic trees of gelatinosa neurons and the arrival of primary afferents at this time (Coimbra et al., 1986).

The mechanism of A fibre withdrawal from superficial laminae may be closely related to maturation of C fibre synaptic connections within the SG (Fitzgerald et al., 1994). Support for this hypothesis was obtained in experiments whereby capsaicin was used to ablate C fibres. In these experiments, the neonatal loss of C fibres resulted in the failure of A fibre withdrawal to deeper laminae (Torsney et al., 2000). This is further supported by a close parallel in time scale between the maturation of C fibre synapses and A fibre withdrawal.

A fibre terminals can be seen within synaptic glomeruli in superficial lamina including lamina II during the early post natal period (Coggeshall et al., 1996). The large number of synaptic terminals with round clear vesicles and labelled with B-HRP in lamina II provide evidence for functional contacts between large primary afferents...
(Aβ fibres) and lamina II dendrites at this time. C-type afferent terminals, on the other hand are not observed until the fifth post natal day within lamina II (Pignatelli et al., 1989). A gradual increase of these axodendritic contacts occurs from P5 to P20 and is suggested to be the basis of the delayed onset of reflexes mediated by unmyelinated fibres.

### 1.5.7 The maturation of descending fibre tracts to the dorsal horn

Descending pathways to the 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 post-natally (Leong et al., 1984). Post-natally maturing tracts include the trigemino-spinal, solitariospinal, tectospinal and cerebellospinal tracts- some of which are only seen clearly some 10-20 days after birth. Age related increases in cell counts are also seen in the lateral vestibular nucleus and nucleus of the posterior commissure. 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). Evidence for this comes from studies using intrathecal 5HT or its antagonists (e.g. methysergide, ketanserin).

Further the functional effects of nucleus raphe magnus stimulation on dorsal horn cells (lamina I and II) is correlated with the distribution of 5HT-immunoreactive contacts on these cells (Miletic et al., 1984).

Despite anatomical connection as early as postnatal day 6 (P6), physiological maturity of the fibre tract (i.e. its ability to inhibit dorsal horn activity) 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 local interneurons of the SG. No 5HT immunoreactivity is discerned 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 postnatal day 14 (cervical cord) and day 21 in the lumbar cord (Bregman, 1987). Responses in motor neurons to 5HT are also consistent with the above maturation sequence. Responses mature from
slow-rising, prolonged depolarizations at E16-17, to fast rising, high frequency potentials at E18 with further significant increases in amplitude after birth. The involvement of multiple receptor types may partly explain the diverse actions seen (excitation of developing motor neurons and inhibition of newly established glutamate-mediated synapses). These findings suggest a complex physiologic role for serotonergic descending fibre tracts during development that correlate with the growth of 5HT projections into the cord at a time of synapse formation (Ziskind-Conhaim et al., 1993).

1.5.8 Human spinal cord development

Within human foetal spinal ganglia, cell grouping according to size is marked, the distinction being greater than that in the rat (Hughes, 1976). Early in development (as early as 6 weeks gestation), the central processes of the larger dorsal root ganglion (DRG) cells (large myelinated cutaneous afferents) enter the cord and occupy both the deep dorsal horn and superficial laminae (Okado, 1981). The peripheral extensions of these DRGs reach the skin between 11 and 20 weeks.

The early entry of dorsal root afferents into the dorsal grey matter (some axons reaching the motor pools by 8 weeks gestation) has been confirmed using lipid soluble fluorescent dyes (the carbocyanines, Dil and DiA). Between 11 and 18 weeks gestation axonal boutons appear in proximity to motor neuron cell bodies. Unfortunately, these studies did not clearly define the developmental sequence of fibres innervating the superficial dorsal horn (Konstantinidou et al., 1995). If not a technical problem, this may represent a delayed entry of fine primary afferents into the grey matter (Konstantinidou et al., 1995).

By 18-19 weeks gestation, axons entering the cord medially are observed to descend into deep lamina and recurve upward in classic flame shaped arborizations. Others, entering laterally traverse without branching and arborize in lamina III. Overall, this patterning is very similar to the sequence in rats therefore allowing the assumption that the two arborization patterns described may represent those for hair follicle afferents and low threshold mechanoreceptors respectively (Konstantinidou et al., 1995).

Although synapse-like contacts can be discerned electronmicroscopically in human foetal spinal cords as early as 4.5 weeks gestation, these are likely to represent transient structures of indeterminate function (Okado, 1981). The first wave of regular synapse formation occurs at about 8 weeks gestation. Early in synaptogenesis axodendritic contacts predominate, followed later by axosomatic and dendrodendritic
contacts by 17-18 weeks. A mature pattern (multisynaptic arrays of all three types) can be observed by 25 weeks (Rizvi et al., 1986).

Even though spinal cord synapses have been documented as early as 6 weeks gestation, neurotransmitter vesicles only begin to form at 13 weeks. Myelin formation within the lumbar spinal cord commences at 14-15 weeks (120mm CR) and proceeds cranially (Gamble 1969). It is during this period (last embryonic week and first two foetal weeks) that the spinal cord undergoes distinct maturational changes. These are manifest as growth in neuronal cytoplasmic organelles, differentiation of glial cells and the formation of synaptic contacts (Wozniak et al., 1980).

Spinal cord ventrolateral tracts become fully myelinated to the level of the thalamus by 30 weeks and thalamo-cortical tracts make synaptic connections at about 24 weeks (Kostovic and Rakic 1990). As descending inhibitory controls mature, the early period of increased excitability of spinal reflexes gradually regresses (Wolf, 1997).

Overall the distribution pattern of the peptides and amines is similar in both species but rats show a greater number of immunoreactive fibres and cell bodies than humans. In general, a number of peptides appear at later stages of development in humans than in rats. In particular, TRH (thyrotrophin releasing hormone), TH (tyrosine hydroxylase) and 5-HT positive neurons are not evident in humans before the 6th post natal week (Marti et al., 1987).

The anatomical and molecular events described above lead to several characteristic developmental patterns in both human and animal spinal reflex excitability. These are described in Chapter 2.
1.6 Pharmacology of spinal pain pathways

1.6.1 Overview of transmitter systems in the adult spinal cord

The pharmacology of spinal cord neurotransmission is normally divided on the basis of results from electrophysiological recording. The simple classification of ligand-receptor systems into either excitatory or inhibitory pathways may prevent an appreciation of the complexity that multiple transmitters, as yet undefined circuitry and neuronal plasticity must confer.

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 substance P, calcitonin-gene-related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide, galanin, bombesin and neurotensin. Of these, somatostatin and galanin are thought to possess inhibitory actions. Two of these neurotransmitters - glutamate and the peptide, substance P assume prime importance in excitatory transmission between primary afferent fibres and dorsal horn neurons in the spinal cord. The pharmacology of glutamatergic neurotransmission is dealt with in Chapter 5.

Substance P- (containing 11 amino acids) acts on the G-protein coupled neurokinin 1 receptor (NK-1) (Hokfelt et al., 1975). It has excitatory effects on both peripheral and central neurons and a variety of actions on non-neural tissues. Substance P belongs to the tachykinin family of peptides which in mammals includes also neurokinin A and B and neuropeptide K and γ (Hershey and Krause, 1990). It is synthesized by small diameter C sensory fibres and released into the dorsal horn of the spinal cord. Though only 10% of all lamina I 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).

Both in vivo and in vitro experiments on dorsal horn cells indicate that this peptide can enhance the response to short acting neurotransmitters such as glutamate. Furthermore, it appears that a post synaptic mechanism may be sufficient to account for the enhanced responsiveness. Studies with intrathecally administered sP and Neurokinin (NK) receptor agonists and neurokinin antagonists suggests that activation of peptide receptors is a requirement for nociceptive responses (Haley and Wilcox, 1992).

Neuropeptide receptors all tend to be over-expressed in early development. Despite the density of NK-1 receptors being maximal at P11, levels of the peptide are initially low and take two 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 NK-1 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). Further, developmental studies in mice lacking the NK-1 receptor suggest that
this receptor system does not play a major role in nociception to heat, mechanical or
chemical stimuli within 3 days of birth but does so by post-natal day 21 (King et al
2000)

Three further “non-classical” transmitters have recently been implicated in spinal
nociceptive signalling these include brain-derived neurotrophic factor (BDNF), nitric
oxide (NO) and spinally-generated prostanoids. The latter two may both represent
down-stream mediators of glutamate (specifically NMDA-receptor) activity
(Dickenson, 1995).

**Brain derived neurotrophic factor (BDNF)** is now known to be
synthesized and constitutively expressed by nociceptors expressing CGRP and trk-A in
dense core vesicles. It is probably released from the central terminals of these fibres in
the superficial dorsal horn in an activity dependent fashion, moreover its release can
code temporal features of presynaptic neuronal activity (Balkowiec and Katz, 2000).
Notably, tissue trauma and conditions that increase peripheral NGF production
(inflammation) result in the up regulation of BDNF receptors. BDNF is able to
modulate spinal reflex excitability via a post-synaptic mechanism quite 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).

**Nitric oxide** is synthesized 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. Dorsal root
ganglion 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-amino-butyric acid (GABA)
system, the opioid system and the monoamines -serotonin and noradrenaline.

In adults, GABA is known to have inhibitory actions mediated via an increase
in chloride conductance through post-synaptic GABAa and GABAb 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 inhibitory action (Schnaffner 1993, Riechling 1994). The depolarizing 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).

A preferential localisation of \( \mu \) opioid receptors on pre-synaptic C afferent fibre terminals explains the success of opioid drugs as analgesics. Both pre-synaptic and post-synaptic actions have been documented, the former results in reduced transmitter release following effects on calcium channels while the latter results in membrane hyperpolarization following potassium channel activation (Dickenson 1994). This system interacts significantly with the cholecystokinin (CCK) receptor system and \( \alpha-2 \) adrenoceptors. (CCK-B receptor activation counters \( \mu \) receptor activity while \( \alpha-2 \) agonists potentiate opioid actions). An interesting dissociation of the effect of \( \mu \) and \( \kappa \) receptors on heat nociception has been observed (Mu but not kappa agonists are able to alter thermal thresholds (Yaksh 1983). During early development, the opioid system is characterised by a changing pattern of receptor (\( \mu, \delta \) and \( \kappa \)) distribution. Mu receptor binding is widespread throughout the spinal cord in neonatal rats and becomes gradually restricted to superficial laminae with increasing age. In contrast \( \delta \) opioid receptors are expressed in low densities at birth (autoradiographic studies detect binding from P7 onwards) and increase with age to a peak at P25 in rats (Rahman et al., 1998; Kar 1995).

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 rostroventral medullary (RVM) neurons. The pontine nuclei mostly secrete noradrenaline while the RVM projections to the spinal cord use serotonin (5-HT) and neurotensin (Urban et al., 1996). Serotonin receptors comprise at least 16 subtypes most of which are G-protein coupled. In the dorsal horn, 5-HT2 receptors have been implicated in anti-nociceptive actions via activation of phospholipase C (Coskun and Anand, 2000).

### 1.6.2 The Excitatory Amino Acid- glutamate

Most excitatory neural pathways including corticocortical, corticofugal and sensory pathways employ an excitatory amino acid (EAA) as a neurotransmitter (Cotman et al., 1987). The anatomy of these pathways has been studied using various techniques including autoradiography following high affinity uptake of labelled aspartate, immunocytochemistry and radioligand binding. Though functionally
heterogeneous and widely distributed, EAA receptor sites show distinctive organization within the brain suggesting that individual pathways represent distinct, anatomically organized subsystems of EAA mediated transmission (Fitzgerald, 1997).

Glutamate is the major excitatory amino acid neurotransmitter in the central nervous system as the primary fast neurotransmitter between primary afferent and dorsal horn sensory neurons. It acts by gating at least three different ion channels namely, receptors for AMPA (α-amino-3-hydroxy-5- methyl isoxazole propionic acid), NMDA (N-methyl-D-aspartate), and kainate, a structural analogue of glutamate. Glutamatergic synapses achieve functional variety through the use of multiple combinations of these receptors - the most well characterized being the NMDA type (Cotman and Iversen, 1987).

### 1.6.3 Developmental issues

The postnatal ontogeny of excitatory amino acid (EAA) systems has been studied mainly in the rat. EAA receptors undergo evolving patterns of change possibly reflecting developmental roles in the survival, migration and growth of neurites. Brain maturation is therefore, characterized by a significant changes in the pattern of excitatory synaptic transmission. Similarly, the maturing spinal cord is characterized by developmental regulation of the expression of various receptors.

In the immature brain synaptic transmission is weak and extremely plastic. A large proportion of it occurs via NMDA-type glutamatergic receptors (Durand et al., 1996). 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; Wu et al., 1996).

High affinity glutamate uptake studies (Kvale et al., 1983) and receptor binding studies (Baudry et al., 1981) show that both uptake and binding increase until postnatal day 25. Concomitant with this, the expression of mRNA for various subunits of the NMDA receptor reaches a peak at postnatal day 20 (Monyer et al., 1994) A general pattern of overproduction of synaptic terminals during development appears to be common to all EAA systems. The overshoots relate temporally to periods of heightened synaptic plasticity and consolidation of synaptic connections (McDonald and Johnston, 1990). Most studies suggest that the ontogeny of the glutamate system in rats is
complete by the start of the post-weanling period (P25) or roughly equivalent to the earliest stages of adolescence in humans (Benes, 1995).

The early foetal cord and hippocampus have a widespread and high density of NMDA (N-methyl-D-Aspartate) receptors. These receptors have a different subunit composition to that of their mature counterparts. This results in enhanced affinity for ligand and a different channel open time. NMDA receptors are heteromeric ion channels composed of NR1 and NR2 subunits. Varying functional properties of the receptor are conferred by subtypes (A-D) of the NR2 subunit (Ishii, 1993). The neonatal forebrain predominantly contains receptors which are formed with the NR2B subunit. During development these are gradually replaced or supplemented with NR2A-containing receptors (Sheng et al., 1994). This change effectively results in a shortening of the NMDA mediated synaptic currents with age (Monyer et al., 1994) (Flint, 1997).

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 post natal life, AMPA receptors become largely restricted to the substantia gelatinosa in adults (Jakowec et al., 1995). Dramatic changes in the pattern of expression occur during the first 3-4 weeks of post natal life 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 three ionotropic glutamatergic receptors (AMPA, NMDA and kainate). This transient expression diminishes in early post natal life in all regions but least so in the substantia gelatinosa until the adult pattern emerges (Kalb 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. It is also noteworthy that the period of high expression in both rodents and humans follows the stage of greatest programmed cell death perhaps making it unlikely that the high expression is directly related to cell loss (Kalb 1997).

Particularly dynamic changes in the relationship between NMDA receptors and those for AMPA and GABA suggest that these three 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 GABAa channel results in depolarization 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 GABAb-receptor activation only mature later, GABA acts as an excitatory neurotransmitter in early development. The depolarizing action of GABA is also associated with increases in intracellular calcium and provides an important excitatory inputs to immature neurons. At these early stages of development, glutamatergic synapses lack functional AMPA receptors and are therefore quiescent at resting membrane potential (Wu et al., 1996). 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 pre-eminence of NMDA receptors in excitatory neurotransmission during early development is the result then of i) a high density of these receptors, ii) the slow decay of glutamate-induced currents and iii) the synergistic action of GABA. The latter undergoes an abrupt change as GABAa receptors switch from an excitatory to an inhibitory synaptic role (Ben-Ari et al., 1997).
1.7 Animal models in pain research

The effort to model human pain states in animals is driven by the need to define the precise molecular and physiological processes that form the adequate and essential substrate of pain perception. Models must allow assessments of analgesic interventions and allow extrapolation of these to human subjects.

The logistic and ethical difficulties in studying nociception in human subjects are obvious and are even more difficult in the study of pain in early development (Abram, 1997). Below is a list of the more common models of nociception together with some advantages and disadvantages of each. (Chronic pain models are not dealt with here.) The models have been divided into those that measure transient pain and those that measure tonic pain. This division may have parallels in an older debate rooted in the early investigation of motor function in as much as the measures of transient pain are based on reflex movements whereas those of tonic pain are based on complex motor behaviours. At the heart of this debate is whether complex motor patterns are fully integrated from the outset of development or whether they are the product of secondary integration of local reflexes. This is discussed further in Chapter 2.

1.7.1 Acute nociception and reflex assessments

Several laboratory test systems are able to quantify the immediate response of an animal to a noxious stimulus. Typical examples include:

1) Hot plate test: Either the temperature required or latency for limb withdrawal is measured following direct contact with a hot plate (Woolfe et al., 1944).

1) Tail flick latency: the latency to withdrawal of the tail from a radiant or contact heat source. (D'Amour et al., 1941)

2) Flinch jump test (Evans 1961)

3) Vocalization thresholds: either the pressure required or latency for vocalization induced by local pressure on a limb.

4) Randall-Sellito: either the pressure required or latency for limb withdrawal in response to local pressure.

These models all test some aspect of reflex movement evoked by an acute, unconditioned noxious stimulus. Activation of nociceptive afferent fibres results in a spinaly mediated reflex response and, in some cases supraspinally organized escape behaviours. In each case either a threshold or latency is recorded. In most cases,
latency is a proxy measure of threshold as the time taken to record a response is a reflection of the cumulative effect of stimulus energy. An increase in threshold from control values is typically interpreted as analgesic effect. The rationale for this assumption derives from psychophysical experiments in human volunteers that show a close parallel between self reported pain thresholds and the thresholds for activation of the withdrawal reflex (Willer, 1977).

While these tests have the advantage of clear, objective end points and reasonable consistency within groups of animals they also have some disadvantages:

a) they each model only transient pain which may be seen as a lesser priority than tonic pain from a clinical point of view. Ongoing spontaneous pain cannot be assessed.

b) some degree of restraint is required in each of the tests above. The aversive reactions generated are liable to influence results of studies that attempt to define mechanisms as well as studies of drug pharmacodynamic effects.

c) as animals are awake and subject to environmental distraction, the variability in response (both intra and inter individual) is likely to be large.

d) time courses of analgesic interventions cannot be assessed without overly repetitive testing.

e) they all assume an intact motor system. As peripheral nerves are typically mixed nerves, interventions aimed at sensory nerves often also effect motor function.

f) these test assume that either measured thresholds and/or reflex latency are good surrogate measures of perceived pain. Wall has recently warned against the uncritical acceptance of this assumption (Wall, 2000).

Given these shortcomings, valuable data are still obtained through manipulations of experimental design. In particular, the combination of one of the above tests with models of inflammation or neural damage may increase the scientific and clinical utility of the test. This is dealt with in greater detail in Chapter 4.

A further insight into the interpretations of thresholds, latency and reflex responsiveness is given in Chapter 3. Under certain experimental conditions, reflex thresholds are a good measure of anaesthetic depth, latency may be a measure of the maturity and integrity of synaptic pathways and the last- reflex responsiveness (response to suprathreshold stimuli) may render a better correlation with tonic pain states.
1.7.2 Spontaneous pain behaviour and algesic agents:

In the attempt to model nociceptive behaviours that represent more than just an acute (reflexive) nociceptive response, several further tests have been devised. These tests each allow some assessment of defined spontaneous motor activity that can be interpreted as pain behaviours. They are based on the injection of pain-producing chemicals into subcutaneous or deeper tissues in awake, freely moving animals. Examples include:

1) The formalin test: dilute (1-2%) formalin in injected subcutaneously into a limb and pain behaviours (flinching, licking, shaking) are scored in awake, freely moving animals (Dubuisson and Dennis, 1977)
2) The acetic acid test: as above but using acetic acid (Finck 1988).
3) Intradermal capsaicin: as above with capsaicin
4) Quinolone writhing test: the number of writhing movements are scored following an intra-peritoneal injection of quinolone.

These tests in contrast to the acute reflex tests, model persistent activity in nociceptive afferents that typically accompanies tissue injury and the inflammatory response. Although these tests may induce some degree of tissue damage, they are not considered to be specific models of inflammation. (Models of inflammation are specifically dealt with in Chapter 4).

The essential features of these tests, as pointed out by Dubuisson and Dennis in their description of the formalin test are
i) the algesic substance produces a distinctive and consistent response. At least some experience of the substance in humans must be available and documented.
ii) the time course of the pain behaviours are convenient for laboratory experimental design (1/2 hr to 2 hrs)
iii) objective behavioural responses are recorded as categorical or ordinally ranked data. From this, a numeric scoring system can be developed.

Further particular advantages of the formalin test are that animals need minimal restraint allowing on going behaviours to be monitored for autonomic changes and idiosyncratic behaviours that may be associated with analgesic interventions. Furthermore, in rats the pain ratings appear to be bi-phasic suggesting that two different nociceptive mechanisms are being modelled- acute direct nociceptor activation and a second inflammation-induced nociception.

(The formalin test is dealt with in more detail in Chapter 4)
Allodynia and hyperalgesia are modelled by carrageenan, capsiacin, mustard oil and formalin. Allodynia is quantified by measuring a drop in mechanical or thermal thresholds for eliciting a withdrawal reflex, while the measurement of hyperalgesia requires some form of quantifying the size of the reflex withdrawal response.

Novel applications of some of the above algesic substances have allowed referred visceral pain to be modelled. Intravesicle turpentine (McMahon 1987) and colonic capsaicin and mustard oil application each result in a reduction in cutaneous sensory thresholds measured in related dermatomes (Laird 2000).

An obvious disadvantage of all these tests is the potential for severe tissue damage and severe or prolonged pain. This prompted Wheeler-Aceto et al to compare a range of possible algesic chemicals in the search for the optimal agent. They noted that the original scoring method described by Dubuisson was difficult to complete (Dubuisson described a calculation in which the time spent in each of four behaviour categories was weighted and summed) and chose to simply compare the time spent in each of two behavioural categories (flinching and licking). Their results show that both formalin and acetic acid produce intense nociceptive responses but that the response to formalin is of longer duration and more distinctly bi-phasic than that of acetic acid. Yeast also produces a prolonged nociceptive response but of a much lower intensity than that of formalin. Carrageenan, yeast and platelet aggregating factor (PAF) each cause marked oedema with only minimal nociceptive behaviour. Suprisingly, mustard oil applied topically produces neither oedema nor nociceptive behaviour.

These results are summarised in the following table:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intensity of response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flinching</td>
</tr>
<tr>
<td>Formalin</td>
<td>+++</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>+++</td>
</tr>
<tr>
<td>Yeast</td>
<td>+</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>+/-</td>
</tr>
<tr>
<td>Serotonin</td>
<td>-</td>
</tr>
<tr>
<td>PAF</td>
<td>-</td>
</tr>
<tr>
<td>Kaolin</td>
<td>-</td>
</tr>
<tr>
<td>Mustard oil</td>
<td>-</td>
</tr>
<tr>
<td>Saline/control</td>
<td>-</td>
</tr>
</tbody>
</table>

Of the agents tested, the authors suggested formalin to be the best for pain testing as it produces the most robust spontaneous response with the most convenient time course. Its bi-phasic response pattern lasts for about an hour (Dubuisson and Dennis, 1977). Carrageenan on the other hand induces no flinching activity but does
induce hyperalgesia to both thermal and mechanical stimuli that peaks between 2 and 4 hours after injection. Yeast and carrageenan may be better used to model peripheral inflammation than tonic pain. This is consistent with the finding of Heapy et al (1987) who showed that formalin but not carrageenan or yeast caused an immediate and intense increase in the spontaneous activity of C-fibre afferents. The other agents could not be recommended as either algiesic substances nor as inflammogens. A further description of both formalin and carrageenan is given in Chapter 4.
Chapter 2  THE WITHDRAWAL REFLEX AS A MEASURE OF NOCICEPTION IN NEONATAL RAT PUPS

2.1  Aims of Chapter 2

The utility of electromyographically recorded withdrawal reflex responses as a tool for investigating spinal cord sensory processing has been established. Application of this tool has included its use to determine organizational patterns of the spinal reflex arc (Holmberg and Schouenborg, 1996b), investigation of changes induced by inflammation and acute noxious inputs (Solano and Herrero, 1999) and assessment of drug pharmacodynamic effects (Herrero and Headley, 1996).

Significant confounding factors related to the use of differing forms of anaesthesia at each developmental stage, the necessity for spinalization and the use of electrical stimulation rather than natural stimulation suggested that the technique had severe limitations (Jiang and Gebhart, 1998).

An attempt to overcome some of these limitations is presented in this chapter. The use of an anaesthetised intact rat for measurement of the withdrawal reflex EMG has advantages over the simpler sensory threshold test in awake animals as a stimulus response function can be determined rather than just a threshold. Statistical variation is reduced by reducing the amount and number of non-specific factors that influence the reflex (Banks et al., 1988).

The aims in this Chapter are to demonstrate a stable in vivo preparation for recording this reflex using a consistent form of anaesthesia that allows direct comparison of responses across a developmental range from 3 to 21 postnatal days.

The EMG responses have been characterised by threshold, duration, amplitude and latency primarily using mechanical stimuli (electrical stimuli were used to measure latencies) in un-conditioned rat pups. This work provides control data for the investigation of an inflammatory pain state (Chapter 4).
2.2 Definition of the withdrawal reflex

The withdrawal reflex is a response to painful cutaneous stimuli that results in the removal of limbs from sources of potential tissue damage. It is a protective response involving coordinated muscle contractions at multiple joints through polysynaptic spinal pathways. The size and strength of the reflex muscle contraction is graded according to the intensity of the stimulus. The duration of the motor activity also reflects stimulus intensity and always outlasts it.

With flexion of the stimulated limb, extensor muscle groups in the opposite limb are excited and flexors inhibited thus enhancing postural support when withdrawing from painful stimuli. This spread of reflex muscle activity to the contralateral limb remains appropriate in what is termed the crossed extensor reflex. (Gordon 1991)

As the above description implies, the withdrawal reflex has generally been thought to be organized as a flexion reflex. That is, it involves simultaneous activation of predominantly flexor muscles and inhibition of extensor muscles from large receptive fields. More detailed studies in rats suggest that the withdrawal reflex is organized in a more task-specific manner.

The anatomical organization of the withdrawal reflex allows nociceptive cutaneous receptive fields to be linked to specific muscles and muscle groups such that the reflex results in the appropriate withdrawal of the area of stimulated skin (Weng and Schouenborg, 1996). Reflex responses within individual muscles and muscle groups vary in both spatial and temporal characteristics and can be differentially inhibited by skin stimulation. Each muscle therefore has both inhibitory and excitatory cutaneous receptive fields suggesting that the reflex is organized in a modular fashion, each module being concerned with a particular muscle. The withdrawal reflex can therefore be thought of as an integrated series of parallel reflex pathways each subserving different muscles (Schouenborg et al., 1995).

2.2.1 The reflex arc- an historical note

As part of his dualistic doctrine of the nature of man, Rene Descartes (1596-1650) did provide a purely mechanistic account of involuntary movement. This, to some extent comprises the first formulation of the concept of reflex action. Much time
and debate (much of it acrimonious) were to pass before physiological reflexes were to achieve their current status i.e. a basic aspect of physiology and psychobiology. The following three quotes are taken from the early history of the development of the concept.

"External sensory impressions are carried at great speed along the whole length of the sensory nerves to their origins, where they are reflected in accordance with a certain law and pass into certain corresponding motor nerves through which they are transmitted very swiftly to the muscles where they produce certain definite movements".

Jiri Prochaska (1749-1820)

there is a property of the sentient and motor system of nerves which is independent of sensation and volition; a property of motor nerves independent of immediate irritation....a principle of action in the animal economy....which I have ventured to give the designation of the reflex"

Marshall Hall (1832-1857)

"The extent of reflex movement depends principally on the strength of the stimulus and on the degree of the excitability"

A.W. Volkmann (1838)

At the turn of the century, meticulous drawings from histological preparations allowed Ramon y Cajal to question the prevailing reticular theory of the nervous system and develop the theory of individual neurons. The influence of this work on the understanding of reflex spinal activity can be seen in Diagram 3.

Diagram 3 Changing concepts of sensori-motor connections in the spinal cord.

During the last two decades of the nineteenth century, the work of Sir Charles Sherrington (1857-1952) established the reflex as the most "elementary functional unit of integrative activity" of the nervous system. His work took advantage of the discovery of the neuron, saw the development of the concept of the synapse and included the analysis of much anatomical data regarding muscle innervation.
The spinal reflex arc can now be considered to be the first stage in integration of nociceptive information. Interposed between an afferent and efferent limb the spinal cord segment integrates information from both the periphery and higher centres and facilitates motor neuron responses, autonomic responses and ascending pathway activation.

The neurons interposed between dorsal root fibres and motor neurons, no doubt form a complex system with an elaborate pattern of convergence both excitatory and inhibitory. At any point in time, the system is most probably in a dynamic equilibrium - dependent on i) the convergent activity of multiple afferents ii) the history of neuronal firing and iii) tonic and phasic inputs from descending pathways (Wall 2000)

2.3 The withdrawal reflex and pain

Although cutaneous reflexes are not necessarily absolute and direct indicators of pain perception, they do provide information about the sensitivity and selectivity of the nervous system to nociceptive stimuli. Any reflex response can be used as a measure of pain provided that the reflex was elicited by stimulation of pain nerve endings only (Goetzl 1943). Four criteria have been suggested for selection of a standard response:

1) clearly perceptible to the observer
2) clear distinction between minimal and sub-minimal stimuli
3) reproducibility (constant appearance during repetitive application of identical stimuli)
4) clearly definable onset.

Implicit in the above criteria is the assumption that the threshold of the withdrawal reflex is directly correlated with pain perception. Establishing 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 (paediatric and developmentally delayed) humans.

Critical experiments aimed at establishing this association were performed by Willer in which he showed that the intensity of stimuli that reach the pain threshold is almost identical to that which activates the flexion withdrawal reflex in human volunteers (Willer, 1977). (see Diagram 4).
Diagram 4 Reflex, sensory and pain thresholds.

The strict relationship between the threshold of the withdrawal reflex and pain perception in humans lies at the centre of all animal models of pain that are based on a measurement of reflex activity. Wilier demonstrated this relationship in adult volunteers using an electrically evoked reflex. Here he showed that the electromyographic appearance of the reflex coincided with the threshold for pain. The heavy arrows indicate the threshold of the R-III reflex response. Further he showed that the intensity of the stimulus (in mA) bore a linear relationship to the pain intensity (VAS score). A relationship between the amplitude of the reflex EMG and pain intensity was not demonstrated as no measure of amplitude was made.

from Wilier (Pain 1977)
The apparent clarity of these experiments must be viewed with caution. Wall argues that these very artificial experiments may suffer from a systematic error in design and that generalizations from sensory thresholds to pain perception may be an unwarranted oversimplification (Wall, 2000).

The complexity of the issue is re-affirmed in studies of thermal nociception. In these, pain intensity and withdrawal magnitude (measured electromyographically) are each well correlated with stimulus strength, but the magnitude of withdrawal does not reliably reflect the intensity of pain sensation (particularly at higher temperatures). In contrast to the studies of mechanical and electrical stimuli, the threshold for withdrawal from radiant heat far exceeds the pain threshold (Campbell et al., 1991).

The withdrawal reflex may therefore be considered a reasonable model for the study of spinal nociceptive processing but not necessarily of pain perception. In specified animal preparations it can be termed the nociceptive withdrawal reflex and its basic anatomy and function can be described in terms of the underlying reflex arc i.e.

i) peripheral nociceptors and nociceptive primary afferents,
ii) neurotransmission between primary afferents and dorsal horn neurons,
iii) somatotopically and “musculotopically” organized interneurons within the dorsal horn of the spinal cord (these project either intraspinally or supraspinally),
iv) motor neurons and muscle groups that act synergistically to effect limb withdrawal.

The value of the withdrawal reflex in investigating spinal nociceptive processing was demonstrated by Woolf et al in chronic decerebrate rats wherein the effects of peripheral injury were shown to result in prolonged changes in reflex excitability (Woolf, 1983). In these experiments tissue inflammation or brief high-threshold afferent inputs were able to change the receptive field properties of flexor motor neurons. The recognition that these activity-dependent changes represented the central component of post-injury pain hypersensitivity and were basis of many pain behaviours confirmed the withdrawal reflex a powerful tool in pain research(Woolf, 1984; Woolf and McMahon, 1985).

Further, the reflex has been used extensively to evaluate “antinociceptive” agents, an increase in the threshold of the response being interpreted as positive analgesic action. More broadly, the withdrawal reflex arc is the first stage in sensory processing of nociceptive information and as such may reveal the basic principles involved in higher order processing and of the processing of other sensory modalities. Its utility as a model of spinal sensorimotor integration must lie in its being a “looped” system i.e. it has an “output” that directly alters its “input”. Like any feedback loop, its function (response characteristics) can be controlled by balancing excitatory and inhibitory influences.
2.4 Stimuli required to evoke reflex withdrawal

In spinalised rats maintained innocuous pressure, noxious cold (1°C) and heat (45°C) and mustard oil all produce reflex withdrawal. On the other hand, vibration (10-100Hz), non noxious temperatures (17-41°C) and intradermal histamine produce either weak or no reflex response. These results confirm that withdrawal reflex pathways involve a selective set of cutaneous receptors and that individual muscles are activated by the same type of inputs (Weng and Schouenborg, 1998).

The most sensitive nociceptive fibres innervating nonsensitised glabrous skin in the rat have a threshold of 45mN (Leem et al., 1993). Reflex mechanical thresholds however, are dependent on experimental conditions. Acute spinalisation results in markedly raised thresholds (300mN) (Woolf and Swett, 1984) whereas in the delayed phase after spinalisation (7-13 hours later) thresholds may be as low as 29mN (Weng and Schouenborg, 1998). In awake, non-stressed rats mechanical thresholds for eliciting the withdrawal reflex average 90mN (Holmberg and Schouenborg, 1996b).

Although some forms of innocuous stimulation can evoke reflex withdrawal, nociceptors clearly provide the major input from skin to the reflex pathways. Non noxious pressure and hair movement is observed to evoke mild responses when compared to noxious pressure and heat. Importantly, skin inflammation and tissue injury can significantly increase the size of the response to normally innocuous stimuli. Similarly, repeated application of heat to mechano-nociceptors results in a reduction of their threshold to thermal stimuli (Hendry, 1999).

Reflex thresholds also show a striking developmental pattern. In both young rat pups and human infants, reflex withdrawal can be elicited by much weaker stimuli than those required in adults (Fitzgerald et al., 1988). Both the mechanical (Stelzner, 1971) and electrical (Collier and Bolles, 1980) thresholds for eliciting the withdrawal reflex are lower in rat pups compared to adult animals (Fitzgerald, 1985). In pups responses are also much more prolonged. These differences are unlikely to be due entirely to changes in skin mechanics (Evans and Rutter 1986).

Thermal thresholds too are lower in 3 day old (Postnatal day 3, P3) rats and rise steadily until postnatal day 15 (P15) from when they resemble adult values. These changes in thresholds are distinct from changes in the temperature-response function or "system gain". The gain appears to be higher in younger pups. Significant habituation of the response is noted during the first week of neonatal life and this is thought to reflect the inability of a maximally excitable (sensitive) spinal neural system to maintain organized reflex responses in the face of repeated inputs (Falcon et al., 1996).
2.5 The reflex arc -from peripheral nociceptor to motor neuron

2.5.1 Nociceptive primary afferent fibres

Stimulus transduction through the skin is a function not only of the histological structure of specialised receptors but also of the position of these structures within the skin, their arrangement across the skin surface and the conduction characteristics of the peripheral nerve fibres that innervate them.

Histologically, nociceptors do not possess a cellular capsule and are therefore described as bare endings of nociceptive cutaneous fibres. The first step in signal transduction at these “bare endings” is thought to be mediated by a variety of membrane receptors/ ion channel complexes. Thermal stimuli may be transduced by the vanilloid receptor (which can also transduce low pH) while tissue damage may be transduced by a variety of receptors including: DRASIC (dorsal root acid sensing ion channel), the kinin (BK2) receptor and the purinergic (P2X) receptor. Unmyelinated fibres expressing these receptors/channels appear to be regulated by the neutrophin -nerve growth factor (NGF) (Lewin 1994b). Noxious mechanical signal transduction may be accomplished by an ion channel belonging to a superfamily of sodium channels that includes epithelial amiloride-sensitive sodium channels (ENaC) and neuronal acid-sensing ion channels (ASICs) (Driscoll 2000).

Within the latter group, the BNC1 channel is emerging as a prime candidate for a mechanosensory channel as it possesses the greatest homology with the degenerin family of channels which have been implicated in touch-transduction in the nematode, C. elegans (Waldmann, 1997). It is likely though that mechanosensitivity in mammals is conferred by multiple protein complexes-most of which are as yet undefined and may include a sodium channel subunit. Regulation of at least some of these gene products has recently been shown to be influenced by the neurotrophins, in particular BDNF (Carroll et al., 1998; Snider, 1998).

Nociceptive fibres can be divided into two functionally distinct groups: those that respond to intense mechanical stimuli- (high threshold mechanoreceptors) and polymodal nociceptors. The former are subserved by axons in the Aδ range (conduction velocity 15-30 m/s) and in humans possess a receptive field distributed as 5-20 small spots over an area of about 2-3 mm in diameter in humans. Polymodal nociceptors respond to intense mechanical stimuli as well as noxious heat and chemicals. Their axons -unmyelinated C fibres, make up the majority of the slowly conducting fibres in a peripheral nerve and in the dorsal roots of all mammalian species (Willis and Coggeshall, 1978). By sheer weight of numbers, nociceptive primary
afferents (intense stimulus and damage sensing fibres) are able to distribute cutaneous inputs over several spinal segments. The withdrawal reflex generated by this input therefore involves multiple muscle groups and joints. In the rat hindlimb these nociceptive fibres are carried in several peripheral nerves including the saphenous, sural, lateral sural, superficial peroneal and tibial nerves. These nerves collectively innervate the entire skin surface of the foot and most of the skin surface of the leg. (A description of the anatomy of these nerves and the cutaneous afferent fibre types in the rat hindlimb is given in Appendix 1).

In addition to the cutaneous afferent fibres above, muscle group II-IV fibres and joint fibres (both low and high threshold) are also involved in the flexion reflex.

2.5.2 Non nociceptive primary afferents

A variety of fibres mediate non-noxious stimuli. These include fibres innervating G- hair cells, Merkel domes, pacinian corpuscles, rapidly adapting mechanoreceptors (RAMs) and slowly adapting mechanoreceptors (SAMs), D cells and cold fibres. These all tend to be myelinated Aβ fibres with conduction velocities between 42 and 72m/s (Hendry, 1999). Activity in these fibres does not appear to generate the withdrawal reflex in the intact awake animal despite significant (oligosynaptic) contact with motor neurons. However, in decerebrate spinal rats, the fact that sustained innocuous pressure but not vibration can elicit reflex activity suggests that slowly adapting but not rapidly adapting mechanoreceptors do contribute to withdrawal pathways (Weng 1998). These inputs are most likely inhibited in by descending spinal circuits in the intact rat.

2.5.3 Dorsal horn neurons

The location of the cell bodies of the flexor reflex interneurons is not defined though some neurons in the substantia gelatinosa are known to have ventrally directed axons and some neurons in laminae 4 and 5 have terminal dendritic fields in the superficial dorsal horn (Woolf and Swett, 1984).

Electrophysiologic study of the interneurons involved in this reflex has helped to define both a somatotopic and “musculotopic” pattern of cells within the dorsal horn. These studies also allow the characterisation of neurons according to their activation stimuli. Confirming similar findings from other species and earlier work by Wall et al (1967), Menetrey et al distinguished three classes of neurons in the spinal cord of spinalised rats. Class I neurons are activated by tactile input only, class II (multireceptive) are activated by innocuous mechanoreceptive and nociceptive inputs and class III neurons are activated by nociceptive inputs only (Menetrey et al., 1977). A
similar and more familiar classification uses the following nomenclature: low mechanical threshold only (class I), wide dynamic range (class II) and nociceptive specific (class III) neurons are identified (Woolf and King, 1990). The classification of dorsal horn (DH) neurons can be extended to include a division of class II neurons into superficial (2S) and deep cells (2D). (The superficial group is found within the region of the Aβ-fibre-evoked field potential) (Schouenborg and Sjolund, 1983). Correlations between motor nerve evoked activity and DH neurons exist for neurons of the 2D type i.e. they have a delayed peak in C-fibre evoked activity that precedes and overlaps the onset of the late motor reflex. Furthermore their prolonged discharge is, like the motor reflex, strongly potentiated by repetitive stimulation. This class of DH neurons (2D and/or 2S) are most likely intercalated in the late (withdrawal) reflex pathway. As class III neurons have a significantly different time course and class I neurons display only weak C-fibre evoked responses with little potentiation, it is unlikely that these particular neurons play a major role in driving the withdrawal reflex (Schouenborg and Sjolund, 1983).

On the basis that the receptive field of the hindlimb withdrawal reflex always includes part of the plantar side of the paw, neurons directly involved in this reflex can be localised to the 4th and 5th lumbar segments. These neurons have a dorso-ventral distribution pattern reflecting the input stimulus that activates individual neurons. Wide dynamic range (WDR) neurons (class II: those responding to mild nociceptive stimuli as well as non-noxious mechanical stimulation) are found in laminae III-IV. Similar (WDR) neurons with greater noxious stimuli thresholds are located slightly deeper (laminae V) while nociceptive specific (class III) neurons are preferentially located in the most superficial laminae (I-II). Interestingly, the latter group of neurons, though small in number, display a pattern of receptive fields that is different to the neurons situated in deeper layers of the dorsal horn. Receptive fields of these nociceptive specific neurons tend to be smaller than for those of deeper laminae. Neurons in the superficial dorsal horn maintain a somatotopic organization whereas those of the deeper layers may be organized in a musculotopic arrangement (Schouenborg et al., 1995)).

This musculotopic pattern is traced in a medio-lateral direction starting with plantar flexors of the digits and in succession, the plantar flexors of the ankle, the pronators, the dorsiflexors of the ankle and most laterally the flexors of the knee.

A group of these neurons show a spatial input-output relationship similar to that of the reflex pathway to single muscles i.e. there are identifiable interneurons whose receptive fields appear to match those of single muscles. This finding becomes the basis of the organizational hypothesis proposed by the Schoenbebog: the existence of nociceptive interneurons that exhibit task-specific receptive fields and can thereby
encode the spatial organization for single muscles supports a basically modular pattern of organization for the withdrawal reflex (Schouenborg et al., 1995).

