# Cutaneous afferent evoked activity in the postnatal rat spinal cord

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# Abstract

The postnatal development of afferent evoked activity in single extracellularly recorded dorsal horn cells, was examined in urethane anaesthetised rats aged 3, 6, 10 and 21 days old. Responses to single and repeated stimuli to primary afferent fibres, and "natural stimulation" were investigated. Cells responded to natural stimulation from the first age tested, but the convergence of inputs increased with age.

A fibre strength evoked long lasting, variable, responses in the youngest animals, particularly in superficial cells. Mean latencies decreased with age, from 33.1±2.78ms at P3 to 7.3±0.3 ms at P21. No long latency spike responses were evoked in response to C fibre stimulation in pups aged P3 (postnatal day 3) or P6 while by P10, 35% of cells had a C fibre response. The size of the peripheral receptive field decreased with age. At P3 the mean receptive field occupied 50% of the plantar hindpaw, this decreased to 15% by P21

Repeated stimulation of cutaneous A fibres at 0.5 Hz at twice threshold produced sensitisation in a population of dorsal horn cells in the neonate. The direct A fibre evoked activity did not increase but the background activity increased during repetitive stimulation leading to a prolonged afterdischarge beyond the stimulation period. At P6, 33% cells were sensitised displaying a mean afterdischarge of 70.6±18 s. At P10, only 6% were sensitised with a mean afterdischarge of 63 s and by P21, it was no longer observed.

Injection of carageenan (an inflammatory agent) into the hindpaw of P10 rats caused an increase in background activity which peaked at under 100mins, less than in the adult. Peripheral receptive fields were larger and stimulation thresholds lower, as is the case in adults.

These results demonstrate that dorsal horn activity undergoes considerable postnatal development, this will affect sensory processing in the newborn.

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# List of abbreviations

| | |
|---|---|
| 5HT | serotonin |
| ADP | activity dependent plasticity |
| AMPA | α-amino-3-hydroxy-5-methylisoxazole-4-propionate |
| AP5 | 2-amino-5-phosphonovaleric acid |
| BSTC | brain stem trigeminal complex |
| $Ca^{2+}$ | calcium |
| CFA | complete freund's adjuvent |
| CGRP | calcitonin gene related product |
| CNS | central nervous system |
| DLF | dorsolateral funiculus |
| DREZ | dorsal root entry zone |
| DRG | dorsal root ganglia |
| E | embryonic day |
| EAA | excitatory amino acid |
| EPP | endplate potential |
| EPSC | excitatory post-synaptic current |
| EPSP | excitatory post-synaptic potential |
| FRAP | fluoride resistant acid phosphotase |
| GABA | γ-amino-butyric acid |
| GAP-43 | growth associated protein-43 |
| GluR1-4 | AMPA receptor subunits |
| HRP | horse radish peroxidase |
| HTMR | high threshold mechanoreceptors |
| LTP | long term potentiation |
| $Mg^{2+}$ | magnesium |
| NA | noradrenaline |
| $Na^{+}$ | sodium |
| NGF | nerve growth factor |
| $NK_1$ | neurokinin 1 receptor |
| NKA | neurokinin A |
| NMDA | N-methyl-D-aspartate |
| NR1/NR1 | NMDA receptor subunits (NR2 has A,B,C&D forms) |
| NRM | nucleus raphe magnus |
| P | postnatal day |
| PAG | periaqueductal grey |
| PGP | protein gene product |
| RT97 | a pan neuronal marker |
| S1 | primary somatosensory cortex |
| SG | substantia gelatinosa |
| SP | substance P |
| TTX | tetrodotoxin |
| VRP | ventral root potential |
| WDR | wide dynamic range |

# Chapter 1:

## General Introduction

### *1.1 Aim of the thesis.*

The aims of this study are to examine the postnatal changes in the cutaneous afferent evoked activity in the dorsal horn of the rat spinal cord, in both the natural state (naive) and after a peripheral inflammatory insult. While there have been a considerable number of studies in this area in adults, there is very little information on how these processes function in the newborn and how they mature in postnatal life.

Only one previous study has examined the postnatal development of the cutaneous primary afferent input to the spinal cord dorsal horn (Fitzgerald, 1985a).

The aim here was to directly record from single dorsal horn neurones that are part of the functional sensory pathways to provide a better understanding of connectivity in developing central sensory pathways. These anatomical and behavioural studies indicate an enhanced input role for the low threshold A afferents, when comparing neonates to adults. The focus of many previous behavioural studies has been on the postnatal development of spinal reflexes (Andrews and Fitzgerald, 1994; Angulo Y Gonzalez, 1932; Ekholm, 1967; Fitzgerald and Gibson, 1984; Fitzgerald et al., 1988; Lewin et al., 1993) and as such concentrate on the motor as well as sensory connections. Here we examine the cutaneous sensory pathways directly. There are *in vitro* studies that have predominantly examined the pharmacology of the young postnatal spinal cord; the results from these experiments are often reported without

fully taking into account the postnatal developmental changes that other studies have highlighted.

In the first part of this study the acute afferent input from the hindlimb (millisecond (ms) to second range of events) to lumbar dorsal horn cells under normal physiological conditions, is examined. A second, related aim, was to look at the central effects of peripheral inflammation in the neonatal dorsal horn. In adults inflammation has been used extensively as a model for persistent pain, and it has been shown that sensory processing alters demonstrated by spontaneous pain and exaggerated responses to noxious and innocuous stimulation (hyperalgesia and allodynia). Neonatal rats also have a strong behavioural, flexor withdrawal response to formalin from birth, with an augmented response in the first postnatal week (Guy and Abbott, 1992). The second part of this study therefore examines afferent input to dorsal horn cells in the presence of an altered sensory background due to inflammation of the hindpaw.

## *1.2 Introduction to the spinal cord.*

Since somatosensory processing in the adult spinal cord has been thoroughly reviewed (Wall and Melzack, 1994; Willis and Coggeshall, 1991), only key relevant points will be highlighted in this introduction. This section contains a brief description of the peripheral and central anatomy and physiology of the mature somatosensory system entering the spinal cord. The next section will focus on the developmental changes that relate to the studies in this thesis.

### 1.2.1 Peripheral receptors

Central recognition of a stimulus, at the spinal cord level, depends on the type and intensity of stimulus. The low threshold mechanoreceptors and hair follicle afferents initiate action potentials that travel in the large diameter thickly myelinated Aβ fibres, (see Willis & Coggeshall, 1991). The peripheral receptors that are innervated by Aβ fibres include the slowly adapting Merkel and Ruffini cells and the rapidly adapting hair follicle or skin receptors as well Meissner's and Pacinian corpuscles, Aβ fibres also appear to innervate high threshold mechano receptors (HTMR) (Koerber et al., 1988).

Most high threshold mechanoreceptors receptors are innervated by the thinly myelinated Aδ fibres, and can become sensitised to heat stimuli. The delta hair follicle afferents are also innervated by Aδ fibres. The Aδ peripheral terminals invade the epidermis, but remain ensheathed in Schwann cells so are not considered to be free ending whereas, those of the fine, unmyelinated C fibres, have genuine free endings (Kruger et al., 1981). These polymodal C fibres respond to noxious mechanical, chemical and thermal stimuli, and are also referred to as nociceptive afferents, i.e. conveying information from nociceptors, which are defined as sensory endings that respond to stimuli that threaten or damage tissue (Sherrington, 1906).

These are the afferent fibre types carrying somatosensory information from the periphery to the spinal cord. Collectively these nerves are referred to as primary afferent fibres and can be found in the mixed peripheral nerves together with the motor nerves.

## 1.22 Dorsal Root Ganglia (DRG)

The dorsal root ganglia house the cell bodies of these afferent fibres. The main function of the dorsal root ganglia is to relay the environmental stimuli and transmit the resulting information centrally. The cells in the DRG are classified initially into small and large cells. There is a correlation between the type of information carried and the cell body size, with large cell bodies giving rise to large, myelinated axons (Aβ) and the small cell bodies giving rise to small diameter unmyelinated axons (C fibres) and possibly to the small myelinated Aδ fibres (Willis and Coggeshall, 1991). The small cells in the DRG and the unmyelinated axons are both lost in mature rats after systemic treatment with capsaicin at birth (Fitzgerald, 1983). The large cells are born earlier at E12-14 than the small cells, E13-15 (Lawson and Biscoe, 1979).

There is no absolute correlation between conduction velocity and axon size (Cameron et al., 1986; Willis and Coggeshall, 1991). It has been reported that other physiological parameters of the DRG show a better correlation with the type of information transmitted than the conduction velocity does There is a characteristic spike duration for each receptor type examined. When examining somata, which receive peripheral input from one class of peripheral fibre, there is a differentiation in the spike duration depending on the receptor supplied. Somata whose axons that supply low threshold cutaneous receptors show spikes with a shorter duration than those that innervate high threshold cutaneous receptors. The width of the spike is positively correlated to receptor type in a given primary afferent fibre type (Koerber et al., 1988).

All neurones in the DRG show broad action potentials in the newborn, only some of which maintain these into adulthood (Fulton, 1987). This has led to a theory that high threshold mechano receptors have an arrested development, despite myelination, and are possibly in an immature state (Koerber et al., 1988).

## 1.23 Central Projections

The main functions of the primary afferents are to convey modality of the peripheral sensation to the spinal cord and to be able to discriminate the position of the stimulus, on the periphery. These are coded for by the spatial organisation of the primary afferent central terminals in the dorsal horn (Woolf, 1987). The two aspects of the input coded for by location in the dorsal horn, are:

i) modality of stimulus, coded for by lamina (i.e. dorso-ventral position in the cord).

ii) site of peripheral receptive field, coded for by mediolateral and rostrocaudal position (also called somatotopy).

The primary afferent fibres have their cell bodies in the dorsal root ganglia (DRG) and their central projections travel in the dorsal root to the spinal cord. Many dorsal root fibres bifurcate on entering the cord giving off rostral and caudal branches, these in turn give off collaterals which penetrate the grey matter, along its entire border with the white matter to terminate in different laminae, depending on the fibre type. C fibres generally travel in the most lateral part of the white matter (including Lissauer's tract) and the A fibres travel more medially. The dorsal roots give off most collaterals in the segment in which they enter the cord, although the rostro caudal spread is up to six segments in the cat L3-S2 segments(Wall and Werman, 1976). The number of collaterals per axon to achieve this rostro caudal spread is 8-12 (Brown et al., 1981; Woolf, 1987). The rostrocaudal extent of the C fibre projection is less in the C afferents than in the A fibres, only 2 segments in the kitten and several segments in the rat (Chung et al., 1979; Szentagothai, 1964).

*LAMINA TERMINATION PATTERNS:*

There are some central projections that travel in the ventral root, most of these fibres are unmyelinated. Many are blind ending, but some do enter the ventral horn, and these do not appear to form synapses and if dorsal root transmission is blocked, only minimal effects can be seen through the ventral roots (Coggeshall et al., 1974). The newborn kitten has relatively few unmyelinated fibres in the ventral roots, but by the age of seven months there are the same percentage of unmyelinated fibres (of the total fibres in the ventral root) as there are in the adult (Risling et al., 1981). The collaterals of primary afferent neurones terminate in distinct regions of the dorsal horn

The spinal cord grey matter is a heterogeneous mix of cells, that have been subdivided into regions that have distinct morphological and physiological characteristics (Rexed, 1952; Willis and Coggeshall, 1991). The majority of spinal cord neurones are interneurones (Chung et al., 1984).

The large diameter myelinated afferents enter the grey matter of the spinal cord either through the medial part of substantia gelatinosa (SG) or from the medial part of the grey matter, the collateral axons having curved around the dorsal horn grey matter (Fig 1.1). Cajal (1909) first described the course of these collaterals using Golgi stains, in the spinal cords of newborn kittens and dogs (Ramon y Cajal, 1909). According to Cajal these fibres reach lamina IV, then turn back on themselves to penetrate laminae III and lamina II inner (IIi) from below, forming flame shaped arborisations, first described as such, by Scheibel & Scheibel, 1968. This has been confirmed in later studies (Light and Perl, 1979a; Woolf, 1987). However, studies on physiologically identified, HRP (Horse Radish Peroxidase) filled A fibres in the cat and rat show that these terminate almost entirely in lamina III (Brown et al., 1981; Shortland et al., 1989). This result suggests that the more superficial terminations of A cells found in earlier studies were as a result of using younger animals. More recent studies have shown that in young rats, the A fibre collaterals do indeed terminate in lamina II and I (Fitzgerald et al., 1994; Mirnics and Koerber, 1995).