Differences in the temporal characteristics of activation of individual muscles may be interpreted to imply that the last order neurons in the withdrawal reflex path are not shared by heteronymous motor-neuronal pools. These last order neurons are likely to receive a large number of convergent inputs (Schouenborg and Kalliomaki, 1990).

Although it has not been possible to date to demonstrate a direct anatomical linkage between the primary afferent inputs and the motor output stage of the reflex, a good spatial relationship between these two limbs of the arc has been shown in lumbar spinal cord section by Woolf (see diagram 5) (Woolf and Swett, 1984).

**Diagram 5**  
**Determination of afferent terminations and motor neuron cell bodies intercalated in the withdrawal reflex**

a) This representation displays the spatial relationship between the afferent fibres of the withdrawal reflex arc and its motor output. The spatial distribution of the terminations of C fibre afferents have been studied using histochemical markers e.g. HRP. Cutaneous C afferents terminate with some precision in laminae I and II (Swett and Woolf 1985).

b) A drawing after Cajal (1909), showing several sensori-motor connections, both direct (left) and indirect (right) in the spinal cord of a newborn rat. Collateral fibres can be seen coursing to the ventral motor nuclei, intermediate grey matter and substantia gelatinosa. The exact topology of the interneurons connecting the substantia gelatinosa and flexor motor neuron pool is not clearly defined.

More important than a strict anatomical linkage between afferent fibre and motor neuron is the fact that the linkage is dynamic. Early experiments in chronic decerebrate rats by Woolf clearly demonstrated that cutaneous receptive fields of motor neurons could be altered by conditioning stimuli such as tissue inflammation (Woolf, 1983).
These electrophysiological studies demonstrated that mechanoreceptive fields for each flexor motoneuron are variable and can be very large implying that the interneurons that subserve the input-output linkage must allow for massive convergence of cutaneous afferent input to each motor neuron (Woolf and Swett, 1984).

Later studies showed that these same dynamic alterations in receptive fields occurred in dorsal horn neurons also and that these changes were also accompanied by changes in firing properties (thresholds and duration of firing) (Cook et al., 1987). Various conditioning stimuli were found to be effective in inducing these changes including electrical stimulation (Cook 1987), local inflammation (Hylden 1989b), injection of various algesic agents either into joints or muscle (Schepelmann 1992; Hoheisel 1989) or noxious heat (McMahon 1984). In each case, the activation of small diameter afferents (C-fibres) during conditioning appears to be crucial and changes are most noticeable in wide-dynamic range (multireceptive, class II) neurons. These changes are not seen in low-threshold only neurons (Woolf and King, 1990). These findings are in keeping with the conclusions of Schoenburg described above, regarding the neurons intercalated in the withdrawal reflex. It is likely that at least in adults, spinal neurons involved in the withdrawal reflex are wide-dynamic range neurons with direct or indirect C-fibre inputs. These neurons are therefore subserved by Aδ and C fibre primary afferents rather than Aβ afferents.

Both the nature of the conditioning stimuli and the time course of the spinal cord hyperexcitability allowed immediate parallels to be drawn with post-injury hypersensitivity states in humans (Raja 1988).

2.5.4 Spinal Motor processing

The production of complex motor patterns is a highly distributed process involving neurons across many brain areas. Recent studies of spinal cord motor processing have confirmed that the spinal cord is an active participant in complex aspects of motor control such as planning, plasticity and organization of movement (Bizzi 2000). In primates, spinal interneurons modulate their activity in the preparatory stages of motor output (Prut and Fetz 1999). Particular spinal neurons (e.g. within the dorsal spinocerebellar tract) have been shown to provide high level sensory processing rather than a simple information relay from the periphery to higher centres (Bosco and Poppele 1996). Experiments in cats involving both denervation of muscle and subsequent spinalization demonstrate that some degree of the adaptive process following denervation takes place at a spinal level (Pearson et al 1997). Developmental studies in rats involving tendon transfers similarly, show that organization of reflex motor withdrawal patterns is a function of postnatal activity-dependent processes that
occur at a spinal level (Schouenberg 1994). Taken together, this data has prompted the hypothesis that co-ordinated movement is mediated by a relatively small set of spinal modules or behavioural units. From this limited number of responses, complex motor activity can be produced through a combinatorial mechanism. Further evidence for such a modular arrangement has been obtained from experiments measuring position-dependent forces generated by spinal microstimulation in frogs (Saltiel 1998). The withdrawal reflex then may be viewed as more than a simple protective escape mechanism but rather one of a set of basic spinal motor modules from which all complex behaviours are assembled.

2.5.5 Flexor motor neurons and motor units

Traditional retrograde chromatolysis studies have demonstrated that individual muscles are innervated by tightly grouped columns of cells in the ventral horn (Kaizawa 1970). Within each muscle, individual fibres are innervated by several interdigitating motor units (Weng and Schouenberg, 1998).

The motor neuron pool specifically involved in the flexion reflex forms a long slender pool of cells in laminae IX of the central horn, spanning spinal segments L3 to L5. Ninety five percent of these cells reside in the L4-5 segment. Significantly, the spatial distribution of the afferent fibres travelling in the sciatic and saphenous nerves lies in the medial portions of the superficial dorsal horn from caudal L2 to caudal L5. This distribution achieves its maximum width in mid L4, occupying the medial 3/4 of laminae I and II. In essence, afferent fibres from the skin distal to the knee are distributed over the same spinal segments from which hamstring motor neurons issue.

Skeletal muscle consists of fibre types with characteristic contractile properties (slow, fast-fatigue resistant and fast-fatiguable). Firing patterns in individual motor units also correspond closely to the fibre type (Hennig and Lomo, 1985). This allows control and modulation of muscle activity appropriate to postural tone, locomotion and fast withdrawal/acceleration (Hennig and Lomo, 1985).

The typical pattern of activation then, is “slow” followed by “fast-fatigue resistant” and finally “fast fatiguable” muscle fibre types. In both humans and cats the synaptic inputs resulting from cutaneous stimulation differentially inhibit small motor neurons (and therefore slow muscle fibres) and excite high threshold (fast fibre) motor neurons. The consequence of this is that reflex muscle activity in response to (noxious) cutaneous afferent activity will be characterised by early activation of fast muscle fibres in preference to slow fibres whose slow relaxation phase may be disadvantageous when rapid motor responses are required (Crone and Nielsen, 1989; Rudomin, 1990).
2.6 Developmental Aspects

2.6.1 The development of reflex activity in rats - in vivo

The early investigation of motor function often focussed on a debate on the origin and development of integrated motor patterns of higher vertebrates. Two approaches were apparent in the early work on mammalian motility. The first assumed behaviour to be integrated from the earliest stages through to the appearance of complex action patterns (Coghill, 1929). The second approach stressed the primacy of local reflexes. Complex action patterns were then viewed as the product of secondary integration of local reflexes (Hooker 1930 and Windle 1940).

In experiments that are unlikely to be repeated, Narayanan et al addressed this issue by observing spontaneous and evoked activity in rat foetuses from E16 to E20. Their general findings were that foetuses are intermittently active and that total activity shows phases during development - commencing at E16, rising to a peak around E18 and then declining to a lower level prior to birth (Narayanan et al., 1971). This suggested the appearance of spontaneous movement prior to reflex activity which in much earlier experiments on rat foetuses "in vivo", could only be evoked by cutaneous stimulation from E17.5 (Angulo y Gonzalez 1932). In later experiments though dorsal root stimulation was found to evoke a polysynaptic response in the ventral root as early as E 15.5 "in vitro" and a monosynaptic response at E17.5 (Saito, 1979). Narayanan et al described three forms of motor activity - generalized or mass activity, regional and local activity. General mass activity is noted early in development and appears to be un-integrated and aimless (in the sense of "random"). When resulting from stimulation, these movements appear as a "startle" response. Evoked responses occur first in the neck and forelimb in response to stimulation of either the snout or forelimb. In later stages, these responses become discrete and local. The progression of these local responses is not perfectly cephalo-caudal and proximo-distal but appears to reflect a precocious development of patterns that are relevant to immediate post-natal suckling.

In conclusion, these researchers suggest that early mass activity contributes little to the gradual emergence of complex action patterns and that the ontogeny of integrated patterns is essentially an increase in complexity and expansion of originally weak and simple local responses. The build up of complex behavioural patterns then must be intimately tied to the closure of local reflex neuronal circuits. Experiments on decapitated foetuses suggests also that much of this behaviour and the related circuits must occur at a spinal level. Higher neural centres, rather than being generators of fully integrated motor activity, are more likely to act by modulation and inhibition of local reflexes (Narayanan et al., 1971).
At the time of birth evoked motor activity still tends toward a generalised or mass pattern. Wriggling and hyper-reactivity can be elicited by punctate and noxious stimuli but as yet, newborn rats lack a discrete “local sign” i.e. motor activity is not oriented with respect to the site of stimulation. This together with the gradual development of diagonal progression across limbs, consistent scratch frequency and opposite limb support all support the progressive postnatal closure of propriospinal reflexes and the late maturation of supraspinal connections. Discrete placing and fine adjustments of the hindlimb which are dependent on corticospinal connections are only observed from 16 days onward (Stelzner, 1971).

Cutaneous flexor reflexes in newborn rat pups can be elicited with much lower (non noxious) mechanical stimulus strengths than in adults and show more synchronized and persistent responses. Up to postnatal day 8 (P8), the reflex is sensitized by repetitive stimulation and only after this time does it show the more mature pattern of habituation (Fitzgerald et al., 1988). Thermal thresholds too are lower in neonatal rat pups compared to adults (Marsh et al., 1999). Responses to noxious mechanical and electrical stimuli are also characterised by prolonged durations that outlast the stimulus duration. In contrast, the responses to C-fibre chemical irritants are often weak in young rat pups.

With increasing age, rats display increasing thresholds to electrical stimulation and differential intensity-dependent behaviours. Only by P20 is the full range of graded responses shown i.e. flinching at detection (threshold) levels through to well developed escape, running and attack responses at high intensities. In conclusion, while sensitivity decreases with age, both intensity discrimination and the range of behavioural responses develops gradually over the first two weeks of neonatal life (Collier and Bolles, 1980). This pattern may be a manifestation of the generally less well developed organization of cutaneous nociceptive reflexes before the second postnatal week in rats (Fitzgerald, 1995). It has also been interpreted as a reflection of relatively weak intrinsic or descending inhibitory mechanisms in the neonatal spinal cord (Jiang and Gebhart, 1998). While significant maturational processes occur in peripheral nociceptors, primary sensory afferents, dorsal horn interneurons and spinal inhibitory pathways, possibly the most significant factor contributing to the developmental pattern of reflex excitability is the maturation of spinal cord connections that occurs postnatally. In early postnatal life, prior to the completion of C-fibre ingrowth and synaptic contact, large diameter myelinated fibres (Aβ) transducing low threshold stimuli make synaptic contacts within the substantia gelatinosa of the superficial dorsal horn. This excitatory input may be the basis of exaggerated low threshold reflexes described above (Fitzgerald, 2000).
Maturation of the withdrawal reflex is a process of increasing functional adaptation which results in appropriate reflex limb movement (i.e. away from a noxious stimuli). Altered muscle attachments (tendon transfers) and sensory inputs (selective nerve lesions) in early development have only minimal effects on the withdrawal pattern in adulthood (Holmberg and Schouenborg, 1996a). This suggests that the developmental process must be experience-dependent rather than being pre-determined and inherent in neural and neuro-muscular connections. Developmental regulation of the withdrawal reflex appears to take into account both the spatial localisation of sensory inputs as well as the actual pattern of limb movement (Holmberg et al., 1997).

The abolition of descending controls during early development results in disrupted organization with persistent neonatal features including erroneous nocifensive movements towards the stimulus. This suggests a critical role for supra-spinal structures in the post natal tuning of the withdrawal reflex (Levinsson et al., 1999).

2.6.2 Developmental patterns of reflex activity in humans

The earliest reflex movements in humans are perioral reflexes (Hooker 1952) and are noted as early as 8 weeks of gestation (20-23 mm CR length). More caudal segmental reflexes appear some weeks later. Bergstrom (1963) first observed stretch reflexes in human foetus at 98 days gestation. Spinal reflex activity was said to be slow and characterised by tonic responses that lacked reflex inhibition. A pronounced radiation to adjacent muscles and antagonists was also described. The results of these studies—completed in exteriorized foetuses being aborted (and therefore in a poor physiological state), have been better demonstrated using real-time ultrasound intrapartum. These later serial studies demonstrated the earliest, “just discernible” movements at 7.5 weeks gestation. A “startle” movement—(a quick generalised movement initiated in the limbs and spreading to neck and trunk) was the next recognisable pattern and first occurred between 8 and 9.5 weeks. Isolated spontaneous limb movements are noted from 9.5 to 10 weeks gestation - a week before the exteriorized foetus shows evoked limb movement. While some agreement with the findings of Hooker are noted, the major finding of the in-utero ultra-sound studies is that spontaneous movements are common and appear to be a fundamental expression of early neural activity (de Vries et al., 1982). The timetable of these movements does bear a reasonable relationship to that of the reflex movements demonstrated in the ex-utero experiments of Hooker (1952) and is presented graphically in Diagram 6a.
The advent of real-time ante-natal Ultra-sound scanning allowed unprecedented observation of the human foetus in its undisturbed environment. In this study, de Vries et al (1982) studied qualitative aspects of foetal motility and plotted the onset of 16 specific movements against gestational age. The first movements were observed at 7.5 weeks and by 15 weeks all the patterns could be observed. Spontaneous foetal movements are specific, consistent and recognisable. Evoked (reflex) movements are more difficult to study as the base-rate of spontaneous movements is high and prolonged observation times are required.

Evoked reflex activity has been utilised for many years in clinical neonatal practice to assess the maturation of the developing nervous system. Several well characterised reflex movements show strict developmental patterns. "Primitive reflexes" are detected from birth and disappear with age whereas other reflexes become apparent later in neonatal life. A reversion to more primitive reflexes often accompanies nervous system damage. This plot of the appearance of various reflexes (taken from Gingold et al 1998) shows a striking maturation process occurring at 4-6 months of age.
The spontaneous movements do not appear to be uncoordinated and random but are specific, recognizable and have a consistent ontogeny. As spontaneous movement has a high frequency, movements in response to stimulation (trans-abdominal) is difficult to discern. The qualitative aspects of many of the in-utero movement patterns closely resemble those observed in preterm and fullterm newborns.

Cutaneous reflexes in postnatal human infants can be clearly elicited with von Frey filaments from as early as 23 weeks post conceptional age (PCA). These infants typically display low mechanical thresholds that then display a striking age related increase (Fitzgerald et al., 1988). Apart from low thresholds which results in reflex responses to non-noxious stimuli, withdrawal reflexes in human infants are also characterised by being prolonged and receptive fields are often large.(Andrews and Fitzgerald, 1994). Exaggerated responses result from synchronous activation of agonist and antagonist muscles and the spread of excitation to adjacent muscle groups.

Repeated stimulation (at 5 second intervals) results in a striking build up of the withdrawal response at earlier ages (Fitzgerald et al., 1988). This pattern of sensitisation is gradually replaced by one of habituation to repeated stimulation as infants reach 29-35 weeks post-conceptional age. A flexor reflex response to mechanical stimulation in human infants can often be elicited by what would be considered innocuous stimuli in the adult and results in a diffuse non-localised response (Andrews and Fitzgerald, 1994).

Further, limb withdrawal in neonates involves not only the knee flexors (biceps femoris etc) but also the ankle dorsi-flexor, tibialis anterior muscle. Spread of the reflex to this muscle becomes restricted with increasing age (Andrews 2000).

Many of these findings find parallels in the developmental patterns already documented for non-nociceptive reflexes. Several studies of the maturation of the stretch reflex in man demonstrate features in common with those described for the withdrawal reflex above. These include: a) a tendency for exaggerated responses in the early newborn period (Issler and Stephens, 1983), b) the synchronous activation of both agonist and antagonist muscles. Electrically evoked forearm responses in neonates to non-noxious currents applied to the fingers result in strong synchronous flexor and extensor contraction (Issler and Stephens, 1983). Inhibition of antagonist muscles appears to be developmentally regulated (O'Sullivan et al., 1991). Finally, c) low thresholds and large receptive fields during early development undergo gradual restriction with age (Myklebust, 1986).

The pattern of reflex development in humans has many parallels with those in rats. It is quite possible that changing synaptic configurations in the superficial dorsal horn, as in the rat, are an important explanation of the exaggerated reflex excitability
noted in human neonates. Specifically, the early input of low threshold afferents (Aβ fibres) to substantia gelatinosa and their gradual withdrawal in the face of C-fibre maturation may be the basis of the behavioural patterns observed. The much greater influence of higher centres on the human spinal cord compared with the rat though, are likely to make the maturation of descending inhibitory fibres relatively more important in human development. Certainly, postnatal maturation of descending tracts is thought to underlie the striking reflex developmental patterns seen during the first year of human infancy. During this time, maturational patterns take the form of a series of well characterised reflexes some of which disappear with age (and are therefore termed “primitive”) and others which appear with increasing maturity. Among the former are the Moro, Tonic neck and Crossed adduction to knee jerk reflexes as well as the Extensor (Babinski) response. Reflexes that appear with increasing maturity include the Neck righting, Landau, Parachute, Hand grasp and Flexor plantar response. The time frame of change for these reflexes is presented in Diagram 6b. In all cases, the changes in appearance of the reflexes are thought to reflect maturation of suprasegmental pathways (Gingold et al., 1998). In as much as primitive reflexes are simply inhibited by these later maturing spinal inputs, the pattern of changing reflexes may represent a change in balance of reflexes rather than “dissappearance” of primitive reflexes.

Importantly, local tissue injury results in significant reductions in reflex thresholds indicating that hypersensitivity states can develop even in the neonatal period. Repeated heel lance in human premature neonates has been shown to result in measurable allodynia (Fitzgerald et al., 1989). A similar finding has also been documented following ischaemic leg injury in infants (Andrews and Fitzgerald, 1999).

The abdominal skin reflex in neonates, like the limb withdrawal reflex also shows significant changes with age and more importantly, the ability to become sensitised in clinically relevant situations of tissue injury (Andrews 2000).

### 2.6.3 Maturation of nociceptors and primary afferents

Cutaneous reflexes can be elicited in the rat as early as embryonic day 17, implying that sensory receptors are functional at this time (Angulo 1932). More recently, foetal primary afferents have been characterised electrophysiologically and found to have small defined receptive fields from E17 onwards and are responsive to mechanical stimulation. Cutaneous afferents can be divided into rapidly and slowly adapting pressure receptors and rapidly adapting low threshold mechanoreceptors. Some afferents are also chemosensitive (respond to mustard oil). Rapidly adapting pressure receptors are the most common foetal afferent (Fitzgerald, 1987a).
By the time of birth skin innervation is apparent and all afferent receptors are present in rat pups though many sensory end organs such as Merkel cells and Meissner's corpuscles continue a maturation process postnatally. Myelinated sensory axons leave the dermal plexus 3 days after birth (P3) and commence innervating hair follicles from P7 in a process that achieves the adult pattern only after 19 postnatal days. Interestingly, a pattern of exuberant innervation and subsequent restriction (typical of muscle innervation) is not apparent during hair follicle innervation. Further, the elaboration of mature central terminals of these fibres appears to occur prior to completion and independently of the peripheral termination (Payne et al., 1991).

Consistent with the presence of sensory terminals at birth is the demonstration of clear receptive fields for dorsal horn cell to both low and high intensity stimulation in neonatal rats (Fitzgerald, 1985). Neonatal mecanoreceptors (both slowly adapting and rapidly adapting) have thresholds similar to those in adults—though their firing frequency (particularly that of low threshold mecanoreceptors) is limited (Fitzgerald, 2000). High threshold mecanoreceptors similarly can be distinguished though firing patterns are not totally mature (Fitzgerald, 1987b). The frequency limitation of these fibres reflects both the immaturity of the end organ and the degree of myelination. Polymodal nociceptors are totally mature at birth as judged by their thresholds and specificity for pinch, heat and chemical stimuli as well as their frequency and pattern of response. Although C fibres appear to mature early their reduced ability to produce neurogenic oedema in neonatal rats perhaps denotes some immaturity in release mechanisms for substance P (Fitzgerald, 1987a).

In essence, the main cutaneous receptors appear to be developed at the time of birth in the rat. Cutaneous mechanical thresholds of primary afferent fibres do not show a marked change with development. These fibres also do not possess particularly large receptive fields in neonates nor do they display long after discharges that are characteristic of neonatal dorsal horn neurons. This inevitably leads to the conclusion that the increased excitability of neonatal reflex activity must have its origin in more central processing mechanisms (Fitzgerald and Jennings, 1999).

Clues to the mechanisms responsible for developmental patterns in reflex activity have been sought in the biochemical phenotype of primary afferent fibres and changes in their expression of surface receptors. Changes in the expression of growth factor receptors and therefore changing sensitivity to the effects of these trophic factors possibly reflect and parallel the phenotypic changes that underlie developmental changes in function. Particular interest has centred on the family of neurotrophins (Snider, 1998). During the first three weeks of rat neonatal life Trk-A expression on a subpopulation of unmyelinated DRG cells is down regulated—resulting in a reduction in their responsive to NGF. In the late prenatal period and up to 1 week post natally, these
cells (which bind the lectin IB4 and do not express calcitonin-gene related peptide (CGRP)) begin to express receptors for glial cell-line derived neurotrophic factor (GDNF) and in postnatal life are dependent on and are possibly regulated by this growth factor (Molliver et al., 1997). Just how this developmental change may alter reflex activity is uncertain but it is of note that the laminar termination of the two populations of unmyelinated fibres differ. Whereas the TrkA expressing (NGF sensitive) population project to lamina I and outer lamina II (IIo), the GDNF receptor (Ret) expressing (IB4 positive) population project to inner lamina II (IIi). The changing pattern of neurotransmitter and receptor expression on primary afferent central terminals is dealt with in greater detail in Chapter 5.

2.6.4 Dorsal horn maturation

Dorsal horn cells first respond to cutaneous electrical stimulation as early as embryonic day 17 (E17) but synaptic efficacy is low and evoked neuronal activity is variable and delayed. Neuronal activity evoked in response to pressure and pinching only becomes apparent 2 days later (E19) (Fitzgerald, 1991). At this early stage, receptive fields are small and both amplitudes and frequencies of spike activity are low. A large increase in responsiveness and receptive field size is seen by embryonic day 20 (E20). From this point, cutaneous mechanical thresholds are noted to be low and natural stimulation can evoke long lasting excitation in dorsal horn cells and reflex muscle contractions that are synchronized and long lasting (Fitzgerald and Gibson, 1984).

In the first post natal week dorsal horn cells display increased excitability. The peripheral cutaneous receptive fields of neonatal DH cells are initially large and therefore necessarily overlap to a greater extent than in the adult. Mean peripheral receptive field size (expressed as a percentage of total hindpaw area) reduces rapidly during the first post-natal week (Fitzgerald, 1985).

Although fine Aδ and C fibre (slow) conduction within peripheral nerves can be detected as early as postnatal day 2 (P2) these fine fibres do not appear to make an obvious contribution to dorsal horn cell responses at either P2 or P5. Importantly, C-fibre evoked activity is not observed in the dorsal horn cells of neonatal rat pups within the first week of postnatal life and repetitive C-fibre peripheral stimulation has no observable effect (Fitzgerald, 1988). During this time A-fibre afferent input assumes a much larger significance in the dorsal horn. Immature substantia gelatinosa (SG) neurons receive inputs in almost equal proportion from Aβ, Aδ and C afferents (51, 46
and 36% respectively) whereas in maturity A\(\delta\) and C fibre inputs predominate (84 and 86% respectively) (Nakatsuka et al., 2000).

Only by P9 is an adult-like pattern of C fibre evoked responses i.e. clear long latency burst of activity observed (Fitzgerald, 1988). Though spike activity is not induced by C fibres, a long latency (2-5 seconds) depolarization of dorsal horn cells can be induced by pure C fibre activity that remains subthreshold (Yanasigawa 1985). Though not immediately functional in transmitting nociception then, the C fibres may alter the sensitivity of other nociceptive and non-nociceptive afferents (Fitzgerald, 1988).

A-fibre evoked responses on the other hand, can be recorded from dorsal horn cells as early as P3. A clear reduction in response latencies to A fibre stimulation is seen with increasing age and with this, the absolute variation in latencies progressively declines also (Fitzgerald and Jennings, 1999). This suggests that the larger fibres form functional synaptic connections more rapidly than the later arriving unmyelinated C fibres. In neonates, repeated stimulation at A fibre intensity (or low intensity mechanical stimulation) results in a sensitisation of dorsal horn cells akin to C-fibre wind-up in adults (Jennings 1998).

In the first week of life, only a few DH cells demonstrate convergent inputs (responses to both innocuous and noxious stimuli) and the proportion increases progressively so that by P21 the adult ratio (57% of cells receive convergent inputs) is evident (Fitzgerald and Jennings, 1999). Cutaneous receptive field sizes are also large in the newborn and undergo a gradual restriction with increasing age (Fitzgerald and Jennings, 1999). This change in the pattern of inputs to SG neurons is likely to be a crucial determinant in the changing pattern of reflex activity observed during development.

A “wind-up like” phenomenon can be seen in electrically evoked EMG responses in 8 day old pups. A slow build up in muscle activity in response to repetitive stimulation is followed by habituation after about 55s (Fitzgerald and Gibson, 1984). Only after the 10th postnatal day, when C-fibre input can be observed, does classical “wind-up” of the type seen in adults, occur and this only occurs in a small percentage of cells initially. The response to A-fibre strength stimulation on the other hand, does display significant sensitization of dorsal horn cells even in the neonate. Its character differs from wind-up in the adult, taking the form of an increase in background activity and prolonged after discharges. Interestingly, this pattern of sensitisation is more marked at younger ages and the percentage of cells displaying the pattern gradually decreases with increasing age (Fitzgerald and Jennings, 1999).
The overall result of the developmental changes described above is that low intensity (non-tissue damaging) cutaneous stimuli give rise to exaggerated central responses and marked motor reflexes in neonatal pups. This occurs despite the delayed function of the unmyelinated fibre system suggesting that unrestricted inputs from larger diameter fibres together with a lack of inhibitory control are the basis of exaggerated neonatal responses. The presence of synaptic inputs from low threshold mechanoreceptive Aβ fibres within the substantia gelatinosa in early development (described in Chapter 1) is likely to be a significant contributing factor. The sequence of development allows innocuous stimuli to elicit in neonatal pups, spinal activity that in the adult would represent a response to noxious stimulation (Fitzgerald and Jennings, 1999). Further evidence for this is obtained from cFos studies. During the period of early development (characterised by the presence of both A and C fibres in lamina II), c-fos expression within laminae I and II can be induced by innocuous and Aβ strength skin stimulation (Jennings and Fitzgerald, 1996). Following the withdrawal of A fibres to deeper layers, only noxious (Ad or C fibre) stimulation will normally evoke the expression of the immediate early gene (Ma and Woolf, 1996).

2.6.5 Motor neuron and motor unit maturation

EMG records of the withdrawal reflex a day after birth are prolonged in response to single pinch, noxious heat and electrical stimulation. The exaggerated response persists until the second week of life and becomes more adult-like in the third (Fitzgerald and Gibson, 1984). Before conclusions can be drawn from these motor responses about spinal cord sensory processing and/or nociception or pain perception, consideration must be given to the developmental changes in motor neurons, neuromuscular transmission and muscle function.

Functional synapses are formed within minutes between motor neuron axons and newly contacted myofibrils which then mature over a prolonged period. The maturation process involves continued arborization as well as withdrawal and editing of synaptic connections. With changes in the efficacy of the synapses, an initial exuberance of contacts is gradually restricted (Hall and Sanes, 1993). Myofibres are initially contacted and innervated by the axons of multiple motor neurons. With maturation, this polyneuronal innervation is rapidly restricted. In the rat soleus and diaphragmatic muscles this process occurs after the first postnatal week so that by the second postnatal week, any one myofibre is innervated by only one motor neuron (Brown et al., 1976).
Less mature motor neurons are characterised by high input resistance (probably related to cell size changes), pronounced after-depolarizations and after-hyperpolarizations, a lack of a clear threshold for repetitive firing and a low maximum firing frequency (Fulton and Walton, 1986). These findings though important do not overshadow the general similarity with adult motor neurons. Electromyographic studies of motor unit activity in unrestrained rat pups confirm a gradual developmental pattern in the first postnatal week. During this time motor unit firing frequency and tonic postural activity gradually increase to resemble the adult pattern by 3 postnatal weeks of age. During this same developmental period, interspike interval variability reduces and only after 10 days does a graded recruitment order become discernible (Navarrete and Vrbova, 1983).

### 2.6.6 Influence of descending pathway on reflex activity in early development

As described in Chapter 1, most major descending tracts to the dorsal horn are present at birth though some specific tracts are known to mature post-natally (e.g. trigemino-spinal, solitariospinal, tectospinal and cerebellospinal tracts). Anatomical tract development of itself does not imply functional (synaptic) maturity which is probably achieved much later (Leong et al., 1984). Spinal cord transection in early postnatal life (before P15) in the rat results in very different behaviour responses compared to lesions in later life. Following early lesions, postural responses recover rapidly and only small deficits in reflex patterns remain. This suggests that descending fibre tracts only provide major modulation of reflexes at later stages of development (Weber and Stelzner, 1977).

This is borne out in the pattern of reflex excitability following spinal cord transection in adult life. Following a period of flaccid paralysis, reflex excitability is greatly enhanced. This is in keeping with a loss of descending inhibitory influences on segmental spinal activity (Weber and Stelzner, 1977). In a parallel clinical situation, anencephalic foetuses, who fail to develop cortical descending pathways also display exaggerated reflexes.

Of particular interest are descending fibres from the brainstem travelling in the dorso-lateral funiculus (DLF). These fibres originate within the rostral ventromedial medulla (RVM) including the midline nucleus raphe magnus and adjacent reticular formation (Urban et al., 1996). Manipulating the output of the RVM through electrical stimulation, microinjection of transmitters (glutamate, neurotensin, cholecystokinin) (Urban 1999a) or soma selective lesions (ibotenic acid) reveal dual- both inhibitory and
facilitatory influences on spinal nociception -measured in a variety of model systems (colorectal distension, carrageenan inflammation and topical mustard oil) (Urban 1999b). High intensity electrical stimulation, high dose glutamate and neurotensin each inhibit nociceptive responses via a descending projection in the DLF (Urban 1997). This action appears to be mediated through spinal cholinergic and monoaminergic receptors. In contrast, facilitatory influences from the RVM are mediated by projections in the ventrolateral funiculi that are dependent on receptors for serotonin and cholecystokinin (Urban 1996).

Despite anatomical connection as early as postnatal day 6 (P6), physiological maturity of the dorsolateral funiculus (DLF -a tract known to strongly inhibit activity within the dorsal horn) is delayed until at least P10-12 (Fitzgerald and Koltzenburg, 1986). Possible explanations include a delay in the development of neurotransmitters (serotonin, noradrenaline), the delay in the maturation of local interneurons of the SG or a delay in expression of specific receptors on target neurons (Bregman, 1987). Noradrenergic neurons only become detectable in the dorsal horn of neonatal rats 4 days postnatally and reach adult levels at 3 weeks of age (Commissiong, 1983).

Further confirmatory evidence of delayed descending inputs to the lumbar cord is obtained from studies of stimulus-produced analgesia (SPA). Periaqueductal grey stimulation is able to produce analgesia in a variety of species via the DLF and in part through opioid receptors. This phenomenon is demonstrable in rats only after 21 postnatal days and even then it remains immature- requiring greater stimulus intensities compared to adult animals (van Praag and Frenk, 1991).

### 2.6.7 Reflex development -conclusion

Overall, the newborn rat pup shows clear responses to noxious stimuli though these are often unpredictable and generalized mass movements. The cutaneous withdrawal reflex appears exaggerated and has low thresholds. This is unlikely to be due to changes in skin mechanics as the time frames of these changes do not correspond (Evans and Rutter 1986). Nor are the differences likely to be wholly attributable to maturation in cutaneous receptors and primary afferent fibres as these appear reasonably functional at birth. Moreover, cutaneous sensory end organs and primary afferent nociceptor fibres undergo a postnatal maturation that gradually increases efficiency of transduction and can therefore not of themselves provide an explanation of the greater reflex amplitude in early development. That exaggerated responses can be recorded from flexor motor axons argues against immature muscle and neuromuscular junctions being prime factors in neonatal excitability. Although
motor neurons are more excitable in early development (Fulton and Walton, 1986) they
do not appear to "overdrive" newborn motor units (Navarrete and Vrbova, 1983), tonic
stretch reflexes (Skoglund 1960) or proprioceptive reflexes (Ekholm 1967) and
therefore are unlikely to represent the reason for flexor reflex sensitivity in neonates.

The most likely mechanism of increased reflex excitability in early development
must revolve around the changing pattern of afferent synaptic contacts in the superficial
dorsal horn, increasing maturity of interneuronal activity and interactions with
increasingly functional descending modulatory pathways within the spinal cord
(Fitzgerald et al., 1988; Coskun and Anand, 2000).
2.7 Using the EMG as a measure of withdrawal reflex activity

In this work, electromyographic recording has been used principally to gain a clear, quantitative and objective measure of the withdrawal reflex evoked by cutaneous stimulation.

2.7.1 Human studies

EMG recording in any species may be accomplished using either surface or intramuscular electrodes. Surface electrodes have the advantages of being simple, cheap and non-invasive. Typical examples are Ag-AgCl surface adhesive electrodes. The main disadvantages are "cross-talk" and poor signal to noise ratios (Laver, 1997).

A typical EMG recording set-up in clinical human practice may involve surface electrodes placed, one each over the muscle belly and tendon with the resulting signal amplified x100-1000. The raw signal can be analysed after filtering frequencies below 0.01 Hz and those above 1 kHz (usually with a notch filter set to remove "mains noise" e.g 50 Hz). A differential amplifier allows "common mode" rejection reducing the problem of background noise. Cross talk- the contamination of the signal with activity from nearby muscles, limits the amount of interpretation in terms of "recruitment" and "rate-coding" that can be made (Macefield, 1997; Andrews and Fitzgerald, 1999).

Intramuscular recording of the EMG allows better interpretation of the signal particularly when larger muscle contractions are being measured.

Single unit recording necessitates the use of insulated tungsten microelectrodes with an inherently high impedance and limited recording field. Recording of potentials from single fibres within individual motor units requires an electrode whose recording surface is typically limited to a small window of about 25 μm diameter.

Recorded EMG signals whether from surface electrodes, intra-muscular electrodes or single unit electrodes, are most usually filtered before analysis. Critical to the choice and setting of filters is the expected mean frequency of the signal. Firing rates recorded from single motor units in different muscles during maximal voluntary contractions in humans range from 10 to 30 Hz (Bellemare 1972). These low firing rates are accompanied by a low (approx 8 Hz) firing rate during weak voluntary contraction. Taken together, this means that the range of discharge frequencies over which typical human EMG recordings are made is small (Macefield, 1997).

The stimulus evoked EMG has been utilized in several experimental models in the investigation of nociceptive signalling. Classic work by Willer (1977, 1983, 1985) describes several variations of reflex recordings in human subjects.
In a typical experimental protocol, stimulation was performed percutaneously in the distal receptive field of the sural nerve while recording the EMG from the biceps femoris muscle. This muscle was chosen as it generates the earliest reflex activity in the lower limb of normal man. The reflex response to electrical stimulation comprises two components. The first (RII) is of short latency and low threshold and is possibly initiated by activation of myelinated large diameter cutaneous fibres. The second (RIII) is of longer latency and appears at higher stimulus intensities. It is most probably initiated by smaller, unmyelinated nociceptive-specific fibres. The threshold of the late response was found to be identical to that for perceived pain.

Surface EMG recordings of withdrawal movements in human neonates (in a clinical setting) have been used to study developmental patterns and the effects of cutaneous injury on the lower limb spinal reflex (Andrews and Fitzgerald, 1999). Both mechanical (von Frey hairs) and electrical stimulation (train of five pulses, 0.1ms width, 2kHz) have been used to study thresholds and stimulus-response characteristics. The latter involved quantifying the EMG response by integrating the area under the rectified EMG signal. This study demonstrated an increase in reflex thresholds (both mechanical and electrical) with increasing post conceptional age. Interestingly, only for mechanically evoked reflexes was the size of the response correlated with stimulus strength. In general, mechanically evoked responses tended to have a longer latency, lower amplitude and be less synchronous than those for electrically evoked ones. The failure to demonstrate a clear stimulus-response relationship with electrically evoked reflexes though, most likely arose from ethical difficulties in applying higher stimulus intensities in neonates (Andrews and Fitzgerald, 1999).

2.7.2 Animal studies

EMG recording of reflex responses in animals have proved useful in investigations of several aspects of sensory processing. The activity of whole muscles (composite or muscle myograms) has been used to assess the overall output of motor neurons (Schouenborg and Kalliomaki, 1990; Falinover 1994).

Typically, non-insulated platinum-iridium needle electrodes are inserted transcutaneously 0.5cm apart in biceps femoris muscle. The reflex, evoked by electrical stimuli (square wave 2ms duration, 0.17Hz) comprises two components - possibly reflecting activation of myelinated afferent fibres and a C-fibre reflex respectively. (Gozariu et al., 1997).

Finer bi-polar recording needles of high impedance (tungsten) allow resolution of single motor units and definition of individual firing patterns (Hennig and Lomo, 1985). Studies of single motor units (SMUs) are generally well correlated with findings
obtained from whole muscle myograms. Differences between muscles when noted have been ascribed to differing involvements in the withdrawal reflex (Solano and Herrero, 1997).

Developmental studies are made possible by recording the EMG from hamstring muscles following subcutaneous electrical stimulation of the hind foot in rats of varying ages. For example, stimuli consisting of single square wave pulses (10mA, 1 ms) are delivered every 3 minutes for up to 120 minutes. With this stimulus pattern the rectified and averaged (over ten trials) signals reveals two distinct components- early and late. Data analysis yields latency and duration parameters for control and experimental groups across an age range from neonatal (P2) to adult rats (Jiang and Gebhart, 1998).

The withdrawal reflex arc can also be measured proximal to muscle by recording activity within flexor alpha motor neurons. In acutely decerebrated and spinalized adult rats, exposing the nerve to the biceps femoris and semitendinosus in the popliteal fossa allows the dissection of fine filaments and identification of alpha motor neurons involved in the withdrawal reflex. The excitability of the reflex can be assessed by counting the total number of spikes elicited by a test stimulus (Woolf and Wall, 1986).

The spinal reflex arc has also been studied by recording evoked potentials in the ventral root. Three components of this reflex have been identified in neonatal rat pups. This model confirmed a post natal reduction of reflex latencies and suggested that (after considering the central delays for each component) unmyelinated slow fibres do not contribute significantly to the first two components of the ventral root reflex (Fitzgerald et al., 1987).

Inflammation causes an increase in receptive field size, lowering of thresholds and an increase in firing rates. In general differences between individual muscles that are apparent in the naive state become less noticeable during inflammation resulting in a more homogenous pattern of firing in all motor units (Solano and Herrero, 1999, Herrero and Cervero, 1996a, Herrero and Cervero, 1996b).

The utility of this animal model has made it suitable for drug pharmacodynamic studies. To distinguish between antinociception and antihyperalgesia, drug effects are studied in both naive and carrageenan inflamed rats (Herrero and Headley, 1996).
2.8 The preparation

Sprague-Dawley rat pups were used for all experiments. These animals are bred in-house (UCL) and housed in plastic solid bottomed cages (56x38x18 cm) on sawdust floors (Gold Chip Lilyco). Temperature is maintained at 20-22 °C, humidity at 50-55% and the light cycle is maintained in a continual breeding mode i.e. 12 hrs full light separated by 1 hour half light from a 10 hour dark phase. Animals were fed a rodent breeding and maintenance diet (TRM 9607, Harlan Teklan) and allowed water ad libitum.

2.8.1 Preparation for EMG recording

Anaesthesia was induced using halothane (1-3%) in oxygen to allow creation of a tracheostomy using a tapered plastic cannula (Argyle Medicut) while the animal breathed spontaneously from a nose cone. Tracheostomies in larger pups (P21 and P10) were created through the cricotracheal membrane in a routine fashion. Tracheostomies in small pups (P3) involved retrograde laryngeal intubation with a stylet through the cricotracheal membrane followed by the passage of an 20G plastic cannula (Ohmeda venflon) through the mouth and then into the distal trachea. This arrangement provided greater airway security and by virtue of the cannulas’ inherent t-piece, allowed minimisation of the dead space associated with the endotracheal tube. Airway procedures in all pups were completed under halothane anaesthesia while the pups breathed spontaneously.

2.8.2 Ventilation

Intermittent positive pressure ventilation (IPPV) was achieved without the use of muscle relaxants. A T-piece system was used in conjunction with a Harvard small animal lung ventilator pump (Harvard Apparatus Ltd) in a “thumb-occlusion” mode. This system affords control of the inspired gas mixture (halothane concentration and oxygen, air and/or nitrous oxide fractions) inspiratory flow rate, respiratory rate and peak inspiratory pressure. A simple water manometer placed in the inspiratory limb provides a monitoring and pressure limiting device with a visual display.

Using an inspiratory flow rate of 1.2 l/min and adjusting the peak inspiratory pressure to between 12 and 15 cm water an appropriate tidal volume was easily
delivered. Adjustment of the respiratory rate and therefore inspiratory time allows adequate ventilation for pups as small as 10gms in weight.

2.8.3 Temperature control and Positioning

A thermostatically controlled heat source was developed especially for the recording set up. This arrangement avoids the placement of an electric heating blanket near the recording apparatus and removes the possibility of inadvertent overheating.

Once anaesthetised and tracheostomised, pups were placed in a Kopf small animal stereotaxic frame. In order to prevent noxious stimulation and undue force being placed on the animal’s skull, a mould was fashioned (Impregum F, ESPE) to support the animals head within the frame. The animal’s rear was gently suspended on a 23G needle through the skin, effectively suspending the animal in a lower body “sling” of skin. This allowed secure animal placement with minimal noxious stimulation, unobstructed lower limb venous drainage and rather fortuitously, a standardisation of passive muscle-stretch within the secured hindlimb. This was important for consistent EMG recording.

The hindlimb used for EMG recording was secured in slight knee extension and ankle plantar flexion on a fixed platform using a double sided self adhesive pad. This arrangement avoided holding sutures and optimised EMG recording while exposing the plantar surface of the paw for cutaneous stimulation.

2.8.4 Monitoring

All rat pups had ECG monitoring when placed within the recording rig. Peripheral perfusion was visually monitored by assessing capillary return in the hindpaws. During preliminary work, transcutaneous oxygen and carbon dioxide tensions were monitored using a combination probe (TCM3, Radiometer). Once adequate ventilation parameters were set, recording of transcutaneous gas tensions was carried out only in selected experiments. Neither end-tidal expired carbon dioxide tensions nor halothane vapour pressure measurements were available for these experiments.

The basic preparation and recording set up are shown in Diagrams 7 and 8.
Diagram 7. A photograph of the set up for EMG recording of the hindlimb withdrawal reflex in rat pups. Rats are anaesthetised with halothane and ventilated via a tracheostomy. Rats are supported in a cranial mould and suspended on a transverse bar (23G needle) through the lumbar skin. Temperature is maintained using a heated water mattress and the ECG is continuously monitored.

Diagram 8. The bipolar recording electrode is placed percutaneously into the belly of either the biceps femoris or semitendinosus muscle. Single shot epidural injections can be made after a simple dissection over the lower lumbar vertebrae avoiding the need for laminectomy. After amplification (x10K) and filtering (<1Hz, >1KHz) the raw signal is digitised (2KHz Maclab ADC) and stored electronically. Data was analysed using Maclab Chart software.
2.8.5 EMG recording

EMG recording was commenced no sooner than 30 minutes after reducing the inspired halothane concentration from the surgical levels (2%) to age-related recording levels. For 21, 10 and 3 day old rats these were 0.7%, 0.9% and 1.0% respectively. These levels represent equivalent anaesthetic depths (equi-MAC values) and are fully described in Chapter 3. This was to allow time for equilibration to steady state-alveolar halothane concentration and hence a stable plane of anaesthesia.

Bipolar EMG electrodes (Ainsworks, London) comprising stainless steel needles (0.33mm external diameter) with a central insulated copper wire core were place percutaneously into the belly of the biceps femoris muscle. The total cross-sectional area of the recording needle was at least 0.45mm², while that of the inner copper core was at least 0.01mm². Raw signals were amplified using a headstage amplifier (NL100, Neurolog, Digitimer) with an input resistance of 10⁸ohms.

Preamplification (x1000) and filters (low pass: 1Hz; high pass: 1KHz; notch: 50Hz) were used (NL104, NL125) before display of the EMG on a digital storage oscilloscope (Hameg HM205). The signal was fed to an analog-to-digital signal converter for further data analysis (Maclab/4s). EMG recordings from the hamstring muscles in response to mechanical (von Frey hairs) and electrical stimulation of the hindpaw were made when the animals were under steady state anaesthesia.

Von Frey filaments were chosen as a stimulus as they are practical, simple and could be used in parallel behavioural experiments on awake, freely moving animals. Electrical stimulation was chosen as it allows synchronous activation of afferent fibres in a fashion that guarantees C-fibre activation. Measures of reflex latency are also made possible using this stimulus modality. Heat was not chosen as a stimulus because the heat threshold for eliciting the withdrawal reflex in halothane anaesthetised animals would almost certainly be above that for causing significant tissue damage (i.e. tissue burns).

Von Frey hairs of graded gram weight were pressed onto the plantar surface of the hindpaw for 1 sec. A 2 minute rest interval was observed before repeated stimulation if reflex withdrawal was elicited. Up to three von Frey hairs above threshold were sequentially applied. The range of mechanical stimulus strengths used was regularly between 5 and 50 gm weight. The calibration of the hairs is shown in Diagram 9.

Electrical stimulation was applied via percutaneous electrodes placed at the medial and lateral borders of the paw. Controlled-current stimuli (Stimulus Isolator, Digitimer) were applied as a train of 15 square wave pulses of 2ms duration at a frequency of 100Hz. A range of 1.5mA to 10mA was used and up to three electrical stimuli above threshold were recorded.
Reflex muscle activity was defined to occur when the EMG signal increased at least three times above baseline noise (S:N ratio 1:3). This level of activity always resulted in visible limb contraction. All experiments were terminated using an overdose of intra-peritoneal thiopentone.

**von Frey hair calibration**

Diagram 9. von Frey hair calibration

von Frey hairs were used as a mechanical stimulus to elicit a withdrawal reflex response. Calibration was completed regularly using an electronic bench top weighing scale. A calibration scale for the full set of filaments is shown in a). The two insets show details for the lower range (b) and the range used in these experiments (c). The stress developed in nylon filaments in compression is known to be influenced by temperature and humidity. Three selected calibration values in (d) are plotted over time. A seasonal variation can just be discerned. Heavy blue bars denote the winter months.
2.8.6 Analysis

Raw data was digitized at a frequency of 4kHz and 12 second epochs were stored electronically (including 2 seconds pre-stimulus). The latency and duration of the response were determined directly from recordings of the raw data. Data for analysis of latency was obtained from reflexes evoked by electrical stimulation only. Integrating software (Chart, Maclab ADI) calculated the peak response and area under the rectified (RMS) signal. The area under the RMS signal was plotted against stimulus strength and the resulting curve integrated to calculate an area under the curve. This value termed "Reflex Responsiveness" was used as a summary statistic for much of the pharmacodynamic analysis and reflects the overall sensitivity and responsiveness of the withdrawal reflex.

The use of this summary statistic that combines individual data obtained over a range of stimulus strengths (i.e. a range of von Frey hairs) has the effect of increasing the discriminative power of the recorded data. The use of this measure has several advantages including simplicity and some degree of intuitive relevance to the reflex being measured (Mathews et al., 1990). The latter can be obtained from a visual representation of a volume on a three dimensional plot of time (ms), RMS signal amplitude (mV) and stimulus strength (gm) see Diagram 10. More importantly, the distribution of this measure is not obviously non-Gaussian and therefore lends itself more easily to parametric statistical analysis.

Critical to the use of this measure (reflex responsiveness) is the range of stimulus strengths used for the calculation. The lower limit must obviously be below the threshold for eliciting the withdrawal reflex. The selection of an upper limit to the stimulus strength is based on the change from a simple (single limb) withdrawal to a more complicated crossed and ascending reflex response. This cut-off lies just below the strength at which a stimulus ceases to be merely "potentially noxious" and becomes overtly tissue damaging. This range (5-50gm weight) was found to be consistent across the age groups. Of interest is the relationship between ranges used in different experimental preparations. The range of stimulus strengths used in these experiments is almost identical to that used by Pertovaara et al (Pertovaara et al., 1998) in experiments on pentobarbitone anaesthetised adult rats undergoing dorsal horn cell recording. The range chosen by Pertovaara was specifically chosen to be supra-threshold.
Diagram 10. A visual representation of the summary statistic used to quantify the responsiveness of the flexor withdrawal reflex. The area beneath the stimulus-response curve may be represented by a volume contained within the axes- "Time", "Stimulus strength" and "RMS signal amplitude". The volume is drawn as a triangular pyramid as both signal duration and signal peak appear to be linearly related to stimulus strength (see Fig 16).
2.9 RESULTS

A typical example of an EMG response to graded mechanical and electrical stimulation is shown in Figure 1. Each recording is made over a 12 second period including a 2 second “pre-stimulus period”. These traces were obtained using a 30G co-axial bi-polar recording needle and represent multi-unit recordings rather than single motor unit recordings. Both the raw EMG signal and the root mean square (RMS) of the signal are depicted.

Anaesthetised neonatal rat pups proved robust in-vivo electrophysiological preparations. With attention to ventilation, fluid homeostasis and temperature maintenance, the preparation proved remarkably stable. Figure 2 shows the heart rates and EMG responses to mechanical stimulation in two 21 day old rat pups over time.

The compound EMG recorded in flexor muscles can be analysed in terms of peak, duration and area under the RMS signal. Each of these parameters code for stimulus strength over a limited though consistent range of stimuli (see Figure 3). The range of stimuli chosen is between subthreshold stimuli and that which is actually tissue damaging. For mechanical stimuli this range is 3 to 47gms. For electrical stimuli a range of 0.25-8mA was chosen. Peak, duration and area under the RMS signal each appear to be linearly related to stimulus strength. Therefore for all further analysis, stimulus strength was expressed in grams or milliamps without any further (e.g. logarithmic) transformation. As the area under the RMS signal incorporates aspects of both amplitude and duration, it was chosen as the primary parameter on which to base pharmacodynamic studies.

Analysis of the same parameters (peak, duration and area under the RMS signal) obtained in naive rat pups at two younger ages (P10 and P3) reveals similar results (see Figure 4a). The ability of the electromyographically recorded withdrawal reflex to code for stimulus strength appears to be present as early as P3. Recordings were made at equivalent anaesthetic depth.

A less clear though broadly similar pattern was seen for electrical stimulation (see Figure 4b). Electrical stimulation may represent a more complex input for the withdrawal reflex arc for several reasons. Firstly, although reliable and easily controlled, the instantaneous, direct and synchronous activation of afferent axons is likely to represent an input with unnatural spatial and temporal characteristics (Gracely 1994). The depolarization of cutaneous nerve endings by electrical fields is subject to
the geometry and placement of the electrodes. The local currents generated are then subject to changes in the fluid environment surrounding the electrodes. It is possible that even a small amount of tissue oedema may alter current density around nerve terminals and thereby change the effective stimulus strength.

Although the stimulus-response function for mechanical stimuli was clearer than that for electrical stimuli, latency measures could only be reliably made using the latter. The changes in latency of the withdrawal reflex were measured from EMG recordings made at suprathreshold stimulus strengths. The effect of increasing age on this latency is shown in Figure 5. Latency for withdrawal is greatest in P3 pups (0.96 sec.) and has the greatest variance (s.d. 0.3). By P10 this delay is significantly reduced. Latency in P10 pups is 0.34 sec. (s.d. 0.06) and in P21 pups 0.3 sec. (s.d. 0.14).

Reflex responsiveness - a summary measure calculated from the area beneath a stimulus-response curve, has been used as the main outcome measure for most of the current experiments. The changes in reflex responsiveness that occur with increasing age can be clearly seen in Figure 6. Reflex responsiveness (measured involts. grams. seconds) is greatest for the youngest pups (1.44 V.g.s (s.d. 1.02). It decreases to 1.09 (s.d. 0.67) by P10 and falls markedly again to 0.39 (s.d. 0.26) at 21 postnatal days.

Thresholds for eliciting neuronal responses are traditionally used as a direct measure of the excitability of those neurones. This same interpretation may not apply to the model being described here. Neither mechanical nor electrical thresholds show a change with age in the current experiments (see Figure 7). This is likely to be a direct reflection of equivalent anaesthetic depth across the three ages tested.
2.9.1 Results: The raw EMG signal.

**Mechanical stimulation**

Raw data

RMS signal

Stimulus: 10g, 15g, 27g, 47g

**Electrical stimulation**

Raw data

RMS signal

Stimulus: 1.5mA, 2mA, 4mA, 6mA, 8mA

Figure 1

Typical raw data recording showing the relationship between EMG response and stimulus strength. Raw data is amplified (x10k) and filtered (<1Hz and >1kHz) and digitised at a sampling frequency of 4kHz. Latency, peak, duration and area under the curve are calculated from the RMS signal.

The electrodes used for this study record compound potentials from several motor units rather than single motor units. Both the raw EMG signal (red) and the root mean square (RMS) of the signal (blue) are depicted.
2.9.2 Stability of the preparation.

The stability of the preparation is shown here as a plot of heart rate and EMG response over time for two P21 pups. “EMG areas” refer to the area under the RMS signal obtained after eliciting the withdrawal reflex with a mechanical stimulus— in this case, a von Frey hair calibrated to 47 gm weight. Time “0” is taken to be when the inspired halothane concentration is reduced from surgical requirements (typically 2%) to fixed, predetermined recording levels (0.7% for P21 rat pups). An initial period of about 25 mins is required for a steady anaesthetic state to be reached. For all further experiments, recordings of the withdrawal reflex were made after an ‘equilibration time’ of 30 mins and completed within a 25 minute period.
2.9.3 Stimulus strength coding (Peak, Duration and Area).

Figure 3
The ability of the peak, duration and area of the recorded EMG response to code for mechanical stimulus intensity is demonstrated here. Eleven P21 rat pups were studied. Plotted data represent means and standard errors (SEM).
2.9.4a  EMG Coding of mechanical stimulation at three ages.

Figure 4a
A comparison of the stimulus-response curves in naive pups at three ages. Average peak, duration and area below the RMS signal are plotted against stimulus strength. Graphs for both mechanically (Fig 4a) and electrically (Fig 4b overleaf) evoked withdrawal reflexes are shown (mean and SEM displayed). Here, a trend toward a higher threshold and lower gain with increasing age may be seen.
2.9.4b EMG Coding of electrical stimulation at three ages.

Figure 4b
A comparison of the stimulus-response curves in naive pups at three ages. Average peak, duration and area below the RMS signal are plotted against electrical stimulus strength. In contrast to mechanically evoked responses, electrically evoked responses do not show age related trends.
2.9.5 Withdrawal reflex latency.

A clear pattern of reducing latency with development is seen. The reflex latency (in seconds ± s.d.) in P3 pups was 0.96 ±0.30, P10: 0.34 ±0.06 and in P21 pup: 0.31 ±0.14. (Box and whiskers show median, quartiles and range).

One way analysis of variance: are the means significantly different? p<0.0001; post-test for linear trend: p<0.0001.

Though several orders of magnitude greater, the pattern of change demonstrated here is similar to that shown by Fitzgerald (1987) in recordings of the ventral root reflex i.e. greatest change seen in the period between birth and P14.
2.9.6 Reflex responsiveness changes with age.

Figure 6

The summary measure “reflex responsiveness” was calculated for individual pups and expressed in the units: V.g.s. Results for naive pups at three ages are displayed here. (Box and whiskers show median, quartiles and range). Tests for normality (albeit with small numbers in each group) reveal KS distances for all the groups shown to be less than 0.26. On this basis, statistical tests for normally distributed parametric data were used when comparing control with experimental conditions. One way analysis of variance: are the means significantly different? p=0.0048; post-test for linear trend: p=0.0013.

The most marked change in reflex responsiveness appears to occur between ages P10 and P21.
2.9.7 Mechanical and electrical thresholds.

Figure 7

Thresholds recorded for both mechanical and electrical stimuli at three ages are displayed here. No significant differences between age groups was seen. These results are thought to reflect the fact that anaesthetic depth was equivalent in the three age groups tested. The influence of anaesthetic depth on recordings is discussed further in Chapter 3.
2.10 DISCUSSION

These experiments show that the anaesthetised rat pup can be used to record responses over a convenient time frame in rat pups as small as 10gms (typically 3 postnatal days). This together with the ability to quantify the withdrawal response (amplitude, duration and latency) makes the model well suited for the study of developmental patterns in spinal nociception.

Particularly important is the fact that measurable parameters of the withdrawal reflex correlate well with stimulus strength. This distinct stimulus-response relation has been documented in other preparations. The magnitude and inverse of latency of reflex withdrawal from a radiant heat stimulus in human subjects appears to be an exponential functions of stimulus temperature (Campbell et al., 1991). This relationship is comparable with electrophysiological data obtained from recordings of nociceptors (Beck, 1974) and dorsal horn responses to graded heat (Kenshalo et al, 1979) wherein neuronal firing frequency increases with stimulus strength. This relationship may be the result of increased synchrony and spatial summation of individual motoneuron responses as stimulus strength increased (Dimitijevic and Nathan 1970). More recently, Pertovaara showed strong stimulus -response relationships in dorsal horn neuronal firing frequencies using mechanical stimuli (Pertovaara et al., 1998).

However, this is the first time that clear stimulus-response functions have been demonstrated for the flexion withdrawal reflex in young rat pups.

2.10.1 Age related changes in the withdrawal reflex

The EMG recordings made here (representing a baseline in the absence of any conditioning inflammatory stimulus) showed a significant developmental pattern. The withdrawal reflex response in 21 day old pups was much smaller than that of either P10 or P3 pups. This reduction in the size of the response was observed despite the reduction in the concentration of inhaled halothane with increasing age. Recordings were made at what were felt to be equi-anaesthetic depths i.e equivalent MAC values. This was judged by overall response to handling, placement in the rig and the ability to ventilate all the pups without muscle relaxants. It is therefore concluded that the change in baseline EMG recordings did reflect a genuine change in the excitability of the reflex rather than differences in anaesthetic depth.
The experiments described in this chapter demonstrated the following age related effects:

i) response latency decreased and became less variable with increasing age.

ii) reflex responsiveness decreased with increasing age.

iii) mechanical and electrical thresholds did not showed significant changes with age.

2.10.1a Latency

Latency as measured in this preparation will depend on changes in cutaneous afferent fibre conduction velocity, maturation of spinal synaptic contacts and maturation of motor unit activation patterns.

Afferent fibre conduction velocity is a function of axonal diameter, the degree of myelination and the pattern of saltatory conduction. All three of these display a developmental pattern. Myelination of the rat sciatic nerve commences within the first three days of birth and advances rapidly over the following two weeks (Webster, 1971). Axon diameter too continues to increase until 6 months of age. The most rapid changes occurring in the larger fibres between P5 and P20 (Webster, 1971). The maturation of nodes of Ranvier, marked by the clustering of Na+ channels has been studied by Vabnick et al and shown to be a process occupying the first post natal week of rat life. Clustering was thought to be closely associated with and possibly induced by Schwann cells (Vabnick et al., 1996).

Age related changes in conduction velocity in neonatal rat pup sciatic nerves have been documented by Fitzgerald and Gibson (Fitzgerald and Gibson, 1984). Stimulating hindfoot skin and recording volleys in the dorsal horn, two peaks are discernible from P2, both with slow conduction velocities (<1m/s). By P10 the pattern resembles that of the adult though the latency is still prolonged (i.e. an early peak travelling at 10- 20 m/s presumably in myelinated fibres and a late peak travelling at 0.5-1 m/s). In a closer study of the afferent volleys evoked in dorsal roots, Fitzgerald documented the gradual maturation of conduction velocities of both large (capsaicin resistant) and fine (capsaicin sensitive) fibres. Calculated velocities confirmed the results obtained earlier. Between P4 and P6, intermediate conduction velocities of presumably myelinating fibres resulted in complex patterns of multiple peaks within the dorsal roots. In young pups (up to P5) the long latency peaks (C fibre activity) did not appear to contribute to dorsal horn responses suggesting that central synaptic contacts of these fibres were as yet immature (Fitzgerald, 1988).
Synaptogenesis in the spinal cord follows axodendritic growth and a large proportion of it is completed postnatally (Cabalka et al., 1990).

Receptor expression too, shows developmental patterns. Of principal concern here is the expression and maturation of excitatory glutamatergic systems- both AMPA and NMDA sub-types and the peptidergic-sP system.

The density of expression of both of the glutamatergic receptors is high in the early post natal period and maturation involves a change in the roles played by these receptor subtypes. The early dependence on NMDA receptors for excitatory transmission gradually makes way for a more mature AMPA-mediated transmission. This change of itself brings about a change in the kinetics of synaptic currents. (AMPA currents having much faster rise times and decay time constants). Further, though more subtle changes in synaptic currents are brought about by changes in NMDA receptor composition. In particular, the replacement of the NR2C and NR2D subunit by NR2B and NR2A subunits would be expected to result in a shortening of typical NMDA receptor mediated currents (These are dealt with in more detail in Chapter 5). Overall, it is not unreasonable to expect these synaptic changes to partly underlie the developmental changes in reflex latency observed here. A developmentally regulated reduction in the duration of synaptic currents may also contribute to the reduction in reflex responsiveness seen here.

2.10.1b Reflex responsiveness

The finding that reflex responsiveness changes with age actually comprises two results. Both the magnitude and the duration of the recorded EMG signals displayed this age related pattern.

Several maturational processes within the first three weeks of rat neonatal life are likely to play a significant role in the finding that reflex responsiveness declines during this period. It is unlikely that any single factor can be isolated and nominated as a pre-eminent cause. Three issues can be considered: firstly, the anatomical arrangements of the central terminals of neurons intercalated in the withdrawal reflex arc, secondly, the pattern and strengths of synaptic connections as well as the intrinsic membrane properties of the neurons in this arc and finally, the role of modulatory influences on the arc.

An evolving pattern of laminar organization in the spinal cord has been well described in the rat. The central terminals of primary afferent fibres enter the dorsal horn and elaborate synaptic connections in a sequential pattern in which initial connections are made between Aβ fibres and substantia gelatinosa cells. During the first two postnatal
weeks these undergo gradual restriction to deeper laminae (Fitzgerald et al., 1994; Coggeshall et al., 1996). Prior to P5 then, 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. These inputs are the probable trigger of the vigorous responses of both superficial and deep dorsal horn cells to cutaneous electrical stimulation in the early neonatal period (Fitzgerald, 1988) and the prolonged responses in the withdrawal reflex at P1 recorded from spinal ventral roots (Fitzgerald, 1985). As fine-fibre (A\(\delta\) and C) afferents come to predominate the SG inputs, the reflex is more likely to be driven by wide dynamic range afferents (Nakatsuka 2000).

In early development synaptogenesis and synaptic efficiency undergo marked age-related changes. The timetable for synaptogenesis as proposed from histological studies confirms that a large proportion occurs postnatally. Further, the maturation of these synapses is a gradual process that is more difficult to study (Stelzner, 1971). The gradual maturation of C fibre synaptic glomeruli within the dorsal horn has been inferred from both electron microscopic evidence and immunolocalisation of nerve terminal proteins and is noted to commence only from P5 (Cabalka et al., 1990; Pignatelli et al., 1989).

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 adults (Hori and Kanda, 1994). NMDA receptors in early life are uniformly and densely distributed through the dorsal horn before becoming restricted. Substance P 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 1983).

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. Receptive field sizes of dorsal horn neurons have been shown to undergo striking age related changes. Cutaneous receptive fields rapidly reduce during the first post natal 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 (proportion of cells responding to both innocuous and noxious stimuli) is seen (Fitzgerald, 1985).

In parallel with the maturation of intra-spinal inhibitory influences, 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 P8-10 (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 detectable only after three post-natal weeks (van Praag and Frenk, 1991). The results of the current experiments are in keeping with the timetable of change documented in the other models cited here in that a major reduction in reflex responsiveness occurs between 10 and 21 post-natal days.

Consideration of developmental data from peripheral nociceptors and primary afferent fibres allows the conclusion that these proximal 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 efferent (motor) limb of the reflex arc, though does undergo significant postnatal maturation. In particular, the pattern of inputs to ventral horn motor neurone pools may result in a changing order of recruitment of motor units with increasing age (Ben Ari 1997). Further, the neuromuscular junction also shows striking maturational changes. The greatest changes occur just prior to birth in the rodent (e.g. metabolic turnover increases ten fold and clustering density increases). More subtle post natal maturation continues over 2-3 weeks and includes a subunit switch in the acetylcholine receptor (Hall and Sanes, 1993) as well as the elimination of polyneuronal innervation of myofibrils (Brown et al., 1976). Neither a change in motor unit recruitment order or the neuromuscular junction changes are likely to have been major factors in the greater reflex excitability of P3 pups as the EMG responses being recorded have a duration several orders of magnitude larger than the time scales of these factors.

A greater reflex excitability in early development is consistent with findings from several other experimental models (Stelzner, 1971). There is widespread agreement that immature rats are hyperresponsive to noxious stimuli including mechanical (Stelzner, 1971) chemical (Guy and Abbott, 1992) and thermal (Falcon et al., 1996) stimuli. Most of this data has been obtained from behavioural tests and measurements of reflex thresholds e.g. thermal thresholds in rats (Falcon 1996) and mechanical thresholds in human neonates (Fitzgerald 1987). Electrophysiological studies of dorsal horn cell activity adds further support to the idea of a largely un-inhibited 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).
2.10.1c Thresholds

To some extent, thresholds for reflex withdrawal were used as a measure of depth of anaesthesia and as such, halothane concentrations were adjusted to maintain similar reflex thresholds across the age groups. This use of thresholds finds precedent in the concepts and experimental designs involved in determining potency of anaesthetic agents. The determination of MAC values for volatile agents has traditionally involved determining the concentration at which 50% of a population displays reflex movement to a noxious stimulus. As this outcome is an “all or nothing” response, both concept and design reflect a threshold. That threshold measurements are directly related to anaesthetic depth is evidenced by the direct relationship between alpha alphaxalone infusion rates and measured reflex thresholds in a study of the jaw opening reflex in cats (Clarke and Mathews, 1985; Le Bars et al., 1979). A brief comparison with other anaesthetic agents (methohexitone, ketamine, α-chlorolase and halothane) appeared to confirm the relationship within the limits of the known differences in kinetics of each of these agents.

In a study of the effects of general anaesthetic agents on reflex activity (tail flick latency) Banks et al compared reflex latency at various anaesthetic depths. Under pentobarbitone anaesthesia, latency was not significantly changed by increasing anaesthetic depth until deep surgical (stage III4) planes were reached. Although these researchers did not measure reflex thresholds, the results are consistent with the premise that anaesthetic depth is better reflected by threshold values than latencies of evoked activity (Banks et al., 1988).

In a similar study into the effects of light pentobarbitone anaesthesia, Archer et al (2000) did show significant reductions (32%) in reflex latency caused by sub anaesthetic doses (30mg/kg) of the barbiturate. Importantly, no long lasting (post-recovery) reflexive or behavioural changes were seen after anaesthesia with this agent.

Under the conditions set out for the current experiments, reflex thresholds appear to be a reasonable measure of anaesthetic depth, latency changes in parallel with maturity and possibly reflects the integrity and efficiency of synaptic pathways and the last-reflex responsiveness (response to suprathreshold stimuli) may render a better correlation with tonic pain states. For this reason, reflex responsiveness has been chosen for the pharmacodynamic studies described in Chapter 5.

As described in the introduction of this chapter, the value of threshold measurements in awake subjects though, is entirely different. Changes in sensory threshold with age have been well documented in both human and animal models (Fitzgerald et al., 1988; Andrews and Fitzgerald, 1999). A gradual increase in mechanical
threshold (for eliciting the flexion reflex) with age was been documented in neonates commencing from a post conceptional age (PCA) of 29.5 weeks. This maturational process was correlated with PCA rather than post natal age indicating its genetic (rather than epigenetic) basis. The same researchers demonstrated a similar developmental pattern in rat pups over the first four post natal weeks with much of the increase occurring in the second post natal week.

2.10.2 Analysis methods

The described analysis method allows calibrated von Frey hairs (which typically render ordinally ranked data i.e. single values representing a mechanical threshold) to render parametric data (area under the stimulus-response curve). This effectively allows the analysis to avoid all the problems associated with logistic regression which typically is required in pharmacodynamic studies of quantal outcomes (Lu and Bailey, 2000). The use of two control measurements i.e. non-inflamed and carrageenan-inflamed pups, effectively standardises recordings between age groups and within limits, precludes the age related changes in MAC being a confounding factor. Pharmacodynamic effects across varying ages can then be quite reasonably expressed as a “percentage of maximal possible effect” (MPE) where a 100% effect represents a return of the response to naive values and where a 0% effect reflects no change from carrageenan-inflamed responses. This method avoids the use of arbitrary cut off values that typically complicate behavioural tests such as the tail flick latency analysis.

The method has a comparable discriminative power to simpler behavioural tests such as tail flick latency as the withdrawal response is quantified over a range of stimulus strengths. This is better demonstrated by sample size calculations for the two experimental techniques. Choosing a significance level of 0.05 (alpha), a power of 80% (beta= 0.2), mean and s.d. for tail flick data= 3.5s (1.7) (Hargreaves et al., 1988) and reflex responsiveness data= 1.56 (0.44) respectively and a minimum relevant change equal to half the difference between naive and carrageenan-inflamed values, then the number of animals required equals 2.6 for the tail flick experiments and 3.1 for the EMG based experiments.
Chapter 3 THE EFFECTS OF ANAESTHESIA ON THE WITHDRAWAL REFLEX

3.1 Aims of Chapter 3

The influence of anaesthetic agents on the electromyographically recorded withdrawal reflex is presented in this chapter. The aim here was to study the age related changes in sensitivity to halothane in rat pups at three developmental stages (post-natal days 3, 10 and 21). This required the determination of halothane dose-response curves for each age group. As the response being recorded was the withdrawal reflex, the determinations were closely allied to MAC finding studies. (MAC i.e. “minimum alveolar concentration” is the concentration of volatile agent required to prevent gross purposeful movement in response to a standard noxious stimulus in 50% of subjects). Completion of these studies allowed the confirmation and choice of equi-anaesthetic regimes for each age group in all further experiments.

Two further experiments were also completed:

a) to determine if the efficacy of nitrous oxide changes significantly with age.

b) to determine the dose of urethane that produces an equivalent depth of anaesthesia to that produced by 0.7% halothane in P21 rat pups.
3.2 Introduction

Recent research suggests that general anaesthetics probably exert their effects on synaptic transmission—rather than on neuronal membranes and axonal conduction (Franks and Lieb, 1994). The precise nature and location of these synaptic (and possibly other non-synaptic) effects have an important bearing on the interpretation of electrophysiological studies of nociceptive pathways which being invasive in nature and often involving inflammatory agents and noxious stimuli, necessitate the use of anaesthetic agents.

3.2.1 The molecular basis of general anaesthetic drugs

For many years the correlation between hydrophobicity and anaesthetic potency of the alcohols and alkanes described by Meyer and Overton had suggested that anaesthesia resulted from a general disturbance in neuronal membrane function and therefore axonal conduction (Meyer, 1899; Overton, 1901). The simplicity of the correlation between anaesthetic potency and the octanol/water partition co-efficient could be interpreted as evidence that anaesthetic action is essentially independent of the detailed size, shape and chemical nature of the anaesthetic molecule. The attraction of this theory lay in its biophysical basis which avoided a classic drug-receptor pharmacologic model providing a ‘unitary site’ of action for the large variety of structurally unrelated anaesthetic compounds.

Three interesting phenomena became useful tools in the investigation of the anaesthetic mechanisms of action— that of pressure reversal (general anaesthesia induced by volatile agents can be reversed by increased barometric pressure), temperature dependence (the gas phase potency of these agents decreases with increasing temperature) and the “cut-off” effect (anaesthetic potency in a homologous series of agents increases to a cut-off chain length and then disappears).

The first provided comforting re-assurance for lipid-based theories, in particular a theory known as the “critical volume” hypothesis. In this hypothesis, anaesthesia results from absorption of an otherwise inert molecule by a hydrophobic membrane which then expands beyond a critical volume. Above this volume the membrane becomes functionally (electrically) inactive. Although this theory was internally consistent and led to impressive predictions of experimental data, it was difficult to corroborate using non-gaseous anaesthetic agents.

The temperature dependence of anaesthetic potency became a major stumbling block for lipid based theories which predicted a positive correlation between temperature and potency. Reviewing the available data, Franks and Leib pointed out the
problem and suggested a solution. Proposing a simple drug-binding model, they showed that both the pressure reversal data and the temperature dependence data could be consistently explained (Franks and Lieb 1982).

These researchers then went on to prove the likelihood of a drug-protein interaction by demonstrating a mirror cut-off effect in inhibition of a soluble protein (using firefly luciferase enzyme and a homologous anaesthetic series). They postulated that a protein binding site of circumscribed dimensions and amphiphilic nature would explain the selective binding of relatively apolar (though containing at least one polar group) anaesthetic agents (Franks 1985).

Lipid based theories of anaesthetic action became even more difficult to sustain on finding that at clinically relevant concentrations, anaesthetic agents induce changes in lipid bilayer structure no greater than those induced by very modest changes in temperature (Raines 2000). Neither bilayer fluidity, investigated with magnetic resonance nor gel to liquid crystalline phase changes nor membrane dimensions are significantly or consistently altered by relevant concentrations of anesthetic agents.

Studies of ion permeability of lipid membranes have shown that while cation fluxes across lipid bilayers can be significantly augmented by anaesthetic agents at relevant concentrations, these results are not easily replicated in biological membranes. Evidence to date suggests that relevant concentrations of several anaesthetic drugs have only small effects on the voltage-gated ion channels mentioned above making them all unlikely primary targets for halothane and other common volatile agents.

Because general anaesthesia in humans occurs at concentrations of halothane 4 to 30 times lower than that required for significant lipid perturbation or inhibition of either sodium channels, calcium channels or delayed rectifier potassium channels, the Meyer-Overton theory has been largely superseded by theories postulating a protein-based interaction and by implication, a synaptic site of action. A prime indicator that these anaesthetic agents interact with proteins is the consistent stereo selectivity displayed by many intravenous agents and also the inhalational agent, isoflurane (Franks and Lieb, 1991).

Biophysical studies into the influence of volatile anaesthetic agents on protein molecules are yet to define the exact nature of the interaction but direct binding of anaesthetic agent with protein molecules, at protein-lipid interfaces and between protein subunits have been demonstrated in various models. Several possibilities exist, including altered tertiary or quarternary structure (information to date suggests that secondary structure is not altered) or altered amino-acid side-chain dynamics. It may be that anaesthetic agent binding holds particular protein molecules in a low energy sub-state which effectively prevents the conformational changes required for normal activity (Johansson and Tanner, 2000). This “protein-binding” model to some extent bridges the gap between a purely biophysical explanation and the “lock and key” model of
classical receptor pharmacology. Good evidence exists however, that many intravenous agents such as the barbiturates, midazolam and the steroids all act with classic drug-receptor interactions but the data is less clear for the volatile agents. While the biophysical aspects of volatile anaesthetic action remain unresolved and limited by the available techniques, accumulating physiological evidence continues to support an important interaction with receptor proteins and associated neurotransmitter systems.

In particular, the superfamily of fast, neurotransmitter gated ionotropic receptors including those for GABA, glycine, acetylcholine and 5-hydroxytryptamine appear to be the most likely targets for the common general anaesthetic agents. The general consensus is that the common volatile agents and many intravenous agents generally depress excitatory synapses and potentiate inhibitory synapses through interaction with receptor protein molecules in particular, ligand-gated ion channels such as those linked to receptors for gamma- amino butyric acid (GABA) (Franks and Lieb, 1994).

GABA receptors expressed on Xenopus oocytes, dissociated rat neurons and cultured rat DRG cells are all potentiated by a variety of anaesthetic agents at appropriately low concentrations (Cheng and Brunner, 1981). This potentiation is manifests as an increase in peak currents. In intact in-vivo or in vitro preparations, a prolongation of inhibitory synaptic potentials or currents can be induced by relevant concentrations of volatile agents (Gage, 1985; Pearce RA, 1996). Anaesthetic agents including the volatile agents, aliphatic alcohols, propofol, alphaxalone and the barbiturates can all enhance the affinity of the GABA receptor for the natural ligand.

The GABA receptor exists in two forms- each a pentameric complex which mediate a change in chloride conductance thereby prolonging and enhancing inhibitory membrane potentials. The GABAa receptor is present on both synaptic and extrasynaptic neuronal membranes. The synaptic form (denoted α1β2γ2S) mediates “phasic inhibition” in response to pulsed high concentrations of GABA released into the synaptic space. In contrast, extrasynaptic GABAa receptors (α6β2δ) are responsible for “tonic” inhibition of neuronal membranes in response to low concentrations of GABA present in extrasynaptic space. This action is facilitated by a high affinity for GABA binding conferred by its particular subunit composition (Soltesz 2000).

The degree of potentiation induced by anaesthetic agents such as the barbiturates and volatile agents (i.e. their potency) is also dependent on the subunit composition of the GABA receptor. Variations in anaesthetic drug action on the other hand, may possibly be a function of receptor distribution within the CNS. The GABAa receptor appears to represent the most sensitive conformation of subunits and is therefore thought to be the most likely target. Single channel studies confirm that anaesthetic agents increase the open probability of the channel without changing its conductance-
finding consistent with enhanced GABA binding and possibly also altered channel gating. This molecular site of action is presumed to underlie a general enhancement of inhibitory neurotransmission throughout the CNS. More precise data regarding the molecular determinants of anaesthetic action are obtained from mutation studies of individual receptor subunits. Mutations within the residues of the second transmembrane domains (TM2) of each of the three subunits -α, β and γ have proven to markedly effect anaesthetic action though other sites may also be important. Residues within the N-terminal region of the α and γ subunits are known to be important for benzodiazepine binding and their subsequent sedative and anxiolytic actions (Walters 2000).

The direct effect of anaesthetic agents on excitatory synaptic pathways has also been investigated. Ionotropic glutamatergic receptor channels have generally proven to be insensitive to the common volatile agents arguing against this being a major mechanism of action of these drugs (Nishikawa and Maclver, 2000).

However, the NMDA receptors does show sensitivity to the dissociative anaesthetic agent ketamine. Ketamine at relevant concentrations is able to block the excitatory effects of NMDA in mammalian brain including NMDA-induced seizures (Anis et al., 1983; Thomson et al., 1985). This synaptic site of action is confirmed by its ability to reverse NMDA-induced behavioural effects in rats (Bennett et al., 1988) and its' stereoselectivity in both the whole animal and at the NMDA receptor in vitro.

The role of the NMDA receptor in general anaesthesia has also been highlighted in recent studies of the anaesthetic actions of the traditional inhaled agent, nitrous oxide (discussed below) and of the noble gas Xenon. Both gases selectively reduce the slow component of the excitatory post synaptic current mediated by NMDA receptors. There is a clear similarity in the pharmacological profiles of ketamine and Xenon (de Sousa et al., 2000; Harrison, 2000).

3.2.2 Urethane (ethyl carbamate)

Urethane was first introduced as an anaesthetic agent by Schmiedeberg in 1885, who noted that urethane induced profound narcosis with little change in respiration and circulation. The agent soon came to be widely accepted as an anaesthetic agent in small rodents undergoing non-recovery experiments. Its' primary advantage at doses of 1g/kg is the production of stable cardiovascular and respiratory parameters with little CNS stimulation. Untoward side effects appear to be related to the route of administration. While intra-arterial and intravenous injection appear relatively safe, intramuscular and sub-cutaneous injection may be locally toxic and intraperitoneal
injection leads to haemoconcentration, transudation of plasma into the peritoneum, hypotension and local tissue damage. It is a mutagenic and carcinogenic compound and hence has been avoided in some institutions (Green, 1982a). Induction of tumours has been demonstrated in mice, rats and hamsters both in topical and systemic administration. It is likely that the actual carcinogen is a metabolite of the parent drug (WHO Committee on Cancer Research, 1974).

The similarity of tissue (including brain) concentrations of urethane to blood concentrations following s.c. injection (Boyland 1949) suggest that urethane achieves a rapid and widespread distribution in rats. It is then metabolized slowly to ethanol and carbamic acid before being excreted (Boyland 1965; Strobel 1969; WHO and Committee on Cancer Research, 1974). The kinetics of repeated injections however have not been elucidated.

Being known to produce hyperglycaemia and hypertension, several studies have investigated the action of urethane on the sympathetic system and concluded that urethane at doses of 1.2g/kg or more activate central sympathetic structures (hypothalamic and limbic) resulting in enhanced sympathetic fibre discharge and raised circulating catecholamines (Reinert 1964).

At doses of 1g/kg given i.p. urethane leads to massive peritoneal exudation with resulting haemoconcentration and hypotension. It is possible that the sympathetic activation ascribed to the direct action of urethane may in fact be a secondary effect of widespread peritoneal irritation and inflammation (Van Der Meer et al., 1975).

In their review, Maggi and Meli note that anaesthetic concentrations of urethane do not inhibit spinal ventral root reflexes (induced by dorsal root stimulation) in frog preparations but that depression is significant at marginally higher concentrations. They note also that urethane does not influence unit activity in rat diencephalon at doses of up to 1.3 g/kg (Cross and Dyer, 1971). In keeping with this finding, urethane does not prevent hypothalamic responses to thermal, painful and auditory stimuli, allowing these stimuli to provoke hypertension and reflex bradycardia and more significantly, activation of the electrocorticogram. Under urethane, wind up displays marked variability, a dependence on baseline responses (which are also markedly variable) and is sometimes characterised by "wind down" (Svendsen 1998; Dickenson et al., 2000).

In contrast to the apparent lack of effect of urethane on subcortical neurons, effects on higher order neurons have been documented (Angel et al., 1980). A dose dependent depression of the cortical response amplitude with an increase in response latency starts at doses of 1.25g/kg. Slight depression of neuronal function at various CNS sites, perhaps more particularly at the thalamic nuclei and within certain cortical cells remains the proposed mechanism of action of urethane (Maggi and Meli, 1986).
EEG recordings under urethane anaesthesia closely resemble those of the normal sleeping state lacking a marked depression of brain activity (Lincoln 1969). Frontal cortex recordings in urethane anaesthetised animals do not differ significantly from awake controls unlike the suppression of activity seen with barbiturates (Pichlmayr et al., 1984).

Though little data are available on its' molecular mechanism of action, it has been shown to cause hyperpolarization of isolated nerve cell membranes and also possesses some anticholinesterase activity. Urethane is able to reverse the antagonistic effects of bicuculline on GABA-induced depolarizations in-vivo (Bowery and Dray, 1978).

Unfortunately, in-vitro investigation of the effect of urethane on GABAergic neurotransmission has added only a small amount of additional information. The evidence favouring a GABAergic action is balanced by a variety of data failing to support a significant interaction with GABA receptors but Schofield (1980) and others found urethane to be the least potent of a series of anaesthetic agents in producing GABAergic inhibitory potentials in guinea pig olfactory cortex.

It therefore appears that anaesthetic doses of urethane (plasma concentrations of 10-20 mM) produce only minimal or no enhancement of GABAergic neurotransmission within the central nervous system (Maggi and Meli 1986) making it markedly different in its mechanism of action to other common anaesthetic agents e.g the barbiturates and volatile agents.

3.2.3 Halothane and other inhalational agents

Inhalational techniques have followed the introduction of calibrated vapourisers. The ease of administration combined with the ability to maintain a constant and adjustable depth of anaesthesia is balanced by the need for more expensive equipment and vigilance over both respiratory and cardiovascular parameters. Both spontaneous ventilation and positive pressure ventilation are possible though the latter can be technically challenging in small rats (Green, 1982b).

Significantly improved laboratory animal survival times have been documented for an anesthetic regime involving paralysis and ventilation with halothane when compared with spontaneous ventilation techniques with either urethane, halothane or alphaxalone-alphadalone (Holder, 1992).

Any distinction between anaesthetic and analgesic actions of the volatile agents is difficult to make. The analgesic effects of the volatile agents can only be studied at sub-hypnotic doses. To date, only methoxyflurane and perhaps isoflurane are generally thought to possess any clinically relevant analgesic effects. At least some of this effect is thought to be mediated at the level of the spinal cord (Namiki 1980). Difficulty in
distinguishing anaesthetic effects from analgesic actions has prompted greater interest in possible pre-emptive and antihyperalgesic actions of these agents.