Electrophysiological studies have confirmed that in the adult the large A fibre afferents have terminals predominantly in laminae III to V. Cells in these laminae respond with short latency to light mechanical stimulation (Brown et al., 1973; Wall, 1967). The terminals are arranged in sheets arranged along the longitudinal (rostro-caudal) axis of the dorsal horn, compressed medio-laterally. There is dense branching except at the extreme rostral and caudal ends of the central terminal field where the ends are simple with no apparent synaptic boutons (Beal, 1979; Scheibel and Scheibel, 1968; Woolf, 1987). A population of cells in the medial parts of laminae III-IV, respond monosynaptically to Aβ fibre afferents (Armett et al., 1961)

Aδ fibres physiologically identified and filled with HRP have been examined and found to enter the spinal cord and sometimes, but not always, bifurcate, travelling rostrally just medial to Lissauer's tract (Light and Perl, 1979b). Both high threshold mechanoreceptors (HTMR) and low threshold Down hairs (D hairs) were examined in two species; the cat and monkey. The central terminations of the HTMRs in the two species were essentially identical, terminating in the marginal zone of lamina I and lamina V on the ipsilateral side, with some terminals in the midline just dorsal to the central canal and a few terminals in laminae I & V on the contralateral side. The D hair afferent central terminals were found in lamina III and IIi, arranged in longitudinal columns. HTMRs respond to increasing intensities of noxious mechanical stimulation with incrementally graded activity. The neurones in lamina I are activated by volleys in cutaneous Aδ fibres and sometime C fibres (Christensen and Perl, 1970; Fitzgerald and Wall, 1980; Light et al., 1979; Woolf and Fitzgerald, 1983), there are some reports of activation of these cells by Aβ fibres (Christensen and Perl, 1970; Woolf and Fitzgerald, 1983). The D hair afferents have slower conduction velocities and are at least as responsive as the other hair follicle afferents (Aβ) (Light and Perl, 1979b).

Nociceptive unmyelinated C fibres penetrate the dorsal horn from the medial Lissauer's tract and terminate exclusively in the superficial dorsal horn grey, as

shown by degeneration studies (Ralston and Ralston, 1979) and HRP labelling of lateral rootlets, which largely contain C fibres (Light and Perl, 1979a). Data from electrophysiological studies confirm that C fibres terminate in the superficial dorsal horn. There are some cells in laminae I and II that respond exclusively to C fibre input (Fitzgerald and Woolf, 1981; Kumazawa and Perl, 1978). Furthermore C fibres can only be antidromically activated by stimulating their terminals in SG (Fitzgerald and Woolf, 1981). Further evidence can be gained from the neurochemical anatomy of the dorsal horn. Substance P (SP), which is known to be localised in the C fibre terminals is concentrated in laminae I and II (Hökfelt et al., 1975) and these areas are depleted following axotomy (Barber et al., 1979). Somatostatin and FRAP (fluoride resistant acid phosphotase) is also concentrated in laminae I and II (Hökfelt et al., 1976; Nagy and Hunt, 1982), and the FRAP band disappears following root section (Knyihar et al., 1974). These SP containing terminals disappear following neonatal capsaicin treatment (Nagy et al., 1981).

The collaterals terminate on SG interneurones that are longitudinally orientated with respect to the cord. These do not generally project outside of SG (Gobel, 1978), although some ventral axonal projections take some information to the deeper layers of the dorsal horn (Gobel et al., 1980; Light and Kavookjian, 1988). Neurones in laminae III & IV with dorsally projecting dendrites can also receive input from the afferents terminals in lamina II (Wall, 1965).

Cells in the SG are primarily activated by C fibre input, although they can be excited by A fibre (Aδ & Aβ) input too (Bennett et al., 1979; Bennett et al., 1980; Fitzgerald and Woolf, 1981; Kumazawa and Perl, 1977; Wall et al., 1979). Since A fibres do not appear to project directly to laminae II, and dendrites of lamina II neurones do not leave SG, this A fibre activation of SG neurones is probably dependent on a polysynaptic pathway (Willis and Coggeshall, 1991). Neurones in lamina $II_o$ are more likely to receive inputs from C fibre nociceptors and thermoreceptors, whilst those in $II_i$ are likely to receive inputs from mechanoreceptors. (Willis and

Coggeshall, 1991). *In vitro* electrophysiological studies suggest that there is a significant monosynaptic input from the Aδ afferents in the SG (Yoshimura and Jessell, 1990). These investigators report that about 70% of cells in the SG receive direct monosynaptic input from Aδ afferents, whilst they find that only 25% of SG cells receive direct monosynaptic inputs from C afferents, in the slice preparation.

The other group of afferents, with terminations in the spinal cord are the Aα (Ia, Ib) afferents. These also give rostral and caudal branches on entering the cord and give off a number of collaterals (5-11) which penetrate the deep dorsal horn grey (Ishizuka et al., 1979). Most collaterals terminate in the aforementioned intra axonal HRP tracing study, make monosynaptic contact with the motorneurones in lamina IX, although some terminate in laminae VI & VII, making contact with the small and medium cells here.

*SOMATOTOPY:*

Afferent fibres grow into specific laminae of the dorsal horn. C fibres terminate in lamina II, Aδ in laminae I and V and Aβ in Laminae III and IV in the adult. Each forms a characteristic map of the skin that it represents, and these horizontal maps lie above or below one another in the dorsal horn, (Shortland et al., 1989; Swett and Woolf, 1985; Wall, 1967; Woolf and Fitzgerald, 1986). The somatotopic organisation of the lumbar dorsal horn was first described by Wall, who reported that the toes and distal structures were represented medially in the dorsal horn, whilst the lateral aspect of the leg and more proximal structures were coded for by cells in the lateral dorsal horn (Wall, 1967).

These somatotopic maps are conserved throughout the somatosensory system, at spinal, brainstem, thalamic and cortical levels (Kandel et al., 1991).

The maps are present in neonatal rats from birth (Fitzgerald and Swett, 1983; Smith, 1983; Wilson and Snow, 1988).

Bryan and colleagues suggested that the somatotopic organisation of the dorsal horn reflects the embryological development of the innervation of the limbs (Bryan et al., 1973; Bryan et al., 1974). The embryonic cord initially has a simple somatotopic arrangement in which dorsal horn cells lying medially receive afferents from peripheral receptive fields in the dorsal part of the corresponding dermatome, whilst those cells in the lateral dorsal horn receive projections from afferents with receptive fields in the ventral part of the dermatome. This situation is maintained in the adult thoracic cord (Smith, 1983). In the lumbar and cervical enlargements, the simple dermatomal pattern is distorted, as the when the limb bud grows out it rotates resulting in the more complex dermatomal and neurotomal patterns (Willis and Coggeshall, 1991).

*Dorsal horn cell activation by afferent inputs*

The activity of populations of neurones in the dorsal horn can be visualised in a number of ways. Field potentials measured from the surface of the cord (cord dorsum potentials) or from within the cord have been recorded in a number of species, including man (Willis and Coggeshall, 1991). The negative waves caused by the incoming primary afferent signals can be measured, and have been used to locate the areas in the dorsal horn where the collaterals from a particular afferent fibre type terminate, this is worked out by stimulating the nerve at intensities sufficient to excite the type of fibre of interest.

A more recent technique for examining the activity of populations of neurones in the dorsal horn is the technique for staining the c-fos expression (Hunt et al., 1987). C-fos is an immediate early gene which is thought to act as a transcription factor at the AP-1 site found on a variety of genes, including those of nerve growth factor (NGF) and dynorphin (Morgan and Curran, 1989). C-fos is selectively expressed in

response to noxious stimulation in the adult spinal cord and it is largely confined to the superficial laminae of the dorsal horn, although it is also found in deeper laminae. Furthermore, the expression can be reduced in a dose dependent manner following the pre-emptive morphine treatment. (Bullitt, 1989; Fitzgerald, 1990; Menetrey et al., 1989; Tölle et al., 1990).

Dorsal horn cells can be classified by determining whether they are excited by just myelinated cutaneous afferents or by both myelinated and unmyelinated (Gregor and Zimmermann, 1972). Mendell (1966) introduced a scheme that looks at the intensity of the stimuli that activate the neurones. Cells that are excited by innocuous stimuli only are termed *narrow dynamic range*, those that receive inputs from both A & C fibres are called *wide dynamic range* (WDR). Mendell & Wall (1965) and Mendell (1966) report that 60% of neurones in the nucleus proprius (lamina V) could be activated by both A and C fibres, the remaining 40% could only be activated by A fibre inputs. By giving stimuli of graded strength neurones with convergent inputs from all somatosensory afferent types were demonstrated (Wagman and Price, 1969).

Repetitive stimulation of C fibres at frequencies of 0.3Hz or greater facilitate the discharge rates of many dorsal horn neurones. This phenomenon has been termed "wind-up" (Fitzgerald and Wall, 1980; Mendell and Wall, 1965; Mendell, 1966; Wagman and Price, 1969; Woolf and King, 1987). This type of central sensitisation is thought to be behind some of the longer term physiological and molecular changes in the spinal cord following sustained noxious stimulation, such as central hyperalgesia and c-fos expression (Dray et al., 1994), this is discussed in more detail in Ch 4.

## 1.24 Spinal cord pharmacology

The fast synaptic transmitters in the SG are the excitatory amino acids (EAAs), and the "synaptic transmission inhibitors" glycine and GABA ($\gamma$-amino-butyric acid). In the presence of antagonists to all of these there is very little, or no evoked current remaining, and miniature synaptic events are absent, demonstrating that these are the major fast excitatory transmitters present in the SG (in a slice preparation) (Grudt and Henderson, 1998).

*Excitatory amino acids*

The two classes of excitatory neurotransmitters involved in the transmission of nociceptive information are the EAAs and the neurokinins. The fast excitatory post synaptic potentials (EPSPs) at synapses between Aδ and afferents and cells in the superficial dorsal horn are probably mediated by L-glutamate (Yoshimura and Jessell, 1990). It has been previously shown, in the newborn rat spinal cord, that Ia fibres release an L-glutamate type transmitter (Jahr and Yoshioka, 1986). Furthermore, synaptic transmission between DRG and dorsal horn neurones in culture can be blocked by EAA antagonists (Jahr and Jessell, 1985). The transfer of nociceptive information from the periphery is thought to be mediated by the action of glutamate on dorsal horn neurones, antagonists of L-glutamate reliably reduce pain behaviours (Hunter and Singh, 1994; Meller et al., 1993). Glutamate has also been localised immunohistochemically in the small cells in DRG, sometimes co-localised with substance P (De Biasi and Rustioni, 1988), and in the terminals of the superficial dorsal horn (Miller et al., 1988). After electrical stimulation of a peripheral nerve, glutamate is released in the rat dorsal horn (Hopkin and Neal, 1971), in a $Ca^{++}$ dependent way (Potashner and Tran, 1984). Investigators examining the spinal dialysate after noxious stimulation have shown large increases in the release of glutamate and aspartate (Skilling et al., 1988) and SP (Smullin et al., 1990). Schneider & Perl have shown that the majority of neurones that are excited by glutamate and

aspartate in an *in vitro* preparation, are located in the superficial laminae of the dorsal horn and are predominantly excited by the C fibre component of the dorsal root volley (suggesting nociceptive input) (Schneider and Perl, 1985; Schneider and Perl, 1988).

There are three main EAA receptor subtypes, *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, (named after the synthetic agonists that act on them). The non-NMDA receptors are selectively activated by AMPA ($\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate (Kleckner and Dingledine, 1989; Verdoorn and Dingledine, 1988). There is also metabotropic glutamate receptors (which involves coupling through a G-protein). The NMDA and non-NMDA receptors are ligand operated ion channels. AMPA and kainate admit $Na^+$ and $K^+$ and the NMDA operated channels admit $Ca^{++}$, and appears to blocked at resting potentials by $Mg^{++}$ (MacDermott et al., 1986). At physiological concentrations of $Mg^{++}$ and membrane potential of -70mV, neurones are not very responsive to NMDA. This voltage dependent block can be overcome by prior depolarisation by another agent, eg. AMPA. This channel will then allow $Ca^{++}$ into the cell, so that the increase in excitability, increase in firing, exceeds that produced by other receptors (Dickenson and Sullivan, 1990). Comparisons of the effects of NMDA and non-NMDA receptor antagonists on the A$\delta$ and C fibre evoked EPSPs suggest that the initial component is mediated by the non-NMDA receptors (Yoshimura and Jessell, 1990). The NMDA receptors contribute to the later phase of the EPSP, as the duration of the EPSP is decreased by the NMDA antagonist AP5 (DL-2-amino-5-phosphonovaleric acid) (Forsythe and Westbrook, 1988; Yoshimura and Jessell, 1990).

Following brief mechanical or thermal noxious stimuli, C fibre terminals release glutamate which acts via AMPA receptors to produce short term excitations (Yoshimura and Jessell, 1990. The activation of the NMDA channel has been shown to be implicated in the phenomena of wind-up and long term potentiation (LTP).