Volatile agents (isoflurane, enflurane, halothane and desflurane) and nitrous oxide significantly suppress the second phase of the formalin test. Assuming that the second phase of the formalin response is a reflection of altered spinal transmission, this may suggest that these anaesthetic agents significantly attenuate dorsal horn neuron sensitization. Interestingly, nitrous oxide antagonises the depressant effect of halothane i.e. the combination of nitrous oxide with halothane returned the second phase of the formalin test to control levels (O'Connor and Abram, 1995; Goto et al., 1996; Goto et al., 1992).

Only a few studies have compared urethane and halothane directly within the same experimental design. Halothane anaesthesia (1% in 66% N₂O) results in significantly larger baseline C-fibre responses from spinal neurons than urethane anaesthesia (1.3-1.7g/kg) in spontaneously breathing adult rats. This has obvious implications for the calculations of wind-up but interestingly, the sensitivity of this wind-up to NMDA antagonists (AP5) was not significantly different between the two anaesthetic regimens (Svendsen et al., 1999).

Long term enhancement of C fibre-evoked activity can be demonstrated in the presence of volatile anaesthetic agents but in contrast to urethane anaesthetised animals, A fibre-evoked responses are not enhanced with typical conditioning stimuli (tetanic electrical sciatic nerve stimulation) in the presence of halothane (Svendsen et al., 1998; Rygh et al., 2000).

3.2.4 Nitrous oxide

Nitrous oxide has long been accepted to possess significant analgesic properties in both clinical practice and animal experimental models of acute pain. Its continued use, though entrenched in clinical practice and accepted by many laboratories, is regularly questioned for a variety of reasons, not least of which is an uncertain mechanism of action (Shaw and Morgan, 1998).

There are considerable experimental data suggesting that N₂O interacts with the opioid system directly or indirectly, not only with mu receptors but possibly also with the delta, kappa and sigma receptors (Gillman, 1998). The evidence includes the demonstration of cross tolerance to morphine in both mice and rats (Berkowitz et al., 1979), evidence for the direct release of opioid peptides and the demonstration of the reversibility of effects by naloxone (Berkowitz et al., 1976). Systemic (but not spinal) naloxone, ablation of the peri-aqueductal grey matter and spinal cord transection each abolish the antinociceptive effect of nitrous oxide (Guo et al., 1996; Zhang et al., 1999). Human studies also provide data with a mechanism of action involving opioid
receptors (Yang et al., 1980). One proposal is that nitrous oxide induces opioid peptide release in the peri-aqueductal grey of the mid-brain stimulating descending noradrenergic pathways. These in turn, modulate spinal nociceptive signaling through alpha-2 adrenoceptors in the dorsal horn. In this view, the inhibitory actions of nitrous oxide on NMDA receptors are thought to mediate the euphoric effects of the gas only (Maze and Fujinaga, 2000). Importantly, a corollary to this theory is that nitrous oxide has no analgesic action prior to the maturation of descending pathways and is therefore an ineffective analgesic agent in early development.

More recently, nitrous oxide has been found to possess significant actions on NMDA receptors (Franks and Lieb, 1998; Jevtovic-Todorovic et al., 1998). Both nitrous oxide and MK-801 have similar excitotoxic and neuroprotective effects. Neuroprotective effects of nitrous oxide are noted at clinically relevant concentrations (EC50 55%) while the neurotoxic effects occur at much higher concentrations (achieved under hyperbaric conditions- EC50 117%). Electrophysiological recordings of ligand gated currents in cultured rat hippocampal neurons confirm that nitrous oxide has significant NMDA antagonist actions at clinically relevant concentrations (solutions equilibrated with 20-80 vol% N₂O mixtures). Studies using recombinant receptors expressed in oocytes confirm NMDA receptor inhibition by N₂O at clinically relevant concentrations (Yamakura and Harris, 2000).

The relative contributions of nitrous oxides' two proposed mechanisms of action for clinically relevant analgesia deserves further examination. The assessment will depend on the appropriate use of models of pain and hyperalgesia and a careful distinction between anaesthetic and analgesic actions (Gillman 1989, de Lima et al., 2000).
3.3 METHODS

The basic method of EMG recording described in Chapter 2 was used for this series of experiments. For the study of anaesthetic effects, rat pups at three ages were studied in a naive (non-inflamed) state. No lumbar dissection was carried out in these pups.

EMG recording was commenced no sooner than 30 minutes after reducing the inspired halothane concentration from surgical levels (required for tracheostomy and positioning within the frame).

Whenever changes to the inspired gas composition were made (halothane or nitrous oxide concentration), 15 minutes of undisturbed ventilation were allowed to elapse before EMG recording was re-commenced (halothane vapour pressure measurements were not available during the course these experiments). Nitrous oxide fractions were controlled using a standard “bobbin in glass” rotameter while halothane concentrations were controlled using a single plenum vaporiser dedicated to this entire series of experiments. Urethane dissolved in sterile water (250mg/ml) was injected intraperitoneally at the doses stated, 20 minutes prior to tracheostomy formation (30 minutes prior to EMG recording).

Data was analysed as described in Chapter 2 using analysis of variance.
3.4 RESULTS

An appropriate anaesthetic depth for EMG studies was defined using the following behavioural criteria:
- stable heart rate (270 - 400/ min)
- lack of spontaneous movement,
- easy control of ventilation without respiratory upset,
- no gross purposeful movement in response to mechanical stimulation except the reflex withdrawal of the limb being tested.

These criteria were applied to all three age groups (P3, PIO and P21) and the inspired halothane concentrations required to achieve them was documented as follows:

<table>
<thead>
<tr>
<th>Age (post natal days)</th>
<th>Inspired Halothane concentration (vol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>1.0</td>
</tr>
<tr>
<td>P10</td>
<td>0.9</td>
</tr>
<tr>
<td>P21</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Confirmation that these levels of anaesthesia were equivalent across the age groups was obtained post hoc, from the recorded thresholds. Figure 8 shows thresholds for eliciting the withdrawal reflex recorded for both mechanical and electrical stimuli at three ages. Neither mechanical nor electrical thresholds showed any significant change with age. These results are thought to reflect the fact that anaesthetic depth was equivalent in the three age groups tested.

Reflex responsiveness is sensitive to anaesthetic depth (inspired halothane concentration). This dose dependency was systematically studied. (Figure 9) The leftward shift of the dose-response curve with increasing age (younger animals require higher inspired halothane concentrations for the same level of reflex responsiveness) confirms higher MAC values in early development.

The effect of inhaled nitrous oxide on the responsiveness of the withdrawal reflex arc is demonstrated in Figure 10. In each of three age groups (P21, P10 and P3), the addition of inhaled nitrous oxide in concentrations of 33% and 66% is compared with the EMG response obtained under halothane in an air/oxygen mix. Data is presented as mean and SEM. To allow better discrimination of the effect of this inhaled agent, slightly lower halothane concentrations were used than those described above (i.e. P3: 0.8%; P10: 0.75%; P21: 0.6%) The results shown in Figure 10 show that...
nitrous oxide dose-dependently depresses reflex responsiveness in rat pups at all three age groups tested.

In an attempt to determine equivalent anaesthetic doses of urethane, EMG responses were obtained in rat pups anaesthetised with urethane i.p. at three doses—(1.7, 2 and 2.5g/kg, Figure 11) Anaesthetic depth as determined by assessing general responses to handling and noxious pinch as well as the response to artificial ventilation suggested that 2 g/kg urethane was equivalent to halothane 0.7% in P21 rat pups. Comparison of the reflex responsiveness also confirmed that urethane 2g/kg i.p. rendered a similar responsiveness to halothane 0.7% in air/oxygen. Interestingly, urethane anaesthesia abolished the hyperalgesic effect of carrageenan inflammation that was seen under halothane anaesthesia. In fact, the withdrawal reflex responsiveness appeared to be significantly depressed by the combination of carrageenan inflammation and urethane anaesthesia.

3.4.1 Establishing equivalent anaesthesia

**Figure 8**
Mechanical and electrical thresholds were not significantly different across the three ages tested. This confirmed that anaesthetic depth was equivalent at the three ages.
3.4.2 The age-dependent sensitivity of “reflex responsiveness” to halothane.

Figure 9

Reflex responsiveness (RR) is very sensitive to anaesthetic depth. This plot of inspired halothane concentration against reflex responsiveness shows that at any inspired halothane concentration, younger pups display greater responsiveness (i.e. there is a leftward shift in the dose-response curve with age). (Mean and SEM are displayed.) Also, increasing age is associated with a steeper dose-response curve.

Two way analysis of variance: Is there an interaction between age and halothane concentration? p<0.0001. Linear regression for P3 data (mean Y values), Slope = 10.96 ±1.28, X-intercept= 12.19 ±1.10, r²= 0.97

Although, reflex responsiveness has not been specifically interpreted as a measure of anaesthetic depth, these results suggest that it does parallel age related changes in MAC (minimum alveolar concentration) of the volatile agent, halothane. Pharmacodynamic studies were completed at concentrations of halothane that produced similar RR values in naive pups at the three ages tested i.e. 0.7% for P21 pups, 0.9% for P10 pups and 1.0% for P3 pups.
3.4.3 Nitrous oxide depresses reflex responsiveness

Figure 10a and b

The effect of nitrous oxide on the withdrawal reflex. In rat pups at all ages tested, nitrous oxide potently and dose-dependently inhibits the withdrawal reflex. These data are obtained from non-inflamed rat pups ventilated with halothane to a slightly lesser anaesthetic depth than for the other studies. (Mean and SEM are displayed).

Two way ANOVA is reflex responsiveness affected by age and/or concentration?

- Age: p=0.8775
- Concentration: p<0.0001
- Interaction: p=0.916

Linear regression r² 0.4-0.55 for each of the three lines shown in b)
3.4.4 The dose-dependent effect of urethane on the withdrawal reflex—a dose comparison with halothane in 21 day old rats.

**Figure 11**

a) Urethane dose-dependently inhibits the withdrawal reflex EMG response. The relationship suggests a very steep dose-response curve between 1.7 and 2.5 mg/kg making this anaesthetic agent too variable for pharmacodynamic study of epidural drug effects. (Mean and standard deviation are displayed). One way analysis of variance: Are the means significantly different? p=0.0002.

b) In contrast to its effect in halothane anaesthetised pups, carrageenan inflammation appears to depress reflex responsiveness in urethane anaesthetised pups. (Mean and standard deviation are displayed). Unpaired t-test Urethane 2g/kg naïve vs inflamed p=0.017
3.5 DISCUSSION

3.5.1 General

The ideal anaesthetic regimen for animal experimentation in the field of pain research would deliver:

1) Humane anaesthesia
2) Physiological stability over a reasonable time frame- (say 2-6 hours). This must include cardiovascular stability and fluid and electrolyte (including acid-base) homeostasis.
3) Predictable and reproducible effect on afferent sensory pathways
4) Lack of central excitatory effects
5) No independent antihyperalgesic effects
6) Ease of administration
7) Provide a rigid and stable recording field appropriate for precise microelectrode placement
8) No strain variation
9) Immediate relevance to and parallel with human clinical anaesthesia
10) The ability to facilitate reduction in the number of animals used and the refinement of experimental techniques.
11) Strong and simple correlation with behavioural and in vitro experimental preparations e.g. tail flick latency studies and studies on perfused tissue samples.

The model described above does provide for humane anaesthesia with reasonable stability for electrophysiological recording. It has direct relevance to clinical human anaesthesia and being based on an inhaled volatile agent, allows direct correlation with in-vitro experimental techniques. The analysis of a measured response to noxious stimulation as used here has much in common with the basis of determining potencies of anaesthetic drugs (e.g. determination of MAC values). Unfortunately, of itself this does not help in distinguishing between analgesic and anaesthetic actions of a drug. The measurement of drug effects on the facilitated withdrawal reflex (i.e. carrageenan inflamed) may better reflect analgesic effects.

The main disadvantage of inhalational anaesthesia is the requirement of expensive anaesthetic equipment including calibrated, temperature-compensated vaporizers and end-tidal agent monitors. Also, the use of halothane in ventilated animals without a muscle relaxant requires greater vigilance over cardiorespiratory
parameters when compared with the use of urethane. This may be the result of the
former technique lacking both the sympathomimetic actions of urethane and the volatile-
sparing effect of muscle relaxant techniques.

The avoidance of muscle relaxants (essential for EMG recording) also
represents a distinct advantage of this preparation as this helps ensure that experiments
remains humane. The depth of anaesthesia is more easily assessed and controlled when
reflex activity (to both noxious and non-noxious stimuli) is visible. This combined with
the readily adjustable concentration of inhaled volatile agent ensures that the
experimental preparation "fails safe".

### 3.5.2 Depth of Anaesthesia

Anaesthetic depth can be assessed in the following ways (Drummond, 2000)

a) Guedels criteria for human anaesthesia

b) Physiological variables (craniofacial EMG, Respiratory sinus arrhythmia,
heart rate variability).

b) EEG derivatives (e.g. spectral edge frequency, median power frequency,
bis-spectral index).

c) Cortical evoked responses (e.g. auditory evoked responses, P300)

c) Behavioural criteria in animals like those outlined in the methods above.

d) Withdrawal reflex thresholds

Only the last two have any real practical value in animal
experimentation. The use of halothane anaesthesia for EMG recording requires the
careful control of anaesthetic depth. The hindlimb withdrawal reflex like most other
reflexes is very sensitive to the inspired concentration of the volatile agent. Reliability
of the recordings are facilitated by regular maintenance of the vaporizer and end-tidal
concentration measurement. The use of high quality polytetrafluorethylene tubing for all
anaesthetic gas tubing would help minimize loss and therefore variability of volatile
agent.

MAC (minimum alveolar concentration) values for adult rats (Sprague-Dawley)
have been determined for a variety of agents:

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>MAC</th>
<th>Bracketed MAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane</td>
<td>1%</td>
<td>(0.95)%</td>
</tr>
<tr>
<td>Enflurane</td>
<td>2.2%</td>
<td>(2.45)%</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>1.4%</td>
<td>(1.58)%</td>
</tr>
<tr>
<td>Desflurane</td>
<td>7.7%</td>
<td></td>
</tr>
</tbody>
</table>

(from Mazze et al., 1985 - bracketed data from Cole et al., 1990)
Data have been collected by several investigators using only partially standardised techniques (White et al., 1974; Maze et al., 1985).

The combination of nitrous oxide with volatile agents does not follow the previously widely accepted principle of simple additivity. The parabolic deflection of MAC values induced by nitrous oxide (i.e. N2O makes a proportionally greater contribution to MAC at lower concentrations) may reflect opposing neurophysiologic effects between nitrous oxide and the volatile agents and/or a biphasic pharmacodynamic effect for the combination (Cole et al., 1990).

While the use of halothane anaesthesia for in vivo electrophysiological studies in rats is widespread, few experimenters make specific mention of the dose-related effects of this anaesthetic on results. A range of inspired halothane concentrations are tolerated and do not appear to have a systematic influence on neurons of the dorsal horn. For example, Morgan adjusted halothane concentrations between 0.6 and 1% to achieve a depth that allowed reflex withdrawal from noxious stimuli in the absence of spontaneous or prolonged movements (Morgan, 1998). Schouenborg and Kalliomaki measuring reflex EMG responses in rat hindlimbs maintained stable recordings using 0.5-0.6% halothane in spontaneously breathing rats. In their preparation, non-paralysed and un-restrained rats were kept atonic but with weak withdrawal reflexes (no other motor responses evoked by noxious stimulation of the skin) (Schouenborg and Kalliomaki, 1990). These researchers, without systematically studying the effect of anaesthetic depth on the EMG recordings, did concede that it might “conceivably have affected the properties of the withdrawal reflex” being studied. However they argued that the organizational principles found and characteristics such as “local sign” and “irradiation” were not significantly altered.

All available rat data are obtained from Sprague-Dawley rats and no data appear available on strain variation of MAC values. No data are available on MAC values in rat during early development. (White does comment on the inverse relationship between anaesthetic requirements and increasing age, noted in unpublished data from adult rats and its agreement with data from man (White et al., 1974)).

A discussion of anaesthetic depth necessarily includes a debate on what comprises anaesthesia and how it might differ from analgesia. Diagram 11 attempts to show some factors that contribute to each of these two processes together with lists of common classes of anaesthetic and analgesic agents. A fuller discussion is presented in Chapter 5.
Anaesthesia and Analgesia

### Anaesthetic agents
- GABAergic agents
- Local anaesthetics
- NMDA antagonists

### Analgesic agents
- Opioids
- NMDA antagonists
- NSAIDs
- Adrenergic agents
  - Others: e.g. cannabinoids
  - low dose local anaesthetics
  - purinergic antagonists

### Anaesthesia
- Unconsciousness
- Amnesia
- Reduced muscle tone
- Autonomic depression
- Increased withdrawal reflex threshold

### Analgesia
- Reduced central sensitization
- Reduced peripheral sensitization
- Reduced hyperalgesia

#### Diagram 11

The common classes of anaesthetic and analgesic drugs are listed here together with a list of physiological alterations that can be used to define each pharmacological state. In this scheme, an increase in reflex withdrawal threshold is classed as an anaesthetic effect while analgesia is defined specifically as a reduction in hyperalgesia.
3.5.3 Nitrous Oxide: efficacy during development

Nitrous oxide has been in clinical use for over 150 years. It is used as a supplement and carrier gas for typical volatile anaesthetic agents and to reduce requirements of the latter. It is thought to have significant analgesic actions of its own that make it a useful either when used alone or as an adjunct to other analgesic agents in particular clinical situations.

Debate about the mechanism of action of this widely used gas continues. Though several molecular targets have been identified, two dominant hypotheses share current favour. One of these suggests that nitrous oxide causes the release of opioid peptides from midbrain neurons (Maze and Fujinaga, 2000). Importantly, this theory predicts that N2O will have no antinociceptive action prior to the maturation of descending noradrenergic pathways (4 weeks in rats and toddler age in humans). This has obvious implications for paediatric anaesthetists as the conjecture that N2O does not provide any measurable analgesia in human infants under two years old would be quite surprising.

Much of the data supporting the opioid/noradrenergic theory rely heavily on the tail flick latency test in rats, a test which models acute nociception rather than post-injury hypersensitivity. Significantly, the data preclude a direct action of N2O on spinal nociceptive neurons (Zhang et al., 1999; Guo et al., 1996). It is difficult to resolve these data with the now well accepted action of nitrous oxide on NMDA receptors (Jevtovic-Todorovic et al., 1998) - given that a) spinal nociceptive processing does involve NMDA receptor mediated transmission (Woolf and Thompson, 1991); b) that NMDA receptor antagonists such as ketamine and MK-801 do have significant antinociceptive actions at subanaesthetic doses and that these actions are at least partially mediated at a spinal level (Hao et al., 1998; Dickenson and Sullivan, 1990) and c) that the kinetics of nitrous oxide do not preclude a spinal cord biophase (i.e. there is no reason to expect spinal cord tensions to be less than cerebral tissue tensions) (Stenqvist, 1994) - a direct antinociceptive action of nitrous oxide at a spinal level would be predicted. The experiments in which antinociceptive actions of nitrous oxide were abolished by supraspinal intervention require careful re-interpretation.

Perhaps a key to this re-interpretation is the distinction between acute antinociception and anti-hyperalgesia. For nitrous oxide, the first may depend on an opioid/noradrenergic mechanism while the latter may be mediated by NMDA receptor blockade.

Data on the efficacy of nitrous oxide in early developmental stages are critically important for the continued rational use of the agent in paediatric anaesthetic and analgesic practice. Data confirming a spinal site of action for N2O in rat pups prior to
the maturation of descending noradrenergic pathways would argue against the importance of the opioid/noradrenergic hypothesis. The data presented here while confirming efficacy of the gas in early development stages, require further development before they can be used in the debate outlined above. Experiments testing the efficacy of nitrous oxide in carrageenan inflamed pups and its efficacy in spinally transected rat pups will prove more valuable.

In view of the data confirming an interaction between nitrous oxide and glutamatergic neural transmission—in particular, the NMDA receptor system, an anaesthetic regime that did not include this agent (i.e. Oxygen/Air/halothane) was used for all pharmacodynamic studies of antinociceptive agents.

3.5.4 Urethane anaesthesia

The trials involving the use of urethane revealed three preliminary findings:

a) that the anaesthetic dose in 21 day old rat pups (approx. 2g/kg) is greater than that typically reported for adult pups (1-1.25g/kg).

b) that urethane dose-dependently inhibits the withdrawal reflex as measured electromyographically and this relationship is defined by a very steep dose-response curve between 1.7 and 2.5 g/kg.

c) following peripheral inflammation, sensitivity of reflex excitability to urethane anaesthesia appears to be increased.

The age related differences in effective dosage may be the result of kinetic factors (e.g. slower absorption, an altered volume of distribution or greater clearance in younger pups) or dynamic factors reflecting age related changes in neurotransmission (in particular, the receptor-ligand systems on which urethane may act). No data are available regarding the kinetics of urethane in early development and there is no consensus yet regarding the exact site of action of the drug. Little further comment is possible.

The steep dose-response relationship between urethane and reflex responsiveness imply that this form of anaesthesia is innappropriate for the study of epidurally administered analgesic drugs in this model.

The surprising finding of increased sensitivity to urethane in the face of peripheral tissue inflammation— if not a spurious finding, is open to speculative explanation. The finding suggests that urethane may act on a mechanism that is more prominent/up regulated in the inflammatory state e.g. the NMDA receptor system or the sP peptidergic system. Both of these possibilities await clearer definition of the mechanism of action of urethane. Another difference between the naive and inflamed state is the activation of the sympathetic-pituitary-adrenal axis. Urethane is known to
activate this important homeostatic system (Reinert 1964). This system may have significant inhibitory effects on nociceptive processes via release of "stress hormones". An increased sensitivity to urethane may then conceivably be due to greater stress response in pups that have been "primed" by a prior inflammatory insult. This idea has parallels with the finding of tachyphylaxis to carrageenan described by Battacharya (Battacharya et al 1987).

Further study of these issues may allow clearer interpretation of much experimental data already gathered from animal models in which urethane anaesthesia has been utilised.
Chapter 4 MODELS OF INFLAMMATION AND INFLAMMATORY PAIN

4.1 Inflammation- a general overview

4.1.1 Definition
Inflammation is a coordinated tissue response to cellular debris and substances or antigens recognised as foreign to the organism. The response may be either localised or systemic. Local inflammation, which is the focus of this discussion, is defined in clinical practice by the appearance of redness, induration, heat and pain. To these original features of Celsus (30BC), Virchow added “loss of function” of the inflamed tissue (usually implying a loss of movement in an inflamed limb).

Histologically, it is characterised by vascular dilatation, pavementing and then migration of leucocytes and an increase in tissue fluid (inflammatory exudate).

Physiological processes involved include: vasodilation and oedema formation (exudation), chemotaxis, leucocyte degranulation, phagocytosis by neutrophils and sensitization of peripheral nerves. A later proliferative process precedes the resolution of inflammation (Walter, 1987).

Central in the initiation and control of the response is the immune system.

Activation of the response can be achieved through

a) degranulation of mast cells residing within extravascular tissue spaces releasing histamine and other mediators (NGF, histamine and 5HT). On activation, mast cells are able to metabolise arachachidonic acid to mediators such as the leukotrienes, prostaglandins and thromboxanes.

b) leucocyte-endothelial adhesion with subsequent release of lysosomal compounds.

c) the release of arachadonic acid metabolites from damaged cells notably, endothelial cells.

d) enzyme cascade activation, in particular activation of Hageman factor (FXII) eventually leads to the activation of the coagulation, kinin, fibrinolytic and complement systems.

Endothelial cell and mast cell reactions are probably the crucial steps in the triggering of most inflammatory responses (Underwood, 2000; Walter, 1987).
4.1.2 Types

Inflammation is most usually classified into acute and chronic forms. Chronic inflammation differs not only in its time frame but more importantly in the molecular and cellular processes that underlie it. An older classification into “phagocytic and non-phagocytic” forms was probably just a reflection of the division between the pattern of acute and chronic inflammation. The following discussion is focused on acute inflammation.

The entire acute inflammatory response is characterised by co-operativity between inflammatory and immune cells together with a large latent potential. This is represented by the storage and sequestering of cells and mediators and the plasma store of inactive precursors. Once triggered, the process is sequential and involves several powerful amplification systems whose spatial and temporal spread are under constant control by inhibitory mechanisms. The process therefore reflects a changing equilibrium between often competing processes (Underwood, 2000).

Some of these characteristics are shared by the nervous system. This observation may be more than just incidental as a close inter-relationship between the CNS and the immune system is being increasingly recognized (Watkins and Maier, 1999; Perry and Gordon, 1997). Interaction between these two systems is bi-directional and especially evident in what is now termed the “acute phase response” or “sickness response” (Salzet et al., 2000).

4.2 Models of Inflammation

Models of inflammation have been used extensively for the screening and evaluation of anti-inflammatory drugs. Various agents have been used in the past including brewers yeast, formalin, dextran and egg albumin. Unfortunately the anti-inflammatory drug effects (measured as a reduction in oedema induced by these agents) are often non-specific and have failed to demonstrate clear dose-response relationships at clinically relevant concentrations. During the sixties several newer agents (carrageenan, kaolin, mustard and formalin) were tested and carrageenan was found to possess distinct advantages over previously used inflammatory agents (Winter et al., 1962; Woolf and Thompson, 1991).

Three of the more common models of inflammation in animal experimental work are described below. A brief description of formalin and Freund’s adjuvant is followed by a fuller description of carrageenan.
4.2.1  Formalin

The use of formalin grew out of experiments that utilised hypertonic saline in human experimental subjects to produce brief, intense laboratory pain (Lewis and Kellgren 1939; Frankstein, 1947). Dubuisson and Dennis gave the test its definitive form and used it to evaluate the analgesic effects of morphine, pethidine and periaqueductal grey matter stimulation (Dubuisson and Dennis, 1977).

Formalin is the aqueous solution of 37% (weight/weight) formaldehyde often with methanol as preservative. It produces a sterile injury if injected into living tissue and has been used in many animal models of pain. Injected into human subjects it produces a poorly localized pain with burning and stinging qualities which then gives way to a steady throbbing ache lasting up to 60 minutes. In rats, the behavioural response takes a biphasic pattern. Initial pain behaviours after paw injection (elevation, licking and shaking) last about 5-10 mins and are followed by a brief period (5-15 mins) during which the rat ignores the paw. A second phase of pain behaviour then re-emerges and lasts for up to 2 hours (Dubuisson and Dennis, 1977; Guy and Abbott, 1992).

The test has the following advantages over tests of acute nociception (such as the tail flick test):

a) the relatively continuous nature of the induced pain compared to transient laboratory pain induced by heat, electric shock or skin deformation. The pain being modelled is tonic rather than phasic (short lasting). This may be of greater clinical utility.

b) its convenience as the test obviates the need for animal restraint, allowing unhindered observation of the behavioural responses. This represented a major advance from the use of the tail-flick test, the flinch-jump test and the hot-plate test.

c) the use of formalin concentrations between 0.05 and 0.2% avoids major permanent tissue damage. (Although originally suggested to be repeatable, formalin can lead to blister formation and is now rarely applied more than once in any animal).

d) the formalin test appears to distinguish between acute nociception (first phase) and sensitization (second phase) and much has been made of this distinction in pharmacologic studies utilizing the formalin test (Yamamoto and Yaksh, 1992). A further advantage of the formalin test is its ability to model spontaneous pain whose behavioural correlate is flinching and licking (Tjolsen et al., 1992).

e) some correlation with human experience is possible (at least in volunteers) in contrast to the models like the “quinolone writhing test”.

f) it does not appear to induce a systemic illness (in contrast to complete Freund’s adjuvant).

g) the test has a limited duration (1 hour) and is therefore ethically acceptable.
Disadvantages of this model include: a) tissue temperature dependence- especially in smaller rodents (mice). b) susceptibility to environmental cues and stress and c) requirement for scoring of multiple behavioural parameters.

4.2.2 Complete Freund's Adjuvant (CFA)

This is a suspension of heat killed and dried Mycobacterium Tuberculosis (H 37Ra 25177). It is made up in mineral oil and mannide monooleate and can be emulsified in saline. It appears as a clear amber liquid containing brown particles.

Adjuvants are admixtures of compounds which are capable of stimulating the immune system in a non-specific fashion (Allison and Byars 1991). Adjuvants may act in any of the following ways:

i) stimulation of macrophages to secret cytokines such as IL1 and IFN. This follows either direct (for particles and emulsions) or indirect (requiring interaction with complement) phagocytosis by tissue macrophages.

ii) as an antigen vehicle to facilitate long-term presentation of an antigen or to protect an antigen from degradation.

iii) direct stimulation of T cell carrier function. This is achieved by inclusion of T cell epitopes from bacterial proteins in the admixture.

iv) direct stimulation of B cells. This is achieved by incorporating mitogens e.g. muramyl dipeptide, lipid A or bacterial polysaccharides.

v) reduced lymphocyte circulation with resulting improvement in lymph node responses (Allison and Byars 1991).

Freund's adjuvant is prepared by the addition of heat killed mycobacteria (+/- 0.5 mg/ml) to a water in oil emulsion (WIO). This emulsion has, in the past included clear mineral oils such as paraffin or plant oils such as peanut oil. When mixed with an emulsifier and water the result is known as Freund's incomplete adjuvant (FIA). WIO emulsions have been used in veterinary practice for some time either alone or as an adjuvant with various microorganisms. Beneficial effects are noted with a large range of adverse effects, the two being difficult to separate. Local inflammation is consistently present at sites of inoculation and may be crucial to proper functioning as an adjuvant. The nature of the oil phase and the emulsifier appear to determine the degree of local irritation and is similar across species.
The addition of microorganisms (e.g. M. mycoides, ActinoB pleuropneum., Bovine rota virus) and bacterial proteins and cell wall components increases the immune response to specific determinants thereby creating a vaccine. CFA enhances the immune response aspecifically and is still considered an adjuvant and not a vaccine. It finds regular use in the manufacture of specific immune system derived products (antibodies, cytokines, T cells etc.).

Inflammation induced by CFA (complete Freund’s’ adjuvant) also involves joints and deep tissues (Calvino et al., 1987) i.e. it induces a local and systemic arthritis (Fawcett 1990). Significant side effects of CFA evident in various animal models (both commercial and experimental) have prompted reviewers to demand careful consideration of ethical issues and animal welfare regulations (Claassen et al., 1992). The range of local side effects include: granuloma and sterile abscesses, ulceration, fistulous tracts and necrotizing dermatitis (Johnston 1991), sterile peritonitis (Toth 1989), splenomegaly (Toth 1989) and muscle atrophy.

Systemic side effects include: “metastatic granuloma” e.g. pulmonary (Schiefer 1979), lymphoid hyperplasia, autoimmune polyarthritis and uveitis (Petty 1989), peripheral nerve demyelination (rabbits) (Mizisin 1987).

Despite these limitations, the injection of CFA into the root of the tail subcutaneously became established as a model of polyarthritis in the rat (Pearson and Wood 1959). This adjuvant-induced arthritis (AIA) model was subsequently validated in a variety of behavioural and pharmacological studies (Calvino et al., 1987; Colpaert, 1987).

Because the complexity of the polyarthritic model makes it less useful for the study of chronic pain states (i.e. observed changes in behaviour could not be definitively and solely ascribed to nociceptive input) an attempt to develop a model of unilateral, localized inflammation using CFA was made. An intraplantar injection of CFA into the hindpaw (rather than into the root of the tail as in the AIA model) was reported to produce just such a localized inflammatory insult (Stein et al., 1988). This report remains unconvincing however, as a systemic response was still recorded (reduced weight gain, reduced food and water intake and a disruption of circadian temperature regulation), follow up was only to 34 days, neither clinical nor histological study was made of supposedly un-involved joints, data on sensory thresholds involving un-involved joints was inadequate and the researchers admitted that in a proportion of animals the arthritis did appear in other joints. The persistence of this model in studies of nociception may partly be a result of uncritical acceptance of this study.
Several more recent studies using the CFA model have shown long term consequences of the induced inflammatory response. The sensory effects of CFA persist well beyond those of carrageenan; cold allodynia for example persists for 70 days in rats (Perrot, 1993). A few studies have documented long term changes in nociceptive responses to mechanical stimuli (Butler, 1992) and central changes that persist for 30 days have been reported (Goff, 1998; Jasmin et al., 1998).

Recently, long lasting changes in central afferent terminal fields and behaviour in adult rats following neonatal inflammation with CFA have been reported (Ruda et al., 2000). Some of these changes may be due to non-neural processes (Anand, 2000), but it is equally likely that an intense inflammatory response may result in neural damage and that the behavioural changes are a result of neonatal neuropathy.
4.3 Carrageenan

An inflammatory pain state was modelled in the current study because of its potential clinical relevance. Defining this inflammatory state across a range of ages in rat pups was necessary before studying any developmental pharmacology. Several issues prompted the choice of carrageenan-induced inflammation as the basis of the model:

(i) As described in Chapter 1, carrageenan does not induce marked behavioural signs of pain yet is known to reliably induce hyperalgesia to both mechanical and thermal stimuli.

(ii) Subcutaneous carrageenan injection results in an inflammatory process with a convenient time frame - i.e. onset within 2-3 hours and persistence for up to 6 hours. In this respect, the time course for formalin was thought to be too short and that of CFA, too long.

(iii) Subcutaneous carrageenan injection results in a reliably unilateral and local inflammatory process. In contrast CFA is likely to result in a progressive systemic inflammatory disease.

4.3.1 Description

Carrageenan is a mucopolysacharride derived from Irish sea moss, Chondrus. This algae contains a mixture of at least 2 two forms (κ and λ) of this polysacharride that each have molecular weights in excess of 100,000 (Smith, 1954). The polymers consist of units of galactose and 3,6-anhydrogalactose which are esterified with sulphuric acid (Rees 1963).

Lambda carrageenan is prepared as a 1-2 % non gelating -hydrocolloid in saline and has been injected subcutaneously, intra-pleurally and intra-articularly to induce inflammation where it appears to produce only local inflammatory effects, is non-antigenic and is devoid of systemic side effects (Gardner, 1960). Furthermore it does not appear to cause the release of either histamine or serotonin. Good reproducibility and detectable regression of induced oedema in response to clinically relevant anti-inflammatory drug doses confirmed carrageenan as a “phlogistic agent” of choice.
4.3.2 Carrageenan-induced inflammation

In an extensive study of inflammatory agents such as formalin and mustard oil, only carrageenan was found to give a reliable degree of oedema (Winter 1962). Following injection of 0.1ml carrageenan (0.1% in 0.9% saline) in the rat, mean paw volume increases and peaks by a similar amount over the next one to three hours after injection. Paw volume remains roughly constant over the following 24-96 hours and thereafter declines, being barely detectable after 7 days (Bhattacharya et al., 1987; Kayser and Guilbaud, 1987). The oedema follows two phases, an early transient phase starting immediately after injection and lasting 20-60 minutes, followed by a delayed phase lasting several hours (van Arman 1965; Vinegar et al., 1969). The early phase of the response is due to the trauma of injection and is independent of the quantity of carrageenan injected. Further, this early phase is not seen following the pleural injection of carrageenan (Doherty and Robinson, 1975). Studies with labelled albumin suggested that protein extravasation occurred throughout the whole response to carrageenan and was not restricted to the early phase as was previously suggested by Garcia Leme et al (Garcia Leme 1968).

Tachyphylaxis to Carrageenan injections is noted within the first 24 hours following an initial injection. This phenomenon resolves after 7 days. Experiments in rats either surgically adrenalectomized or chemically sympathectomised suggested that tachyphylaxis to carrageenan may be due to activation of the sympatho-adrenal axis after initial induction of inflammation (Bhattacharya et al., 1987).

Twenty minutes after injection of carrageenan a few perivascular neutrophils are observed in histological sections and by 60 minutes after injection there is a 10-fold increase in the relative number of neutrophils. These are mostly perivascular but a few do migrate into the extravascular space. Though the greater volume of oedema fluid at 180 minutes results in physical disruption of tissue, a 50-fold increase in the relative number of neutrophils is still evident and these are noted to be primarily in the extravascular space (Vinegar et al., 1976).

Pharmacological studies following sub-plantar injection and the analysis of pleural exudate fluid following intra-pleural injections allows several conclusions. Firstly, that serotonin, bradykinin, arachidonic acid, PGE1 or PGE2 do not mediate the main (second phase) inflammatory response to carrageenan (Vinegar et al., 1976). These early studies using less potent and specific histamine receptor blockers also suggested that histamine had no role to play.

Secondly, that the delayed phase of carrageenan-induced oedema is directly and causally related to the number of neutrophils mobilised. Exudation of fluid occurs only
after neutrophils are mobilised and have moved from the perivascular space and into the extracellular space. This is in contrast to the oedema generated by formalin or dextran. (Di Rosa, 1971)

The chemotactic agent in acute carrageenan inflammation has not been definitively identified but must result from an interaction between the polycarbohydrate and the cellular elements of the injected tissue.

In summary then, the initial phase of oedema (first 20 minutes) may be accepted as the result of a vasodilating co-compound in the carrageenan preparation or the release of an autocoid such as serotonin from tissue mast cells. The second and main phase of tissue oedema only becomes apparent after neutrophil localisation, a process that itself presupposes the generation and release of a neutrophil chemotactic agent from a tissue source. Following the migration of neutrophils to the extracellular space, the phagocytosis of carrageenan results in lysosomal enzyme release and the activation of the prostaglandin biosynthetic pathway. A highly reactive intermediate species possibly an unstable hydroperoxide, endoperoxide or a thromboxane moiety is the most likely candidate as the mediator of capillary permeability changes that underlie the oedema of the second phase of carrageenan inflammation. (Vinegar et al., 1976)

More recent pharmacological investigation suggests a role for the prostanoids in carrageenan-induced oedema but also confirmed a role for histamine acting largely through H2 receptors (Al-Haboubi and Zeitlin, 1983).

Peripheral nerve terminals are able to release trophic factors and neuropeptides in response to noxious stimulation suggesting a role for these ligands in the inflammatory response. Tissue levels of nerve growth factor (NGF) have been shown to increase twofold following carrageenan inflammation. Interestingly, this increase is not prevented by doses of indomethacin and salicylate that inhibit the oedema formation. The rise in NGF is however, prevented by a dosing schedule of dexamethasone that inhibits oedema formation. This suggests that NGF may be a necessary but not sufficient mediator of the oedema in carrageenan inflammation (Amann and Schuligoi, 2000).
4.3.3 The use of carrageenan inflammation in behavioural studies of pain and hyperalgesia

Some years after the introduction of carrageenan-induced oedema in the screening of anti-inflammatory drugs, Hargreaves and Dubner adapted it as a model of cutaneous hyperalgesia. Initial behavioural studies were followed by extensive electrophysiological and pharmacological studies that attempted to elucidate the mechanisms of both peripheral and central sensitisation.

In their study, Hargreaves and Dubner described the time course and dose-responses of three behavioural correlates of hyperalgesia, each induced using a thermal stimulus. Both the latency of the withdrawal reflex and the presence of licking were scored and recorded. Their results indicated that the thermal method provided reliable quantitation of hyperalgesia and that carrageenan inflammation produced dose dependent behavioural changes. The major advances of this work included the ability to use un-restrained animals, the greater bioassay sensitivity of the thermal method compared to the mechanical tests and the sensitivity of the technique to analgesic agents (morphine and indomethacin). The work not only provided a powerful adjunct to mechanical sensory tests for the study of behavioural hyperalgesia but also established carrageenan as an agent of choice for the induction of experimental hyperalgesia (Hargreaves et al., 1988). Thermal hyperalgesia reaches a maximum 2 hours after carrageenan injection and has been found to sensitive to spinal prostaglandin formation via a cyclooxygenase pathway (Yamamoto and Nozaki-Taguchi, 1997).

Intra-plantar carrageenan also induces mechanical hyperalgesia. The hyperalgesic effect as detected by vocalisation thresholds, begins 15 minutes after injection and appears stable from 60 to 120 minutes. Eighty percent of rats become hyperalgesic (that is, have vocalisation thresholds that fall by at least 16% of control values) an hour after carrageenan injection. The number of rats continuing to display reduced vocalisation thresholds after this time gradually falls until less than 50% of rats are hyperalgesic after 72 hours (Kayser and Guilbaud, 1987). Decreases in the vocalisation threshold are not limited to the injected paw, but are also observed for the other paws (Kayser and Guilbaud, 1987).

The thermal and mechanical hyperalgesia induced by carrageenan appear to follow slightly different time courses. Both are maximal at 2-3 hours after injection but thermal hyperalgesia resolves at 20 hours while mechanical hyperalgesia persists until then (Hedo et al., 1999).
4.3.4 Electrophysiological studies of carrageenan inflammation

Electrophysiological studies of the carrageenan-induced inflammatory state have included measured responses of i) primary afferents (dorsal root ganglion cells) (Kocher et al., 1987) ii) spinal cord dorsal horn cells (Stanfa and Dickenson, 1992, 1994) iii) thalamic neurons and iv) the entire reflex recorded at the motor neuron, ventral root (Hedo et al., 1999) or electromyographically (Solano and Herrero, 1999).

Primary afferent

Early work with carrageenan in animal models suggested that oedema formation is independent of factors (neuropeptides) released from primary sensory neurons (Gamillscheg, 1984). Sensitisation of peripheral nerve fibres due to carrageenan (50μl 2% sol.) results in a reduction in thermal threshold, an enhanced response to stimulation and a shift in peak discharge to lower stimulation strengths. This sensitisation occurs in nociceptive C fibres at 1-2 hours after cutaneous injection - almost an hour before pain behaviours are observed in adult rats. Sensitisation is seen with thermal stimuli (ramp-shaped radiant heat stimulus) only and not with mechanical stimuli (von Frey hairs) suggesting that in the periphery, only mechano-heat sensitive C fibres are significantly affected by inflammation. Sensitisation also is confined to fibres whose receptive fields are within the area of inflammation and does not spread to fibres with adjacent receptive fields (Kocher et al., 1987).

Dorsal horn cells

In extensive studies of electrically evoked dorsal horn cell activity following carrageenan injection into the paw, Stanfa et al found that spontaneous activity does not change significantly but that the magnitude of C-fibre evoked responses is altered. These changes have been documented 3 hours after the induction of carrageenan so as to match the time course of changes seen in paw oedema and behavioural hyperalgesia. Almost equal numbers of cells display increases as do decreases and the direction of the change is significantly correlated to the degree of wind-up seen in the cell prior to inflammation (Stanfa et al., 1992; Stanfa and Dickenson, 1994). Cells showing little wind-up prior to inflammation subsequently show a greater enhancement of evoked responses whereas cells with significant degrees of initial wind-up are more likely to be inhibited with
inflammation. This complex bi-directional effect of inflammation on dorsal horn cell activity may be the result of the activation of inhibitory interneurons. A-fibre evoked responses show much smaller alterations with inflammation. In pharmacological dissections of this phenomenon, Dickenson et al have demonstrated that opioid sensitivity at a spinal level is altered during carrageenan-inflammation (Stanfa et al., 1992) and that this is possibly due to alterations in the dynorphin system (Stanfa and Dickenson, 1994). Further, nitric oxide (NO) appears to be central in spinal cord processing of both acute noxious signals and nociceptive inputs in the carrageenan-induced inflammatory state (Stanfa et al., 1996). Taken with the earlier finding that the NMDA receptor is critical to the development of wind-up (Dickenson and Sullivan, 1987), these authors suggest that during inflammation there are complex interactions between the NMDA receptor and the opioid system and that NO may represent a downstream amplification mechanism following NMDA receptor activation.

When inadvertent (pre-carrageenan) sensitization of dorsal horn cells is minimised, the mean C-fibre response is more consistently, though modestly increased (7%) by carrageenan inflammation. Again, no significant alterations in A-fibre evoked responses, post-discharge or wind-up are induced by inflammation. In both the normal and inflamed state, evoked responses are sensitive to the application of NMDA antagonists (aminophosphonovaleric acid-AP5). Interestingly, sensitivity of wind-up to AP5 is seen only in carrageenan inflamed animals. This finding suggests that the proposed mechanism(s) of the phenomenon of wind-up may differ between the normal and inflamed states (Svendsen et al., 1999).

The spinal reflex arc

Using an isolated cord preparation that allows recording of a ventral root potential, Hedo et al showed progressive changes in spinal reflex responses up to 20 hours after hindpaw carrageenan injection. Significant increases in wind-up were noted from 6 hours after injection. At 20 hours after injection, increased responses to both trains and single high-intensity stimuli and a novel low intensity (Aβ) trains were observed (Hedo et al., 1999).

In spinalised, decerebrate unanaesthetized rats, carrageenan injection induces an initial weak and brief EMG discharge followed by a delayed and gradual increase in reflex excitability. The mechanical threshold for eliciting the withdrawal reflex in reduced from 30 minutes after injection and the magnitude of the reflex is significantly increased 120
minutes after injection. These findings are in contrast with those following formalin injection which induces immediate and intense motor activity (5-10 mins) and a second phase of relatively weak motor activity (20-70 mins) but appears to depress reflex excitability from 90 minutes after injection (Xu et al., 1995).

Further electromyographic studies have shown that carrageenan injection into the rat hindlimb results in an increase in receptive field size, lowering of thresholds and an increase in firing rates of single motor units (SMUs). In general differences between individual muscles that are apparent in the naive state are reduced during inflammation resulting in a more homogenous pattern of firing in all motor units (Solano and Herrero, 1999).

Exploiting the utility of this animal model, drug effects can be studied in both naive and carrageenan inflamed rats allowing some distinction to be made between acute antinociception and anti-hyperalgesia. The antinociceptive effect of intravenously administered opioids (fentanyl) and non-steroidal drugs (Flunixin) and its reversibility with naloxone have been documented in both naive and carrageenan inflamed rats. (Herrero and Headley, 1996).

In the attempt to distinguish primary from secondary hyperalgesia, various researchers have compared the effects of intra-plantar injection of carrageenan to intra-articular injection. The former studied with stimulation of the plantar surface of the inflamed paw serves as a model of primary hyperalgesia. Intra-articular injection with subsequent stimulation of the plantar surface of the paw serves as a model of secondary hyperalgesia.
4.4 METHODS

Two series of experiments are described here. Features of an inflammatory pain state have been described in awake freely moving rats as well as in the halothane anaesthetised rat model described earlier. These features have been characterised across three age groups.

4.4.1 Behavioural studies

In initial pilot work young pups (P3), the injection of even small volumes of carrageenan (5μl) frequently resulted in severe leg oedema with redness and tense over-stretched skin. This gross inflammation appeared to be accompanied by a loss of movement of the injected limb making it impossible to record a meaningful reflex threshold. This prompted the study of differing degrees of inflammation through a novel means of administering carrageenan. By inoculating carrageenan into the epidermis with a depth limiting lancet, greater control of the degree and area of inflammation was obtained. Various degrees of inflammation could be produced by varying the number of inoculations in any paw.

To validate this technique a behavioural study was designed with the following four aims:

A) to describe the time course of the mechanical sensory threshold changes induced by moderate inflammation.

B) to compare the mechanical sensory threshold changes induced by mild, moderate and severe inflammation.

C) to compare the pattern of sensory threshold changes induced by moderate inflammation in the two younger age groups used during pharmacodynamic studies.

Separate litters of each age group of rat pups (P3 and P10) were used to document the changes in mechanical sensory thresholds following subcutaneous carrageenan administration. Determination of the mechanical threshold was done using calibrated von Frey hairs. These were applied perpendicular to the surface of the skin with a pressure just sufficient to bend the hair. The dorsal surface of the paw was tested while the rat pups remained in a normal weight-bearing posture. Each hair was applied in turn three times at about 30 second intervals. The lowest calibrated hair that elicited a clear withdrawal movement on at least two occasions was considered to be the mechanical sensory threshold. All testing was carried out in a warm environment and after allowing the pups to become accustomed to being handled.
Pups at each age were divided into four groups.

**P3 litters:**

1) Control (no paw intervention) \( n=14 \)

2) Severe inflammation: s.c. injection of 5μl 2% carrageenan \( n=13 \)

3) Moderate inflammation: 6 inoculations 2% carrageenan \( n=8 \)

4) Mild inflammation 2 inoculations 2% carrageenan \( n=13 \)

**PIO litters:**

1) Control (no paw intervention) \( n=10 \)

2) Severe inflammation: s.c. injection of 10μl 2% carrageenan \( n=8 \)

3) Moderate inflammation: 10 inoculations 2% carrageenan \( n=8 \)

4) Mild inflammation 2 inoculations 2% carrageenan \( n=5 \)

Sensory thresholds were tested in the fashion described above at hourly intervals for 5 hours and than again 24 hours later. Data were analysed by calculating both the mean and the median threshold recorded for any group at any one time. Data have been displayed in graphical form.

### 4.4.2 Problems of statistical analysis of data obtained from behavioural experiments on hyperalgesia during development.

Experiments described in this chapter are typical of many behavioural experiments in the area of developmental pain research. The design of these experiments have the following features in common:

- Unilateral hindlimb inflammation compared with naïve controls (or with the contralateral paw).
- Repeated measures in the same group of animals over time either short term (hours) or long term (weeks).
- Comparison of data across 2 or more age groups.

This design is used to answer one (or all) of at least three possible questions:

1) Does inflammation result in a significant depression of the sensory threshold at each age?

2) At what time points following inflammation is the depression of sensory threshold significant at each age?

3) Is the degree of depression of sensory threshold significantly different when comparing the age groups?
Several potential problems arise during data analysis including:

i) vFh data (recorded in grams) are not parametric nor are they normally distributed

ii) data are categorized in three ways: treatment (naïve or inflamed), time and age.

iii) control data (sensory thresholds) change with age during the course of long term experiments.

iv) the potential effects of inflammation on the contralateral side may make this matching of data inappropriate.

v) sample sizes are small (usually 4-8 animals per group). Non-parametric tests lack statistical power in such small groups (e.g. the Mann-Whitney test always produces a P value >0.05 (two tailed) when the total number of observations is less than or equal to seven).

vi) variance may differ between treatment groups.

vii) data collected at each time point are not independent observations.

Several possible approaches to analysis are available:

A) Data transformation
   a) by using a von Frey hair number rather than a gram weight an effective log transformation is achieved (Cole 2000).
   b) use a change or difference in vFh threshold (from the pre-inflamed value)
   c) logistic regression analysis allows vFh threshold data to be analysed as a binary variable (reflecting its true yes-no nature) (Lu and Bailey 2000).

B) Alter experimental design so that timed observations are obtained from independent groups of animals (i.e. each group of animals can only be tested once).

This would increase the number of animals required for each experiment to unacceptable levels.

The results in Chapter 4 do not use any form of data transformation but report and analyse mechanical thresholds in grams. Results are then analysed using the following approaches:

D) Allow unwarranted assumptions:
   i) that data is parametric
   ii) that data is normally distributed
   iii) that variance in differently treated groups is equal

E) Two way ANOVA with data categorized by time and condition. This analysis is then repeated for each age group. It may help answer the question: “Does inflammation result in a significant depression of the sensory threshold at each age?”
F) One way Analysis of variance (ANOVA) for each condition categorized according to time. Pairs of group means are then analysed using a post-test such as Dunnett's test which allows a control group to be selected for comparison with all other groups. This is repeated for each experimental group. It may help answer the question: “At what time points following inflammation is the depression of sensory threshold significant at each age?”

G) Use a summary statistic to represent set of serial measures (Mathews and Altman 1990).

e.g. the nadir of the measured sensory threshold for each animal.

the time to reach the nadir

a single time point for analysis.

In some behavioural experiments, data collected 2 hours following the inoculation of carrageenan is used as summary statistic. This time point has been chosen because it correlates with the experimental protocol of electromyographic experiments described later in Chapter 4.
4.4.2 Electromyographic studies

The basic method of halothane anaesthesia and EMG recording described in Chapter 2 was used for this series of experiments. In these experiments, unilateral hindpaw inflammation was induced using carrageenan as follows:

Induction of anaesthesia and Carrageenan inflammation

Carrageenan inflammation was induced 2 hours prior to EMG recording. This was completed under brief (5min) halothane anaesthesia. The plantar surface of the left hindpaw was inoculated with a 2% solution of lambda carrageenan (Sigma) made up in 0.9% saline. A depth limited skin lancet (allergy testing lancet) was used for inoculation and dosing was adjusted across the age groups by adjusting the number of inoculations performed on the paw i.e. fourteen inoculations for P21, 10 for P10 and 6 for P3 pups. These ratios were based on the changes in the average plantar surface area of the paw with age and on the data collected from the behavioural studies described above. The inflammation induced by this regimen was consistently of moderate intensity and reasonably restricted to the plantar surface of the hindpaw. Carrageenan inflammation was allowed to develop for 2 hours before commencing EMG recording. Older pups (P21) were kept in warmed cages within the laboratory during this time, younger pups (P10 and P3) were returned to their mothers for feeding and recovery during this time.

Epidural saline injections

These experiments were used to control and normalise the epidural drug pharmacodynamic studies described in Chapter 5. All pups therefore had an injection of epidural saline (1ml/kg) administered before EMG recording. The technique of epidural injection is described in full in Chapter 5.
4.5 RESULTS BEHAVIOURAL STUDIES

Hyperalgesia produced by carrageenan inflammation in adult rats has been well documented (see section 4.3.3 –Carrageenan inflammation in behavioural studies). Here, the pattern of hyperalgesia induced by carrageenan has been studied in younger pups (P10 and P3).

Moderate carrageenan inflammation consistently resulted in a significant reduction in the mechanical sensory threshold in both P3 and P10 rat pups. The effect developed within two hours of inoculation and persisted for at least 5 hours. In P10 pups the sensory thresholds had returned to normal 24 hours after inoculation whereas in P3 rat pups a residual effect was still evident at 24 hours. Figure 12 a and b shows the mean sensory thresholds over the testing period in P10 and P3 rats during moderate inflammation.

Mild inflammation in both P3 and P10 rat pups did not result in a statistically significant change in sensory threshold. Figure 13 a and b shows the mean sensory thresholds over a 5 hour period in P10 and P3 rats during mild inflammation.

Severe inflammation in P10 rat pups caused a relatively larger reduction in threshold compared to moderate inflammation. In contrast, no significant reduction in threshold could be detected following severe inflammation in P3 rat pups (see Figure 14 a and b).

Data displayed in Figures 12 through to 14 have been re-presented in Figure 15 to allow better comparison between groups of pups at both age groups.
4.5.1 Time course of moderate inflammation

Figure 12a and b
The course of sensory changes induced by carrageenan inflammation is shown here. Mechanical sensory thresholds were determined using calibrated von Frey hairs on an hourly basis for 5 hours. Sensory thresholds had returned to normal at 24 hours in P10 pups but remained lower than normal in P3 pups at this time. (n= 8 for both P10 and P3 inflamed pups, 10 naïve P10 pups and 14 naïve P3 pups were used as controls. Mean and SEM are displayed) One way analysis of variance P10 data (excluding 4 and 5 hours post inoculation) p=0.0017, Dunnett’s multiple comparison test p<0.05 for control vs 1, 2 and 3 hours post inoculation. P3 data: p=0.0034, Dunnetts post test p<0.05 for control vs 3 and 4 hours post inoculation.
4.5.2 The effect of mild inflammation

Mild inflammation was induced by making two inoculations of carrageenan into the paw of the rat. No significant drop in mechanical sensory threshold could be detected following this degree of inflammation. (n=5 for P10, n=13 for P3 inflamed pups. 10 naïve P10 pups and 14 naïve P3 pups were used as controls. Mean and SEM are displayed).

**Figure 13a and b**

Mild inflammation was induced by making two inoculations of carrageenan into the paw of the rat. No significant drop in mechanical sensory threshold could be detected following this degree of inflammation. (n=5 for P10, n=13 for P3 inflamed pups. 10 naïve P10 pups and 14 naïve P3 pups were used as controls. Mean and SEM are displayed).
The effect of severe inflammation

a) P3

![Graph showing vFlh threshold (gms) over time (hrs) for Naive and Severe inflammation in P3 pups.]

b) P10

![Graph showing vFlh threshold (gms) over time (hrs) for Naive and Severe inflammation in P10 pups.]

Figure 14 a and b

Severe inflammation was induced by subplantar injection of carrageenan. A significant fall in reflex threshold was observed in P10 rat pups. (Repeated measures ANOVA p<0.0001. Dunnett’s post test p<0.05 for control vs 0.5, 1,2,3,4 and 5 hours post inoculation.) No significant drop in mechanical sensory threshold could be detected in severely inflamed P3 pups. (n=8 for P10 and n=13 for P3 inflamed pups. 10 naïve P10 pups and 14 naïve P3 pups were used as controls. Mean and SEM are displayed.)
4.5.6 Summary graph  P10 and P3 data displayed in Figures 12 through 14 have been re-presented here.

**P10** Comparing the inflamed paw with paws in naive pups

Severe Inflammation
- Naive
- Severe inflammation

Moderate Inflammation
- Naive
- Moderate inflammation

Mild Inflammation
- Naive
- Moderate inflammation

**P3** Comparing the inflamed paw with paws in naive pups

Severe Inflammation
- Naive
- Severe inflammation

Moderate Inflammation
- Naive
- Moderate inflammation

Mild Inflammation
- Naive
- Mild inflammation

Figure 15 Mechanical reflex thresholds in ipsilateral hindlimbs following mild, moderate and severe inflammation.
4.6 ELECTROMYOGRAPHIC STUDIES

The effect of Carrageenan inflammation on the withdrawal response of P21 rat pups is shown in Figure 16a and b. The peak, duration and area under the RMS signal (mean and standard deviation) are each plotted against stimulus strength (mechanical: Figure 16a and electrical: Figure 16b). In inflamed pups a clear leftward shift of the stimulus-response curve is seen. These data demonstrate the reduction in mechanical threshold (from 27gms to 7gms) as well as the increased size of the response to suprathreshold stimulation following carrageenan inflammation. At the highest stimulus strength (47gms), carrageenan-inflammation resulted in a 3-4 fold increase in the duration and area under the RMS signal.

The peak, duration and area under the RMS signal in response to electrical stimuli are displayed in Figure 16b. A broadly similar pattern to the results obtained with mechanical stimuli is evident but the variability (standard deviation) is consistently greater. For this reason, pharmacodynamic analysis (Chapter 5) was limited to the mechanical stimulus-response curves. The data shown in figures 16a and b together with data from P10 and P3 rat pups have been summarised and displayed in Figure 17. Reflex responsiveness (as described in chapter 2) has been calculated for both naive and carrageenan-inflamed pups at three ages.

Carrageenan inflammation was also studied in 10 and 3 day old rat pups. Using the same measure of reflex responsiveness outlined above, a comparison of naive with carrageenan inflamed pups was made at all three ages and is shown in Figure 17a. Reflex responsiveness calculated from recordings evoked by electrical stimuli show much greater variability (s.d.). Differences in the response to electrical stimulation between the two younger age groups (P3 and P10) are not significant. These data are displayed in Figure 17b.

The data used to calculate the means and standard deviations of mechanically evoked reflex responsiveness (Figure 17a) have been plotted again in Figure 18a and b, so as to display their distribution. This distribution is Gaussian i.e. KS numbers ranged from 0.12-0.25. Carrageenan increased reflex responsiveness in all three age groups though the proportional increase was greater in older pups. The relative degree of facilitation of the withdrawal reflex, expressed as a percentage increase from naive controls was 78% for P3 pups, 111% for P10 pups and 300% for 21 day old pups. These age comparisons were completed at equivalent anaesthetic depths (halothane at equi-MAC concentrations) as described in Chapter 3.
In the hyperalgesic state thresholds to both mechanical and electrical stimuli were reduced in all age groups tested (see Figure 19a and b). There was no age related effect on threshold measurements in either the naive or inflamed state.

The latency of the withdrawal reflex on the other hand did show a clear age related trend in both naïve and inflamed states. (see Figure 20). Carrageenan inflammation did not have a significant effect on latency.

**Statistical analysis**

Two way analysis of variance (ANOVA) of reflex responsiveness evoked by mechanical stimulation (see Fig 17a).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
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<td>0.0148</td>
<td>0.03</td>
<td>0.967</td>
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<td>0.450</td>
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</tr>
<tr>
<td>Total</td>
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</table>

Two way ANOVA of reflex responsiveness evoked by electrical stimulation (see Fig 17b).

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<th>Mean square</th>
<th>F value</th>
<th>p value</th>
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<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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</table>
4.6.1 The effect of Carrageenan on the mechanically evoked withdrawal reflex.

**Figure 16a**

The peak (a), duration (b) and area under the RMS signal (c) are plotted against the gram weight of the von Frey hairs used to elicit a reflex withdrawal response. Two separate groups of rat pups (n=11 for each) were used to define the relation between stimulus and response in un-inflamed pups (blue) and carrageenan inflamed pups (red). Mean and standard deviation are displayed. Carrageenan inflammation reduces the reflex threshold and induces a greater response at each given stimulus strength i.e. a leftward shift of the curve. The area under the last (c) curve has been used as a summary measure (reflex responsiveness) for all further comparisons across age and drug doses.
4.6.2 The effect of Carrageenan on the electrically evoked withdrawal reflex.

**Figure 16b**

The peak (a), duration (b) and area under the RMS signal (c) are plotted against the current strength used to elicit a reflex withdrawal response. Two separate groups of rat pups (n=11 for each) were used to define the relation between stimulus and response in un-inflamed pups (blue) and carrageenan inflamed pups (red). Mean and standard deviation are displayed. Carrageenan inflammation produces a leftward shift of the stimulus-response curve. Variability of the response is generally greater for electrical stimuli than for mechanical stimuli.
4.6.3 Effect of Carrageenan at different ages.

Figure 17a
The effect of carrageenan inflammation on "reflex responsiveness" (the area under the RMS signal-stimulus response curve using mechanical stimuli) has been calculated across three ages. Mean and standard deviation are displayed. Un-inflamed pups show an age related decrease in responsiveness. Carrageenan increases responsiveness at all ages - the relative increase being greatest in the older pups. Two way ANOVA: Is responsiveness affected by age and/or inflammation? Age: p<0.0001, Inflammation p<0.0001, interaction p=0.965.
4.6.4 Effect of Carrageenan at different ages (electrical stimuli)

![Graph showing reflex responsiveness to electrical stimuli for different ages and inflammation states.]

Figure 17b

In this case, "reflex responsiveness" has been calculated from the stimulus response curve generated using electrical stimuli. Carrageenan increases responsiveness at all ages - the relative increase being greatest in the older pups. Two way ANOVA: Is responsiveness affected by age and/or inflammation? Age: p=0.047, Inflammation p < 0.0002, interaction p=0.704.
4.6.5 Effect of Carrageenan at different ages - data distribution

Figure 18 a/b
These graphs show the same data as on Figure 17a. Results for individual pups are plotted so as to better display the variance. The greatest age related changes occur between P10 and P21.
4.6.6 Effect of Carrageenan on reflex thresholds

Figure 19 a/b
Mechanical threshold for eliciting the withdrawal reflex are plotted for individual rat pups. Little effect of age on the threshold is observed (reflecting equivalent anaesthetic depth). Carrageenan reduces thresholds at all three ages. Variability in recorded thresholds is greater in younger pups. Two way ANOVA: Is threshold affected by age and/or inflammation Age: p=0.498, Inflammation p< 0.0001, interaction p=0.258.
4.6.7 Effect of Carrageenan on reflex latency.

Figure 20

The latency of the withdrawal reflex following electrical stimulation is plotted for individual rat pups. Latencies become shorter with increasing maturity. Carrageenan did not have a significant effect on reflex latency. Two way ANOVA: Is latency affected age and/or inflammation? Age: \( p < 0.0001 \), Inflammation \( p < 0.066 \), interaction \( p = 0.789 \).
4.7 **DISCUSSION**

Carrageenan inflammation enhanced the withdrawal reflex in all pups. Not only did it result in a significant reduction in both electrical and mechanical reflex thresholds but the responses to suprathreshold stimuli were also augmented in both amplitude and duration. These parallel changes are reflected in the calculated measure of reflex responsiveness. Reflex latency (to electrical stimulation) was not altered by inflammation.

Most probably as a reflection of the rather larger control values obtained in naive (non-inflamed) P3 and P10 pups, the proportional increase in reflex responsiveness due to carrageenan was greatest in P21 pups. Calculated as a ratio of naive values, carrageenan inflammation induced an almost fivefold increase in mean reflex responsiveness in P21 pups. In P10 pups the increase was two fold while in P3 pups carrageenan induced an increase in reflex responsiveness of 65%.

Several potential mechanisms may give rise to the pattern of enhanced reflex excitability documented including sensitisation of primary afferent nociceptors, altered excitability of dorsal horn neurons and altered central descending facilitatory systems. Two methodological issues will be dealt with before discussion of the potential mechanisms.

### 4.7.1 Methodological issues- Inoculation vs injection

In these experiments, inflammation was induced by inoculating 2% carrageenan into the plantar surface of the hind paw. This technique, representing a change from the more traditional subcutaneous injections- was made so as allow greater control of the degree and area of cutaneous inflammation in small pups. In pilot studies of the behavioural effects of carrageenan in young pups, sub-cutaneous injection yielded variably inflamed hindpaws with a significant number of pups developing severe reactions including whole limb oedema and redness. In these experiments, the degree of inflammation appeared to influence the subsequent behavioural measures von Frey hair mechanical thresholds. While moderate inflammation reduced thresholds, severe inflammation consistently raised thresholds- to a point where motor dysfunction was suspected and the measurement quite possibly did not reflect a true sensory threshold.

Limiting the inflammatory process to cutaneous tissues may have some bearing on interpretation of the final results. Wall and Woolf (1984), measuring flexor motor neuron responses noted differences in response depending on whether cutaneous or
deep-tissue afferents were stimulated. The increase in spinal cord excitability induced by brief input was more prolonged if C fibres were stimulated in muscle rather than skin (Wall and Woolf, 1984). This was characterised by a decrease in reflex threshold as well as an increase in the size of response at any given stimulus strength.

### 4.7.2 Inadvertent noxious input as a confounding factor

Segmental processing of noxious stimuli is well known to be strongly modulated by both segmental and suprasegmental inputs. Common to many forms of this modulation (TENS, acupuncture, dorsal column stimulation, peri-aqueductal stimulation) is an effect on dorsal horn nociceptive neurons especially on convergent units (WDR neurons). Inhibitory modulation in anaesthetised intact animal preparations is often termed “diffuse noxious inhibitory controls (DNIC)”. It typically describes inhibition of lumbar dorsal horn neurons (receiving both C and Ad fibre inputs) by noxious stimuli applied to sites remote from the excitatory receptive field (Le Bars et al., 1979). A wide variety of noxious stimuli are effective and that these stimuli are effective when applied over a widespread area. These modulatory effects are felt to be different to segmental inhibitory effects previously demonstrated to arise from activation of large diameter fibres. Interestingly some degree of spatial and temporal summation appears crucial in the mechanism of DNIC.

These effects can have significant influences in experimental data (Svendsen et al., 1999). Severe and long lasting depression of excitability of neurons in the trigeminal nuclei has been documented during routine preparation that included vascular cannulation, tracheostomy, stereotaxic frame support and craniotomy in cats (Clarke and Mathews, 1985). Possible mechanisms for this “trauma-induced inhibition” may include both DNIC and influences from the lateral reticular formation of the brainstem. Importantly, threshold measurements were thought to be a reflection of depth of anaesthesia and the researchers warned that anaesthetic and technical aspects of electrophysiological preparations may be the source of misleading results (Clarke and Mathews, 1990).

Significant advantages of a “low trauma” acute preparation have been demonstrated in animal preparations. The modifications introduced include:

a) sponge mattress support for animals while in a stereotaxic frame, b) atraumatic ear bars only, c) lignocaine ointment to all skin wound edges. (Clarke and Mathews, 1990)

The relatively light plane of anaesthesia required for EMG recording of the withdrawal reflex also imposes limits on the level of background noxious afferent input that can be tolerated during recording. A carefully controlled and stable plane of
anaesthesia is important for both reasons given above i.e. to prevent both inadvertent inhibition of dorsal horn neurons and also their inadvertent sensitisation.

Studies of central sensitisation usually require baseline or control data from preparations in which little or no sensitisation is likely to occur before recording begins. The provision of stable anaesthesia unfortunately often involves interventions that may do just this. Possible sources of sensitising inputs include: i) handling stress during induction of anaesthesia, ii) i.p. injection of induction agents and peritoneal irritation of delivered anaesthetic drugs, iii) surgery for tracheostomy, iv) stereotaxic frame positioning: head bars, pelvic bars, v) laminectomy and calcaneal holding sutures, vi) peripheral stimuli- mechanical and electrical, and vii) acute phase reactants induced by tissue trauma ("sickness response").

The preparation described allows for the minimisation of many of the factors described above. Recording of the elicited withdrawal reflex was limited to two recordings per stimulus. This minimised the chance of stimulus induced sensitisation and habituation of the reflex. It also meant that each pup was used in only one experimental condition (i.e no before and after recording). Data have therefore been expressed as means and s.d. rather than SEM.

4.7.3 Hyperalgesia documented in early development

Despite a gradual progression of segmental, intraspinal and supraspinal connections, the results described demonstrate that from 3 post-natal days spinal mechanisms do allow adjustment of withdrawal reflex sensitivity in the face of tissue inflammation and that these adjustments appear to be well lateralized. These results confirm the existence of mechanisms responsible for hyperalgesia in early development.

The size of the withdrawal reflex response is a function of the sensitivity of peripheral nociceptors, the anatomical arrangement of central terminals of primary afferent fibres, the dendritic distribution and membrane excitability of dorsal horn neurons and the pattern and strength of synaptic connections between neurons driving the reflex arc and descending modulatory pathways. Some of these factors, notably sensitivity of peripheral nociceptors, membrane excitability and synaptic efficacy can be altered in a use dependent manner in adults and result in enhanced excitability. During early development the anatomical arrangement of central terminals and the maturation of descending modulatory pathways will also influence the degree of reflex enhancement induced by persistent activity or tissue injury.
Peripheral sensitisation results from the action of several pro-inflammatory substances including bradykinin, prostaglandins, serotonin, ATP and hydrogen ions on peripheral nociceptors (Dray, 1995). In tissue injury the substances are either released by mast cell and other immune competent cells, produced de novo by local tissues or are the product of plasma precursors. These mediators interact with ligand-gated receptors (e.g. BK1, PGE2, VR-1, P2X3 receptors) which may in turn alter voltage-gated channels (e.g. acid sensitive ion channels (ASIC), TTX-resistant sodium channels) (Khasar, 1998) to alter primary afferent excitability. Apart from altering excitability, these mediators may also trigger phenotypic changes within the population of nociceptors (Neumann et al., 1996). The maturation of these receptor systems during early development has yet to be investigated though phenotypic changes in neonatal primary afferents have been documented in response to inflammation in neonatal rats. In this model, carageenan inflammation resulted in the expression of neuropeptides in previously non-peptidergic neurons in pups as young as 3 postnatal days (Beland, 1999).

Central sensitisation induced by peripheral inflammation is thought to result from co-operative interaction between NMDA and substance P (sP) receptors (Iversen, 1998). Both glutamate and sP are found within primary afferent terminals in the dorsal horn. Glutamate is co-contained with aspartate in clear vesicles, whereas sP is contained within dense core vesicles (Rustioni and Weinberg, 1992). The role of the NMDA receptor in central sensitisation is dealt with in the following chapter. Substance P is a peptide containing 11 amino-acids and acts on the neurokinin 1 receptor. The latter is a G-protein coupled receptor expressed by neurons involved in motor, autonomic and sensory neural systems. These neurons are abundant in lamina I of the spinal cord and in deeper laminae (III-V) whose neurons possess dorsally directed dendritic arbors traversing the substantia gelatinosa. These neurons are thought to be central in transmission of nociceptive signals (Brown et al., 1995).

By studying sP receptor internalisation, Honore et al were able to describe different patterns of sP release (assuming receptor internalisation is directly correlated with sP release) in different forms of inflammation. Immediately following (10 mins) carageenan hindpaw injection, sP release is observed in dendrites and a few lamina I neurons but by 3 hours after injection this release had ceased. This is in contrast to the ongoing release (sP-receptor internalisation) of sP that is seen with either capsaicin application or formalin injection. Notably though, 3 hours after carageenan injection, normally non-noxious mechanical stimulation of the inflamed paw (which would not induced sP receptor internalisation in naive animals) induces massive receptor internalisation in lamina I. Further, noxious stimulation of the inflamed paw causes receptor internalisation not only in lamina I but also in lamina I-II and III-IV.
Considering the time frame of these changes, the authors suggest that the pattern is most likely due to peripheral sensitisation of primary afferents that manifests as an increase in sP release in lamina I with subsequent diffusion into deeper laminae. This implies that carrageenan-induced inflammation results in a change not only in the degree but also in the nature of synaptic neurotransmission within the dorsal horn (Honore et al., 1999).

Substance P release, binding and receptor internalisation results in the formation of diacyl glycerol and inositol triphosphate within postsynaptic neurons. These in turn induce increases in intracellular calcium which facilitates protein kinase C activity. This enzyme, once translocated from cytoplasm to membrane, is able to phosphorylate the NMDA receptor, thereby countering the magnesium block and allowing the NMDA receptor to operate at a more negative potential (Urban et al., 1994).

Although the interaction between sP and the NMDA receptor appear to play the major role in determining excitability in inflammatory states, the interaction is not unique. The NMDA receptor is also modulated by other peptides such as the opioids and CGRP (Aniksztejn, 1991).

The ontogeny of neuropeptide-dependent sensitization is the subject of intense investigation in the light of the fact that C-fibre nociceptive function undergoes a postnatal maturation process in the rat- i.e. mustard oil does not elicit reflex withdrawal until postnatal day 10-11 (Fitzgerald and Gibson 1984), and does not induce cFos expression at P3 (Williams 1990). Also, neurogenic oedema is not apparent until P11 (Fitzgerald and Gibson 1984).

Neuropeptide expression in early life is characterised by low foetal levels and rapid increases around the time of birth. Adult levels are not achieved until around 10 post natal days (Marti et al., 1987). The expression of receptors on the other hand is characterised by over-expression and wide distribution in early life with gradual restriction during the third post natal week. Both CGRP and Substance P/Neurokinin-1 binding sites though are relatively stable until P14 after which they decline (Kar and Quiron, 1995). Substance P release dependant ventral root potentials have been documented in the neonatal rat implying functional release mechanisms and receptors (Agaki 1982) but C-fibre evoked slow potentials involving the NK-1 receptor are only apparent by postnatal day 12 (Nagy 1993). Response to heat, mechanical and chemical stimulation (formalin) in neonatal rats lacking the NK-1 receptor also suggest that this peptide system becomes involved in nociceptive processing more than 3 day after birth (King 2000).

The findings of the current experiments are consistent with data from other sources. Sensitisation (increased excitability in response to repetitive stimulation) of limb reflexes has been documented in very young pups. A “wind-up like” phenomenon
was seen by Fitzgerald and Gibson in electrically evoked EMG responses in 8 day old pups. In these experiments, a slow build up in muscle activity in response to repetitive stimulation was followed by habituation after about 55s (Fitzgerald and Gibson, 1984).

Behavioural responses of pre-weanling rats to acute inflammation have been well characterised by Guy and Abbott (Guy and Abbott, 1992). In their experiments, the development of nociceptive patterns of behaviour have been studied following the injection of various concentrations of formalin into the hindpaw of neonatal rat pups. The data collected has tended to be essentially observational and is made semi-quantitative by a strict time-based sampling protocol. The development of motor responses to formalin is described as a progression from non-specific (squirming, kicking and convulsive jerking) to specific (licking and shaking the paw). Also notable is the decrease in response with increasing age. Furthermore, in comparison with adult responses, pups under 15 days old show little evidence of the characteristic bi-phasic response to formalin.
Chapter 5 THE ROLE OF NMDA RECEPTORS IN NEONATAL INFLAMMATION-THE EFFECT OF KETAMINE AND AP5

5.1 NMDA PHARMACOLOGY

5.1.1 Glutamate receptors and synaptic activity

Glutamate is the major excitatory neurotransmitter in the CNS. Together with substance P, it assumes prime importance in neurotransmission between primary afferent fibres and dorsal horn neurones. It is formed within neuronal terminals from a precursor glutamine. The latter is provided by adjacent astrocytes via the "glutamine-glutamate shuttle" that is central to the co-operative neuron-astrocyte metabolic unit. Aspartate too has been observed in immunochemical studies to co-exist with glutamate in primary afferent terminals in the substantia gelatinosa raising the possibility that aspartate may be a neurotransmitter in small DRG neurons. The functional significance of this is yet to be determined but may revolve around aspartate being a relatively specific ligand for the NMDA receptor (Rustioni and Weinberg, 1992).

Like glutamate, aspartate is contained in small clear vesicles as opposed to the large dense core vesicles (LDCV) that typically contain peptides. This histological distinction between the amino acids and the peptides may underlie differences in physiological roles as each may be released under different circumstances and LDCVs preferentially exocytose away from the active zone. The possible implications of this are that the release of endogenous peptides or glycoproteins away from the active zone may act as a central signal by acting outside of the synapse (Rustioni and Weinberg, 1992).

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. Belonging to the family of G-protein linked receptors they can alter cell function and may then indirectly alter membrane responsiveness.
The following table shows the classification of glutamatergic receptors.
(Gonzalez et al., 1993)

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Distribution and main characteristics</th>
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| 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 EPSP's |
| Kainate       | concentrated in a few specific areas (e.g. striatum lucidum of hippocampus)  
complimentary distribution pattern to NMDA/AMPA receptors (e.g.  
specific presence on some dorsal root C fibres and DRGs) |
| Metabotropic  | linked (positively) to inositol triphosphatase or (negatively) to cAMP  
not antagonized by NMDA antagonists  
may be involved in developmental plasticity |

Under normal synaptic conditions (i.e. at normal membrane potentials and in the presence of Mg and normal synaptic inhibition), low frequency responses at glutamatergic synapses are mediated by AMPA and/or kainate receptors with very little contribution from NMDA receptors. Despite this, the NMDA receptor subtype has been more intensively investigated because this receptor system appears to play a crucial role in synaptic plasticity (activity dependent changes in synaptic efficacy). Because of this feature it has been assumed to be the basis of learning and information storage within the CNS. A further impetus for the study of NMDA receptors is its apparent involvement in many pathological (neurotoxic) brain states. The earlier availability of specific antagonists for these receptors has allowed better characterisation of the NMDA system than of the other glutamatergic receptors.
5.1.2 The distribution and classification of NMDA receptors within the brain

NMDA receptor sites (as identified by NMDA-displaceable labelled glutamate binding studies) are found predominantly within the telencephalon. Notably high densities are found within the hippocampal areas (strata oriens and radiatum or CA1 area-Sommers sector). Moderate levels are found in the dentate gyrus and CA3 region while the mossy fibre termination zone (stratum lucidum) has only a low density of NMDA receptors. Significantly, the NMDA dependence of LTP generated within these areas of the hippocampus closely follows the density of NMDA receptors.

Cortical areas of the brain with high densities of NMDA receptors include the frontal, anterior cingulate and pyriform cortices. The neocortex tends to show high levels of NMDA receptors within outer layers and within minor zones of the deeper layers. For example, in the parietal cortex layers 1-3 and 5a show high NMDA receptor densities.

Non cortical areas with high NMDA receptor densities include the basal ganglia (nucleus accumbens and caudate/putamen), the dorsal lateral septum and the amygdala.

More caudal regions with significant NMDA receptor densities include the dorsolateral and dorsomedial geniculata, the superficial grey of the superior colliculus, the dorsal cochlear and medial vestibular nuclei, the external plexiform layer of the olfactory bulb and its anterior nucleus, the solitary tract nucleus as well as the substantia gelatinosa of the spinal cord. All these latter sites are of course closely involved with sensory processing. A further description of NMDA receptors within the spinal cord is given in the discussion section of this chapter.

The general importance of EAAs and more specifically the NMDA neurotransmitter system in sensory processing is supported by the prominence of NMDA receptors in ascending projections (spinofugal pathways). Spinobulbar, lemniscal and thalamocortical fibre paths have all been shown to contain glutamate and/or glutamate-synthesizing enzymes (Ericson 1995; De Biassi 1994). Excitatory activity in the thalamo-cortical system is mediated by both NMDA and non-NMDA glutamatergic receptors. Neurons of the ventral posterior lateral (VPL) nucleus of the thalamus can be immunolabelled for both GluR2/3 and NMDAR1.

In contrast, structures associated with motor function which tend to be more ventrally located all tend also to have low densities of NMDA receptors, such areas include the molecular layer of the cerebellum, the red nucleus, pontine nucleus, mammillary bodies and the brain stem motor nuclei.
Binding studies with radiolabelled antagonists (e.g. AP5, CPP) generally confirm the binding distribution described above. Subtle differences observed in these studies may be the result of labelling of NMDA receptor subpopulations or differences in binding kinetics. Studies of allosteric binding sites on the NMDA receptor such as those for phencyclidine and glycine also correspond well with the patterns of NMDA receptor distribution already described.

5.1.3 Subunit composition and distribution

The genes encoding the subunits of the receptor have been identified, cloned and classified into three related families of subunits. These are the NMDAR1 (NR1) and NMDAR2 and the most recently identified NR3 group. The NR1 family consists of a single member existing as several splice variants. The NR2 family includes the NMDAR2A, 2B, 2C and 2D (NR2A-D). The NR3 group is also known as the NR-like and Chi-1 receptor. Sequence homology between NR1 and NR2 subunits is only 20% whereas that between NR2A and NR2B is about 70% and that between NR2A and NR2C is about 55% (Monyer et al., 1992; Moriyoshi et al., 1991). Within the rat neocortex, there is considerable heterogeneity of heteromeric complexes (Sheng et al., 1994). Despite this, distinct distributions of five NMDA receptor subunits have been demonstrated throughout the mouse central nervous system (Watanabe et al., 1993). In these studies, the NMDAR1 subunit RNA is distributed ubiquitously in the brain together with the NR2A subunit. In contrast, the NR2B subunit appears to be confined to the forebrain. The NR2C subunit is predominantly found in the cerebellum (granule cells) and weakly in the olfactory bulb, vestibular nuclei and the thalamus. Only faint NR2D subunit expression is detected in adult animals and this occurs in the olfactory bulb, the thalamus and the midbrain (Watanabe et al., 1993).

NMDA receptors can be broadly grouped into agonist-preferring (NMDA-displaceable [3H]L-glutamate binding sites) and antagonist preferring (CPP binding sites) receptors. In the forebrain the NR2A distribution is very similar to that of the [3H]CPP binding sites while the NR2B distribution is similar to the [3H]L-glutamate binding site distribution. The former has a high density in the hippocampus and cerebral cortex and in the ventral posteromedial thalamic nucleus. The NR2B receptor, while also appearing in high density in the hippocampus and cortex is also well represented in nuclei of the olfactory bulb, amygdala, caudate/putamen and many thalamic nuclei. It appears then, that the identity of the NR2 subunit underlies the distinction of the two pharmacological isoforms of the NMDA receptor in the forebrain.
NMDA receptors are also found on glial cells. The NR2C receptor subunit appears to make important contributions to putative astrocyte receptors. As these cells possess processes that closely ensheath the mammalian synaptic cleft, these receptors may be involved in control of the functional state of astrocytes which in turn may influence neuronal excitability and synaptic transmission (Watanabe et al., 1993).

The distinct regional variability in the expression of receptor subunits has been confirmed using reverse-transcriptase polymerase chain reactions. These studies suggest that the most marked differences exist between the motor regions of the isocortex and the piriform region of the allocortex. In the latter, the predominant transcript is that of the NR1 subunit with the NR2A subunit being expressed at just more than 50% of the NR1. In contrast the motor regions predominantly express the NR2B transcript (135% of NR1 levels) and does not express either the NR2D or NR3 subunit. In the olfactory bulb (a structure that retains many features of the immature nervous system and may therefore reflect the general pattern of receptor subunit expression during early development) the NR1 and NR2B subunits are equally expressed and appear to be the most abundant transcripts. The NR2A transcript is expressed at 80% of that of the NR1. The distinguishing feature of the olfactory bulb is its relatively high levels of expression of the NR2D transcript whose expression is similar to that of the NR3 subunit (Sun et al., 2000). Progressive alteration in subunit composition in the postnatal period is suggested to contribute to changing synaptic plasticity during cortical development. This is dealt with in greater detail in the discussion section of this chapter.