NMDA antagonists have been shown to reduce wind-up (Davies & Lodge., 1987; Dickenson and Sullivan, 1987; Woolf and Thompson, 1991), and NMDA receptor activation is an absolute requirement of LTP (Collingridge and Singer, 1990)

*Neurokinins:*

The undecapeptide, substance P (SP) is a member of a family of related peptides called either tachykinins or neurokinins. Of the three classes of neurokinin receptors currently classified, SP has been shown to be the preferred transmitter of the neurokinin-1 receptor ($NK_1$) (Otsuka and Yoshioka, 1993). Immuno studies have shown that SP and CGRP coexist in capsaicin sensitive primary afferents (De Biasi and Rustioni, 1988; Wiesenfeld Hallin et al., 1984), and that they occur with somatostatin and bombesin in dorsal roots of unmyelinated dorsal afferents (McNeill et al., 1989). SP and CGRP have also been shown to coexist with galanin and dynorphin in varicosities in the superficial dorsal horn (Tuchscherer and Seybold, 1989). The release of neurokinin A, as with SP, is increased following peripheral inflammation (Duggan et al., 1988)

Of the neuropeptides found in primary sensory neurones, SP and CGRP are the only ones of which there are substantial levels synthesised and stored under normal conditions. Neuropeptide Y, galanin and vasoactive intestinal peptide, are synthesised and stored in very low levels under normal conditions and require a stimulus for synthesis upregulation (Hökfelt et al., 1997). Calcitonin gene related peptide (CGRP), is a peptide released by primary afferents in response to noxious peripheral stimulation, and has the effect of exciting dorsal horn neurones. It has been shown that SP and CGRP share the same breakdown enzyme, which may lead to the situation where SP can diffuse into large areas of the dorsal horn in the presence of CGRP, and escape breakdown as the enzyme is occupied with CGRP

SP has been found to co-exist with glutamate in the afferent fibres that respond to noxious stimulation (De Biasi and Rustioni, 1988), but the contribution of SP to the

processing of noxious information is unclear. SP is not necessary for the experience of pain, and is considered to be a neuromodulator that alters the excitability of the afferents that respond to noxious stimulation, in the dorsal horn (Rusin et al., 1992). SP appears to be required in different conditions to those that evoke the release of glutamate exclusively. Higher frequencies of primary afferent stimulation are required to evoke the release of SP compared to glutamate (Duggan et al., 1995; Marvizon et al., 1997). It has also been demonstrated in dorsal horn neurones that internalisation of the $NK_1$ receptor (a marker of SP release) occurs in the normal animal only when the stimulus intensities are well above the threshold for pain (Abbadie et al., 1997; Mantyh et al., 1997).

SP is synthesised by small diameter nociceptive sensory fibres (McCarthy and Lawson, 1989) and is released into the dorsal horn following intense peripheral stimulation (Duggan et al., 1988) promoting central hyperexcitability and increased sensitivity to pain (Laird et al., 1993; Ma and Woolf, 1995; Neumann et al., 1996; Xu et al., 1992).

Small diameter neurones appear to be split into two distinct neurochemical phenotypes, those that stain for neuropeptides and those that contain FRAP or thiamine monophosphatase (Hunt and Rossi, 1985). These phenotypes also terminate in different laminae, the peptide containing small diameter neurones terminate in laminae I and $II_o$, and the FRAP neurones terminate largely in $II_i$. A recent paper by Lawson and colleagues (Lawson et al., 1997) has thrown new light onto this issue. Approximately two thirds of small diameter neurones are nociceptors, and one third of small diameter neurones contain SP. All SP containing neurones are nociceptors, although not all nociceptors contain SP. Neither of the two main classes of cutaneous afferent nociceptor, polymodal C nociceptors, and Aδ high threshold mechanoreceptors stain strongly for SP. The SP staining afferents appear to have their peripheral terminals in the deeper layers of the skin, and would appear to signal about deep pressure from the skin and muscle, whilst the FRAP containing

SP/CGRP negative neurones had peripheral terminals in the superficial layers of the skin (Lawson et al., 1997).

Two recent *Nature* papers have shed further light on the role of SP in somatosensory processing. Cao and co-workers (1998) have disrupted the preprotachychinin gene which codes for both SP and neurokinin A (NKA). Although the mice lacked both SP and NKA, they showed normal thresholds for reactions to painful stimuli, but had a blunted response to higher intensity pain stimuli. De Felipe and colleagues (1998) have examined a $NK_1$ knockout and these mice again did not show any changes to acute stimuli at noxious thresholds when compared to wildtype, but again their responses to more intense stimuli were blunted. They point to a role for SP in mediating the more intense noxious stimuli, as has been demonstrated by the ability of $NK_1$ antagonists to block wind-up without affecting acute pain thresholds (Laird et al., 1993; Longmore et al., 1997; Ma and Woolf, 1997).

Immuno studies on the distribution of the $NK_1$ receptor in the dorsal horn have shown it in lamina I, III and IV. Some cells in laminae III and IV with long dendrites projecting dorsally, through SG to reach lamina I, also stained for $NK_1$. These sections were double labelled to examine their GABA immunoreactivity, and there was no colocalisation in 90% of neurones. Those that did show colocalisation of GABA or glycine and $NK_1$ were mainly in the deep dorsal horn. The authors conclude that SP acts through $NK_1$ receptors on mainly excitatory neurones (Littlewood et al., 1995). A study on young rats P12-20, has found that $NK_1$ receptors are only present on lamina II and IV neurones, although some of these have long, dorsally projecting dendrites, which reach through lamina II to reach lamina I (Bleazard et al., 1994).

*GABA and glycine:*

GABA and glycine are the main inhibitory neurotransmitters in the CNS, both mediate synaptic transmission in the SG and other parts of the spinal cord (Grudt and Williams, 1994; Todd and Spike, 1993; Yoshimura and Nishi, 1995). Furthermore, virtually all SG neurones receive inputs from both GABA and glycine containing afferents, although it is not clear whether these are in the same fibres or separate (Grudt and Henderson, 1998).

GABA appears to have a role in the modulation of the incoming EPSPs, for example, spinal administration of the $GABA_A$ receptor antagonist enhances response of dorsal horn neurones to touch and activation by Aβ fibres, and decreases the threshold for activation by innocuous mechanical stimulation (Sivilotti and Woolf, 1994).

There are two main sources of spinal GABA. The first is from the GABAergic interneurones, which are thought to be the principle source of GABA in the dorsal horn (Todd and Mckenzie, 1989). GABA, and receptors from $GABA_A$ and $GABA_B$ occur in high concentration in the superficial laminae of the dorsal horn (Bowery et al., 1987; Waldvogel et al., 1990). That GABA is involved in the modulation of nociceptive afferent information has been demonstrated in various ways. Firstly, treatment of neonatal rats with capsaicin, and the subsequent loss of a large proportion of C fibres, results in a significant loss of the presynaptic $GABA_A$ and $GABA_B$ receptors, which have been estimated to make up 20%-40% of the total numbers of receptors in the SG (Price et al., 1984; Singer and Placheta, 1980). Intracellular recordings from DRG neurones have demonstrated that both $GABA_A$ and $GABA_B$ receptors are present on the central terminals of Aδ and C fibres (Désarmenien et al., 1984). There is some anatomical evidence for a presynaptic presence of GABA receptors, GABA immunoreactive axons and vesicle containing dendrites in the SG have been demonstrated to be presynaptic to the type I synapses

arising from unmyelinated primary afferents neurones (Todd and Lochhead, 1990), and many of these presynaptic boutons contain both GABA and glycine (Todd and Spike, 1993).

This anatomical evidence appears to be contentious, as other studies demonstrate GABA immunoreactive vesicle containing dendrites to be more frequently situated postsynaptic to the primary afferent (Carlton and Hayes, 1990). These investigators have shown that primary afferents containing CGRP make synaptic contacts with the dendrites of GABAergic interneurones (Carlton and Hayes, 1990; Hayes and Carlton, 1992), suggesting that GABA is released in the dorsal horn as a result of input from small diameter nociceptors (but not directly from the primary afferent). The $GABA_B$ agonist baclofen, inhibits both the spontaneous and evoked EPSPs recorded from SG neurones in slice preparations (Kangrga et al., 1991), and inhibits the C fibre evoked activation of dorsal horn neurones *in vivo* (Dickenson et al., 1985). Stimulation of primary afferent fibres in the dorsal root elicits polysynaptic inhibitory synaptic potentials mediated by glycine, acting at glycine receptors and GABA acting at $GABA_A$ receptors in the spinal cord SG (Yoshimura and Nishi, 1995). Application of glycine and $GABA_A$ antagonists result in the enhancement of the excitatory evoked responses recorded in SG neurones after stimulation of the primary afferent fibres (Grudt and Williams, 1994).

The second main source is from the spinal projections of the GABAergic neurones in the ventromedial medulla (Antal et al., 1996; Jones et al., 1991; Reichling and Basbaum, 1990). The antinociception from produced by these descending neurones can be antagonised by intrathecal (i.t.) administration of $GABA_A$ receptor antagonists (McGowan and Hammond, 1993). Other evidence shows that the activity in the bulbospinal pathway is increased during the development of acute inflammation (Schaible et al., 1991)

Descending control, mainly through the dorsal lateral funiculus (DLF), carrying axons from the peri-aqueductal grey (PAG) via the nucleus raphe magnus (NRM) to the dorsal horn. The bulbospinal pathways arise from the nucleus paragigantocellularis, which also receives input from the PAG and also a noradrenalin input produced in the nuclei lateral to nuc paragigantocellularis (Fields and Basbaum, 1994). As well as transporting GABA to the dorsal horn these pathways transport much of the noradrenalin and serotonin (5HT) to the dorsal horn (Basbaum et al., 1982). The supraspinal effects of opiates are thought to work through this pathway, and the 5HT rich bulbospinal pathway. Antagonists to both 5HT and NA block the analgesia produced by intracerebral morphine (Wall et al., 1979). Additionally iontophoresis of 5HT inhibits the response of dorsal horn neurones to noxious stimulation (Headley et al., 1978; Randic and Yu, 1976).

At the spinal level lumbar intrathecal injection of NA, causes a profound analgesia in nociceptive neurones (Headley et al., 1978), although at the level of the medulla, microinjection of a NA antagonist into the NRM produces analgesia, showing that at this level in the CNS, NA can inhibit the analgesia system (Hammond et al., 1980).

## 1.3 Development of the spinal cord (an outline).

The first two postnatal weeks of development are particularly busy for the rat pup. Suddenly after birth it is subjected to a barrage of somatosensory stimuli. Its eyes only open towards the end of this period. There are also many other postnatal developmental changes occurring during this period, some of these, which affect the processing of somatosensory information, are highlighted below.

### 1.31 Anatomy

*Peripheral innervation:*
At lumbar levels in the spinal cord primary sensory neurones are generated from around E11 to E15, the smaller cells being formed last (Altman and Bayer, 1984; Nornes and Das, 1974). In the rat foetus the first A fibre afferent collaterals from the hindlimb grow into the spinal cord at embryonic day (E)14 were RT97 positive (Jackman, 1997). RT97 is expressed almost exclusively in large cells of the DRG in the adult rat (Lawson et al., 1984) and Jackman has constructed size frequency histograms from foetal tissue and confirms that this relationship is the same at E18 and in the neonate (Jackman, 1997).

The reorganisation of the peripheral terminals of cutaneous afferents still takes place for some weeks after birth, in contrast to that of the human where hair follicle afferent growth is complete by 5 to 6 months gestation. (Payne et al., 1991). Due to this continued growth in the rat neonates the density of hair follicles remains consistent, during the perinatal period.

A striking feature of adult hair follicles is their polyneuronal innervation where a single follicle can be innervated by up to 15 nerves (Millard and Woolf, 1988). During development this pattern is achieved gradually over the first three postnatal

weeks. This is in contrast to the neuromuscular junction where polyneuronal innervation of end plates occurs initially and subsequently withdraws to leave one axon per end plate (Purves and Litchman, 1985).

*Spinal cord development:*

The spinal cord develops ventrodorsally, beginning with the motorneurones, the intermediate neurones, the deep dorsal horn projection neurones and finally the superficial neurones in lamina I and II (Altman and Bayer, 1984; Nornes and Das, 1974). The axonal and dendritic growth of these SG neurones appears to have two phases (Bicknell and Beal, 1984), the first prenatally when projection and propriospinal neurones develop their axons and dendrites and the second postnatally where axodendritic growth takes place in the non projection neurones (the majority). Those collaterals positive to protein gene product (PGP) 9.5 and growth associated protein (GAP) 43 entered the spinal grey matter at E15-E16 in the rat foetus. All of theses early axon collaterals grew ventrally and laterally into the dorsal grey matter, locations consistent with 1a and Aβ fibres. Jackman's (1997) results are consistent with those from previous studies (Fitzgerald et al., 1991; Mirnics and Koerber, 1995; Smith, 1983).