5.1.4 AMPA and Kainate receptors

The AMPA receptor is a tetra- or pentameric ion channel composed of four different subunits (GluR-A,B,C,D). The cloning of cDNAs for each of these molecular species was completed shortly after the first kainate receptor protein was identified (Keinanen et al., 1990). Each subunit exists in two alternative splice variants (flip and flop), the expression of which is developmentally regulated. The flop variant is expressed later in development (from P8 onward, reaching adult levels at P14) and is likely to facilitate a reduction in receptor conductance and therefore neuronal excitability with increasing age (Monyer et al., 1991).

Radioligand binding studies of AMPA-type receptors confirm a similar pattern of distribution to that of the NMDA-type receptor. The highest concentration of AMPA-type receptors being found in the hippocampus (CA1 stratum oriens and radiatum and over the pyramidal layer). Cortical binding has a similar preference for outer layers and other
concentrations are found within the olfactory nuclei, nucleus accumbens, caudate/putamen, lateral septum and amygdala. This parallel distribution appears to strengthen electrophysiological evidence that these two glutamate receptors work in concert. In only a few sites, AMPA receptors appear relatively more abundant than the NMDA-type, these areas include the stratum pyramidale of the hippocampus, indusium griseum and the cerebellar molecular layer.

Selective non-competitive blockade of AMPA receptors by 2,3 benzodiazepines such as GYKI 53655 (LY300168) allows unmasking of neuronal kainate receptors (Lerma, 2000). Kainate receptors are able to modulate transmitter release by pre-synaptic mechanisms, in particular they appear to be able to reduce the efficacy of inhibitory connections (e.g in the hippocampus). This regulation of GABA release by kainate receptors is independent of ion channel activity but involves the activation of phospholipase C and protein kinase C through a G-protein mechanism. Like both the AMPA and NMDA receptors, kainate receptors are homo/heteromeric assemblies of 5 subunits (GluR5,6,7 and KA-1 and KA2).

While the roles of AMPA and NMDA type receptors in synaptic plasticity are well accepted, the physiologic roles of Kainate-type receptors have been more difficult to elucidate. This may have been partly due the difficulty in pharmacologically isolating the kainate receptor in experimental models (CNQX- a non-NMDA antagonist blocks both AMPA and kainate receptors.) (Lerma et al., 1997). Selective activators (ATPA) and inhibitors (LY 294486) of the GluR5 subunit are now available and allow elucidation of the roles played by GluR5 containing receptors (Collingridge, 2000). Despite the earlier limitations, the kainate receptor has an accepted role in hippocampal mossy-fibre pathways where repetitive activation generates a slow kainate-receptor mediated excitatory postsynaptic current. This current appears to augment the excitability of pyramidal cells in response to repetitive granule cell activity (Castillo et al., 1997). This suggests that the kainate receptor provides a mechanism for NMDA-independent synaptic plasticity in the CNS (Vignes and Collingridge, 1997). Kainate-receptor mediated synaptic transmission is important between primary afferent fibres and dorsal horn neurons in the spinal cord. In particular, synapses which receive inputs with a high activation threshold such as those from C and/or A\(\delta\) fibres are more likely to include kainate receptors than those with low threshold inputs. The fact that the EPSPs of this transmission can be blocked by selective \(\mu\)-opiate receptor agonists adds weight to the hypothesis that kainate receptors are important in nociceptive primary afferent processing at the level of the spinal cord (Li et al., 1999).
5.1.5 Models of the NMDA Receptor-ionophore complex

NMDA receptors like other native glutamate receptors appear to be pentameric complexes of NMDAR subunits. Each subunit has a molecular mass of about 97 kDa (about 900 amino acids) making the whole receptor complex almost twice the size of an nicotinic-acetylcholine receptor. Each subunit comprises four putative transmembrane segments (M1-M4) - a characteristic of most neurotransmitter-gated ion channels. The subunits share amino acid sequence homology with each other and with subunits of the AMPA- or kainate-selective glutamate receptor channels. NMDA subunits (both NR1 and 2) carry an asparagine residue in the putative channel forming region as opposed to either arginine or glutamine in AMPA receptors. This particular amino acid substitution appears to determine important electrophysiologic properties of the NMDA receptor (high Ca/Cs and Ca/Mg permeability ratios and a near linear current-voltage relationship) (Monyer et al., 1992).

Also characteristic of NMDA receptors is a voltage-dependent Mg block and the relatively slow onset and offset time course of the current response to high glutamate concentrations when compared to AMPA receptors. Some of these electrophysiological properties are displayed in Diagram 12.

Some controversy exists though as to the exact topology of the subunits. A more recent model includes an M2 transmembrane segment that does not traverse the membrane fully but folds back into the cytoplasm. This radically changes the overall structure of the subunit bringing to it similarities to the pore forming domain of voltage activated K+ channels. The difference lies in the position of the carboxylic acid terminal (which is particularly long in the NR2A and B subunits). If this terminal is actually located intracellularly, it might provide an additional target for cellular constituents (Monyer et al., 1992).

The NMDA receptor bears some resemblance to the GABA-receptor in that it possesses an elaborate array of regulatory binding sites apart from its agonist recognition site and ion pore. Notably, glycine is known to enhance NMDA-induced responses and a binding site for glycine is co-distributed in the brain with the NMDA receptor. This site is distinct from the strychnine-sensitive glycine receptor and can be considered as an independent allosteric regulatory component of the NMDA receptor system.
Diagram 12.  **NMDA receptor electrophysiology**  

Conductance and ion permeability properties have been studied in recombinant NMDA receptor subtypes by Monyer et al (1992). Typical results for the NR1-NR2A heteromer are summarised here. A) whole cell current in response to 100μM NMDA in the presence of 10μM glycine. The membrane potential is held at -60mV and the inhibitory effect of Mg (0.5mM) is clearly seen. B) Whole cell currents displayed so as to demonstrate the slow onset and offset times of glutamate activated currents (300ms pulse, 100μM glutamate with 10μM glycine). C) In the absence of glycine glutamate does not evoke a current. The brief addition of glycine (10μM) increases the current as long as glycine is present. D) The high divalent-ion permeability of the receptor is demonstrated by a shift in the reversal potential to more positive values caused a change in the extracellular solution from high Na to high Ca.). E) Steady state I-V relations measured during voltage ramps and 100μM glutamate activation. Both the voltage and concentration dependance of the Mg block is demonstrated here.
Currently, the NMDA receptor is thought to have i) an agonist recognition site, ii) a modulatory glycine site which is cooperative in opening and iii) an ion channel that permits transmembrane flux of Na, K and Ca. Within the ion channel are: a Mg$^{2+}$ ion, the phencyclidine (PCP) binding site and an MK-801 binding site. There is also a modulatory binding site for Zn and certain polyamines (spermine). It appears that the glycine site and PCP binding site are on the same subunit and that occupation of two agonists and two glycine sites (i.e. full occupation of two individual subunits) is required for receptor activation. Divalent ions may be an integral part of the glutamate/ glycine recognition sites.

### 5.1.6 The post synaptic density

This large (approximately 1$\mu$m diameter and therefore visible under EM) protein complex represents a specialization of the submembrane cytoskeleton of neurons that functions to provide a precise spatial arrangement of cellular signalling apparatus associated with synapses. It is particularly prominent in excitatory synapses and probably regulates receptor adhesion, clustering and function. The various proteins included in the structure include the cytoskeletal proteins- tubulin, actin and fodrin; the signal transduction protein calmodulin and the $\alpha$-subunit of Ca-calmodulin kinase type II (CAMKII). Three further protein component have also been identified. These include PSD-95 (a protein that binds a K-channel and the NR2B sub-unit of the NMDAR), the NR2B subunit itself and finally a glycoprotein- densin 180 that functions as an (extra-cellular) adhesion molecule (Kennedy, 1997).

The resolution and scope of investigations into the molecular structure of the post-synaptic density (PSD) has recently been increased by the application of proteonomic analysis combined with mass spectrometry and immunoblotting of the NMDA receptor protein complex. These studies suggest that the complex is comprised of at least 77 proteins arranged in an extended protein framework that includes polymerized scaffolding molecules (e.g. myosins, AKAP-150 and yotiao), possibly multiple receptors and receptor subunits (e.g. mGluR, GluR6/7), adhesion molecules (e.g. L1 and N-cadherin) and signalling proteins (protein kinases- A and C, phosphatases and Raf, MAP and Rsk kinases) (Husi et al., 2000). Only some of the detected proteins were previously known to be associated with the NMDA receptor (e.g. nNOS, Citrin, Shank, and Homer). The
heterogeneity of the complex suggests that the complex contains multiple effector pathways that may be differentially regulated to complete various tasks such as: second messenger signalling, trafficking and phosphorylation of AMPA receptors, dendritic-spine structural changes, regulation of local protein translation and gene transcription (Sheng and Lee, 2000). An impression of the structural complexity of the NMDA receptor and post-synaptic density is given in diagram 13.

Diagram 13 The NMDA receptor complex

The NMDA receptor complex is a subcellular structure comprised of a ligand gated channel embedded in a post synaptic density. It is made up of a large number of proteins some of which are represented above. Cytoskeletal and scaffold proteins are shown in green, signalling enzymes are shown in yellow. More recently identified components are shown in open circles. Overlapping circles implies a known protein-protein interaction (From Sheng 2000).

5.1.7 Electrophysiological characterisation

Glutamate can cause an increase in cation permeability (Na+/ K+ currents) across a post-synaptic membrane in proportion to the net driving force of the prevalent ionic gradient. This action is mediated by AMPA type receptors. Electrophysiological studies of glutamate transmission show it to consist of two components. Rapid transmission can be
blocked by the non-NMDA-receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). A slower component appears to be mediated by NMDA receptors that are sensitive to D(-)-2-amino-5-phosphonovaleric acid (AP5). CNQX blocks both AMPA and kainate receptors and thus does not distinguish the contributions of these two receptors subgroups (Yoshimura, 1990). Research to date suggests that NMDA receptors, by virtue of their voltage dependent Mg sensitivity, play a modulatory role in most synaptic pathways for which AMPA receptors exercise the primary transmitter role.

A characteristic feature of the NMDA-type receptor is a high calcium permeability and its sensitivity to the prevailing membrane potential. The latter allows the post-synaptic currents generated by NMDA type receptors to be modulated by the transmembrane voltage. The size of excitatory post synaptic potentials (EPSPs) increase as the cell is depolarized from the resting potential. This makes the NMDA-induced current qualitatively different. It is longer in duration and allows both calcium and sodium ions to traverse the membrane. In comparison to AMPA receptor currents, the NMDA mediated currents display slow activation and deactivation kinetics.

The voltage dependence (effected by a voltage sensitive Mg+ ion block of the ion channel near the resting potential) makes the NMDA post-synaptic current regenerative- i.e it increases with increasing depolarization of the postsynaptic membrane. This in effect, ensures that the channel will conduct in response to pre-synaptic transmitter release when there is concomitant post-synaptic membrane depolarization.

These characteristics of the NMDA receptor allow it to play a critical and unique role in activity dependent synaptic processes. These include phenomenon such as temporal integration, rhythmic firing and synaptic plasticity.

The electrophysiological study of NMDA receptors has been greatly aided by the cloning of subunit proteins and the use of recombinant techniques. Studies of recombinant homomeric channels show that channels of the NR1 type do allow ligand gated currents whereas such channels formed from NR2 subunits do not. Heterologous co-expression studies suggest that the NR1 subunit is essential and that the incorporation of various NR2 subunits increases, by several orders of magnitude, the whole cell glutamate-induced currents. Varying the specific NR2 subunit (A-D) confers functional diversity on receptors in-vivo. Biophysical and pharmacological properties such as sensitivity to Mg block, kinetics of desensitization and offset decay, modulation by glycine and phosphorylation as well as the affinity for agonists and antagonists appear to be dependent on the type of NR2 subunit expressed. The NR3 subunit when cotransfected with the NR1 and NR2 subunits
does not of itself allow ligand gated currents but decreases such currents (Sun et al., 2000).

Progressive alteration in subunit composition in the postnatal period is suggested to contribute to changing synaptic plasticity during cortical development. This is dealt with in greater detail in the discussion section of this chapter.

5.1.8 NMDA receptors and spinal neurotransmission

NMDA receptor binding studies in the spinal cord show patterns that parallel higher centres. There is a higher concentration of glutamate in the dorsal roots compared to the ventral roots (Neuroscience, 8 1983; 861-866) and binding sites have a greater density in outer laminae of the dorsal horn where nociceptive afferents terminate (Gonzalez, 1993).

The involvement of NMDA-type receptors in the synaptic transmission of dorsal horn nociceptive neurones was clearly demonstrated using the single unit extracellular recording technique on spinal cord dorsal horn neurons. In these studies, AP5 - a competitive NMDA antagonist is found to reliably reduce “wind up” and the post-discharges of deep dorsal horn neurones in response to repetitive C-fibre stimulation (Dickenson and Sullivan, 1987; Dickenson and Sullivan, 1990).

Consistent with the proposal that NMDA receptors play a modulatory role is the finding that these receptors play a significant role in mediating hyperalgesia but have no obvious role in the responses to acute noxious stimuli in uninflamed somatic tissues. This helps explain the categorization of NMDA antagonists as “anti-hypersensitivity drugs” with little anaesthetic activity (Ma and Woolf, 1995).

In contrast to this, models of acute visceral pain such as ureteric distension in the rat, suggest that NMDA receptors do mediate acute nociceptive signals from viscera (Olivar and Laird, 1999). In addition, it appears that peptidergic afferents may play a more specific modulatory role in visceral pathways and in the development of visceral hyperalgesia (Cervero and Laird, 1999).

5.1.9 Glutamate transmission and synaptic plasticity

Crucial to all models of synaptic plasticity and associative learning is a mechanism of modulation that requires coincident pre and post synaptic activation. The NMDA receptor provides just such a mechanism, allowing current to flow only when presynaptic activity releases neurotransmitter and postsynaptic cells are sufficiently depolarized to relieve the voltage dependent Mg+ block. The slow deactivation kinetics of the NMDA
receptor permit conductance even in the event of slightly asynchronous pre-and post synaptic activity. In effect, the receptor acts as a conditional logic gate—“where Hebb-like conditions are realized at a single synapse” (Cotman and Iversen, 1987).

Classical descriptions of the role of the NMDA receptor in activity dependent plasticity include the formation of ocular dominance columns in kittens (Tsumoto et al., 1987), imprinting in the chick (McCabe 1988) and olfactory learning in the rat pup (Lincoln 1988). These descriptions followed the first demonstration of the role of NMDA receptors in the induction of LTP (long term potentiation) in the hippocampus (Collingridge and Bliss, 1987). In general, each of these developmental models suggest a period of over expression of NMDA-sensitive glutamatergic activity that parallels critical periods when activity/experience can modify the organization and or strengths of inputs (McDonald and Johnston, 1990).

This modulatory role of the NMDA receptor has also been confirmed in preparations that model the cortical mediation of learning processes (Gutierrez et al., 1999) and long term potentiation (LTP) in limbic systems (especially within the hippocampal formation). LTP and the induction of long term depression have been studied in the primary somatosensory cortex, visual cortex, perirhinal cortex as well as the motor area of the cortex. Both phenomenon (LTP and LTD) have been shown to be consistently dependent on NMDA receptor function, confirming the widespread role of glutamatergic NMDAR neurotransmission.

The exact mechanism whereby NMDA receptors are able to facilitate long term changes in synaptic efficacy have remained controversial (Nicoll and Malenka, 1995). Debate has focused on whether or not the mechanism is predominantly pre-synaptic or post-synaptic (Malinow, 1994).

Evidence for a pre-synaptic mechanism has been gained in experiments on hippocampal mossy fibre synapses. Interestingly, these synapses express predominantly AMPA receptors and display an NMDA independent form of LTP (Weisskopf and Nicoll, 1995). It is thought that LTP at these synapses is the result of changes in the probability of transmitter release—an entirely presynaptic event in both induction and expression. On the other hand, LTP at hippocampal CA1 synapses does not appear to be dependent on changes in release probabilities suggesting instead that a post-synaptic mechanism must exist to explain this form of plasticity (Manabe and Nicoll, 1994).

The finding that a high proportion of CA1 synapses transmit with NMDA receptors but not AMPA receptors (i.e. are silent at resting at membrane potential) and that these synapses can acquire AMPA-type responses with LTP inducing protocols provided a novel
explanation for this form of LTP (Liao et al., 1995). In this model, LTP inducing stimuli induce calcium entry into postsynaptic cells through NMDA receptors which in turn leads to the functional addition of AMPA receptors to the post-synaptic membrane. These newly activated AMPA receptors allow a response at hyperpolarized potentials that were not possible in the previously silent pure-NMDA synapses (Scannevin 2000).

The relevance of this form of synaptic plasticity and the existence of silent synapses during development is discussed further in the Discussion section of this chapter.

Irrespective of the exact locus of NMDA-dependent synaptic plasticity, there is general agreement that a high calcium permeability is central to most of the lasting effects of NMDA receptor activation. Calcium influx into post-synaptic cells is a prime physiological signal for several intracellular events including alterations in synaptic efficacy, altered cytoarchitecture and also of cell death. Though the biochemical machinery that mediates LTP remains under intense investigation, several key molecules are thought to play a major role. These include the \( \alpha \)-calcium-calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), the c-AMP-dependent protein kinase A (PKA), the tyrosine kinase Src and mitogen-activated protein kinase (MAPK). Each of these molecules are known to be concentrated in the region of the post-synaptic density. Molecules that may be involved as retrograde messengers and therefore implicated in pre-synaptic mechanisms include nitric oxide (NO), carbon monoxide (CO), arachidonic acid metabolites and platelet-activating factor (Malenka 1999).

5.1.10 Synaptic plasticity and Hebbian learning

The concept of synaptic plasticity is central to models of learning, memory and the development of neural circuits. First suggested by Cajal more than 100 years ago, evidence for it has only been available in the last half century and two broad forms are now defined, homosynaptic or Hebbian (activity-dependent) plasticity and heterosynaptic (input dependent) plasticity. The first was proposed by Donald Hebb in 1949 and is characterised by being homosynaptic, associative (associates pre-synaptic firing with post-synaptic firing) and input specific (only the input synapse is strengthened). Heterosynaptic plasticity on the other hand refers to the strengthening or weakening of a synapse by an independent (third) modulatory neurone. Two models have provided much understanding of each phenomenon. Recording of synapses in the mollusc, Aplysia have allowed the development of the concept of heterosynaptic plasticity (Kandel 1965) while the discovery
of long term potentiation (LTP) in the hippocampus has been the basis of understanding Hebbian mechanisms (Bliss 1993).

Basic to the concept of both heterosynaptic and homosynaptic plasticity are i) activity-dependent modification of synaptic strength and ii) homeostatic regulation of neuronal firing rates (preventing both saturation and complete loss of synaptic transmission).

The electrophysiological properties of the NMDA receptor—i.e. its ability to allow current flow in response to neurotransmitter release only when the post-synaptic membrane is already partially depolarized—make it an ideal candidate for the molecular basis of homosynaptic activity-dependence. The NMDA receptor, though does not of itself, provide a mechanism for any form of competition between synapses. Neural circuit models must invoke other mechanisms for synaptic competition before reasonable accounts of learning are possible. These postulated constraints must ensure competition such that when some synapses are strengthened others are weakened. Such constraints may involve global intracellular signals that limit the sum of synaptic signals received by any one cell or the mean activity of the cell. Other models postulate competition derived from local processes that depend on equilibration of synaptic efficacy at pre-set levels (Turrigiano et al., 1998). The molecular mechanisms of these constraints are entirely speculative and may involve some form of negative input correlation or non-Hebbian synaptic change (growth or decay).

A recent model proposed by Song et al provides a novel mechanism that appears to explain the strengthening of correlated (causally related) pre- and post-synaptic activity as well as synaptic competition. It does this without the need for additional constraints such as global signalling, growth factors or artificially imposed balances of synaptic decay terms. In their scheme, synaptic strengthening occurs together with competition by a mechanism of modification of spike timing termed spike-timing dependent plasticity (STDP). The basis of this model is that the largest changes in synaptic efficacy occurs when the time difference between pre and post synaptic action potentials is small. As the time difference approaches zero and becomes negative, there is a sharp transition from synaptic strengthening to weakening. Though admitting that this mechanism cannot be the sole source of plasticity in Hebbian learning, the researchers point out its several advantages including: i) it allows for a stable distribution of synaptic conductances.

ii) it forces the post-synaptic neuron into a balanced, irregularly firing regime that is sensitive to the timing of the pre-synaptic action potential it receives.
iii) it allows for both the strengthening of short latency inputs as well as the weakening of remaining synaptic inputs.

iv) the critical temporal windows over which the mechanism functions could be influenced directly by variations in the decay times of the NMDA mediated currents.

Further, the diversity of NMDA receptors conferred by the incorporation of different subunits allows the mechanism to be dynamically adjusted in individual brain regions and at different periods of development (Song et al., 2000).

Models of synaptic learning and development have been dominated by homosynaptic mechanisms, particularly those of the hippocampus. Models of heterosynaptic plasticity have been developed in invertebrates and demonstrate that this form of plasticity is recruited in classical conditioning forms of learning. In these models persistent changes in synaptic strength are found to contribute to long term memory and are notable for being mediated by monoaminergic neurotransmission. Both inhibitory and facilitatory changes in synaptic strength have been documented. Importantly, models of heterosynaptic plasticity display a longer time course than typical demonstrations of LTP in the hippocampus. Heterosynaptic mechanisms are able to recruit signalling mechanisms that lead to transcriptional events and the growth of new synaptic connections.

In a recent review of these two main forms of learning-related synaptic plasticity, Bailey et al suggest that homosynaptic and heterosynaptic plasticity not only have different properties but serve different functions. Hebbian mechanisms may be the basis of learning and short term memory while heterosynaptic mechanisms may be more important in long term memory and its maintenance (Bailey and Kandel 2000).

The descriptions given above are focussed on the properties of ligand-gated channels (NMDA, 5HT and dopamine receptors) and have generally assumed neuronal membranes to have stable properties facilitating high fidelity transmission of action potentials. Newer models have suggested that voltage-gated channels may also play a significant role in neuronal plasticity (Stemmler and Koch, 2000). By adjusting intrinsic membrane excitability, e.g. by changing the density of sodium channels, neurons may be able to adjust their firing rate in a homeostatic fashion that matches a neuron's output to its input. As a form of plasticity this is fundamentally different to Hebbian synaptic learning (correlated activity between pre- and post synaptic neurons) in that it can occur within single neurons and provides a mechanism whereby a neuron can respond to the structure of an input while optimising the information carried by its output firing frequency (Spitzer, 1999).
5.1.11 Agonists and Antagonists

Glutamatergic receptors are large (twice as large as Ach receptors) and include multiple recognition sites. Several distinct classes of compounds are able to modulate ligand binding. The NMDA receptor gives rise to at least four distinct classes of NMDA receptor antagonists. These either act competitively at the glutamate recognition site, antagonise the modulatory role of glycine, antagonise the actions of polyamines or block the integral ion channel (Lodge et al., 1994).

Glutamatergic receptors and their pharmacologic classification:

<table>
<thead>
<tr>
<th>Receptor class</th>
<th>Agonist</th>
<th>Competitive Antagonists</th>
<th>Non-competitive Antagonists</th>
<th>Allosteric agonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>NMDA</td>
<td>CPP</td>
<td>MK-801</td>
<td>Glycine</td>
</tr>
<tr>
<td></td>
<td>Aspartate</td>
<td>D-AP5</td>
<td>PCP</td>
<td>Kvurenic acid</td>
</tr>
<tr>
<td></td>
<td>Ibotenate</td>
<td>D-AP7</td>
<td>SKF10047</td>
<td>MRZ/2</td>
</tr>
<tr>
<td>AMPA</td>
<td>AMPA</td>
<td>?GAMS</td>
<td>GYKI-53655 (LY 300168)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quisqualate</td>
<td>?CBP23DA</td>
<td>CNOX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-glutamate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kainate</td>
<td>kainate</td>
<td>L-AP4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-AP4</td>
<td>Domoate</td>
<td>L-SOP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-glutamate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Structure-activity relationships for the agonists can be summarized as follows:

i) L-glutamate has the highest affinity of all agonists tested and agonists of glutamate-length generally have higher activity than those of aspartate length.

ii) The receptor can accommodate both D and L forms of agonists.

iii) The ω-terminal acidic group preference for agonists is CO2H > SO2H2 >> PO3H2 (Watkins and Olverman, 1987).

iv) The most potent antagonists are longer chain acidic amino acids having the D configuration at the amino terminal and a distance between acidic groups of 4-6 atoms.

v) The rank order for potency of the acidic group at the non-amino terminal is PO3H2 > CO2H > SO3H.
Diagram 14 Chemical structures of antagonists and agonists.

**Excitatory Amino acids**

- L-Glutamate
- L-Aspartate

**Selective agonists**

- Amino-methyl isoxazopropionic acid (AMPA)
- Quisqualate
- Kainate
- N-methyl-D-Aspartate (NMDA)

**Antagonists**

- Ketamine
- D-2-amino-5-phosphonovalerate (APV)
- Phencyclidine

**Relative potencies of the various EAA receptor antagonists**

The following table of relative potencies were obtained from studies of the effect of intrathecally administered drug on behavioural tests (tail flick latency) in carrageenan inflamed rats. The dose response curves were created from cumulative dose response experiments.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ED50 (nmol (95% CI))</th>
<th>potency (nmol/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-801</td>
<td>9.3 (2.9-24.0)</td>
<td>2.06 (0.64-5.31)</td>
</tr>
<tr>
<td>AP5</td>
<td>20.11 (9.6-33.1)</td>
<td>3.96 (1.9-6.53)</td>
</tr>
<tr>
<td>CPP</td>
<td>40.42 (10.5-288.3)</td>
<td>10.19 (2.6-72.7)</td>
</tr>
<tr>
<td>7-Cl kvurenic</td>
<td>45.97 (25.9-74.6)</td>
<td>10.28 (5.8-16.6)</td>
</tr>
<tr>
<td>Ketamine</td>
<td>50.43 (13.6-424.2)</td>
<td>11.99 (3.2-100.8)</td>
</tr>
<tr>
<td>CNOX</td>
<td>50.939 (314.8-)</td>
<td>118.26 (73-275)</td>
</tr>
</tbody>
</table>

(From Ren et al., 1992)
5.2 KETAMINE INTRODUCTION

5.2.1 Ketamine as an analgesic drug

In parallel with the increasing appreciation of the role of the NMDA receptor in nociceptive processing, clinical interest in NMDA antagonists has been re-kindled (Schmid et al., 1999). Currently the choice of NMDA antagonists in clinical practice is limited to ketamine and dextromethorphan. Chemically related to phencyclidine, ketamine (formula, 2-(o-chlorophenyl)-2-methylamino cyclohexanone HCL) is an effective analgesic and anaesthetic agent with a shorter (and therefore more controllable) duration of action than phencyclidine (Friesen and Morrison, 1994). A range of adverse effects has prevented widespread use of these drugs but continued interest has resulted in the identification of a dose range in which beneficial analgesia can still be obtained without major side effects.

Delivery of analgesic drugs into the epidural space - in particular, low doses of local anaesthetic agents and opioids are now established as part of most acute pain management protocols in adults and is gaining wider popularity in paediatric practice. The potential for improved outcomes and reduced adverse effects through the addition of NMDA antagonists to epidural solutions is being addressed in both post-operative and chronic pain settings (Chia et al., 1998; Takahashi et al., 1998). Very little data is available on the pharmacologic profiles of either ketamine or dextromethorphan in children whether given systemically or epidurally.

These issues prompted the selection of ketamine as the first NMDA antagonist to be trialled in this developmental model of spinal antinociceptive agents. Documenting the antinociceptive effects of this clinically relevant and effective drug provided confirmation of the ability of the preparation to profile the efficacy of analgesic drugs. The study of ketamine was then followed by a study using a more specific (and "cleaner") competitive NMDA antagonist- AP5. In a parallel experiment, morphine was studied across the same developmental period to provide comparative data with a typical opioid agonist.

5.2.2 Early clinical use

The initial trials in humans (done among 20 volunteers from a prison population) assessed its actions after intravenous administration at doses ranging from 0.1-2mg/kg. At 1mg/kg, coma was induced that lasted for 3-8 minutes (mean 6mins). The pattern of unconsciousness was quite unlike that described for either the halogenated volatile
anaesthetics or the barbiturates. Onset of action was heralded by a feeling of numbness which was then followed by eye opening and simultaneously, the loss of contact with the environment. The authors of this first report of the drug in human subjects suggested the term “dissociative anaesthesia” to describe the state produced by this class of drugs. The most dramatic finding in this study was the intense analgesia produced by a continuous infusion of the drug at a dose rate that did not induce unconsciousness. Psychic phenomenon were noted in the recovery phase following drug administration and included alterations in mood and affect. EEG monitoring showed marked changes in pattern from alpha activity to theta activity during drug administration. The return to alpha activity did not occur until at least half to one hour after drug injection. Delta activity and burst suppression were only rarely seen.

Following the above trials, ketamine was introduced to clinical practice in 1965. It has potent analgesic actions when given in sub-anaesthetic doses. It currently fills several niches in both paediatric and adult clinical anaesthetic practice (Reich 1989).

5.2.3 Perioperative Analgesia including pre-emptive analgesia

Low doses- also called sub-dissociative or non-anaesthetic doses- of ketamine have been the object of clinical study of many years ever since a call for clinical trials was made in 1971 following a meeting of the International Anesthesia Research Society (Bosomworth, 1971). At the time, the analgesic effects of ketamine were becoming apparent in studies of the drug used as an anaesthetic agent. Analgesia in adults for up to 40 minutes after normal (2-3mg/kg) anaesthetic doses of ketamine had been demonstrated quite early (Bovill and Dundee, 1971).

In both experimental pain models, postoperative pain settings (Mathisen et al., 1995) and in procedural pain models (e.g. burns dressings (Slogoff et al., 1974)) ketamine delivered by intramuscular injection produces analgesia equivalent to 1-2mg/kg of pethidine. The effect tends to be of shorter duration than that of the opioid and there is no significant difference in side effects. In particular, psychic phenomenon are reported as being uncommon, minor and non-problematic (Sadove et al., 1971). There are very few studies of the use of ketamine in the paediatric population.

In a controlled trial of the pre-emptive analgesic effect of ketamine, 60 adults undergoing abdominal hysterectomy were given a bolus dose of 1mg/kg ketamine followed by an infusion of 0.01mg/kg/min during the surgical period (until peritoneal closure). This resulted in significant reductions in VAS scores at rest and with movement,
as well as significant reductions (20-50%) in total post-operative morphine consumption. (Hong et al., 1999)

The mechanism of this preemptive effect remains to be elucidated but is likely to involve blockade of NMDA receptors within central nociceptive pathways. A possible peripheral action was suggested by Tuttle et al, after in-vitro studies of the effect of local anaesthetic agents and ketamine on the production of nerve growth factor. At concentrations of 100μM, both lignocaine and ketamine depress the production of NGF from a standard model system of NGF production (bladder smooth muscle cells) (Tuttle et al., 1999).

In a small report (uncontrolled and un-blinded) Forestner (1988) reported the use of peri-operative ketamine (1 mg/kg intravenously) in ten children undergoing minor surgery and described “excellent analgesia and a calm anaesthetic recovery...without detectable cardiovascular depression” (Forestner 1988).

5.2.4 Anti-hyperalgesic actions

Possibly the first human clinical trial to demonstrate the anti-hyperalgesic action of ketamine when given as an adjunct to opioid post-operative analgesia was completed by Stubhaug et al (1997). In a strictly controlled, double blind trial, these researchers demonstrated a clear anti-hyperalgesic effect of a 72 hour intravenous infusion of ketamine following abdominal surgery. Ketamine infusion resulted in a significant reduction in the area of punctate hyperalgesia surrounding a nephrectomy wound in well, living-donor patients at 1, 3 and 7 days post-operatively. Concomitant with this was an reduction in cutaneous temporal summation in response to repeated mechanical stimuli around the wound and a reduction in opioid consumption. Pain intensity scores were also reduced though these improvements were not sustained beyond the first few post-operative hours. The study confirmed also a favourable global satisfaction rating from patients and a reduced incidence of side effects (Stubhaug et al., 1997). Infusion rates were 0.5mg/kg bolus followed by 2μg/kg/min for 24 hours followed by 1μg/kg/min for 48 hours.

5.2.5 Premedication and Sedation

Pellier et al have used ketamine as part of an intravenous sedation protocol in paediatric patients undergoing painful procedures for tumour-related disease (principally haematological). Used in combination with midazolam (0.025mg/kg) as an i.v. bolus adequate sedation was achieved in a large proportion (81.5%) of children in a day care
setting. Ketamine doses varied from 0.5mg/ml to 3mg/ml (mean 1mg/ml) and resulted in sedation with an onset time of 5mins and a mean duration of 10mins. The sedative protocol failed in only one of 92 patients and analgesia was considered poor in two cases. Adverse effects included agitation in 5 of 92 patients though all remained amnesic for the experience.

### 5.2.6 Epidural ketamine

In an attempt to demonstrate the efficacy of epidurally administered ketamine for post-operative analgesia in children, Marhofer et al (1999) compared the duration and efficacy of analgesia following caudal injections of either S(+) ketamine 1mg/kg with that of bupivacaine 0.25% with 1:200,000 adrenaline. Though the study was unlikely to be able to demonstrate subtle differences in the quality of these agents, it demonstrated at least, that the S isomer of ketamine could provide a similar profile of post-operative analgesia to one of the most commonly used local anaesthetic agents (Marhofer et al., 1999).

Semple et al studied the addition of preservative free ketamine to caudally administered bupivacaine in paediatric anaesthetic practice and concluded that it significantly extended the duration of analgesia obtained from the caudal injection of bupivacaine alone. In their dose finding study, they concluded that a dose of 0.5mg/kg was probably the optimal dose of ketamine in single shot caudal epidural injections in the peri-operative setting. This dose avoided behavioural side effects that were significantly more common with higher doses (Semple et al., 1996). These researchers continued their study of epidurally administered ketamine at a dose of 0.5mg/kg and concluded that the addition of ketamine allowed the reduction of the concentration and therefore total dose, of bupivacaine from 0.25% to 0.125% without any significant reduction in the quality of analgesia (Johnston et al., 1999). The addition of ketamine 0.5mg/kg to caudal injections of 1ml/Kg bupivacaine resulted in the effective injection of a 0.05% solution of ketamine.

The study of the efficacy of ketamine in axial blockade in paediatric practice is complicated by a difficulty in selecting an appropriate patient cohort. Many studies are carried out in children undergoing inguinal hernia repair or orchidopexy. Post-operative analgesic prescription for these procedures does not commonly include powerful analgesic drugs (i.e. moderate –high dose opioids) after a single dose of an epidural local anaesthetic and most children are discharged from hospital care within 6-8 hours following surgery. This makes the demonstration of significant differences between agents and protocols very difficult.
5.2.7 Neuropathic Pain Management

Reports of the use of ketamine for neuropathic pain are restricted to several clinical case reports dating only as far back as 1995 (Felsby et al., 1995; Mathisen et al., 1995) (Cherry et al., 1995). These last three studies quoted generally showed beneficial effects of ketamine in terms of pain relief, reduced opioid requirement and reduced alldynia in adults suffering a variety of neuropathic pains. There are very few reports of its use in paediatric patients.

In 1995, Persson et al (Persson et al., 1995, Torrance, 1997) reported the case of a 17 year old girl who developed a pathological pain state associated with a chronic suppurating abdominal wound. Over an 11 month period the effectiveness of systemic ketamine (0.2-0.5mg/kg) was documented for opioid-resistant pain associated with dressing changes. In this case, there was marked mechanical alldynia but no spontaneous pain and little evidence of sympathetic outflow imbalance. Objective measures of thermal sensitivity showed grossly deranged (raised) heat and cold pain thresholds that were only partially normalized by phentolamine administration. In two temporally separated periods of ketamine administration, both symptoms and objective physiological measures (cutaneous temperature thresholds) improved. While the objective improvements related to ketamine administration appear convincing and the findings can be synthesized into a conceptual model incorporating NMDA receptor mechanisms, the report must be considered with caution as several confounding factors could not be eliminated. Ketamine was delivered systemically and did give rise to albeit tolerable psychotomimetic symptoms. Attempts to prevent these necessitated the use of midazolam. The patient had undergone a period of escalating opiate use prior to the commencement of ketamine therapy and a psychological assessment is not included in the report. The report though does clearly show the benefits of the use of objective sensory testing in the setting of clinical neuropathic pain management and suggests that epidural drug administration might make mechanistic interpretations easier.

Takahashi et al (1998) reported the use of ketamine in a 14 year old boy suffering from a complex regional pain syndrome (CRPS), type 2. Spontaneous pain, dysaesthesia and mechanical alldynia following traumatic sciatic nerve injury was associated with signs of sympathetic outflow imbalance (oedema, coldness and atrophy of the affected limb). Failed responses to non-steroidal analgesics, antidepressants and anticonvulsants were all documented while an epidural infusion of local anaesthetic solution was noted to give only partial relief. Systemically administered ketamine (0.3mg/kg) resulted in intolerable side
effects (nausea, headache and discomfort) without any relief of symptoms while the same dose delivered epidurally relieved the symptoms but with the return of side effects.

Ketamine was subsequently administered via an epidural infusion at low dosage (25\(\mu g/kg/hr\)) and continued for 10 days. This resulted in long lasting (up to 8 months) relief of pain though the sympathetic signs remained. While this report appears to demonstrate a striking and specific response of a severe neuropathic pain syndrome to the segmental administration of an NMDA antagonist, it suffers from a lack of objective pain scoring and a lack of detail regarding the timing of other associated interventions. Details regarding the epidural infusion (volume rates, drug concentrations and formulation) are also unfortunately not given. The case highlights the ongoing need to define optimal dosage, timing and duration of such therapy. It demonstrates also that CRPS quite possibly involves two distinct pathophysiologyes, one NMDA dependent and the second involving the sympathetic nervous system.

Insights into the involvement of NMDA receptors in nociception have prompted several studies of the use of ketamine in the palliative care of patients with cancer. This follows the demonstration of reduced opioid tolerance and the enhancement of analgesia in animal models (Mao et al., 1996; Shimoyama, 1996; Dunbar, 1996). Lauretti et al have recently confirmed the efficacy of low dose (0.5mg/Kg at 12 hourly intervals administered orally) ketamine as a co-adjuvant analgesic in the treatment of patients with cancer. In their study, the addition of ketamine to a maintenance dose of oral morphine prevented the escalation of opioid requirements as disease progressed. This opioid sparing effect (interpreted as the prevention of tolerance) was more dramatic than that of dipyprone (a non-steroidal anti-inflammatory drug) and topically applied nitroglycerine (a NO donor) (Lauretti et al., 1999).
5.3 METHODS

The main aim of the experiments described in this chapter was to compare the antinociceptive effects of the NMDA antagonists ketamine and AP5 when delivered epidurally at three developmental stages. Dose-response curves for each of the drugs at each of the three age groups were determined and final results expressed as a comparison of ED50 values.

To ensure reliability, the following four methodological issues needed to be established:
1) that drug effect was tested at an equivalent anaesthetic depth in all rat pups.
2) that the dose range and route of application of drug resulted in local (spinal) drug effects only (i.e. that systemic drug effects did not confound the results).
3) that the measure of drug effect at each group could be meaningfully normalised (i.e. that "nil" and "maximal" drug effect reflect equivalent physiological states across the age groups tested.
4) that variations in the time course of drug effect at each age did not significantly confound the results (i.e. that time to peak effect in all groups were similar so that records obtained exactly 30mins after drug administration could be directly compared).

5.3.1 Anaesthetic depth

The basic technique for electromyographic recording was identical to that described in previous chapters. Data presented in chapter 2 were used to normalise the drug-effect data collected here. Anaesthetic depth as described in chapter 3 was adequately controlled in these experiments at equivalent MAC multiples in the three age groups tested.

5.3.2 Epidural drug administration

The technique of epidural drug administration in small rodents has been developed from initial work by Marsh et al in this same laboratory (Marsh et al., 1999). Following pilot experiments using a percutaneous epidural injection technique, a more reliable technique was developed that involved minimal lumbar dissection.
Immediately after secure placement within the stereotaxic frame and while still under deep halothane anaesthesia (2%), a midline lumbar incision was made to facilitate epidural injection. The lumbar paraspinal muscles were displaced laterally from the spinous processes of L5 and L6 with a combination of sharp and blunt dissection. The iliac crest was used as a surface landmark for identification of the sixth lumbar spinous process. Unilateral dissection (opposite to the side prepared for EMG recording) was adequate for the injection technique. Retracting paraspinal muscles from the underlying paraspinal gutter and lumbar laminae exposes the interlaminar ligament (ligamentum flavum) to direct view. A 30G needle could then be introduced into the epidural space atraumatically if directed from a paramedian approach and slightly cephalad. This paramedian and contralateral approach reduces the risk of intrathecal injection, traumatic cord damage and nerve root damage on the side being studied. A schematic diagram of the injection technique is shown in Diagram 15. During pilot work developing this technique, the reliability of epidural injection was checked by injecting Evans blue dye epidurally. Confirmation that injectate did not spread intrathecally was obtained at post-mortem dissection. This dissection was not a regular part of the experimental protocol as intrathecal spread of injectate was not seen in the pilot injections.

Injection volumes for all drugs were standardised to pup weight (1ml/kg using a 100μl Hamilton syringe) and drug doses adjusted by controlling the concentration of the drug being injected. As soon as epidural drug injection was complete the halothane concentration was reduced to predetermined age-related levels (P21: 0.7%, P10: 0.9%, P3: 1.0%). EMG recording was commenced 30 minutes after epidural injection.

Control experiments confirmed that systemic absorption of drug did not confound the analysis. In these experiments similar drug doses (mg/kg) were injected subcutaneously. No significant effect on recorded reflexes were seen for any of the three drugs under investigation (ketamine, AP5 and morphine). Experiments testing the effect of injectate volume on the measured drug effect also confirmed that drug concentration and not volume was the critical independent variable. For all experiments, epidural drug volumes were standardised against animal weight (1ml per kg). This resulted in volumes of injection of

8-12μl for P3 pups,
25-35μl for P10s and
50-60μl in P21 rat pups.
Lumbar dissection

Diagram 15. The lumbar dissection.
A minimally invasive lumbar dissection facilitates epidural drug injection under direct vision. 1 cm midline skin incision allows blunt dissection of the paravertebral muscles away from the midline on one side of the animal’s back. The iliac crests are used as a landmark to identify the L5- L6 interspinous space. Exposure of the paravertebral gutter brings the spinous laminae into view. A 30G needle can then easily be introduced from a paramedian approach for epidural injection.
5.3.3 General experimental protocol

The following time line diagram explains the general experimental protocol for all the pharmacodynamic experiments.

In essence, carrageenan inflammation was induced at least 2 hours prior to EMG recording. EMG recording was commenced 30 minutes after epidural drug administration.

5.3.4 Normalisation of Drug effect data

Effective normalisation of drug effect data across different age groups requires an exact definition of drug action. For the drugs being tested, the broad definition: “antinociception” is inadequate. The action of interest is the ability of the drug to return the excitability (responsiveness) of the spinal cord to normal from an inflammatory pain state. This may be better described as “Anti-hyperalgesia”. Using this definition, “nil” and “maximal” drug effect can be easily defined as follows:
Nil effect: measured spinal reflex responsiveness equal to that in carrageenan inflamed pups following epidural injection of saline.

Maximal effect: measured spinal reflex responsiveness equal to that in un-inflamed (naive) pups following epidural injection of saline.

These points of reference have been defined for each of the three age groups being studied, in independent experiments described in Chapter 4. The above definition allows a distinction to be made between drugs that depress normal reflex responsiveness and those with specific actions in the hyperalgesic state. Clinically, this may reflect differences in drugs that work as acute anti-nociceptive agents i.e. drugs that reduce acute pain—otherwise termed physiological or procedural pain and drugs that act to reduce the allodynia and hyperalgesia that characterise pathological pain states.

From the above considerations, it is apparent that a drug may, by depressing reflex responsiveness below that of the naive state- be regarded to have a “supra-maximal effect”. This does not present a problem for calculating ED50 values but is open to debate regarding its interpretation.

ED50 values were computed by non linear regression of data on all three drugs (Graphpad Prism v2.0c). The logarithm of drug concentration was used to create a log dose-response relationship following the equation for one-site competition, i.e.:

\[
Y = Bottom + \frac{(Top - Bottom)}{(1+10^{(X-\log EC50)})}
\]

The fit converged for all data sets- although some SE values were large. Each replicate was considered individually. The Top was held constant at 100- (normalising the curve for zero drug effect). The algorithm minimized the sum of the squares of the actual distance of the points from the curve. Convergence was reached when two consecutive iterations changed the sum of squares by less than 0.01%.
5.4 RESULTS

The effect of epidural ketamine on the hyperalgesic state has been assessed in these experiments by quantifying its effect on the withdrawal reflex stimulus-response curve in carrageenan inflamed pups (described for in chapter 4). The response curves for the area under the RMS signal in carrageenan inflamed 21 day old pups is shown in red in Figure 21. Superimposed on this are response curves obtained 30 minutes after epidural ketamine injection in separate groups of similarly inflamed pups. From the four curves displayed it is possible to discern a dose dependent effect of ketamine injection on the slope and position of the response curves. Increasing ketamine concentrations results in a rightward shift in the response curve.

To allow better comparison, the antihyperalgesic effect of epidurally administered drugs has been quantified by calculating the area under the stimulus-response curves. This has been done for the area under the RMS signal to produce a summary statistic (termed reflex responsiveness) that gives an indication of the relative responsiveness of the withdrawal reflex under any given experimental condition. The effect of epidural ketamine on carrageenan inflamed pups is shown in Figure 22 (mean values with standard deviation for each group of pups). This confirms that in carrageenan-inflamed 21 day old rat pups the response was dose-dependently reduced by ketamine.

To ensure that injection volume was not a confounding factor in these experiments, a comparison of three injection volumes of a fixed concentration of ketamine was carried out in P21 pups. At a concentration that produced maximal drug effect (return of reflex responsiveness to naive control value) no significant difference between injectate volumes of 25, 50 and 100μl were seen (see Figure 23).

Using the summary statistic “reflex responsiveness” the dose-response curves shown in Figure 24 have been generated for ketamine, AP5 and Morphine. From this data, the ED50s for epidural ketamine (ED50 = 0.035% sol), AP5 (ED50 = 0.016% sol) and morphine (ED50 = 0.00375% sol) have been estimated. These doses were ineffective if given systemically (intra-peritoneally) (see Figure 25).

Comparative dose-response curves across the age groups tested are shown in Figure 26. To allow a comparison of ED50 values, drug effect data has been normalised for each age group. Reflex responsiveness is normalised between values obtained following carrageenan inflammation (100%) and naive values (0%). Figure 27 shows that neither the NMDA antagonists (ketamine and AP5) nor morphine had a depressant effect in non-inflamed pups (the lower reflex responsiveness values recorded for morphine at all three age groups did not reach statistical significance).
5.4.1 Epidural ketamine in carrageenan inflamed P21 pups (i)

Figure 21

The effect of epidurally administered ketamine on the withdrawal reflex can be seen as a dose dependant reduction of the carrageenan-facilitated response. A ketamine concentration of 0.05 gm% returns the response to the naive (non-inflamed) state. Higher concentrations (0.1%) do not result in any further significant inhibition (see Figure 25). The area under each stimulus-response curve has been used as a summary statistic to aid comparison of experimental conditions (see diagram 10, Chapter 2). (n=6-8 pups for each drug dose; n=11 saline controls. Mean and SEM displayed)
5.4.2 Epidural ketamine in carrageenan inflamed P21 pups (ii)

Figure 22
The effect of inflammation and varying concentrations of epidural ketamine on the withdrawal reflex displayed as comparisons of the areas under each stimulus-response curve (mean and standard deviation displayed (n:bracketed). Inflammation induced by carrageenan results in a fourfold increase in the response while epidural ketamine dose-dependently returns this facilitated response toward the naive state. Systemic administration of ketamine within this dose range is ineffective, providing evidence that the measured effect is due to a local (spinal) action of the drug (One way ANOVA Epidural saline vs Systemic ketamine0.05% p=0.142). b) Epidural ketamine administered to naive pups does not depress reflex responsiveness.
5.4.3 Epidural injection volume

To confirm that epidural injection volume was not a confounding factor in these experiments a study of injectate volume against drug effect was carried out. Varying the volume did not significantly influence drug effect. (Mean and standard deviation displayed.)

**Figure 23**

To confirm that epidural injection volume was not a confounding factor in these experiments a study of injectate volume against drug effect was carried out. Varying the volume did not significantly influence drug effect. (Mean and standard deviation displayed.)
5.4.4 Ketamine, AP5 and Morphine concentration-response curves - P21.

Figure 24
Concentration-response curves for epidurally administered ketamine, AP5 and morphine obtained in carrageenan-inflamed 21 day old rat pups (4-8 pups were studied in each drug dose group; mean and standard deviation displayed). EC50 values estimated from these curves define the concentration of drug required to reduce the response by 50% of the facilitated amount i.e. carrageenan-inflamed value minus the naive paw value.
5.4.5 Systemic drug administration

Figure 25
Reflex responsiveness was determined following intraperitoneal drug administration using doses at or above the calculated ED50 of each of the drugs. (Mean and standard deviation displayed). (One way ANOVA p=0.142)
Ketamine, AP5 and Morphine concentration-response curves: Age comparisons

Figure 26
Concentration-response curves were created for the three drugs under investigation at each of three ages. To take account of the age related changes in both naive and carrageenan-inflamed reflex responsiveness values, curves were normalised between these two points for each age. An RR value of 100% then, was equivalent to the carrageenan-inflamed value (and therefore would define "zero drug effect") while an RR value of 0% was equivalent to the naive pup value (and therefore defined "100% drug effect"). EC50 values were estimated following non-linear regression of log concentration-response curves. These are shown as an inset beside each graph.
EC50 values calculated from the previous dose-response curves are presented here. One way ANOVA: Are the means significantly different? Morphine p=0.509, Ketamine p=0.011, AP5 p=0.049. Post test for linear trend: Ketamine p=0.003, AP5 p=0.015.

<table>
<thead>
<tr>
<th></th>
<th>Morphine</th>
<th>Ketamine</th>
<th>AP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>0.001 (0.003-0.007)</td>
<td>0.002 (0.0004-0.008)</td>
<td>0.002 (0.0005-0.008)</td>
</tr>
<tr>
<td>P10</td>
<td>0.001 (0.002-0.009)</td>
<td>0.008 (0.002-0.038)</td>
<td>0.005 (0.001-0.024)</td>
</tr>
<tr>
<td>P21</td>
<td>0.004 (0.001-0.01)</td>
<td>0.03 (0.009-0.10)</td>
<td>0.016 (0.007-0.03)</td>
</tr>
</tbody>
</table>
5.4.7 Drug effect in the absence of inflammation.

In P21 pups the effect of epidural administration in un-inflamed pups was tested. Concentrations above the calculated E50 were used for these experiments. No drug significantly reduced the baseline (naive) reflex responsiveness. (Mean and standard deviation displayed).

Figure 27
In P21 pups the effect of epidural administration in un-inflamed pups was tested. Concentrations above the calculated E50 were used for these experiments. No drug significantly reduced the baseline (naive) reflex responsiveness. (Mean and standard deviation displayed).
5.5 DISCUSSION

These experiments have shown that the NMDA antagonists, ketamine and AP5 can effectively reverse the changes in the hindlimb withdrawal reflex induced by inflammation. The efficacy of these drugs at low doses (lower than those required to depress the reflex in non-inflamed animals) is consistent with the critical role played by NMDA receptors in inflammatory pain states (discussed in chapter 4).

The principal finding of these experiments however, is that there is a significant developmental pattern in the spinal cord sensitivity to both NMDA antagonists and morphine in rats. No previous studies documenting developmental changes in NMDA antagonist sensitivity in the spinal cord have been published. This study suggests a marked and continuing reduction in sensitivity to both ketamine and AP5 during the first three postnatal weeks of rat life.

The ED50 for ketamine appears to be an order of magnitude lower in P3 rat pups compared to P21 pups. A similar though less marked pattern was noted for AP5. The fact that morphine did not give a similar pattern suggests that these findings are not the result of a methodological problem or general function of changes in drug delivery.

Potential explanations for the changing sensitivity must include a consideration of the developmental patterns of NMDA receptors. A discussion of these patterns follows below after consideration of four points: i) the methodological issue of epidural drug administration and ii) the mechanism of action of ketamine iii) ketamine’s analgesic profile in the laboratory setting iv) definitions of analgesia and anaesthesia.

5.5.1 Epidural drug delivery

A major advantage of epidural drug delivery over systemic drug delivery in pharmacodynamic studies is the avoidance of pharmacokinetic factors that may influence measured drug effect. Absorption, distribution, metabolism and clearance of drug and of potentially active metabolites from the systemic circulation can all directly influence the active drug concentration within the biophase with immediate implications for measured effect. Epidural drug delivery may allow less complicated assumptions regarding estimated biophase concentrations. For this same reason intrathecal drug administration has become very popular in animal pharmacodynamic studies. Epidural administration may offer the additional advantage over intrathecal administration of avoiding significant spread to more rostral neural centres via the cerebro-spinal fluid.
The main factors influencing the biophase concentration following epidural injection are tissue distribution across the dura (blood-brain barrier) and drug clearance from the epidural space. Permeability coefficients for a variety of drugs across the blood brain barrier (BBB) have been studied by Bernards and Hill and found to correlate in a bi-phasic fashion with a drug's octanol: buffer (7.4) co-efficient. Drugs of intermediate hydrophobicity (e.g. alfentanil, lignocaine and bupivacaine) were most permeable. Morphine on the other hand, being quite hydrophilic has a low permeability (Bernards and Hill, 1992).

The maturation of the BBB in rats has been addressed by Butt et al in a study of transendothelial electrical resistance and lanthanum permeability. In essence, important signs of maturity (high resistances, low paracellular shunt and low lanthanum permeability) were noted from just before birth and remained stable in the neonatal period (Butt et al., 1990). Altered drug effects secondary to developmental changes in BBB permeability are therefore unlikely in this study of post natal rats.

As described previously, measurement of the withdrawal reflex was made 30 minutes after epidural drug injection, in this case to allow for stabilisation of anaesthetic depth. This single shot technique while simple and inexpensive necessarily means that epidural drug concentrations do not achieve steady state. (A continuous infusion technique, though technically more demanding and requiring the use of a syringe pump driver may represent a useful advance in the technique.)

### 5.5.2 Ketamine's mechanism of action

The stereoselectivity of the action of ketamine was an early clue to its interaction with an endogenous receptor-ligand system. In an attempt to characterise the pharmacodynamic profiles of the two isomers of ketamine, White et al (1980) compared the effects of the two isomers with the racemic mixture at equianaesthetic doses in surgical patients. This study suggested that the (+) isomer was a more effective anaesthetic than either the racemate or the (-) isomer. The study also suggested that the (-) isomer resulted in a greater incidence of post-anaesthetic emergence reactions and agitation. Animal studies in mice confirm the greater potency of the (+) isomer, while studies in rats showed important qualitative differences in behavioural effects between the two isomers (White et al., 1982).

A spinal site of action was soon recognised and studied in animal models. In electrophysiological recordings of spinal dorsal horn, ketamine and phencyclidine were found to selectively block excitation by N-methyl- aspartate (NMA) in contrast to a much lesser interaction with quisqualate, kainate and inconsistent and non-selective interactions
with glycine and GABA (Anis et al., 1983). Ketamine is now known to block the ion channel of the NMDA receptor in a non-competitive and use-dependent manner through interactions at the phencyclidine (PCP) binding site. This would predict a potent analgesic effect at subanaesthetic doses (which is being increasingly recognised) but because the affinity of ketamine for the NMDA binding site is relatively low (Kd ~1 µM) in comparison to that of MK-801 (Kd of ~4 nM) other pharmacological mechanisms have been postulated and extensively investigated. These include actions such as blockade of voltage gated ion channels, effects on cholinergic, noradrenergic and serotonergic transmission as well as possible interactions at opioid receptors. The predominant sites of each of these actions is uncertain and probably dependent on dose and route of administration.

Local anaesthetic actions have been studied using whole cell patch clamp recordings of dissociated dorsal root ganglion cells. Zhou et al recently showed that ketamine blocks tetrodotoxin-sensitive (TTX-s) sodium channels with a half maximal inhibitory concentration (IC50) of 146.7 µM. This antagonism occurs in a use dependent manner and involves an alteration in the channels kinetics (activation potentials become more positive, inactivation potential more negative). Interestingly, TTX-resistant channels appear to be less sensitive than TTX-sensitive ones (IC50 866.2 µM) (Zhou and Zhao, 2000). It must be noted however, that the IC50 for blockade of TTX-s channels is two orders of magnitude greater than the Kd value quoted for NMDA receptor binding.

In high systemic dosage, Ketamine has central anaesthetic actions. These may be mediated via NMDA or monoaminergic receptors. A complex interaction between ketamine and the opioid receptor system has also been demonstrated in several experimental models. Radioligand binding studies as well as bioassays show this interaction to be stereoselective and specific to µ and κ receptors. The S(+) isomer of ketamine is two to three times more potent than R(-) isomer. Ketamine is able to displace opioid agonists from µ and κ receptors at clinically relevant concentrations and from δ receptors only at supra-clinical concentrations. The kinetics of this interaction in radioligand binding studies are consistent with a simple competitive interaction. Ketamine does not interact with the ORL1 receptor but rather appears to be a competitive antagonist at µ and κ receptors and therefore has anaesthetic and analgesic effects through non-opioid mechanisms (Hirot a et al., 1999).

Further evidence of significant interaction between ketamine and opioid receptors includes the finding that injection of ketamine into the periaqueductal grey region of the rat antagonizes morphine induced analgesia as effectively as naloxone. (Smith, 1985)
In contrast to this, naloxone was shown to antagonize the analgesic effects of ketamine in rats (Smith 1980) and in a human corollary, Stella et al were able to counteract ketamine-induced anaesthesia by injecting naloxone (6μg/kg) in adult patients (Stella 1984).

Tolerance studies in animals also suggest that some element of ketamine analgesia may be mediated through opioid receptors. Morphine tolerant animals, showing up-regulation of μ and δ receptors are cross tolerant to the analgesic effects of ketamine. (Finck, 1988)

Controversy regarding the interaction of ketamine at opioid receptors has been fueled by contradictory findings such as those of Hao et al who were unable to demonstrate any opioid receptor mediated effects in spinal preparations (Hao et al., 1998; Fratta et al., 1980).

In a human experimental setting ketamine’s action in blocking secondary hyperalgesia is not attenuated by opioid receptor blockade with naloxone. In adult volunteers following a first degree burn injury, infusions of naloxone regardless of its timing are not able to significantly reduce the analgesic effects of ketamine (Mikkelsen et al., 1999).

To some extent, the conflicting experimental results described above also share a historical division. Studies completed prior to 1985 are more likely to suggest a clear and clinically relevant interaction between ketamine and the opioid receptor system than are more recent studies. Considering the modern studies alone, allows the slightly more confident proposal that ketamine’s analgesic action is independent of the opioid system but that a simple (clinically irrelevant) competitive interaction at some opioid receptors is possible.
5.5.3 **Ketamine in the laboratory setting**

a) **Acute antinociception or anaesthesia?**

Though introduced into clinical practice as an anaesthetic agent, ketamine has increasingly been recognised to have significant analgesic properties. Attempts to model these latter effects have paralleled its use as an adjunctive agent in many analgesic regimens.

Parenterally administered ketamine is known to prolong tail flick latency in rats in a dose dependent manner at doses above 25mg/kg (Kawamata et al., 2000; Baumeister and Advokat, 1991). Similarly, the withdrawal latency to heat stimuli is prolonged by oral ketamine in rhesus monkeys (France, 1989). Other traditional nociceptive test systems confirming ketamine's effect include the acetic acid test and phenylquinone writhing tests in mice (Ryder 1978; Finck, 1988). Unfortunately, none of these studies investigate the action of ketamine in established pain states and few discuss the difference between anaesthetic and analgesic effects making interpretation of the results difficult.

Studies of the behavioural effects of ketamine on rats after enteral administration of a variety of doses show that central nervous system depression only becomes evident at a dose of 60 mg/ kg and is not observed at 30 mg/kg. Dose dependent depression of the flexor reflex has been demonstrated by Hao et al in a dose range of 1- 8mg/kg following intravenous injection (Hao et al., 1998). These results have been interpreted to mean that ketamine is an effective acute anti-nociceptive agent. Interestingly, these effects of ketamine are not shared with other more selective NMDA antagonists such as MK-801 and AP5 suggesting that the effects of ketamine in these test systems are mediated through non-NMDA mechanisms.

Spinal administration of the drug has produced conflicting results. Tung and Yaksh (1981) found ketamine to result in analgesia as judged by the tail flick test (Tung, 1981). In these experiments, ketamine was deposited spinally in doses of 23.8-714µg (100-3000nmol) directly on the spinal cord. Ahuja showed prolongation of the tail flick latency following i.t. (indwelling sub dural catheter) ketamine at doses above 800µg (Ahuja, 1983). The same model in the hands of Crisp et al also showed a significant though brief effect at doses of 238 and 714µg i.t. ketamine.

Kawamata et al (2000) on the other hand found no such effect following i.t. administration of the drug in the dose range of 10-1000µg. Joo et al similarly, found both
enantiomers of ketamine to be devoid of effects on tail flick latency (the S-racemate did
cause brief motor impairment at the highest dose tested, 300µg) (Joo et al., 2000).

In summary, the anti-nociceptive effects documented above appear to occur within a
dose range that may also result in significant central nervous system depression. Taken
together, these findings suggest that there is no clear distinction between ketamine’s “acute
antinociceptive” effects and its anaesthetic actions when tested in normal (non-
inflamed/non-hyperalgesic) animals.

Some of the above studies attempted to define the mechanism of action by either
pharmacologically or surgically blocking descending neural pathways. Papers by Crisp et
yohimbine and methysergide and by Hao et al (1998) using naloxone in intact and in
spinalized rats, each came to the conclusion that the documented effects of ketamine (in
non-inflamed/non neuropathic rats) were dependent on neuronal activity in descending
pathways, and were probably mediated by monoaminergic systems which interacted
significantly with the opioid receptor system.

b) Antihyperalgesic effects

With an increasing understanding of the distinct algesic states produced by
peripheral inflammation and nerve damage, it is now apparent that ketamine does possess a
true analgesic (i.e. anti-hyperalgesic) action that can be demonstrated at a much lower dose
range than that described above. It appears also that this effect is mediated at a spinal level
and is probably mediated by antagonism of NMDA receptors. The unique pharmacology of
the facilitated sensory states represented by inflammation and neuropathic pain states have
been investigated both electrophysiologically and behaviourally.

Early electrophysiological studies suggested that ketamine’s analgesic actions were
dependent on a spinal mechanism of action whereas its anaesthetic actions may be due to a
supraspinal disruption of somaesthetic information (Collins, 1986). In a study of dorsal
horn cell activity, Collins (1986) demonstrated a selective effect of ketamine on noxiously
evoked activity in wide dynamic range dorsal horn neurons. In contrast, it had no effect on
activity evoked by low intensity stimuli. Although this study did not specifically model a
pain state, it did call for a distinction to be made between anesthetic and analgesic actions.

The distinction was made apparent in electrophysiological experiments that studied
the effects of NMDA antagonists in various algesic states. In 1995, Ma and Woolf
characterised a hypersensitivity state induced by electrical C-fibre stimulation, mustard oil
and i.m.i. bradykinin in anaesthetised rats. Using this model, they demonstrated the ability of intrathecally applied NMDA antagonists to attenuate the enhanced motor neuron responses of hypersensitive rats. The antagonist tested (MK-801) was able to both prevent the development of the hyperalgesic state as well as reverse it if already established, at doses that did not modify baseline responses (Ma and Woolf, 1995). This increase in the sensitivity of dorsal horn cells during inflammation (induced by carrageenan) has been independently documented using AP5 (Svendsen et al., 1999).

Behavioural studies comparing morphine and MK-801 show that both drugs are able to attenuate the behaviours induced by intraplantar formalin injection. Intrathecal morphine suppressed both phases with only a small difference in ED50 (0.5 µg for phase I and 0.3 µg for phase II) while MK-801 displayed a greater differential (ED50 1.6 µg and 0.1 µg for phases I and II respectively) (Yamamoto and Yaksh, 1992).

Similarly, significant antinociceptive effects of both orally and intra-thecally applied ketamine in the formalin test have been demonstrated at doses as low as 30mg/kg (oral) and 100nM (i.t.). These doses did not appear to depress central neural or spinal motor function (Shimoyama et al., 1999).

Reductions in tail flick latency secondary to paw inflammation induced by carrageenan are also effectively inhibited by both intraperitoneal (30mg/kg) and intrathecal ketamine (100µg). Importantly, these effects appear to be independent of monoaminergic systems (Kawamata et al., 2000). Ren et al (1992) showed that intrathecal ketamine caused a similar reversal of thermal hyperalgesia following carrageenan-induced inflammation with an ED50 of 12/µg. This dose did not effect the withdrawal latency in naïve rats.

In further well controlled behavioural experiments in adult rats, Celerier et al demonstrated a clear anti-hyperalgesic action of ketamine using the paw pressure vocalization test. Investigating long-lasting (up to 5 days) hyperalgesia induced by subcutaneous injections of fentanyl (4x400µg/kg), these researchers showed that ketamine pretreatment (10mg/kg subcutis) had no analgesic effect of its own, enhanced the early analgesic effect of fentanyl and prevented the development of long lasting hyperalgesia induced by the opioid (Celerier et al., 2000).

In as much as neuropathic pain states may involve similar mechanisms as do inflammatory pain states, it is reasonable to expect ketamine to have a selective antihyperalgesic action in animal models of neuropathic pain. Qian et al demonstrated dose and time dependent effects of ketamine in doses as low as 0.01mg/kg (systemic) or 3µg
i.t. Reductions in mechanical and cold allodynia, spontaneous pain and mechanical hyperalgesia were documented in rats with spinal nerve ligations (Qian et al., 1996; Mao et al., 1993).

The studies described above have been summarised in the following table:

<table>
<thead>
<tr>
<th>Ketamine dose</th>
<th>Route</th>
<th>Animal model</th>
<th>Effect</th>
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<tr>
<td>25mg/kg</td>
<td>parenteral</td>
<td>Naïve-tail flick</td>
<td>Increased latency</td>
<td>Kawamata (2000)</td>
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<tr>
<td>60mg/kg</td>
<td>i.p.</td>
<td>Naïve-electrically evoked flexor reflex</td>
<td>CNS depression</td>
<td>Hao (1998)</td>
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<tr>
<td>30mg/kg</td>
<td>i.p.</td>
<td>“</td>
<td>No CNS effect</td>
<td>“</td>
</tr>
<tr>
<td>1-8 mg/kg</td>
<td>i.p.</td>
<td>“</td>
<td>Reflex arc depression</td>
<td>“</td>
</tr>
<tr>
<td>24-714μg</td>
<td>i.t.</td>
<td>Naïve-tail flick</td>
<td>Increased latency</td>
<td>Tung</td>
</tr>
<tr>
<td>&gt;800μg</td>
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<td>Increased latency</td>
<td>Ahuja (1983)</td>
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<tr>
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<td>Increased latency</td>
<td>Crisp (1991)</td>
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<td>No effect</td>
<td>Kawamata (2000)</td>
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<td>Joo (2000)</td>
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<td>Reversal of threshold changes</td>
<td>Kawamata</td>
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<td>“</td>
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<td>s.c.</td>
<td>Fentanyl hyperalgesia -vocalization threshold</td>
<td>Reversal of threshold changes</td>
<td>Celerier (2000)</td>
</tr>
<tr>
<td>0.01mg/kg</td>
<td>i.p.</td>
<td>Spinal nerve ligation -sensory thresholds</td>
<td>Reversal of changes</td>
<td>Qian (1996)</td>
</tr>
<tr>
<td>12μg</td>
<td>i.t.</td>
<td>Carrageenan inflammation -Thermal threshold</td>
<td>Reversal of threshold changes</td>
<td>Ren (1992)</td>
</tr>
</tbody>
</table>

In summary, the current experimental results are consistent with ketamine having a clear and specific antihyperalgesic effect mediated via NMDA receptors at a spinal level. It occurs at a dose range that is about 10 fold below that required for anaesthetic effects.
c) **Algesic effects of NMDA antagonists in naïve animals**

In the current series of experiments, an attempt was made to demonstrate anti-hyperalgesic effects of ketamine and AP5 within a dose range that was not perceptibly anaesthetic i.e. doses that did not depress the normal (non-inflamed) reflex responsiveness. The results of these control experiments are displayed in Fig 020 and clearly show that at the doses tested (greater or equal to the ED50 for antihyperalgesic effect) no depression of the withdrawal reflex was seen in non-inflamed rat pups. Moreover, a strong tendency for reflex responsiveness to be augmented by epidural ketamine and AP5 in non-inflamed rats was seen.

This finding, though unexpected is not without precedent. In 1990, Dickenson and Sullivan recording extracellular spikes from dorsal horn cells (non-inflamed rats) noted that even low doses of the NMDA antagonist AP5 caused an excitation of C-fibre evoked activity and an increase in post discharges of substantia gelatinosa cells (in contrast to the inhibitions seen in deeper neurons) (Dickenson and Sullivan, 1990). They concluded that NMDA receptors mediate subst. gelat. neuronal responses in a complex manner which may involve disinhibition of inputs to deeper neurons, positive feedback onto inhibitory interneurons or direct inhibitory influences on deeper neurons. The interpretation of their results necessarily changes in light of the current findings that not only can subst. gelat. neurons be excited by NMDA antagonists but that the excitability of the entire reflex arc is also increased by low concentrations of NMDA antagonists.

### 5.5.4 The distinction between anaesthesia, acute antinociception and anti-hyperalgesia

Ketamine is regularly described as an agent with both analgesic and anaesthetic properties without precise definition of either of these actions. Anaesthesia is broadly defined as a drug-induced reversible depression of the central nervous system resulting in the loss of response to and perception of all external stimuli (Evers, 1996)- in short, a reversible loss of consciousness. Analgesia may also be assigned a simplistic definition: the relief of pain. Clearly these definitions fail to take account of the various components that comprise both complex processes. In routine clinical practice the distinction between anaesthesia and analgesia is generally thought to be obvious and is made intuitively. An explicit distinction is rarely made in current anaesthesia texts (Schwinn et al., 1994) but
rather, some blurring of the boundaries is occasionally implied. In a recent textbook on pain, only three sensory states were said to be intuitively apparent: hyperalgesia, hyperaesthesia (or allodynia) and hypoalgesia. Hypoalgesia was said to be synonymous with analgesia and the following discussion failed to mention the anaesthetic state (Yaksh 1999). Several descriptions of anaesthetic depth suggest that analgesia is an integral part of the action of anaesthetic drugs. Notably, Guedel's original description of anaesthetic planes suggests that analgesia is the first stage of anaesthesia. (Cullen et al have since shown that none of Guedel’s major signs can be correlated with stable alveolar tensions of common volatile anaesthetic agents (Cullen et al 1972).) Similarly, psychopharmacology texts more often comment on dual (anaesthetic and analgesic) drug action than attempt to distinguish precisely how these actions differ.

Despite a lack of precision in definitions, anaesthetists have delivered both anaesthesia and analgesia—separately and in combination, safely and effectively using simple (often intuitive) conceptual frameworks, traditional practices, empirical data and cues from practice settings enlightened by increasing basic knowledge of the physiology and pharmacology of both states. In recent years anaesthetists have paid increasing attention to the provision of analgesia during surgery as a separate pharmacological concept (D. Hatch personal communication). Many gradual improvements in current practice are quite probably due to the ability of clinicians to adjust practice in response to direct patient feedback i.e. self reports of pain and of awareness under anaesthesia. The advantage of direct feedback from subjects is of course lost when dealing with paediatric patients, the developmentally delayed and experimental animals.

The distinction between anaesthesia and analgesia does however present difficulties whenever pharmacological agents appear to have both effects. The commonest examples of these agents are ketamine and nitrous oxide. The more recent use of high dose opioids as a clinical anaesthetic regimen has also raised the need for a lucid conceptual framework that allows a clear distinction to be made between anaesthesia and analgesia. The recent description of intrathecaly administered aminosteroids (alphaxalone) as analgesic agents in rats (Goodchild et al., 2000) as well as the description of analgesic effects of sucrose in human neonates is evidence of continuing confusion in both research and clinical literature (Carbajal et al., 1999). These last two examples demonstrate both the importance and difficulties in distinguishing anaesthetic and analgesic drug effects in non-communicating subjects. A conceptual framework allowing this distinction is urgently needed and critical to current debates on the use of analgesics and anaesthetics in human organ donors and foetus' being surgically aborted. It may well also impinge on the ethical debates surrounding palliative (end-of-life) care and euthanasia.
Both anaesthesia and analgesia represent complex changes in sensory states each with several components. Anaesthesia comprises loss of consciousness, amnesia, reduced muscle tone and reduced autonomic responsiveness. Loss of consciousness (loss of conscious perception) is accepted to mean a loss in the ability to respond to command. Amnesia comprises both loss of explicit memory (and therefore the lack of recall) and loss of implicit memory (sub-conscious learning). Analgesia on the other hand is the return to normal affective disposition and sensory equilibrium from an algesic or pain state.

Ironically, the discussion of these two sensory states is aided by putting aside consideration of the conscious perception involved with each, and by this manoeuvre avoiding philosophical debate about consciousness and perception. Both anaesthesia and analgesia include physiological features that can be objectively measured and modulated without specific consideration of the conscious state. In anaesthetised and non-communicating subjects, the terms nociception and antinociception then replace pain and analgesia respectively. Features measured in laboratory animals can effectively act as proxy measures of anaesthesia and analgesia. Documented correlations with self report measures in adult human volunteers are the justification of use of these proxies and were discussed in Chapter 2 (Quasha et al., 1980) (Willer JC, 1983). These features are conveniently categorized as follows:

- response to non-noxious stimuli.
- threshold for response to noxious stimulus.
- response to suprathreshold (noxious) stimulus.

The measurement of anaesthetic potency has become well established since the introduction of the concept of MAC by Eger et al (1980). MAC is defined as the alveolar partial pressure of a gas at which 50% of subjects will not respond to a surgical incision. In animals, the stimulus used is usually a tail clamp. Because MAC is determined by measuring a quantal response, it is best represented by a cumulative frequency distribution from which a concentration-response curve can only be inferred. Despite this, a typical MAC determination can quite easily be interpreted in terms of a threshold for response to noxious stimulus. Increasing anaesthetic depth would lead to an increase in this threshold. See diagram 16. Importantly, the determination of MAC for anaesthesia (as opposed to MAC for loss of righting reflex or MAC for hypertensive response) has traditionally been based on the response to noxious stimuli. This may be one of the fundamental problems in separating the actions of anaesthetic drugs from analgesic drugs.
Determination of MAC values

The determination and comparison of anaesthetic agent potencies has been greatly aided by the concept of MAC (minimum alveolar concentration). The measurement represents the alveolar gas tension required to prevent 50% of subjects responding to a standard surgical stimulus. In humans, the stimulus is a 5cm skin incision on the lower abdominal wall. Animal experiments typically involve a tail clamp protocol. The measurement is extremely reproducible, constant over a wide range of species and bears direct relation to CNS gas tensions (biophase concentrations)(Evers 1996). The cumulative frequency curves used to determine MAC values for varying end-points (e.g. loss of righting reflex, obtunding hypertensive response to laryngoscopy) appear to be parallel suggesting a stable underlying concentration-response function for volatile anaesthetic agents.

Diagram 16. Determination of MAC in human subjects

Since its introduction in 1963 by Eger et al, the concept of MAC has become firmly entrenched in anaesthetic research and practice. These graphs, taken from Saidman and Eger (1964) show the responses (move/not move) to surgical incision in three groups of patients (upper diagram). An simple non-linear regression was then carried out on the original binary data to produce the cumulative frequency curves (lower graph).
As discussed in Chapter 2- a pain or algesic state can be characterised by the appearance of a nociceptive reflex in response to a previously non-noxious stimuli (allodynia) and an augmented response to suprathreshold stimuli (hyperalgesia). Analgesia (antinociception) by definition then, is a return to normal sensory thresholds (reversal of allodynia) as well as a return to normal responses to suprathreshold stimuli (reversal of hyperalgesia). These changes in sensory equilibrium are represented in diagram 17.

This scheme implies the following:

a) Analgesic interventions do not affect sensory thresholds or responses to noxious stimuli in normal (i.e. not in a pain state) subjects. This is in keeping with the pharmacodynamic profiles of several traditional and experimental analgesic drugs e.g Non-steroidal anti-inflammatory drugs (Yamamoto and Nozaki-Taguchi, 1997) and NK1 antagonists (Malmberg and Yaksh, 1992; Yaksh 1991).

b) Interventions that attempt to reduce acute nociception (i.e reduce the response to noxious stimuli in otherwise normal subjects) must necessarily be anaesthetic agents. The ability of opiates to increase the sensory threshold in normal subjects must then be seen as an "anaesthetic action".

c) A drug-induced increase in the threshold of response to noxious stimuli can only be interpreted in light of the initial threshold value i.e. an increase from normal represents anaesthesia whereas an increase from below normal usually represents analgesia (the latter can be confirmed by demonstrating a lack of effect of the drug on the normal threshold). In this way anti-nociceptive drug effects can be distinguished from anaesthetic drug effects.

d) On the other hand, a decrease in threshold can be interpreted without such constraints as it generally signifies the development of an algesic state. This implies that a pain state and therefore anti-nociceptive drug action can be objectively studied in anaesthetised subjects -with the proviso that the depth of anaesthesia is kept constant.
Diagram 17  Anaesthesia and Anti-nociception

The above diagram attempts to use a stimulus-response graph to illustrate differences between anaesthetic and analgesic drug actions. The stimulus range depicted includes subliminal, non-noxious and noxious stimuli. The transition between non-noxious and noxious stimuli is determined by the choice of measured response. Any reflex response can be used provided it is elicited by stimulation of pain nerve endings only, is clearly perceptible, reproducible and with definite onset (Goetzl 1943). According to Wilier (1984), the threshold for eliciting the cutaneous withdrawal reflex (RIII reflex) in the human lower limb coincides with the subjectively determined pain threshold.

It remains possible to study the hyperalgesic state even in anaesthetised animals so long as the anaesthetic agent used does not possess anti-nociceptive actions. The use of an anaesthetised animal not only allows humane experimentation but also ensures that only responses from noxious stimuli are obtained.
Interpreting the results of the current experiments in light of the above considerations suggests several general conclusions.

i) A significant anti-hyperalgesic action has been demonstrated for both ketamine and AP5. This action appears to be mediated at a spinal level. For both drugs, the anti-hyperalgesic effect is notable at concentrations that are not detectably anaesthetic. This is in keeping with the clinical pharmacodynamic profile of ketamine in humans i.e. the anaesthetic effects of ketamine occur at doses an order of magnitude higher than analgesic doses (Schmid et al., 1999).

ii) Morphine too has been shown to have an antihyperalgesic effect but the EC50 for this effect is close to the concentration (0.005%) that can be considered “anaesthetic” in naive pups. This suggests that the analgesic and anaesthetic doses of this drug do not differ markedly. This is in keeping with the clinical pharmacodynamic profile of opioids in humans i.e. the sedative effects of morphine occur at doses only slightly higher than analgesic doses.
5.5.5 Mechanisms of changing sensitivity of spinal nociceptive pathways to analgesic agents

a) Sensitivity to morphine

Though the primary aim of the current experiments was to investigate spinal sensitivity to NMDA antagonists, the mu opioid receptor agonist—morphine was also studied in directly parallel experiments for the sake of comparison. The fact that the pattern of changing sensitivity differed between morphine and the NMDA antagonists argued against a serious methodological flaw in the experiments.

Epidural morphine did not show an age related change in efficacy. The apparent increase in EC50 after 10 days of age did not reach statistical significance. This pattern is unlike that described by Marsh et al (1999) who used epidural drug delivery and behavioural tests of sensory thresholds. The discrepancy between her findings and these data may reflect a difference in the definition and calculation of ED50 values (Marsh et al., 1999a).

The pattern of change in sensitivity described by Marsh might have reflected developmental patterns in the density and distribution of mu opioid receptors in the spinal cord but may equally have been due to systematic differences in the calculation of drug doses across the age groups. The neonatal spinal cord has been reported to show a greater proportion of autoradiographically identified mu receptors. These receptors are distributed widely at P1 and become more restricted to superficial lamina with increasing age (Rahman et al., 1998) The ontogeny of the opioid receptor system has been extensively discussed by Marsh (Marsh et al., 1999a; Marsh et al., 1999b)

b) Changing sensitivity to NMDA antagonists

Excitatory amino-acid (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 significant changes in the pattern of excitatory synaptic transmission. Similarly, the maturing spinal cord is characterised by developmental regulation of the expression of various receptors. In the immature brain synaptic transmission is weak and extremely plastic. A large proportion of it occurs via NMDA-type glutamatergic receptors. 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).

5.5.5.1 Changes in receptor density

The early foetal cord and hippocampus have a widespread and high density of NMDA receptors (Gonzalez et al., 1993; Baudry et al., 1981). Early studies focused on hippocampal tissue because its simple organization and its importance in learning and memory. The delayed maturation of the hippocampus makes it a valuable model also in the study of brain development. High affinity glutamate uptake studies (Kvale et al., 1983) and receptor binding studies (Baudry et al., 1981) show that both uptake and absolute binding increase until post-natal day 25.

These early studies showed a 40 fold increase in the number of sodium-independent glutamate binding sites (expressed as pmol/hippocampus) between postnatal day 4 and adulthood. (Sodium-independent binding sites give a better indication of synaptic glutamate receptors whereas sodium dependent sites include also extra-synaptic receptors). This increase in the absolute number of binding sites was most rapid during the first 10 days of life and then increases more slowly over the next 2-3 weeks (Baudry et al., 1981). When expressed as specific binding per gram of tissue or protein assayed i.e the density of binding, the developmental pattern is characterised by a peak in density at P9 followed by a progressive decline to adult densities by P23.

NMDA receptor binding studies in the spinal cord show patterns that parallel higher centres. NMDA-displaceable [3H] L-glutamate binding sites are found in abundance throughout the spinal grey matter during neonatal stages (Kalb et al., 1992). Gonzalez et al using the same technique showed a peak in binding around postnatal days 6-8 followed by a decline to adult levels by P20. Some impression of the regional distribution of binding (fmol/mg of tissue) was obtained by comparing ventral horn, dorsal horn and substantia gelatinosa tissue samples. All areas showed a progressive reduction in specific binding with age from P8, except for lamina II which maintained higher binding levels well into adult life (Gonzalez et al., 1993). These patterns are represented graphically in diagram 18a and b.
Ontogenesis of specific Glutamate binding sites

a) Specific binding (MK-801)

![Graph showing specific binding of MK-801 in different brain regions.](image)

- Hippocampus
- Cortex
- Cerebellum

b) Specific Glutamate binding in the spinal cord

![Graph showing specific binding of glutamate in different spinal cord regions.](image)

Diagram 18  The ontogenesis of NMDA receptors

a) The binding of radio-labelled MK-801 can be used as an index of the density of NMDA receptors in neural tissue. This technique has been used to compare densities in different areas of the developing rat brain (Zhong et al 1995). Densities in the hippocampus and cortex increase rapidly after birth and become especially dense in the hippocampus. The number of binding sites is much lower in the cerebellum.

b) Tritium labelled glutamate can also be used to estimate NMDA receptor density. Gonzalez et al (1993) have studied the mouse lumbar spinal cord using this technique and shown developmental changes that are regionally specific. From P12 onward, the substantia gelatinosa shows a significantly greater density of NMDA receptors than either the dorsal or ventral horns.
These studies show that the marked developmental patterns in NMDA receptor density are essentially complete by the start of the postweanling period (P25), roughly equivalent to the earliest stages of adolescence in humans (Benes, 1995).

The pattern of a gradual decline in NMDA receptor density within the spinal cord from P8-9 onwards can at best be only a partial explanation of the result obtained in the current experiments (the age related decline in sensitivity to both ketamine and AP5). The peak receptor density seen at P8-9 might of itself have predicted a peak sensitivity for the P10 rat pups studied.

5.5.5.2 Developing receptor structure/composition

NMDA receptors are heteromeric ion channels composed of NR1 and NR2 subunits. While the NR1 subunit is thought to be an essential component of the receptor, varying functional properties of the receptor are conferred by subtypes (A-D) of the NR2 subunit. (Ishii 1993) Consistent with this, expression of the NR1 subunit is ubiquitous in the adult brain while the NR2 subunits display a restricted, region specific pattern of distribution.