Small diameter afferents, C fibres, reach the white matter over the dorsal horn by E19, and their terminals can be seen entering lamina IIi by E20 (Fitzgerald, 1987b). Other investigators, using different markers shows roughly the same time course of growth (Jackman, 1997; Mirnics and Koerber, 1995; Plenderleith et al., 1992; Smith, 1983), the most recent studies of Jackman show trkA positive fibres first detected in the superficial and lateral grey at E17. TrkA is the tyrosine kinase receptor for NGF, and small diameter, unmyelinated afferentshavebeen shown to be dependent on NGF (

The muscle afferents reach the cord by E13 and grow into the grey matter by E15 (Altman and Bayer, 1984; Smith, 1983; Vaughn and Grieshaber, 1973). The

formation of synapses by primary afferents proceeds in a ventral to dorsal manner, with the terminations on the superficial cells being the last to develop (Smith, 1983). The presumptive large diameter Aβ fibres traverse the cord medially from the DREZ and curve ventrally through the superficial dorsal horn into laminae IV and V, where they turn back dorsally and send out arbours into Lamina III and II (Fitzgerald et al., 1991; Maxwell and Noble, 1987; Ramon y Cajal, 1909; Scheibel and Scheibel, 1968; Smith, 1983). These dorsally directed branches are present during the first few postnatal days, and remain in the superficial laminae for about three weeks after birth in the rat (Fitzgerald et al., 1994; Mirnics and Koerber, 1995). Whilst in the superficial laminae these myelinated afferents form synapses (Coggeshall et al., 1996). The withdrawal of the central terminations to the deeper laminae is presumably due to competition from the maturing C fibres (Fitzgerald et al., 1994). If most of the C fibres are destroyed by neonatal treatment with capsaicin, the A fibres remain in SG until adulthood (Shortland et al., 1990).

These collaterals grow into the spinal cord in a somatotopically appropriate fashion as described in the adult The somatotopic map of the central terminations of afferents is also established by birth (Fitzgerald and Swett, 1983; Smith, 1983; Wilson and Snow, 1988).

The neurone specific protein NT75 is located in nerve terminals synapsing in the superficial laminae of the dorsal horn, in the adult rat spinal cord. In the neonatal rat lumbar spinal cord, synaptogenesis as measured by NT75 expression peaks at P4-5 in the deep dorsal horn and P7-9 in SG (Cabalka et al., 1990). It should be noted that only some of these synapses will be those made between incoming primary afferents and the dorsal horn cells, although in adult cord dorsal rhizotomy, severely depletes the expression of NT75. Slow postnatal synaptic maturation may underlie the delayed onset of C fibre evoked spike activity in the dorsal horn until the second week postnatal, in the rat (Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987; Jennings and Fitzgerald, 1998). In fact formation of C fibre

synapses appears to be an entirely postnatal event. Synaptic glomeruli are not observed on C fibre terminals until P5 (Pignatelli et al., 1989).

### 1.32 Physiology

*Behaviour:*

The earliest, and least invasive, studies into the development of somatosensory physiology were behavioural studies. These show that that reflex responses to somatic stimuli begin at E15 in the rat foetus (Narayanan et al., 1971). These reflexes change considerably over the postnatal period. A widely reported feature is that cutaneous reflexes in newborn rat kitten and human are exaggerated compared to those of the adult. Thresholds are lower, and the reflex muscle contractions last longer (Andrews and Fitzgerald, 1994; Angulo Y Gonzalez, 1932; Ekholm, 1967; Fitzgerald and Gibson, 1984; Fitzgerald et al., 1988; Lewin et al., 1993). Young kittens, rats and humans require only light touch to evoke a flexor reflex (Ekholm, 1967; Fitzgerald et al., 1988), rather than the nociceptive stimulus required by the adults (Willer, 1977; Woolf, 1983; Woolf, 1984).

Studies using electrical stimulation have shown that the RII component of the adult flexor reflex has an Aβ fibre low threshold input (Willer, 1985; Woolf, 1985), but that this input is not activated by natural stimulation in the adult. It has been suggested that this low threshold component may be functional in the neonate (Andrews and Fitzgerald, 1994) and could account for the low thresholds of flexion withdrawal observed in the newborn. An *in vitro* study has shown that in young rat spinal cord, neonatal flexion reflex discharges due to myelinated fiber volleys were exaggerated when compared with those in the adult rats (Hori and Watanabe, 1987), although only the later components of the flexor reflex discharge, which were associated with recruitment of unmyelinated afferents, were depressed by morphine.

Another explanation for the exaggerated flexor reflexes reported in the neonate is the delayed maturation of the descending inhibitory systems travelling in the dorso lateral funiculus. These are ineffective until P10-12 and remain immature until P24, although they are present before birth, they do not extend collaterals into the grey matter of the dorsal horn until later (Fitzgerald and Koltzenburg, 1986). This finding is compounded by the delayed maturation of the SG interneurones (Bicknell and Beal, 1984), and the report that there may be a deficiency in the neurotransmitters associated with this descending pathway 5HT (serotonin) and noradrenaline (NA) (Bregman, 1987). Further evidence for this delayed maturation of the descending inhibitory pathways comes from the report that stimulus produced analgesia from the peri-aqueducatal grey (PAG) is not effective until P21 (van Praag and Frenk, 1991).

Studies examining the behavioural response to formalin, report that before P10 there is little organised response to an injection of the agent into the hindpaw (Guy and Abbott, 1992). Before P10, there is a withdrawal response, along with whole body jerks and squirming, after this age the responses became more specific to the injured paw and shaking and licking could be observed. The response to formalin has a ten fold higher sensitivity in neonatal rats compared to weanlings (Guy and Abbott, 1992; Teng and Abbott, 1998). Thresholds for withdrawal from heat stimuli are also lower in younger animals (Falcon et al., 1996; Lewin et al., 1993)

The use of the specific C fibre irritant mustard oil has been shown to elicit nociceptive responses from P5 (Jiang and Gebhart, 1998), although responses similar to those seen in adults could not be evoked before P34. An earlier study reports that mustard oil does not evoke the flexor reflex before P10-11 (Fitzgerald and Gibson, 1984). These studies suggest that there is little organised C evoked response in the first postnatal week.

*In vitro & in vivo preparations:*

Many of the physiological studies demonstrating the onset of functional central primary afferent connections have been done using *in vitro* preparations. Stimulation of the L4 dorsal root begins to produce long latency (probably polysynaptic) reflexes in the L4 ventral root (Kudo and Yamada, 1987; Saito, 1979), and long latency EPSPs in L4 motoneurones (Ziskind-Conhaim, 1990) between E15,5 and E16. Monosynaptic reflex response was seen later E17.5-E18.5 (Kudo and Yamada, 1987). Postnatal studies, show the maturation of the afferent nerve components to the ventral root reflex. In the first postnatal week, in the rat, two peaks can be recorded and their latencies decrease in older animals, these are thought to be from the Aβ and Aδ fibres, only in the second postnatal week can a third longer latency wave (from C fibre) be seen (Fitzgerald et al., 1987).

*In vivo* recordings show that responses to electrical stimulation can be recorded in individual lumbar dorsal horn cells from E17 (Fitzgerald, 1991a). Natural cutaneous sensory input from the hind paw begins to evoke dorsal horn activity at E19 (Fitzgerald, 1991a). Initially receptive fields were small and response magnitudes and frequencies initially very low but, by E20, bursts of up to fifty spikes were recorded to a single pinch and some cells displayed responses that outlasted the stimulus by 10-15s (Fitzgerald, 1991a).

Whilst both mono and polysynaptic responses can be recorded from A afferents prenatally, both from the dorsal horn and ventral root (Fitzgerald et al., 1987; Fitzgerald, 1987c; Fitzgerald, 1991a), C fibre activation cannot evoke spike activity until the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987; Jennings and Fitzgerald, 1998).

### 1.33 Pharmacology (Chemical Neuroanatomy)

The expression and distribution of excitatory and inhibitory neurotransmitters and their receptors undergo significant postnatal changes in the first weeks after birth.

*NMDA:*

NMDA binding studies in the mouse lumbar spinal cord show that [$^3$H] glutamate binding peaks around postnatal days 6-10 and is labelled fairly evenly throughout the grey matter until P10-P12 when labelling begins to increase in substantia gelatinosa (SG). The labelling then decreases in other areas so that the adult pattern is visible by P30 (Gonzalez et al., 1993). The affinity of receptors for NMDA and NMDA-induced elevations of $[Ca^{2+}]_i$ are also markedly elevated in SG neurones in the first postnatal week while neither the AMPA response nor the resting $[Ca^{2+}]_i$ show these developmental changes (Hori and Kanda, 1994).

NMDA receptors are likely to be heteromers, composed of subunits NMDA receptor 1 (NR1) and NR2 (Monyer et al., 1994; Tölle et al., 1993). Initial studies report that in the mature dorsal horn the predominant form of NR2 is NR2D, when this combines with NR1, it forms an NMDA receptor with lower $Mg^{2+}$ sensitivity and slower kinetics (Monyer et al., 1994). The foetal dorsal horn expresses high levels of the NR2D subunit and low levels of the NR2B subunit. The NR2B subunit, when it combines with the NR1 subunit, forms NMDA receptors with fast kinetics and high $Mg^{2+}$ sensitivity (Monyer et al., 1994). More recent single channel recordings from dorsal horn neurones have demonstrated that channels with different conductance levels are present. The proposed subunit construction of these channels includes units NR2A/B (higher conductance) and NR2D (lower conductance) (Momiyama et al., 1996). Most recently, *in situ* hybridisation and single cell PCR studies in the dorsal horn have shown that the most common subunit composition of NMDA receptor (a cervical levels) is NR1, NR2A, B and D, with the NR2A subunit predominating (Karlsson et al., 1997; Sjödin et al., 1997). Other brain areas and developing neurones, that include the NR2A subunit, have fast kinetics (Flint et al., 1997; Monyer et al., 1994)

This lower sensitivity to the voltage sensitive $Mg^{2+}$ block, in the adult, and embryonic dorsal horn, appears to be particular to this part of the CNS. It has been hypothesised that this is compensated for by having low levels of the GluR2 subunit of the AMPA receptor in the dorsal horn. AMPA receptors without the edited form

of GluR2 have been shown to be $Ca^{2+}$ permeable (Burnashev et al., 1992). Since $Ca^{2+}$ permeable non-NMDA receptors are localised (in embryonic dorsal horn) with NMDA receptors, they can compete with the NMDA receptors, so compensating for the decreased $Mg^{2+}$ sensitivity (Kyrozis et al., 1995). Of the total population of dorsal horn neurones, 30% that expressed $Ca^{2+}$ permeable receptors also expressed GABA immunoreactivity, 24% glycine immunoreactivity and 4.3% NK-1 receptor immunoreactivity, demonstrating that these $Ca^{2+}$ permeable AMPA receptors are confined to specific neuronal populations in the embryonic dorsal horn (Albuquerque and MacDermott, 1998).

It should be emphasised that not all investigators report that neonatal NMDA receptors have a lowered sensitivity to $Mg^{2+}$. In a study examining spontaneous activity in the dorsal horn, a dramatic decrease in this activity was noted in younger preparations after the addition of 1mM $MgCl_2$. (Abdul-Razzak et al., 1994). A 92% reduction was reported at P6, 68% at P10, 48% at P16 and 13% at P28, there was not reduction in the adult.

*AMPA:*

Afferent fibre activation of adult dorsal horn cells involves glutamatergic AMPA receptors (King et al., 1992) and these are also more widespread in the developing spinal cord compared to the adult. The subunit composition of the non-NMDA receptor in the neonatal spinal cord maximises non NMDA $Ca^{2+}$ influx into the cell (Jakowec et al., 1995a).

AMPA receptors are thought to function as a pentameric ion channels made up from a combination of the subunits GluR1, GluR2, GluR3, GluR4 (Wenthold et al., 1992). In the mature spinal cord the distribution of these receptor subunits is largely confined to the superficial dorsal horn, motor neurones, and areas that contain preganglionic autonomic neurones (Tachibana et al., 1994). At the mRNA level GluR1 and GluR2 are dominant in the dorsal horn and GluR3 and GluR4 are dominant in the ventral horn, indicating that subunit composition the AMPA receptors regulating motor neurones is different from that of the AMPA receptors in the spinal sensory neurones. (Furuyama et al., 1993).

The expression of these subunits is developmentally regulated (Jakowec et al., 1995a). GluR1 and GluR2/3 stain strongly in the SG, but also in cells throughout the grey matter, in young postnatal animals. GluR4 is seen in large multipolar cells throughout the grey matter. GluR1 reaches a maximum value at P7, and GluR2/3 between P7 and P14, after which levels decrease to the adult values (Jakowec et al., 1995b). Quantitative autoradiography, shows an overshoot, in expression, of 140-170% of adult levels, in several brain structures (Standley et al., 1995). Different combinations of the subunits infer different functional properties to the receptor. Absence of the edited form of GluR2, in particular, makes the receptor more permeable to $Ca^{2+}$ and monovalent cations, and this subunit decreases during the first postnatal month, suggesting that older cells have less protection from the neurotoxic effects of $Ca^{2+}$ influx (Standley et al., 1995).