Riva et al were able to correlate the time course of glutamate binding with the mRNA expression of several of the receptor subunits. Low levels of mRNA for the NR1 subtype were detected in newborn rats with a progressive increase over the following 2-3 weeks. Developmental profiles for mRNA expression of other subtypes showed varying onset and peak times but in general appeared to reach adult levels by P21 (Riva et al., 1994). Specific profiles in different brain regions are discussed below.

a) higher centres:

The study of regional variations in the expression of NR proteins initially relied heavily on data obtained from Western blotting (Wang et al., 1995) and immunoprecipitation experiments (Portera-Cailliau et al., 1996). These experiments had only limited resolution and have made way for studies such as those of Wenzel et al using a more sensitive histo-blot technique. These techniques have been supplemented with in situ hybridization studies of the relevant mRNAs and have shown consistent results (Wenzel et al., 1997). The increasing sophistication and improved resolution of histological studies of NMDA receptors can be traced from early work by Sheng et al in 1994 who used co-immunoprecipitation with the first subunit specific antibodies available. These studies demonstrated for the first time that the native NMDA receptor population includes a
proportion that are hetero-oligomers containing at least three distinct subunits (NR1/NR2A/NR2B) (Sheng et al., 1994).

Neural development is characterised by a progressive increase in the heterogeneity of NMDA receptors due to the comparatively late appearance of the NR2A and NR2C subunits. Some area-specific rearrangement of NR2B subunits also contributes to the developmental patterns seen (Wenzel et al., 1997).

From as early as the 14th embryonic day all rat neurons express the NR1 gene (Monyer et al 1994). These studies by Monyer et al showed that the neonatal forebrain predominantly contains receptors formed with the NR2B subunit. With development these are gradually replaced or supplemented with NR2A-containing receptors (Sheng et al., 1994).

Immunoreactivity for the NR2A subunit is initially faint and restricted to the hippocampus, cerebral cortex and striatum. Over the first three post natal weeks expression of the NR2A subunit becomes abundant throughout the brain. NMDA-mediated transmission at birth is therefore performed by receptors of the NR1/NR2B composition with a small component performed by receptors containing the NR2D subunit. Shortly after birth there is a rapid increase in the expression of the NR2A subunit in virtually all areas of the rat brain, the NR1/NR2A receptor becomes the predominant receptor subtype in the adult brain (Wenzel et al., 1997). These results are paralleled by consistent findings from mRNA expression studies (Riva et al., 1994). These studies confirm that NR2B subunits mRNA expression is already high in embryonic life and continues to rise rapidly from the time of birth especially in the hippocampus. In the newborn cortical expression levels were already 50-60% of adult levels. On the other hand mRNA expression of NR2A and C subunits rise after about P8 (Zhong et al., 1995)(NR2C- most notably in the cerebellum). These results also suggest that the NR2B subunit will be predominant in determining NMDA receptor properties in the early weeks of postnatal life.

To summarise, the developmental pattern in rodent brain is characterised by an embryonic expression of the NR2B and NR2D subunit while the NR2A and NR2C subunits appear shortly before birth and become more widely expressed postnatally. The most conspicuous post natal changes are the disappearance of the NR2D subunit around P12, the marked increase in NR2C subunits in cerebellum and the replacement of NR2C with NR2A subunits in the forebrain. These patterns are represented graphically in diagram 19a.
Ontogenesis of NMDA receptor subunits

Diagram 19  The ontogenesis of NMDA receptor subunits

a) Brain: Using an RNase protection assay in which protected fragments are separated on polyacrylamide gels and quantified by autoradiography, Riva et al (1994) found low levels of NR-1 mRNA expression in newborn rats with a progressive increase in expression over the next 2-3 weeks. In this diagram, the developmental pattern of expression of the NR-1, NR-2A and NR-2B subunits is plotted as a percentage of values obtained in adult rats. The data for the NR-2D subunit has been obtained from immunoprecipitation studies of rat brain membranes using an affinity-purified antibody (Dunah 1996). It must be noted that absolute mRNA expression levels (moles/µg total mRNA) for the NR2C and NR2D subunits are very low in cortical tissue (Zhong 1995).

b) Spinal cord: Studying the mouse cervical cord using in situ hybridization with specific oligonucleotide probes for subunit mRNA, Watanabe et al (1994) provided semi-quantitative data on the post-natal pattern of NMDA receptor composition. Dunah et al (1996) provided quantitative data for NR2D subunit protein expression in rat spinal cord. The NR1 subunit is ubiquitous at all stages of development, while the NR2B mRNA is widely expressed in the spinal grey from E13 through neonatal stages and then becomes restricted to lamina II by P21. The NR2C subunit is not expressed at any stage and the NR2D is expressed in embryonic and early post-natal life and decreases to background levels by P21.
b) hindbrain:

Early studies suggested that the NR2A subunit expression levels in the hindbrain are low throughout development and that the NR2B subunit is only transiently expressed here (Portera-Cailliau et al., 1996). Transient expression of the NR2D subunit does occur within this region (ventrobasal thalamus, hippocampus, inferior colliculus and brainstem reticular formation) making it and the NR2B subunit the predominant subunits of embryonic life. In the adult, the NR2D subunit shows restricted distribution within this region notably in the globus pallidus, thalamus and superior colliculus (Wenzel et al., 1996). Several studies have confirmed that the NR2D subunit is highly expressed in the above mentioned hindbrain areas and also in the spinal cord. It is highly expressed in embryonic life and after a peak in the first postnatal week, it declines several fold to adult levels (Dunah et al., 1996).

c) spinal cord:

A more detailed description of channels subunit composition in the spinal cord is provided by Watanabe et al although this study was carried out in mice. In situ hybridization with subunit specific probes reveals that in mouse cervical cord the NR1 subunit is ubiquitous across all laminae from embryonic day 13 to postnatal day 56 (adulthood). In contrast, the NR2A subunit was expressed only in the most ventral portion of the cord in embryonic life and came to extend dorsally through all laminae except lamina II during postnatal life. In a reciprocal pattern, NR2B subunits were widely expressed in the embryonic grey and became gradually more restricted to lamina II by P21. The NR2C subunit was not significantly expressed in the mouse spinal cord at any developmental stage while the NR2D subunit was widely expressed in embryonic life and during early post-natal life and decreased to background levels by P21 (Watanabe et al., 1994). These patterns are represented graphically in diagram 19b.

d) subunit switching during development

The ability of receptor systems to alter their properties and function during development by switching subunit composition has been shown for GABA receptor systems (Killisch, 1991), AMPA systems (Monyer, 1991) and nicotinic acetylcholine receptor systems (Sandrick, 1995). The mechanism by which this switching process is accomplished is being investigated. Implicated in the process are a family of widely expressed growth and differentiation factors termed neuregulins. These compounds (a.k.a. ARIA, NDF, heregulin, GGF) are thought to be secreted by neurons, accumulate at synaptic clefts and are able to stimulate
transcription of specific receptors. They mediate their action via ErbB receptors which are tyrosine kinases related to the EGF (epidermal growth factor) receptor. A study of the influence of neuregulin β on the expression of the NR2C subunit in mouse cerebellum by Ozaki et al provided strong evidence for the involvement of this differentiation factor in the ontogenesis of NMDA receptor systems and also underlined the importance of convergent signals in the regulatory process (neuregulin-evoked NR2C expression was selectively blocked by NMDA antagonists). The neuregulin/ErbB system has been proposed as a general neuronal mechanism in receptor system maturation (Ozaki et al., 1997).

Of itself, the pattern of changing NMDA receptor subunits within the spinal cord may partly explain the declining sensitivity to NMDA antagonists seen in the current experiments. The neonatal preponderance of NR2B subunits (with their long excitatory post-synaptic currents, possibly greater ability to modulate synaptic efficacy and therefore greater involvement in the production of hypersensitivity state) may also contribute to the greater neonatal sensitivity to ketamine and AP5 seen. Arguing against this is the fact that within lamina II of the dorsal horn, the changing pattern of subunits, as note above is not marked (i.e. the preponderance of NR2 subunits persists into adult life).

5.5.5.3 Changes in NMDA receptor kinetics

The differences in subunit composition of immature receptors outlined above, results in enhanced affinity for ligand and a different channel open time. Functionally, this results in immature receptors having longer NMDA mediated synaptic currents (Monyer et al., 1994) (Flint et al., 1997).

Demonstrating changes in NMDA receptor-mediated synaptic responses with age is problematic because of the voltage-dependence of the channel. Despite this, significant age related changes in decay times have been documented in rat superior collicular neurons aged between 10 days and 33 days (Hestrin, 1992). Excitatory post synaptic currents show a progressive shortening in the duration with increasing age (Carmignato and Vicini, 1992).

It is tempting to postulate that these long excitatory postsynaptic potentials would allow a greater degree of synchronization of presynaptic inputs in the immature brain, thereby increasing the potential for modification of synaptic efficacy (Wenzel et al., 1996). With increasing maturity, the shortening e.p.s.c.s may impose a more precise temporal coupling between pre-and post-synaptic activity for NMDA receptor activation.
The voltage dependent properties of the channel on the other hand, do not show marked age related changes over this same time period. The studies by Hestrin (1992) (voltage clamping in both slice preparations and excised patches), suggested that the changes in the time course of NMDA e.p.s.ps that occur during development probably reflect changes in receptor structure (rather than changes in voltage sensitivity, transmitter release or transmitter clearance). Fitting exponential functions to recorded e.p.s.cs provided evidence of an intermediate phase in development during which a mixed population of channels is responsible for excitatory currents.

Although Hestrin showed the voltage-dependent Mg block to be stable with age in the superior colliculus, hippocampal NMDA receptors may show age related changes, becoming more sensitive to Mg block with increasing age(Ben-Ari, 1988; Kleckner 1991; Kato, 1993). Certainly, the strength of Mg blockade depends on the particular subunit composition (Monyer et al., 1992) and the lower sensitivity of NMDA receptors to Mg block in early postnatal life may be attributable to the low level of NR2A subunit expression.

Similarly, the NR1/NR2D combination- recognised as a predominant embryonic receptor has been shown to have remarkably long decay times and weak Mg block relative to other NMDA receptor subunits. Its’ expression within the embryonic spinal cord suggests it will have a large bearing on the pharmacodynamic profile of axially delivered NMDA antagonists in early development (Dunah et al., 1996).

The slow gating properties of the NMDA receptor have been systematically studied by Monyer et al using recombinant channels composed of various subunits. The characteristics of all four possible hetero-oligomers were studied in whole cell transfected HEK 293 cells. Calcium permeabilities were not significantly different between the four channel configurations but clear differences in the strength of the Mg block are evident. The Mg block in NR1-NR2A and NR1-NR2B channels displayed a greater voltage sensitivity than did the block in NR1-NR2C and NR1-NR2D channels (Monyer et al., 1994).

Current decay following glutamate activation also showed significant differences between channel configurations. Measured time constants for current offset were shortest for the NR1-2A configuration (118ms), longer for the NR1-2B and NR1-2C channels (400 and 382 ms respectively) and extremely prolonged for the NR1-2D configuration (4.8 s) (Monyer et al., 1994). Some of these patterns are displayed in Diagram 20.
The NMDA receptor is characterised by slow gating. Following synaptic activation, the current rises relatively slowly to a peak and then decays to the baseline with a time constant that is dependent on the subunit composition. Here, the individual currents induced through four different heteromeric combinations are displayed. Current rise times are all similar (20-80% rise times between 12 and 14 ms) but offset time constants vary considerably. That of the NR1-NR2A channel (approximately 118 ms) is three to four times faster than that of the NR1-NR2B or NR1-NR2C channels (each between 380 and 400 ms). The NR1-NR2D channel is strikingly different again, with an offset decay time constant several orders of magnitude greater (approx. 4.8 s). (Monyer et al 1992)

The steady state I-V relationships shown alongside, demonstrate a difference in the strength of the voltage-dependent block by extracellular Mg between the channels. At physiological concentrations of 1mM Mg, the NR1-NR2A and NR1-NR2B channels are characterised by a stronger voltage sensitivity than are the NR1-NR2C and NR1-NR2D channels. For the former two channels, the induced inward current in the presence of Mg finds a maximum at a membrane potential of -25mV whereas for the latter two channels, this maximum is shifted leftward to -45mV. Therefore, the block by magnesium of the Na and Ca ionic movements occurs over a larger range of physiological membrane potentials for the NR1-NR2B and NR1-NR2B channels. (Monyer et al 1994)
Importantly, these studies are based on recombinant channels which are typically hetero-oligomers composed of two subunit types. Native receptors may be composed of more than two subunit types which may lead to greater functional diversity. Also, these recombinant studies do not differentiate between splice variants of the constant NR1 subunit which may further add to diversity. Studies of native receptors generally yield consistent results and overall it appears that the NR2A subunit plays a dominant role in determining the receptor e.p.s.c duration. Further, even low levels of expression of the subunit mRNA in individual neurons are sufficient to alter electrophysiological properties (Flint et al., 1997).

In a developmental study of native NMDA receptors, Carmignato were able to show age related changes in the kinetic profiles of e.p.s.c.s recorded in visual cortical neurons of rats in the early post natal period. Currents elicited from younger rat neurons were of longer duration (larger decay time constant) than those of adult neurons. Current amplitudes and rise times though did not change significantly with age. Interestingly, by comparing results obtained from neurons in dark reared rats, the researchers showed that the developmentally regulated changes in NMDA receptor kinetics could be modified by neonatal experience (dark rearing led to a delayed maturation pattern) (Carmignato and Vicini, 1992).

These kinetic changes suggest that the immature NMDA receptor is “permissive” of calcium influx. Hence only moderate activity is able to induce intracellular changes which may then modulate synaptic efficiency. At later stages of development, a higher activity threshold must be reached in order to effect the same changes. With progressive brain maturation, synchronization of presynaptic inputs is more finely tuned as a consequence of altered subunit composition and shorter e.p.s.p.s.

In a developmental study of native NMDA receptor kinetics in the rat dorsal horn, Bardoni et al confirmed that NMDA receptors contribute to synaptic transmission at many glutamatergic synapses in lamina I and II by birth and that the voltage dependance of maturing native NMDA receptors remains relatively stable. Excitatory post-synaptic potentials had rise times between 10 and 16 ms and decay times between 267 and 357 ms. While interesting differences between pure NMDA synapses and mixed (AMPA and NMDA) synapses were seen, age related changes in receptor kinetics were not significant (Bardoni 1998).

In the spinal cord, the persistence of the NR2B subunit in lamina II (substantia gelatinosa) as well as the presence of (though gradually declining) NR2D subunit may imply that these receptor subunits may play a relatively larger role in nociceptive

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processing. They may also confer a greater degree of plasticity on this sensory modality than is available for other modalities. The changing pattern of subunit composition may also impose a developmental pattern to the pharmacology of NMDA antagonists.

5.5.5.4 Changes in NMDA receptor pharmacologic profile

Parallels between developmental changes in receptor expression and pharmacologic profile have been provided by Williams et al (1993). These researchers showed that during postnatal development, native NMDA receptors (Williams et al., 1993) increasingly express low-affinity ifenprodil binding sites. Ifenprodil- a novel antagonist was shown to displace MK-801 only with a high affinity component in the newborn rat brain. From P7 onward a second (low affinity) binding site became apparent. With a similar developmental time scale native NMDA receptors become less sensitive to glycine (Kleckner, 1991; Hestrin, 1992; Kato et al., 1991).

A developmental change in affinity of the NMDA receptor for ketamine and AP5 is an attractive postulate for an explanation of the current experimental results (declining sensitivity to ketamine and AP5 with age). The change in affinity, as suggested above might be the result of a change in subunit composition. In a study of recombinant NMDA receptors Yamakura et al addressed this issue by comparing drug sensitivities of four different receptor heteromers. Mouse equivalents of the NR1-2A, NR1-2B, NR1-2C and NR1-2D receptor composites were expressed in Xenopus oocytes and the effect of ketamine, PCP, SKF-10047 (also an NMDA antagonist) and MK-801 were measured on evoked whole cell currents. PCP, ketamine and SKF-10047 each blocked the four channel types to similar extents. (MK-801 did show some differential efficacy that may explain its' differential binding in different parts of the brain and lack of psychotomimetic effects in humans.) (Yamakura et al., 1993).

Developmental changes in subunit composition of itself then cannot underlie the developmental changes in drug sensitivity seen in the current experiments.

5.5.5.5 Activity dependence of the glutamatergic systems

While good evidence for the activity dependent-maturation of receptor characteristics exists, it is uncertain if the expression patterns of various NMDA receptor subunits is subject to epigenetic modification or are controlled solely by age-dependent genetic factors (Quinlan et al., 1999).
Maturation of these receptors tends to occur in an activity dependent fashion (Quinlan et al., 1999). The time frame of this activity dependence has been studied in several in vitro models. Surface expression of AMPA receptors is known to change with neuronal activity in cells grown in culture (Lissin, 1998). Turrigiano et al. documented changes in AMPA induced e.p.s.p. amplitude following varying conditioning processes (TTX or CNQX blockade, bicuculline pre-treatment) for time periods between 15 and 48 hrs (Turrigiano et al., 1998).

Postsynaptic NMDA receptor subunit expression and clustering in response to presynaptic activity and particular innervation sources have also been studied (Gottmann et al., 1997; Ozaki et al., 1997). These experiments suggest adaptation of glutamatergic receptors to specific innervation and neuronal activity over a period of days. A more rapid (in the order of 2-4 hours) regulation of NMDA receptors in response to neuronal activity has been demonstrated electrophysiologically in visual cortical slices (Quinlan et al., 1999).

5.5.5.6 Silent glutamatergic synapses

Glutamatergic synapses are able to express both AMPA and NMDA type receptors on the post synaptic membrane and the ratio of the two may vary according to anatomical location and developmental stage. Initial work on hippocampal pyramidal cells suggested the existence of synapses that expressed NMDA receptors exclusively making these synapses quiescent at resting membrane potentials. The finding that a typical LTP (long term potentiation) protocol can convert these synapses to functional synapses in electrophysiological experiments threw new light on the molecular nature of LTP and the mechanism that underlies NMDA-mediated synaptic plasticity (Isaac et al., 1995). Changes in synaptic strength, as observed in both LTP and LTD (long term potentiation and depression respectively) can arise from the modulation of the number of AMPA-type receptors at the surface of the synapse (Malenka 1999; Scannevin 2000).

At least in the hippocampus, glutamatergic transmission is initially purely via NMDA receptors without a significant contribution from AMPA receptors. During the first post-natal week, the percentage of synapses that are purely NMDA-type decreases. These immature synapses (both functionally and morphologically different to adult ones) can still be found up to three weeks post-natally (Durand et al., 1996).

Further evidence of developmental patterns in silent synapses and a possible functional role was obtained by Wu et al. studying the tadpole optic tectum. This structure contains neurons at different developmental stages placed along a rostrocaudal axis. In this model, individual pre-synaptic cells make a small number of synapses onto each post
synaptic cell with purely NMDA receptors. With increasing maturity, these synapses acquire functional AMPA receptors in a mechanism that may involve post-synaptic CAMKinase II activity (Wu et al., 1996).

The existence of silent NMDA receptors within the mammalian spinal cord has also been documented and a developmental pattern established. Studying cells of the superficial lumbar spinal cord in rats, Li and Zhou estimated the percentage of pure NMDA synapse to be almost 90% in P0-2 rat pups, falling to less than 20% after P10. Although the techniques used did not allow the specific study of synapses from high threshold afferents, no anatomical separation of silent and functional synapses was seen and recorded cells were said to receive high threshold inputs also (Li and Zhuo, 1998).

A less striking though similar age related pattern was seen by Bardoni et al in a more considered developmental study. In her study, differences in kinetics between pure NMDA synapses and mixed synapses were also documented. Currents evoked by activity at pure NMDA receptors had slower rise times and faster decay t values than e.p.s.c.s at mixed synapses. From these values, she proposed that pure NMDA synapses are more likely to include receptors with a NR1-NR2A composition while the NR1-NR2B configuration may be more prevalent at mixed synapses (Bardoni 1998).

c) Mechanisms of changing drug sensitivity- final comments

Though this thesis did not set out to define a mechanism for developmental regulation of spinal cord sensitivity to NMDA antagonists, the discussion above provides several possible explanations for the principle findings of the experiments i.e. a greater sensitivity to NMDA antagonists at earlier developmental stages. The discussion has focussed on the the various maturational changes within NMDA receptor function, many of which may be an adequate basis for an age-related decrease in sensitivity to antagonists. In short, a greater density and distribution of these receptors and a more permissive role in synaptic transmission (determined by subunit composition) in early development as well as an age-related change in the number of silent synapses and possible age-related changes in drug affinity may each contribute to altered drug sensitivity of the spinal cord in vivo.

While changes in NMDA receptor function appears to be a prime explanation for changing drug sensitivity, an alternative explanation must also be considered. If in fact,
there were no net change in NMDA receptor function during development, the age-related changes in sensitivity to ketamine and AP5 may yet still have arisen if the nature of the hyperalgesic state (on which the drugs were tested) had changed with age. If in early development, facilitation of the withdrawal reflex by inflammation is predominantly due to NMDA receptor activity and that with age, other novel mechanisms (e.g. peptidergic transmission (King 2000) or brain-derived neurotrophic factor effects (Thompson 1999)) matured and were also able to contribute to spinal cord sensitisation, then this may result in an apparent reduction in sensitivity to NMDA antagonists with age.

It is clear then that no sure explanation of the changing drug sensitivity seen in these experiments is possible until more is known about the maturation and developmental regulation of the entire process of inflammation-induced sensitisation.
CHAPTER 6 CONCLUDING REMARKS

a) The model

The preparation described in this thesis appears to provide a useful tool for neonatal pain research. The measurement of general reflex responsiveness under various experimental conditions such as inflammation, epidural drug administration, varying inhaled gas mixtures at varying developmental stages makes the model particularly flexible.

An attempt to find a relationship between reflex threshold, latency and reflex responsiveness and clinical end points (anaesthesia, analgesia and development) has been made. In this experimental model, reflex thresholds are correlated with anaesthetic depth while reflex latency is a function of the maturity and integrity of the synaptic pathways involved. Reflex responsiveness as described in earlier chapters, quantifies the reflex response to suprathreshold stimuli and as such appears to be an index of the hyperalgesic (tonic pain) state. Of the three parameters mentioned, it was thought to be the most appropriate for pharmacodynamic studies.

The model has attempted to address experimental issues such as inadvertent sensitization and the maturational changes in anaesthetic requirements (MAC). Neither of these potential problems has prevented the collection of dose-response data for a series of analgesic/anaesthetic drugs. Further, the model has allowed developmental changes in pharmacodynamic effect of this same series of drugs to be documented.

b) Sensory states: a need for clear definitions

The original question of this thesis and the model used to answer it both required the use of strict definitions of anaesthesia and analgesia. These were set out in Chapter 3 and adhered to in the discussion of carrageenan-induced inflammation and analgesic drug actions in chapters 4 and 5. These definitions of anaesthesia and analgesia provided an adequate conceptual framework for the questions being asked though this success is likely to be due to the focus on spinal cord sensory processing which can be examined without detailed consideration of perceptual correlates.

Anaesthesia, analgesia, inflammatory and neuropathic pain conditions each represent distinct sensory states - two pharmacological and two pathological. The pathological states have been subject to much description and continuous re-definition as a result of both basic and clinical research. The pharmacologic sensory states on the other
hand, still rely on traditional definitions and have not undergone the same degree of scrutiny (Evers, 1996). Recent clinical literature (notably regarding the mechanism of action of nitrous oxide (Maze and Fujinaga, 2000) and the analgesic actions of sucrose in neonates (Carbajal 2000) as well as media coverage of issues such as anaesthesia for organ donation, foetal surgery and foetal abortion have highlighted the need for lucid definitions of pharmacologically induced sensory states.

Defining anaesthesia and anaesthesia raises the issue of definitions of other sensory states. The pharmacological states of “neurolepsis” and “dissociative anaesthesia” also suffer from empiric and often outmoded definitions and will certainly benefit from a better understanding of the neurophysiological mechanisms underlying them (Bissonnette et al., 1999). The recent suggestion that tachykinin antagonists may act to dissociate the affective component of pain perception from the purely sensory component should provide the impetus for re-consideration and possible re-definition of these pharmacologic sensory states (Hunt, 2000; Hill, 2000).

In parallel, and providing precedence, clearer definitions of the altered sensory states of drug dependence /addiction (Bohn et al., 2000; Robbins and Everitt, 1999) and schizophrenia (Farber et al., 1995) have each resulted from better understanding of their pathophysiological mechanisms.

c) The resurgence of interest in ketamine

Thirty years since its introduction into clinical practice, ketamine remains one of the few NMDA antagonists still in use. Its place as an anaesthetic agent has become limited since the advent of the “balanced anaesthesia” concept and as major advances in the delivery of inhaled agents and post-operative intensive care have occurred. Its place as an agent for brief procedural interventions is also being questioned as better intravenous agents are now available.

The more recent appreciation of the role of the NMDA receptor in the development of inflammatory pain states has led to a resurgence of clinical interest in the drug (Schmid and Katz 1999; Akers 2000). A role for low-dose ketamine in post operative pain management is being increasingly recognised particularly when used as an adjunct to local anaesthetics, opioids or other analgesic agents. This use of the drug co-incides with a change in general attitudes toward pain management. Instead of relying on the sequential addition of increasingly powerful analgesic agents (i.e. the WHO analgesic ladder), clinicians now accept that analgesia is often better provided through multimodal techniques. The implied poly-pharmacy may result in improved analgesia and a reduced incidence of adverse effects. These improvements in the principles of pain management are not
automatically and immediately apparent in paediatric practice for the many reasons outlined in Chapter 1 (1.2 Pain in infancy-a special case). Advances in paediatric pain management may therefore depend more heavily on evidence gained from animal models.

The clinical implication of the current results is that the analgesic dose range of ketamine is dependent on maturational state. The lower ED50 values described for neonatal rat pups compared to older pups suggests that the perinatal period and perhaps early infancy are characterised by an increased sensitivity to the anti-hyperalgesic effects of ketamine and other NMDA antagonists. The preparation of clinical dosing schedules for paediatric patients must therefore take account of this sensitivity. Increased sensitivity and age-related changes in sensitivity may manifest as marked inter-individual variability in drug effect. This unfortunately makes the use of ketamine in infants more difficult, compounding the prevalent clinical skepticism regarding the adverse effects of the drug.

The poor side-effect profile (hypertension, bronchorhoea, psychotomimetic actions and a concern regarding neurotoxicity) of ketamine remains problematic. The use of the drug in low systemic doses or the spinal/epidural delivery of the drug may provide a means of avoiding significant adverse effects. The latter route raises concerns regarding possible neurotoxicity. While further systematic study of this issue in animal models is required, a review of the literature to date suggests that the current clinical use of ketamine in the epidural space does not represent a neurotoxic risk to spinal neurons (de Lima 2000).

While renewed interest in ketamine is unlikely to radically change current paediatric practice, investigation and discussion of the drug in the paediatric setting may help focus attention on newer concepts in the neurobiology of pain and the unique issues of developmental biology.

d) Paediatric pain research

Despite the significant constraints on research directed at paediatric pain outlined in Chapter 1, the effort to alleviate infant pain and distress is vigorous. To some extent, the continued effort may not require justification to be found in evidence-based studies of clinical outcome or economic advantage. Nor does it require a dogmatic assumption about pain perception in infancy or an evaluation of real or potential suffering imposed by accidental or iatrogenic injury.

Motivation to gain an understanding of the neurobiology of paediatric pain and then apply it so as to reduce pain- however that pain is defined, measured and valued, can be found in a simple acceptance of and need to maintain human dignity (McGrath and Unruh, 2000). This dignity imposes a responsibility of compassionate care toward children and is an integral part of being a member of a moral and social community.
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A1.1 Hindlimb musculature in the rat

The muscles of the hindlimb in the rat can be divided into 7 broad groups:

a) The sublumbar muscles- that fix the vertebral column and draw the limb forward. These muscles include iliacus and psoas mm as well as quadratus lumborum.

b) The rump muscles- these gluteal muscles act on the hip joint to either flex or extend it.

c) The muscles of the thigh- form a caudal group (m. biceps femoris, mm. semimembranosus and semitendinosus) and a medial group (mm. gracilis, pectineus and adductores). These muscles generally act to adduct the limb.

d) The inner pelvic muscles- these muscles (mm. obturatorius, gemelli and quadratus femoris) act to externally rotate the limb.

e) Quadriceps femoris group of muscles- these muscles extend the stifle joint and draw the limb forward.

f) The muscles of the tarsal joint - forming flexor (tibialis and peroneus mm) and extensor (gastrocnemius and soleus mm) groups.

g) Digital muscles- both long and short.

A1.2 Hindlimb innervation in the rat

The somatic nerve supply to the hindlimb is provided via the plexus lumbosacralis which carries fibres from the third lumbar (LIII) to first sacral (SI) segments. Three smaller nerves (nn. iliohypogastricus, inguinalis and genitofemoralis) arise from upper lumbar nerves and supply the perineum. Parts of lumbar nerves III, IV and occasionally V form the N. femoralis which gives motor supply to the iliacus, pectineus and quadriceps group. It continues as the N. saphenus to give sensory supply to the medial aspect of the leg and foot.

The medial muscles of the thigh, the obturator exturnus and quadratus femoris muscles are supplied by N. obturatorius which is derived from LIV through LVI. It also sends sensory branches to the medial aspect of the thigh.
The N. Ichiadicus is similarly derived from lumbar nerves IV through VI and before exiting the pelvis sends muscular branches to gluteal, gemelli, piriformis and obturator internus muscles. It crosses the incisura ischiadicus to enter the thigh and splits into its two major branches- the N. fibularis (peroneal) and N. tibialis in the proximal third of the thigh.

N. fibularis gives muscular branches to the flexors of the tarsal joint and long extensors of the digits before dividing into deep and superficial branches. The superficial branch emerges between ext. digitorum and the peroneus mm, enters the dorsal aspect of the foot and divides into terminal digital branches. The deep branch may also contribute to digital innervation.

N. tibialis runs between the two heads of the gastrocnemius muscle and via branches supplies the extensors of the of the tarsal joint and the long digital flexors. Close to the tarsal joint it divides into medial and lateral plantar nerves that then enter the foot. The Medial plantar nerve runs across the medial malleolus and sends digital branches from the medial side of the first digit through to the third interdigital space. The Lateral plantar nerve supplies the fourth interdigital space and the lateral aspect of the fifth digit.

A1.3 Cutaneous sensory receptors in the rat foot

Adult cutaneous mechanoreceptor fibres consist of four different classes, each distinguished on the basis of their thresholds, conduction velocities and response properties. These are all myelinated fibres and include the faster conducting (i.e. Aβ fibres) slowly-adapting and rapidly-adapting mechanoreceptors (SAMs and RAMs respectively) and the slower conducting (i.e. Aδ fibres) D-hair afferents and A-fibre mechanonociceptors (AM). SAMs innervate Merkel cells.

During early development a fifth category of mechano sensitive fibre is evident. Between 14 and 20 days postnatally in mice- some less differentiated fibres with intermediate response characteristics can be found are labelled RA/SA fibres.
The relative percentage of each fibre type is as follows:

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Prevalence of myelinated afferent fibre type in hairy skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adults</td>
</tr>
<tr>
<td>Aβ fibres</td>
<td></td>
</tr>
<tr>
<td>RAM</td>
<td>52%</td>
</tr>
<tr>
<td>SAM</td>
<td>48%</td>
</tr>
<tr>
<td>RA/SA</td>
<td>22%</td>
</tr>
<tr>
<td>Aδ fibres</td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>65%</td>
</tr>
<tr>
<td>D-hair</td>
<td>35%</td>
</tr>
</tbody>
</table>

from Carroll et al 1998

These various mechanoreceptors vary not only in their response and basic physiological properties but also in their dependence on neurotrophic factors. The NT-3/trk-C receptor-ligand system appears to be crucial for postnatal survival of proprioceptive neurons (Klein 1994). SAM type cutaneous mechanoreceptive fibres also show a similar dependence on NT-3 for survival (Airaksinen Neuron 1996) and a striking dependence on BDNF for normal mechanotransduction. Neurons lacking BDNF do not die but rather show a specific reduction in mechanical sensitivity (Carroll 1998). On the other hand, unmyelinated axons- a vast majority of which comprise nociceptors are dependent on the NGF/trk-A system for survival (Smeyne 1994).
Cutaneous afferents in the rat have been systematically studied by Leem et al and differences between the plantar and sural nerves described. While receptive field properties appear to be similar to other mammalian species, differences between the two nerves studied suggest functional adaptation of receptor types.

In short:
The sural Aβ fibre population comprised

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>% of fibres</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-hair cell</td>
<td>41</td>
</tr>
<tr>
<td>Field</td>
<td>11</td>
</tr>
<tr>
<td>Rapidly adapting (RAM)</td>
<td>6</td>
</tr>
<tr>
<td>Slowly adapting (SAM I) mechanoreceptors</td>
<td>7</td>
</tr>
<tr>
<td>Slowly adapting SAM II) mechanoreceptors</td>
<td>35</td>
</tr>
</tbody>
</table>

The plantar Aβ fibre population comprised

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>% of fibres</th>
</tr>
</thead>
<tbody>
<tr>
<td>G- hair cell</td>
<td>3</td>
</tr>
<tr>
<td>RAM</td>
<td>35</td>
</tr>
<tr>
<td>SAM I</td>
<td>30</td>
</tr>
<tr>
<td>SAM II</td>
<td>24</td>
</tr>
<tr>
<td>Pacinian corpuscle mechanoreceptors</td>
<td>8</td>
</tr>
</tbody>
</table>

The sural Aδ fibre population comprised

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>% of fibres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nociceptors</td>
<td>68</td>
</tr>
<tr>
<td>D hair</td>
<td>27</td>
</tr>
<tr>
<td>cold</td>
<td>5</td>
</tr>
</tbody>
</table>

The plantar Aδ fibre population was comprised entirely of nociceptors, a majority of which (73%) were mechanical nociceptors.
APPENDIX 2

Neural toxicity of ketamine and other NMDA antagonists

We would like to commend Schmid et al. (1999) on a timely, comprehensive, and generally balanced review of the use and efficacy of low dose ketamine in acute post-operative pain. The recent interest in the clinical use of this drug is evident in the increasing number of publications concerning its use in clinical anesthesia journals.

We do feel however that the blanket recommendation that 'ketamine should not be injected intraspinaly in humans' is an unwarranted conclusion not supported by current data. By 'intraspinal injection' the authors mean both intrathecal and epidural administration.

In a short discussion of intrathecal use, the authors suggest that ketamine may induce spinal toxicity. This statement has important clinical implications and demands clarification. Four issues are pertinent to a discussion of the neural toxicity of a locally applied NMDA antagonist. First, direct chemical cytotoxicity unrelated to specific receptor binding; second, specific NMDA antagonist neurotoxicity (sometimes termed NMDA receptor hypofunction (NRH)-induced neurotoxicity); third, NMDA antagonist-induced apoptosis and finally, the dose response relationship between permanent cell damage and either intrathecal or epidural drug administration.

Several early studies using both sub-human primates (Brock-Utne et al., 1982) and rabbits (Malinovsky et al., 1993; Borgbjerg et al., 1994) all confirmed the safety of intrathecally administered ketamine. These studies demonstrated the neurotoxicity of chlorobutanol (a preservative) but concluded that intrathecal preservative-free ketamine (including repeated injections) in concentrations up to 1% is devoid of neurotoxic effects. The study of Amiot et al. (1986) in a small number of rats, using a high concentration and dose of preservative-containing ketamine and a flawed experimental technique does not constitute evidence of a significant neurotoxic effect.

The second issue concerns specific NMDA antagonist neurotoxicity. Treatment of adult rats with either competitive (D-AP5) or non-competitive NMDA antagonists (phencyclidine, MK-801, tiletamine, ketamine) consistently leads to pathological changes in neurons of the cingulate and retrosplenial cortices. These changes are found to be quite selective and specific for these cortical neurons (Olney et al., 1989).

Further work in rats by Nehls et al. (1988), Allen and Iversen (1990) and Fix et al. (1993) principally on the NMDA antagonist MK-801 but also involving ketamine, led to the term 'NMDA-receptor hypofunction (NRH)-induced neurotoxicity' (Benes, 1995). In all these studies, the neurotoxicity of NMDA antagonists appears as a specific lesion in the above mentioned corticolimbic areas of the brain in rats and as yet is not found in all species. The data available for ketamine suggests that systemic doses of ketamine above 40 mg/kg are required before even reversible vascular changes are seen (Olney et al., 1989). Extrapolation from data on MK-801 would also support this contention, keeping in mind the five fold lower potency of ketamine compared to MK-801. These high ketamine doses are very unlikely to ever be warranted or achieved in clinical practice.

Ironically, the neurotoxic effects outlined above became apparent during the investigation of the neuroprotective effects of NMDA antagonists. The relative ability of NMDA antagonists to either protect neurons or damage them is best demonstrated by the recent work of Jevtovic-Todorovic et al. (1998) in their investigation of the mechanism of action of nitrous oxide. From their findings it is reasonable to conclude that neurotoxic doses of NMDA antagonists in rats are an order of magnitude higher than neuroprotective doses.

The third issue concerns the relatively recent finding that the rate of programmed cell death (apoptosis) that occurs during normal development may be accelerated by NMDA antagonists (Ikonomidou et al., 1999). Though these findings prompted the researchers to suggest implications for human foetus' exposed to NMDA antagonists through maternal drug abuse and human infants exposed through clinical anesthesia, the evidence remains incomplete. The studies, yet to be repeated in other laboratories and other experimental paradigms, do not control for the effects of the severe physiological disturbance that usually accompanies the dosing schedule outlined. In fact the same researchers had earlier reported the same pattern and striking age dependency of neurodegeneration in response to hypoxic-ischaeemic insults in rat pups (Ikonomidou et al., 1989). Unfortunately, the researchers do not offer an explanation for the failure of NMDA antagonists to induce neurodegeneration in pre-natally exposed rat pups bringing into question their final conclusion. Most importantly, the dosing schedule used was exceptionally high and bears little relation to any clinical analgesic practice (Ikonomidou et al., 1999).

The final issue concerns the relationship between the minimum neurotoxic and minimum effective analgesic dose of ketamine. This is made more complicated by the fact that neurotoxicity data typically refers to intrathecal drug application whereas epidural drug administration remains the focus of much analgesic practice.

Several animal studies of the antinociceptive effects of ketamine confirm that intrathecal doses ranging from 25 to 100 μg in adult rats have significant antihyperalgesic actions (Crisp et al., 1991; Mao et al., 1993). In comparison, the spinal neurotoxicity studies of ketamine quoted above (using a 1% solution) almost certainly represent a neuronal exposure to ketamine at least 6-fold greater than that required for analgesic efficacy.

Thirteen of the 22 studies not included in Schmid et al.'s review
described the use of epidural ketamine for post-operative analgesia. These studies typically involved the epidural use of ketamine solutions ranging in strength from 0.01 to 0.05% (Sandler et al., 1998). Taking into account the concentration gradient that must exist across the spinal meninges (Bernards and Hill, 1992), current clinical use of ketamine in the epidural space would not seem to represent a neurotoxic risk to spinal neurons.

While it would be prudent then to continue to advise caution when administering ketamine intrathecally or epidurally, and to recommend strongly against the inclusion of any preservative/anti-oxidant in such injections, the continued study of low doses of ketamine (in low concentrations) in epidural injections and infusions in both animals and humans should be encouraged and not proscribed.

References


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Reply to de Lima et al.

We welcome the opportunity to respond to Drs de Lima, Beggs and Howard and we thank them for their thorough review of the toxicity of epidural and intrathecal ketamine. Their letter contains information that we did not cover in our review article. Our decision to not recommend the use of spinal ketamine and to call for further research into the issue of spinal toxicity was arrived at after careful consideration. The target audience for the review article was the practicing anaesthesiologist. In our opinion, the risk of the toxic effects of spinal ketamine, however small, warranted a conservative recommendation especially since ketamine has not been approved anywhere in the world for administration by this route. We do not dispute the data and arguments presented by Dr de Lima; we simply have a different threshold for accepting risk. Each anaesthesiologist must decide what is best for his or her patient. The information provided by de Lima et al. will be useful in helping clinicians arrive at an informed decision.

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APPENDIX 3

Nitrous oxide analgesia - a ‘sting in the tail’

The recent editorial entitled ‘Recent advances in understanding the actions and toxicity of nitrous oxide’ (Maze & Fujinaga, Anaesthesia 2000; 55: 311–14) reviews the evidence that nitrous oxide acts on several sites and argues strongly for separate anatomical and molecular mechanisms of action for the analgesic and anaesthetic effects of the drug. Assuming that anaesthetic and analgesic actions of nitrous oxide may be easily modelled and studied separately, Maze et al assign an opioid/noradrenergic mechanism as the basis of the drug’s analgesic actions while relegating nitrous oxide’s NMDA-inhibiting activity as a mechanism for the euphoric effects of the gas. While there is good evidence for the involvement of an opioid/noradrenergic mechanism in nitrous oxide’s analgesic action, it is premature to suggest that this is the exclusive basis of its analgesic effect. Here we wish to point out the importance of NMDA receptor activity in pain and analgesic mechanisms and to highlight the potential for nitrous oxide to interact at a spinal level on NMDA receptor mechanisms.

Perhaps a key to current understanding of pain mechanisms is the distinction between acute nociception and hypersensitivity. The NMDA receptor plays a limited role in acute nociception [1] but is critically involved in postinjury hypersensitivity. The data collected by Fitzgerald and Kolvenburg [8] clearly show significant changes in the function of the descending dopaminergic tracts during early development, their work did not identify specific transmitter systems nor can it be taken to preclude the existence of other functional descending tracts. Furthermore, Maze’s prediction relies on precise developmental correlations between rats and humans that current data cannot provide. Quite apart from the issue of NMDA antagonist activity then, these latter two points would make any dismissal of nitrous oxide’s efficacy in paediatric practice premature.

Future research will no doubt be aided by experimental design that takes into account the distinction between acute nociception and hypersensitivity pain states. The distinction is not purely academic as clinicians are increasingly making decisions based on the ability to alter each independently [9]. Management of procedural pain requires acute antinociceptive intervention while that for postoperative, chronic and neuropathic pain requires antihyperalgesic agents [10].

Nitrous oxide may not be an ideal analgesic agent but having served for over 150 years, a reappraisal of its mechanism of action must take thoughtful account of the evolving understanding of the neurobiology of nociception.

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References