In addition, the receptor subunits exist as *Flip* and *Flop* splice variants (Sommer et al., 1990). These variants impart different pharmacological and kinetic properties on currents evoked by glutamate or AMPA, but not those evoked by kainate. Glutamate activation of the receptors containing the flip variant produces more current than those containing the flop variant (Sommer et al., 1990). The predominant mRNA in the rat lumbar superficial dorsal horn is the Flip variant of GluR2 (Furuyama et al., 1993; Tölle et al., 1995). There were lower levels of GluR1-Flip in lamina I and the superficial parts of II outer (Tölle et al., 1993). During development, mRNA for the AMPA subunits are expressed prominently before birth, and expression of the Flip variants of GluR1-4 is constant until P14 (Monyer et al., 1991). The Flop variants are expressed at low levels at P0, and increase consistently to reach a peak between P8 and P14.

*mGluR:*

Metabotropic glutamate receptors (mGluR) mediate their modualtory responses by coupling to guanine nucleotide binding proteins and are thought to be contribute to acute or long-lasting modulation of synaptic transmission in the CNS (Conn and Pin, 1997). An insitu hybridisation study (using radioactive oligos) in the spinal cord demonstrates that in the adult spinal cord mRNA for mGluR subtypes 1,3,4,5 and 7 were differentially expressed (Berthele et al., 1999). Transcript for mGluR1 and 5 were most abundant and the mGluR5 mRNA was found predominantly in the

superficial dorsal horn. In the neonate however, there were age related changes in the expression. Transcripts for mGluR1 and 7, showed a general decrease in expression over the first three postnatal weeks, mGluR3 and 5 were expressed strongly in the superficial dorsal horn, but also decreased with age (Berthele et al., 1999). An earlier immunoblotting study showed that mGluR5 receptor protein levels were higher at P8 than in the adult rat, and levels could also be discerned at E15 (Valerio et al., 1997). mGluR5 immunostaining was also present in human spinal cord, confined to laminae I and II, strongly suggesting that this receptor has a role in somatosensory processing in both the adult and neonate (Valerio et al., 1997).

*Silent synapses:*

In the developing hippocampus and spinal cord, there are many glutamatergic synapse that have only NMDA receptors. (Malenka and Nicoll, 1997). This poses a problem, as the voltage dependent $Mg^{2+}$ block is present from birth (Bardoni et al., 1998). Their activation, and the subsequent cascade of events is thought to activate AMPA receptors, so as to make a functional glutamatergic receptor at resting membrane potentials. This has been achieved in the neonatal hippocampus with a brief high frequency stimulus, sufficient to induce LTP (Durand et al., 1996). *This subject is fully covered in section 4.22.*

*Neuropeptides:*

Both substance P(SP) and somatostatin are present before birth in the dorsal horn and the DRG, and their levels increase considerably in the second postnatal week (Senba et al., 1982), confirmed by a immunostaining study, which shows that both calcitonin gene related peptide (CGRP) and SP are present in the dorsal horn by P4 but only reach adult intensity at around P10 (Reynolds and Fitzgerald, 1992). Somatostatin and galanin also show this type of expression (Marti et al., 1987).

Levels of SP are regulated by NGF and application of excessive NGF to newborn rat pups results in an upregulation of SP in the DRG (Goedert et al., 1981). The onset of NGF expression in the skin coincides with the onset of SP expression in the

cutaneous sensory neurones. In fact, there is a second postnatal surge of NGF production (P21), and again corresponds with an upregulation in SP levels (Constantinou et al., 1994). Administration of excess NGF (centrally) in neonatal rats causes hypersensitivity and decreased nociceptive threshold, perhaps due to an increase in SP, and its central effects (Lewin et al., 1993).

Maximal density of the NK-1 receptors is in the first and second postnatal week, so that at age P60 (adult), the cord has one sixth the binding sites that it had at P11 (Charlton and Helke, 1986). In the newborn rat the distribution of NK-1 receptors is inverse to that found in the adult. The newborn cord shows a paucity of SP receptors in the superficial dorsal horn, and the high density of receptors in the region which is characteristic of the adult is not seen until the second postnatal week (Charlton and Helke, 1986; Kar and Quirion, 1995). The function of these receptors may not be as straightforward as it first appears, i.e. that an adult type nociceptive response can only be evoked in the second postnatal week, as a result of the appropriate excitatory receptor distribution. In a study of rats aged P12-P20, the NK1 receptors in SG are shown to be on the dendrites from laminae III & IV that project up through laminae II to reach lamina I, rather than on the lamina II neurones themselves (Bleazard et al., 1994)

*GABA:*

GABA is one of the earliest neurotransmitters detected by immunohistochemistry in the developing central nervous system. GABA immunoreactivity (GABA-ir) is observed at E13 in the brainstem and diencephalon and cerebral cortex by E14 (Lauder et al., 1986; Van Eden et al., 1989). In the spinal cord GABA-ir is first seen at E 13 in the cervical region, and only in fibres in the ventral root, ventral funiculus, ventral commisure and dorsal root entry zone (DREZ). At this age there are no cell bodies stained, although, $K_2$ antiserum against $GAD_{67}$ related protein stains the fibres mentioned and cell bodies in the neuroepithelium and presumptive ventral horn. By E14 GABA-ir can be seen in the cell bodies of cervical motoneurons. At E15-17

GABA-ir expands dorsally staining medial areas first and lamina I neurones before neurones in the deeper dorsal horn. Peak intensity of the stain is reached by E18/19, and by the end of gestation in the rat (21.5 days) there is a decline in the ventral horn immunoreactivity, with no motor neurones showing GABA-ir at birth, in the cervical region, although they can still be seen in the lumbar region. This trend continues so that by P28 there is a dense band of GABA-ir in lamina II with moderate immunoreactivity in laminae I & III, with a little little diffuse staining ventral to lamina III and none in motor neurones (Lauder et al., 1986; Ma and Barker, 1995; Ma et al., 1992; Ma et al., 1993).

The pattern of GABA expression follows the ventral to dorsal gradient of neurogenesis revealed by $^3$H-thymadine radiograms (Altman and Bayer, 1984; Nornes and Das, 1974), except that in the dorsal horn, GABA-ir is seen in the long projecting relay neurones of lamina I, before it is seen in deeper laminae of the dorsal horn. This quirk might be explained in terms of an hypothesis put forward by Altman and Bayer, 1984. They postulated that during neurogenesis the long projecting relay neurones of lamina I are located on the dorsal lateral surface of the cord between E13 and E15. For the next two days they are "tied" to the central canal by roof plate glial fibres and become scattered in the dorsal most layer of the spinal cord in a semicircular position around the dorsal horn. If this is the case then the large relay neurones of lamina I express GABA prior to the *later* generated small interneurones of the deeper dorsal horn (Ma et al., 1992).

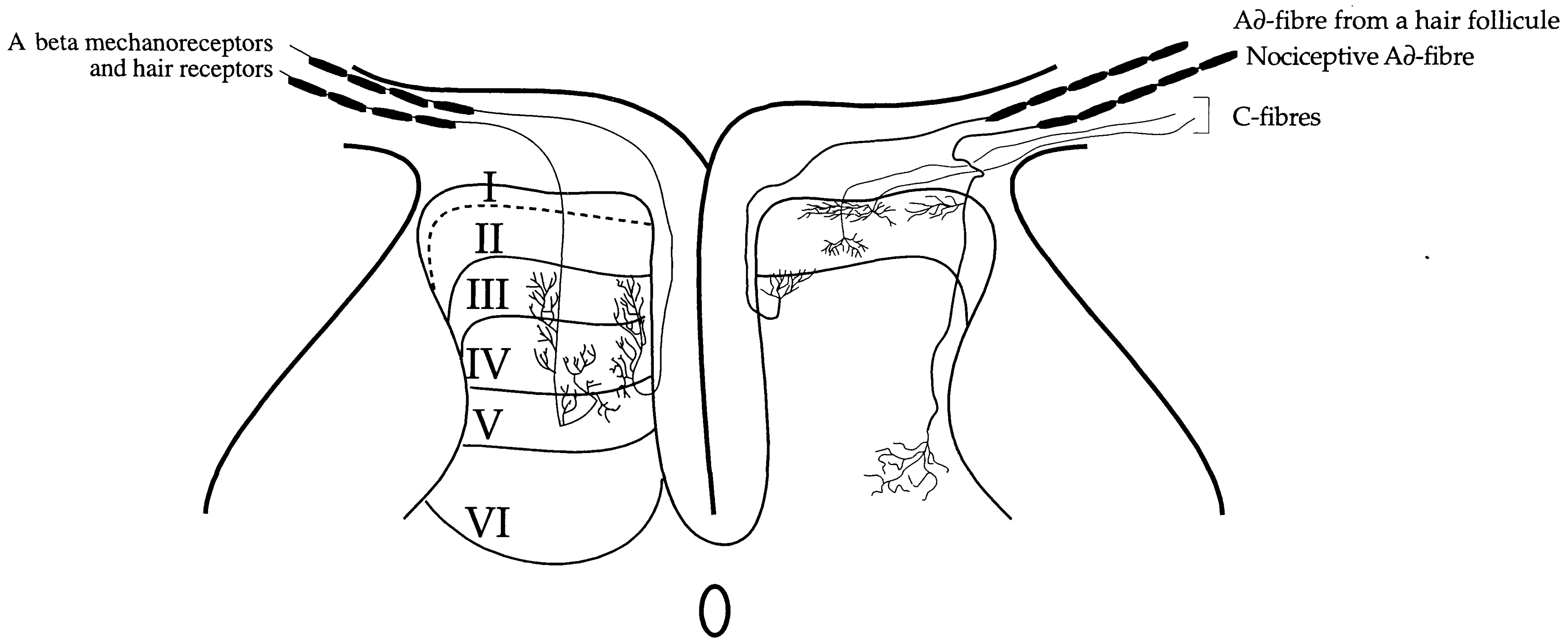
$GABA_A$ receptors develop in approximate spatiotemporal synchrony with the GABAergic pathways in the rat brain (Schlumpf et al., 1992). The co-ordinated development of GABAergic pathways and $GABA_A$ receptors has lead to speculation that GABA may modulate the expression of its own receptor (Schousboe and Redburn, 1995). There is *in vivo* and *in vitro* evidence to support this. Chronic prenatal exposure to $GABA_A$ antagonist differentially regulates expression of individual mRNA transcripts encoding $GABA_A$ receptor subunits in the foetal rat

brainstem (Liu et al., 1998), consistent with other data showing that individual subunits can be differentially regulated (Morrow, 1995; Poulter et al., 1992).

*Opiate:*

Opiate receptors also show developmental differences in their expression. Both the μ and κ receptors are present at birth, whilst the δ receptor is first seen at P7 in the rat (Rahman et al., 1998). At birth the distribution of μ receptor is diffuse throughout the cord, binding peaks in the first postnatal week and then decreases to adult levels. The distribution of the receptors sites gradually regresses and only occupies the superficial dorsal horn (Kar and Quirion, 1995; Rahman et al., 1998). The distribution of the δ receptor remains diffuse, equally spread between the superficial and deep laminae of the dorsal horn. κ receptor binding also remains diffusely spread throughout the dorsal horn (Rahman et al., 1998).The functional sensitivity to spinal morphine, appears to be only partly determined by the number and distribution of μ receptor binding sites. Another factor could be inefficient coupling of the receptors to second messenger G proteins (Thornton et al., 1998). GTPase activity and both $G_o$ and $G_i$ subunits have been demonstrated in postnatal rats (Windh and Kuhn, 1995), although it is still not clear what percentage of the μ receptor population is efficiently coupled to the G proteins, during postnatal development (Thornton et al., 1998). In fact, large numbers of uncoupled μ receptors have been reported in the endoplasmic reticulum of neonatal rats when compared to adult rats (Bem et al., 1991), and receptor ligand studies are likely to include both coupled and uncoupled receptors.

The cutaneous somatosensory system of the neonate is different from that of the mature rat in terms of anatomy, pharmacolokgy and physiology. The aims of this study were to examine the A-afferent evoked avtivity in the dorsal horn during the time when the low threshold Aβ terminate in the superficail laminae (fitzgerald et al., 1994) apparently forming synapses (Coggeshall et al., 1996).




From Fitzgerald 1994, Textbook of pain.

# Chapter 2:

## General Methods

### 2.1 Laboratory Animals

Sprague-Dawley rat pups of both sexes, with postnatal ages 3 days (P3), P6, P10, P21, and P30, (where P0 is the day of birth) were obtained from the University College London animal house for use in these experiments.

### 2.2 General Preparation for the physiological experiments

Pups were weighed and anaesthetised with 2.0mg/kg urethane (intra- peritoneal (i.p.), ethyl carbamate; Sigma). Once the pups were areflexic, a tracheostomy was performed and the airway cannulated with a plastic tube with a snug fit (Portex-FG 3-5), and securely tied into the trachea with surgical silk (Mersilk 3/0 (Ethicon)). The pups were then set up in a small animal Kopf stereotaxic frame, with the head (earbars-non traumatic) and pelvis firmly held, and kept warm with an electric blanket placed under them. Electrodes for a two lead ECG were attached and the heart rate did no drop below in the range 350bpm for the duration of the experiment. The tracheal cannula was attached to the ventilator (an intermittant flow lung ventilator-Harvard Apparatus) set at a rate of 76 cycles per minute, and the pups were paralysed with Flaxedil (gallamine triethiodide; May and Baker Ltd.) 0.1ml i.p. This prevented instability in the preparation from reflex movements due to electrical stimuli. In the younger animals stability of the preparation was particularly difficult to ensure, as their bones are incompletely ossified and the stabilising bars and clamps are therefore less firmly attached. Urethane has been shown to be an effective anaesthetic for this type of study (Maggi and Meli, 1986), giving anaesthesia for at least 8 hours. These experiments never lasted more than six and after four hours, when the effects of the Flaxedil had worn it was possible to confirm that the urethane was still effective, even in the late parts of the experiments.

## 2.3 Surgery

A lumbar laminectomy was performed, giving access to the dorsal surface of the lumbar enlargement of the spinal cord. The dura mater (and arachnoid) was slit over the area of the exposed cord and warm mineral oil (Sigma) was put onto the cord to prevent it from drying out. The upper thoracic cord was clamped near the rostral end of the laminectomy by fine forceps attached to the Kopf frame, for extra stability. Finally the hind limbs were supported with a suture under the archilles tendon, this was to reduce the movement artefact when applying natural stimuli (brush and pinch) to the hind foot. Glass covered platinum coated tungsten microelectrodes (tip diameter ±1µm, tapering to 4µm. Tip length 10-15µm. Impedence 1-2MΩ) (Merrill and Ainsworth, 1972) were used for recording. They were bought from Alan Ainsworth (who now works privately ("*Ainswork*"-London)). The electrodes were attached to a Neurolog (Digitimer) head stage mounted on a microdrive (New Brain-Digitimer). The recording electrode was lowered onto the surface of the dorsal horn and the reference electrode put into the muscle alongside the laminectomy. The depth of the cells from the surface of the cord was measured from the microdrive. Using this method cells were grouped into those recorded in the superficial dorsal horn (I & II) and those recorded in the deeper dorsal horn (III, IV & V). As the cord grows significantly during ages examined in this study the superficial/deep boundary changed with age. The boundary values were measured from Nissl stained lumbar cord sections from animals of the same ages as those used in these studies. The superficial laminae were defined as follows: 200µm from the dorsal surface of the spinal cord at P3 and P6, 250µm at P10 and 300µm at P21.

## 2.4 Stimulation

Once a single cell had been localised, the receptive field was mapped out using touch as a search stimulus, starting with touch (with a finger), and brush (cotton bud), and then pinch (forceps), allowing some initial characterisation of the input modality of the cell. Electrical stimuli at intensities to excite A fibres only or both A and C fibres at different ages were delivered via transcutaneous pin electrodes, implanted into the centre of the receptive field, and were generated by Neurolog modules (pulse buffer (NL510), delay width (NL 402, and stimulus isolator (NL800)). These thresholds

were determined from earlier dorsal root recordings and were within the parameters established previously in the laboratory (Fitzgerald et al., 1987; Fitzgerald, 1985a). The threshold for exciting axons with electrical stimulation is an inverse function of the diameter of the neurone (Willis and Coggeshall, 1991). In the younger animals, however, it was difficult to distinguish between the Aβ and Aδ response by latency, due to the general immaturity of the nerve fibres, including lack of myelination and small diameter in all axons. Here I refer to the *A fibres* collectively, with the threshold defined as the minimum electrical stimulus needed to produce a short latency response from the dorsal horn cell. All cells were also tested at higher stimulus intensities to test for a longer latency C fibre input.

In some experiments, trains of electrical stimuli were applied. The magnitude of the stimulus used in the train of stimuli was twice the magnitude that would evoke the first dorsal horn response. The train of sixteen stimuli was delivered at a frequency of 0.5Hz, (period determined by a Neurolog period generator (NL303)). It also allowed examination of the stability of the afferent evoked response by examining the "latency jitter". The sizes of the evoked spikes recorded were typically in the range 60 - 150μV.

## 2.5 Recording Set-up

The recording rig, including oscilloscopes and Neurolog components, and the animal were all earthed to a single point. The evoked response from single dorsal horn cells were recorded with tungsten electrodes mentioned above, and amplified and filtered with standard Neurolog (Digitimer) modules. An AC preamplifier, amplified the response 2000 times. This signal was then filtered (upper limit, 75 kHz and the lower, 45kHz), and visualised on a digital storage oscilloscope (Gould-4041). It then passed through the spike trigger (NL200) level set on the Neurolog module NL200 to a MacLab/4s analogue/ digital interface (ADInstruments).

Three recording channels were used on the Maclab. The *response* channel, which received its input from the spike trigger (NL200), in the form of standard transistor-transistor logic signals, the threshold for the generation of these having been set on the spike trigger front panel, for each cell individually. The second channel was the *stimulus* channel, which received its input from the period generator (NL303). This signal was also in the form of standard TTL pulses. The same output from the period generator was used to drive the external trigger of the digital storage oscilloscope. The third recording channel fed into the Maclab was the *raw data* channel. This

channel received its input as an analogue signal from the Filter module (NL125) after the raw signal had been amplified by the AC pre-amp (NL104) and the high and low frequency filters (and the 50Hz notch filter) had been applied to this signal. Due to the size constraints of the subsequent computer files, the raw data channel was not active during all recordings occasions. The *stimulus* and *response* channels provided the data for the calculation of the PSTH.

Analysis consisted of measurement of the latencies and magnitudes (number of spikes) of response and construction of a post stimulus time histogram (PSTH), a powerful way of demonstrating the temporal distribution of the evoked response (Budai, 1994).

Data were statistically analysed using the Student's t test and ANOVA factorial. All data are expressed as mean ± SEM unless specified otherwise. (Staview: Abacus Concepts inc.)

Mid way through this study the lab acquired a new computer system for recording this data. The data prior to this time was recorded on chart recorder, although some records were recorded on an older, very unreliable computer system.

## 2.6 Methods for inflammation experiments

The series of experiments on inflammation (Chapter 5) used the same basic recording set up, described above. The only change being that pups of only one postnatal age were examined (P10) and that only one cell was recorded from each animal. In early experiments, once a cell with a stable and well defined response to peripheral stimulation had been isolated, three sets of control stimulations (at twice the *A fibre* threshold) were delivered via pin electrodes in the centre of the receptive field (set being the train of sixteen stimuli 0.5Hz, 10 minute intervals were left between sets). If the magnitude of the evoked response was stable then the inflammatory agent (10μl of 1% carageenan subcutaneously into the plantar aspect of the hindpaw) was injected and the background firing activity and evoked response recorded every ten minutes for the length of the experiment. The inflammatory insult, caused local oedema and erythema. Because of this the method of delivering the electrical stimuli was changed to direct sciatic nerve stimulation via platinum wire hook electrodes, rather than to the hind paw via pin electrodes. The sciatic nerve was exposed just rostral to the popliteal fossa, freed from the underlying tissue and platinum wire electrodes hooked underneath.

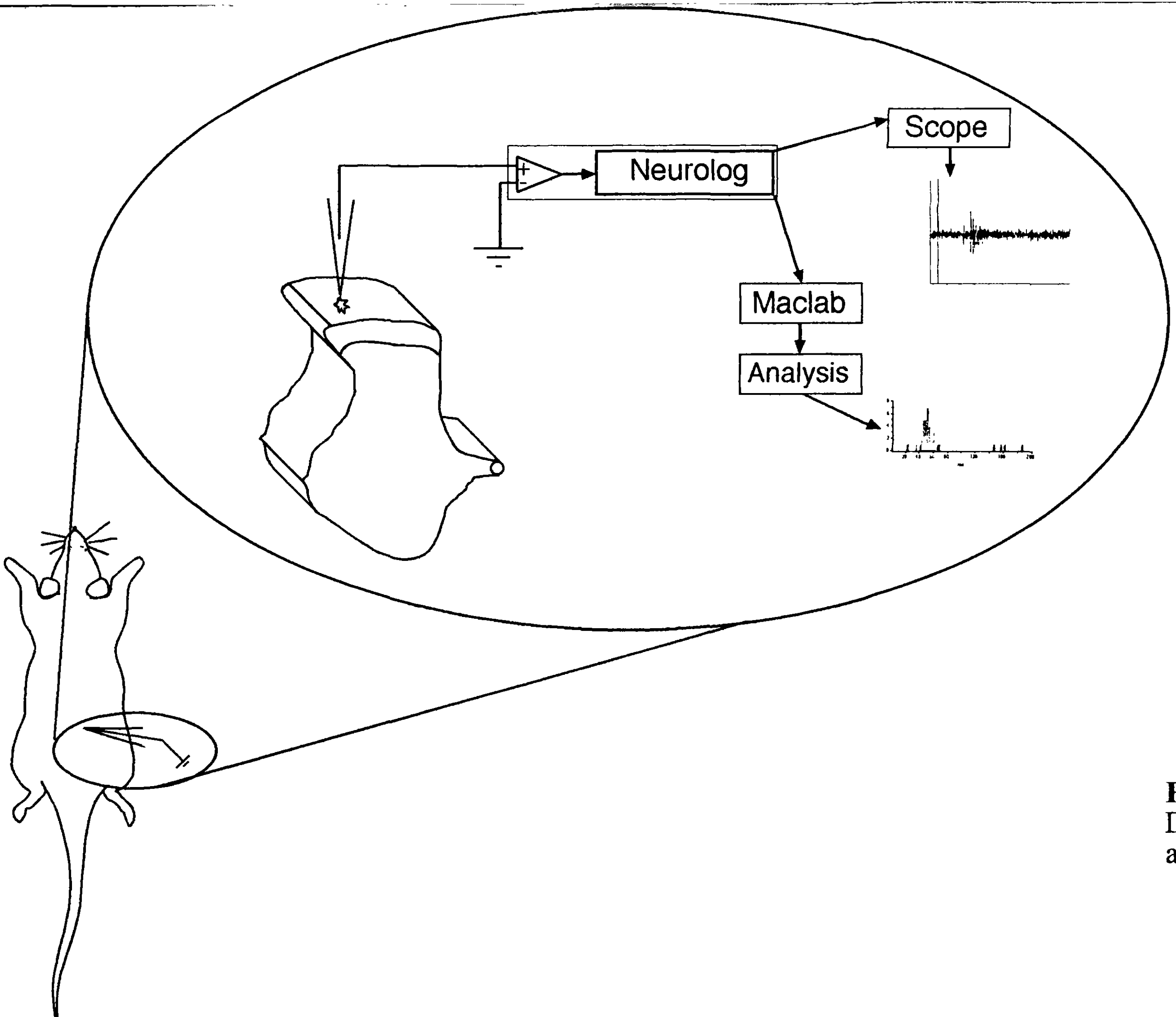



**Fig 2.1: Dorsal horn recording**
Diagram demonstrating the set-up of the recording apparatus, used in these experiments.

# Chapter 3:

## General characteristics of afferent evoked responses in neonatal dorsal horn cells.

### 3.1 Introduction

In the rat foetus, A fibre afferents from the hindlimb grow into the spinal cord at embryonic day (E)15 before C fibres, which do not enter lamina II until E18-19 (Altman and Bayer, 1984; Fitzgerald et al., 1991; Fitzgerald, 1987c; Jackman, 1997; Mirnics and Koerber, 1995; Smith, 1983).

On growing into the dorsal horn, the central terminals of the low threshold A fibres penetrate up into the superficial dorsal horn, and remain there for the first three weeks of postnatal life, before gradually withdrawing to occupy laminae III-V as in the adult. Ultrastructural studies show that whilst in the SG, (Fitzgerald et al., 1994; Mirnics and Koerber, 1995) A fibre terminals form synapses (Coggeshall et al., 1996). In addition, there are enormous postnatal changes in connectivity and pharmacology in the dorsal horn (this has been reviewed in chapter 1). These postnatal developmental changes in afferent fibre synaptic connections are likely to be reflected in a change in the pattern of afferent evoked activity in dorsal horn cells.

Only a few previous studies have looked at dorsal horn cell activity over the postnatal period. Dorsal root stimulation evokes polysynaptic ventral root activity at E15.5 (Saito, 1979), while responses to electrical stimulation can be recorded in individual lumbar dorsal horn cells from E17 (Fitzgerald, 1991a). Natural cutaneous sensory input from the hind paw begins to evoke dorsal horn activity at E19 (Fitzgerald, 1991a). The afferent input and receptive field properties of dorsal horn cells in the newborn rat and cat have been surveyed (Fitzgerald, 1985a; Wilson and Snow, 1988) and compared to the adult. A number of postnatal changes were observed including the delayed maturation of C fibre evoked activity spinal cord which does not evoke spike activity until the second postnatal week, although long lasting subthreshold depolarisations can be induced in the first week (Fitzgerald and Gibson, 1984; Fitzgerald, 1985a; Fitzgerald, 1988; Hori and Watanabe, 1987).

The aim of this study therefore, was to examine the physiological responses of the superficial and deep dorsal horn cells to afferent stimuli during postnatal development. Since all such activity arises from A fibres in the early postnatal period, the study inevitably concentrates on activity evoked from this fibre group, especially in the context of the transient A fibre synapses in the neonatal superficial dorsal horn. The previously reported delayed maturation of the C fibre evoked response (Fitzgerald, 1985a; Fitzgerald, 1988; Hori and Watanabe, 1987) was noted in these experiments and will be included in this chapter. Some of the results in this chapter have been published (Jennings and Fitzgerald, 1998).

## 3.2 *Results*

### 3.21 General properties of extracellularly recorded units

175 single unit recordings were made from the lumbar dorsal horn at different postnatal ages; n=22 at P (postnatal day) 3, n=65 at P6, n=53 at P10, and n=35 at P21. Between one and five cells were recorded from each pup. Cells were recorded from both superficial laminae I and II (≤ 200µm from the dorsal surface of the spinal cord at P3 and P6, ≤ 250µm at P10 and ≤ 300µm at P21) and from deeper laminae III to V. At P3, n=13 were from superficial laminae and n=8 from deep laminae; at P6, n=45 were superficial and n=12 deep; at P10, n=36 were superficial and n=12 were deep and at P21, n=20 were superficial laminae and n=11 were deep.

In addition, at P10 ten deep dorsal horn cells and three superficial cells and at P21 three deep dorsal horn cells were recorded separately as controls for the inflammation experiments described in chapter 5. In these experiments afferents were electrically stimulated directly from the sciatic nerve at mid thigh level. Since latencies were quite different (due to the shorter conduction distances from cells stimulated from the hindpaw skin), the results from this group were not included in the main latency calculations. They have been used, however, for calculating the magnitude of response (number of spikes) at these ages.

### 3.22 Response to "natural" stimulation:

All cells were initially examined for their response to natural stimulation. A response to either brush (innocuous mechanical) or pinch (noxious mechanical) stimulation, was recorded for every cell. Cells were not tested for their responses to heat or cold stimuli. Background activity was generally absent when cells were initially isolated for recording. An example of the response of a deep dorsal horn cell at P21, to brush and pinch of its receptive field is shown in *FIG 3.1*.

Some of the recorded responses were noted to be either rapidly or slowly adapting. Rapidly adapting responses gave a burst response at the beginning of the natural stimulus and sometimes also at the end, (adapting to the stimulus for the duration). The slowly adapting responses fired spikes for the duration of the stimulus. Of the 9 records made, 6 responses were rapidly adapting, following either pinch or brush, and 3 responses were slowly adapting. These response patterns were not studied in

detail here since it has already been reported that 28% of cells were rapidly adapting at P7-8 and 22% were rapidly adapting at P14-15 (Fitzgerald, 1985a).

*CONVERGENCE*

Dorsal horn cells responded to either brush or pinch, and in some cases to both brush and pinch of their receptive fields. Of the entire population of cells the responses to mechanical "natural" stimuli were as follows. 61% were responsive to light touch or brush only, 14% responded to high intensity mechanical stimulation such as pinch, giving a more intense response when pinched than when brushed. The remaining 25% were responsive to both pinch and brush. The numbers of cells responding to these stimuli, at individual ages are shown below (*TABLE 3.1*). The responses recorded from cells in the younger animals were mainly elicited by low threshold mechanoreceptors. There were few cells with convergent input in the first week of life, this gradually increased so that by P21 the percentage of neurones with convergent primary afferent input was similar to that seen in the adult (Fitzgerald, 1982). These results confirm the postnatal trend in convergence reported by Fitzgerald (1985). See *FIG. 3.2*.

| | **Brush** | **Pinch** | **Brush & Pinch** |
|---|---|---|---|
| **P3 (n=22)** | 20 | 1 | 1 |
| **P6 (n=65)** | 54 | 7 | 4 |
| **P10 (n=53)** | 22 | 12 | 19 |
| **P21 (n=35)** | 10 | 5 | 20 |

**Table 3.1:** Shows the modality of the stimulus to which cells responded.

*RECEPTIVE FIELDS:*

The receptive fields of the cells used in this study were located on the distal hindlimb and all had cutaneous mechanoreceptive fields. Most of the receptive fields were on the plantar surface of the hindpaw in the tibial nerve dermatome. One of the cells, at P10, thatresponded to both pinch and brush, had different but adjoining receptive fields.

In order to calculate the size of the receptive fields the map of the peripheral field, recorded on the results sheet of each cell, was scanned into a computer (Adobe Photoshop). This program calculated the number of pixels in the scanned area of the receptive field image. A value for the number of pixels for the entire plantar surface

of the hindpaw was similarly obtained, and the value calculated for the percentage of the plantar hindpaw that the receptive field occupied. Since the receptive field of the majority of cells was confined to this plantar surface, only these were used in this calculation (P3, 19cells; P6, 43 cells; P10, 50 cells and P21, 30 cells). This method of calculating the receptive field as a percentage of the total plantar hindpaw area, has the advantage that the size of the foot (which changes considerably over this period), does not have to be taken into account. *FIG 3.3* shows the peripheral receptive fields of these cells.

*FIG 3.4* shows that at P3 the mean (±SE) peripheral receptive field occupies 50%±5.6 of the plantar hindpaw. This value drops to 36%±2.9 at P6, 20%±1.9 at P10 and 15%±1.6 at P21. The biggest change occurring in the first postnatal week. Comparisons of the mean and SEM of the percentage area values that the receptive field occupied, using the *student t test* yields the following results. Comparing P3 and P6 gives a $p$ value of 0.0397, comparison of the receptive field sizes at P6 and P10 gives a $p$ value of $<0.0001$, and comparison of the receptive field sizes at P10 and P21 suggests that the modest decrease in area is not significant. This data confirms previous reports that the size of the peripheral receptive field decreases with age (Fitzgerald, 1985a).

This result is a reflection of the mean value for receptive field size at each age. There is some variance, where, for example a cell recorded from a P6 pup has a receptive field which occupies just one toe. The extent of this variation is shown in the frequency distribution histograms for this sample *FIG 3.5*.

### 3.23 Responses in response to electrical stimulation

Cells were also tested for their responses to electrical stimulation, via pin electrodes in the plantar surface of the hindpaw.

*LATENCIES OF A FIBRE EVOKED RESPONSES: SINGLE STIMULI*

The spike activity evoked in individual cells to low intensity electrical skin stimulation (100μA-3.5mA, 50-200μs) sufficient to recruit A fibres was investigated at each postnatal age. The latency of response to A fibre stimulation was defined as the latency to the first spike after a single stimulus at twice the minimum threshold to evoke a response, presumably recruiting the larger A fibres. *FIG. 3.6* shows that this latency progressively decreased with age, in both superficial and deep cells. At P3,

the mean latency of the A fibre evoked response was 33.1±2.78 ms (n=22), compared to 19.1±1.32 ms (n=65) at P6, 13.5±0.8 ms (n=53) at P10 and 7.3±0.3 ms (n=35) at P21. There was no significant difference between the mean A fibre evoked latencies of superficial and deep cells. *FIG 3.7* shows post stimulus time histograms of example cells at each of the ages examined in this study. It is clear that the latencies of the spikes evoked decrease with age.

*FIG. 3.6* also shows that the variation in the A fibre latencies within the population of recorded cells decreased with age. The standard deviation of the latencies was taken as a measure of the variation for this comparison (*FIG 3.8*). The standard deviation for the total cell population at P3 was 12.8 compared to 10.0 at P6, 5.3 at P10 and 1.9 at P21. *FIG. 3.8* shows that the A fibre evoked latency variation between superficial cells was consistently greater than that of the deeper cells in the dorsal horn, except at age P3 where the standard deviation for both the deep and the superficial cells was the same and both were very large. This *intercellular* variation in latency, with the variation between latencies in the superficial dorsal horn and those in the deeper dorsal horn, presumably reflects the reported ventral-dorsal maturation of cells in the spinal cord (Altman and Bayer, 1984; Nornes and Das, 1974)

*LATENCIES OF A FIBRE EVOKED RESPONSES: REPETITIVE STIMULATION*

Repetitive 0.5 Hz electrical skin or sciatic nerve stimulation, at twice the A fibre threshold leads to considerable variation in latency in the evoked response or 'latency jitter' of *individual* cells in the neonatal dorsal horn. *FIG. 3.9* shows this variability, expressed as the standard deviation of the latencies over sixteen stimuli, in both superficial and deep cells at P3, P6 and P10. Despite the variation in *initial* latency between individual superficial cells at P3, as shown in *FIG. 3.6*, repeated stimulation of these cells produces significantly less 'latency jitter' than deeper cells. A similar pattern appears at P6 but the difference is not significant at this age and at P10 the latency jitter is very much reduced in both superficial and deep cells.

*LATENCIES OF C FIBRE EVOKED RESPONSES*

The spike activity evoked in individual cells to high intensity skin stimulation (1-5mA, 500μs) which recruits both A and C fibre afferents was investigated at each postnatal age. No long latency spike responses were evoked in response to C fibre stimulation in pups aged P3 or P6, consistent with previous reports (Fitzgerald, 1988). Cells responding with long latency activity to a C fibre electrical stimulus

were found in both superficial and deep laminae in animals aged P10 and P21. At P10, 35% of cells had a C fibre input with a mean latency of 97.65±4.44 ms (n=17), and at P21 the value was 32% with a mean latency of 107.0±10.12 ms (n=10).

### *Magnitude of Afferent Evoked Activity: single stimuli*

*Fig.3.10* shows the mean number of spikes evoked by each of the stimuli in a train of sixteen stimuli, in the first 70ms following each stimulus. The mean response to the first stimulus of each was remarkably consistent between cells at a given age, as shown by the low standard errors. The mean values at P3, P6 and P10 were not significantly different at 5.1±0.6, 3.8±0.7 and 5.64±0.9 spikes per stimulus respectively. The mean evoked response was also not significantly different in superficial and deep laminae at each age. This is shown in *Fig 3.11*

C fibre evoked responses at P10 & P21 were weak, ranging from 1 to 4 spikes evoked by the first C fibre stimulus. In two of the cells that responded to pinch at P10, only 0-1 spike was evoked with electrical stimulation, the other cells gave a greater magnitude of response.

### *Magnitude of Afferent Evoked Activity: repetitive stimulation*

*Fig. 3.10* also illustrates that the mean number of A fibre evoked spikes did not significantly alter on repetitive stimulation at all ages. Despite the latency jitter illustrated in *Fig.3.9* the number of evoked spikes over sixteen stimuli stays remarkably consistent at P3, P6, P10. There is no significant linear relation between the number of spikes evoked and latency of response in cells at P3, P6 and P10 (see *Fig 3.12*). There is a tendency for cells at P6 with longer latencies, to have a greater magnitude of response, but the probability of the relationship between these parameters being greater than zero is p=0.8571 for superficial cells and p=0.1073 for deep cells. The sample size is quite small in P3, but still shows lack of a response pattern for the population of cells probably a reflection of the immaturity of the system, at this age. At P10, however the latencies of response to direct sciatic nerve stimulation, were remarkably consistent. The P10 data does not tie in with the variability of latencies when the animals were stimulated with pin electrodes into the plantar surface of the hindpaw, which is the data which has been used in the previous sections of this results summary. Stimulating the nerve at mid thigh level rather than using pin electrodes in the plantar surface of the hindpaw, shortens the conduction

distance, so narrowing the variability in the system. Stimulating the nerve directly, may also eliminate or shorten and peripheral delay, such as receptor transduction time.

## 3.3 Discussion

The present study demonstrates that the postsynaptic activity evoked in neonatal dorsal horn cells by cutaneous afferents undergoes a number of developmental changes in the postnatal period. C fibre evoked postsynaptic spike activity is absent before P10 and therefore A fibre evoked activity is of particular importance. Despite long and variable response latencies at younger ages, A fibre input to both superficial and deep dorsal horn cells is robust and repeatable.

### 3.31 Afferent evoked responses:

It appears, therefore, that all the direct cutaneous evoked dorsal horn spike activity in the first postnatal week results from activation of A fibres. In the present study electrical stimulation of the receptive field allowed us to clearly identify low threshold A fibre evoked responses in both superficial and deep cells from P3 to P21. Previous studies involving dorsal root recordings of afferent volleys (Fitzgerald, 1985a; Fitzgerald, 1988) show that these responses were evoked by the large diameter, myelinated, A fibres. The mean magnitude of the A fibre evoked response does not change substantially over the postnatal period, but there are considerable changes in the response latencies, which are longer and much more variable in younger animals. These changes in response latencies have been noted in some recent developmental electromyogram (EMG) studies (Jiang and Gebhart, 1998). The authors observe that the latency of the early component of the EMG decreases with the increase in age of the animal, and reached a value similar to that found in the adult at P18. Once again this result is dependent on both conduction velocity changes and central delay.

To some extent the long latencies are due to low cutaneous afferent conduction velocities in the newborn which increase in the rat hindlimb from 3.0 $m.sec^{-1}$ at P3 to over 18 $m.sec^{-1}$ at P21 (Fitzgerald et al., 1987; Fitzgerald, 1985a).

### *A) CONDUCTION VELOCITY CHANGES*

Factors that influence conduction velocity are (Waxman, 1980):

- degree of myelination
- axon diameter
- channel activity
- temperature

*Myelination:*

Myelination is achieved, in the periphery, by Schwann cells wrapping around the axon in a series of spiral turns, a process which is functionally equivalent to increasing the thickness of the axonal membrane, by up to 94 times (Webster, 1971). The capacitance of a parallel-plate capacitor such as the membrane, is inversely proportional to the thickness of the insulating material, myelination decreases $c_m$ (capacitance per unit length of the axon), and thus also decreases $r_a c_m$ ($r_a$: axial resistance), resulting in an increase in conduction velocity (Kandel et al., 1991). (The rate passive spread of depolarisation during conduction of an action potential, varies inversely with the product $r_a c_m$).

In rat sciatic nerves there is no myelin at birth, it appears within 3 days and grows rapidly for two weeks, after this time more spirals turns are formed until the adult complement is reached (Webster, 1971). In Webster's study, at P1, up to one complete spiral was observed; at P3, up to 4 spirals had formed; at P7, it was 28, and by P16, it was 53. In the adult marginal sciatic nerve there were up to 94 myelin spiral turns (Webster, 1971). Others have observed that although there are Schwann cells along the axons at P0-P1 in the rat, they are isolated and with large gaps between them (Vabnick et al., 1996). Part of the process of myelin growth in the first postnatal week is for the Schwann cells to grow along the axon to decrease the gap between Schwann cells, and this process is likely to induce the clustering of $Na^+$ channels (Vabnick et al., 1996).

There is some evidence that myelination follows a proximo-distal gradient (Vabnick et al., 1996). This might partly explain the variability of latency in the early postnatal rats, as the proximal part of the axons start to myelinate before the more distal parts.

*Axon Diameter:*

There is a direct relationship between conduction velocity and core axon diameter. Axial resistance ($r_a$) decreases in proportion to the square of axon diameter, whilst the capacitance per unit length of the axon ($c_m$), increases in direct proportion to the diameter, the net effect of an increase in diameter is a decrease in $r_a c_m$ (Kandel et al., 1991). The diameter of sciatic nerve fibres continues to increase until six months of age, with the largest fibres doubling in diameter between P5 and P20 (Webster, 1971), this age range falls within the parameters of my study, and will influence the conduction velocity.

*$Na^+$ Channels:*

In addition to myelination, which is essential for *saltatory conduction*, the clustering of the $Na^+$ channels at the nodes of Ranvier, is something which continues to mature during the first postnatal week. At P0 there are very few fibres with $Na^+$ channels, by the 1st day after birth there are some clusters of $Na^+$ channels, and at the end of the first postnatal period most of the sites with $Na^+$ clusters have nodal characteristics (Vabnick et al., 1996).

A study examining the developmental expression of sodium channel α-subunit mRNA (using non-isotopic insitu hybridisation) show the following (Felts et al., 1997). Some of the cloned $Na^+$ channel α-subunit transcripts show an early peak in expression followed by a down regulation in the spinal cord. This is true for channel III mRNA, NaG and Na6. It is interesting that PN1 was only found in the DRG (possibly this is only found in the CNS), this was continually upregulated over the period covered in the study (E17-P30) (Felts et al., 1997). The expression of PN1 and NaG at P30, in the DRG, might provide the molecular correlate for the distict types of Na+ channels seen in the adult rat {Caffrey, et al., 1992}.

From E17, both tetrodotoxin sensitive and resistant $Na^+$ channels are present in the DRG. TTX sensitive $Na^+$ channels were found on large light cells in the DRG and the TTX-insensitive $Na^+$ channels were found on small dark DRG cells (Ogata and Tatebayashi, 1992).

The internodal distance is likely to be continually changing during the postnatal growth of the axon and its myelin sheath. This internodal distance does have some

effect on conduction velocity, although in the healthy nervous system this distance is relatively constant. Differences are usually only seen in demyelinating disease and axotomy (Waxman, 1980).

*Temperature:*

The effects of temperature on conduction velocity have been observed in clinical studies, and are especially important in the limbs where temperature can vary by several degrees (Waxman, 1980). Conduction velocities in human subjects have been shown to decrease by 2.4m/s for every degree drop in temperature, in the range 29-38C (J.D. Hendricksen 1956, PhD Thesis, University of Minnesota; quoted in (Waxman, 1980)). To eliminate this variable, we attempted to maintain a constant temperature environment for these experiments, using a heated blanket with a feed back probe (chapter 2).

*Depth of cells:*

Superficial cells generally displayed a wider range of response latencies to A fibre stimulation than deeper cells, while their ability to respond to repeated stimulation was superior, with lower 'latency jitter' than deeper cells. This is presumably either a reflection of the relative immaturity of the superficial cells and synapses (Bicknell and Beal, 1984), or a reflection of the transient nature of A fibre synapses in the superficial dorsal horn and their physiology (Fitzgerald et al., 1994; Mirnics and Koerber, 1995).

### *B) Postnatal maturation of central synapses*

Changes in the conduction velocities is likely to be only part of the explanation of the long latencies. As the cells that I recorded from were second order cells, the long response latencies are also likely to arise from prolonged central, synaptic delays and very slowly rising EPSPs in the neonatal dorsal horn. Such long central delays may also lead to the latency variation between cells particularly in younger animals.

Although little work has been done on the timetable of synaptogenesis in the dorsal horn, it has been reported that the peak of synaptogenesis for deep cells is P4-5, and

that synaptogenesis peaks in the superficial dorsal horn in the second postnatal week (P7-9) (Cabalka et al., 1990). In the rat, the time period over which neuromuscular junction (NMJ) synapses form is about three weeks after the first neuro-muscular contact is made, so can be expected to continue well into the postnatal period (Hall and Sanes, 1993).

Much of what is known of synaptogenesis comes from the large body of work on NMJ formation (Reviewed by: Hall and Sanes, 1993; Navarrette and Vrbova, 1993; Purves and Litchman, 1985). This system is different from CNS synaptogenesis in two ways:

1. In the CNS, the size of the synaptic contact zone rapidly reaches adult size and does not change significantly during synaptic maturation (Vaughn, 1989). This holds true for different regions of the CNS, and across species. The NMJ on the other hand, increases considerably, in size, during postnatal maturation (Banner and Herrera, 1986).
2. In the mature NMJ, there is one presynaptic axon to each muscle cell. Transmission in this system is know as "one-to-one", as every action potential in the nerve leads to an action potential in the muscle fibre (Sargent, 1992). At the NMJ, the nerve terminal of the motor fibre, releases acetylcholine (ACh) as the neurotransmitter, following an action potential. This ACh then binds to the ACh receptors concentrated on the postsynaptic membrane, producing a potential, which in the NMJ is called the *endplate potential* (EPP). In the CNS, however, the major excitatory transmitter is glutamate. Glutamate opens postsynaptic cation selective channel. An important difference between EPSPs and EPPs is that the former are almost always below threshold, usually summing with other EPSPs allowing some integration to occur, whilst the EPPs are suprathreshold, apart from very immature synapses (Sargent, 1992)

Bearing these differences in mind, the factors that affect synaptic efficacy are:

- Transmitter release
- Rate of rise of depolarisations
- Channel properties
- NMDA receptors
- AMPA receptors

*Transmitter release:*

Depolarisation of the presynaptic terminal causes voltage sensitive $Ca^{2+}$ channels on the surface of the terminal to open and $Ca^{2+}$ influx into the terminal. It is the influx of $Ca^{2+}$ to the presynaptic terminal which triggers the release of transmitter by exocytosis at a region of the nerve terminal that directly opposes the postsynaptic membrane, the active zone (Katz and Miledi, 1967).

The transmitter is released in packages called quanta (Fatt & Katz, 1951) The number of quanta released in the developing NMJ are initially low (E15-16), but increases as the synapse matures (Bennett and Pettigrew, 1974), although the quantal content in the first postnatal week, in the intercostals, was not significantly different to the levels at E15-18 (Dennis et al., 1981), suggesting that in this system maturational changes are slow in the first postnatal week. This increase in quantal content is likely to be caused by the increase in the number of quanta available rather than a change in the probability of release (Robbins and Yonezawa, 1971). For the first few days after first neuro-muscular contact there is low quantal content, and the EPPs are subthreshold (Bennett and Pettigrew, 1974).

The ACh receptor channel properties also change during maturation. At immature endplate the channels have longer opening time than they do at mature endplates (Sakmann and Brenner, 1978). The change in channel opening time has now been attributed to the subunit composition of the channel reviewed by (Schuetze and Role, 1987). The ACh receptor with the slow channel properties contains the  subunit and those with the fast properties contain the  subunit. The proportion of slow endplate channels decreases during early postnatal life. For example, in the developing soleus the percentage of slow channels decreases from 100% at birth to less than 20% at P21 (Sakmann and Brenner, 1978). There is a corresponding increase in the mRNA coding for the  subunit, and a loss of mRNA coding for the  subunit, over this time period (Brenner et al., 1990)

*Rate of rise of depolarisations:*

The rate of rise of EPPs is slow at E14/15, as is the rate of decay, this becomes significantly faster after E16 when clustering of the ACh channels begins on the postsynaptic membrane (Dennis et al., 1981; Redfern, 1970). Before E16 spontaneous EPPs have been recorded, whilst after this time EPPs need to be evoked (Bennett and Pettigrew, 1974).

In motoneurones, the amplitude of the average dorsal root evoked monosynaptic EPSP, does not change significantly during the first postnatal week, but the average latency and rise time of EPSPs decreases significantly over this same time period (Seebach and Mendell, 1996), so contributing the decreased central delay. The input conductance of motoneurones is observed to significantly increase when comparing two age groups P1-3 and P7-9 (Seebach and Mendell, 1996). Others (Fulton and Walton, 1986), suggest that this may be as a result of the growth of the motoneurones, during this time. These results are consistent with the reported Ia afferent monosynaptic reflex central delay in P3 lumbar cord of 5.7±0.5 ms and polysynaptic cutaneous reflex delay of 14.2±3.6ms (Fitzgerald et al., 1987).

*Channel properties:*

The rate of turnover of ACh receptors is also developmentally regulated, and is dependent on clustering at the endplate. In chick muscle, about one week after hatching, the half-life of both junctional and extra-juctional receptors is about 30 hours. A few weeks later, then half-life of the juctional receptors has increased to 5 days but that of the extra-junctional receptors remains about the same (Burden, 1977). The function of the receptors in the developing muscle also changes during this period, the average channel opening time of the embryonic receptors is curtailed (Sakmann and Brenner, 1978).

*NMDA:*

NMDA receptor function has been reported to be transiently enhanced during early postnatal life in the visual cortex of kittens (Fox et al., 1991; Tsumoto et al., 1987), and this is thought to mediate plasticity during this period. The duration of NMDA EPSPs in the superior colliculus is longer in the early postnatal development than in

older animals, although the amplitude of the EPSC does not change during development (Hestrin, 1992). The slow time course of the NMDA mediated EPSC in the early postnatal period can be explained either by the slow removal of transmitter from the synaptic cleft or by the intrinsic channel kinetics of the receptor (Hestrin, 1992; Lester et al., 1990). It has been demonstrated in the hippocampus, by looking at the effect of the NMDA antagonist AP5, that this slow time course can *only* be explained in terms of receptor kinetics (Lester et al., 1990).

The ESPC decay time decreases significantly during postnatal development in the superior colliculus. It is recorded as 213±57ms at age P10-P15, 127±79ms aged P16-P22, and 85±37ms in animals aged P23-P33 (Hestrin, 1992). These changes in the NMDA mediated EPSC reflect the changes in channel kinetics and are not influenced by transmitter release (ascertained by examining the miniature spontaneous EPSCs, which are thought to represent the release of transmitter packets at single synaptic contacts) (Hestrin, 1992). Changes in NMDA EPSCs may reflect developmentally controlled expression of NMDA receptor subunits (Moriyoshi et al., 1991). NMDA receptor mediated EPSCs have a role in governing activity dependent synaptic plasticity, as they allow $Ca^{2+}$ influx at synaptic sites. The developmental change in kinetics of the receptor, means that the threshold for activation of NMDA EPSCs changes too. The immature NMDA receptor is permissive, requiring moderate activity to induce the formation of specific neuronal wiring, whilst the mature form of the receptor has an increased threshold of activation (Hestrin, 1992).

NMDA receptors are thought to be heteromers, composed of subunits NMDA receptor 1 (NR1) and NR2 (Monyer et al., 1994; Tölle et al., 1993). Inital studies report that in the mature dorsal horn the predominant form of NR2 is NR2D, when this combines with NR1, it forms an NMDA receptor with lower $Mg^{2+}$ sensitivity and slower kinetics (Monyer et al., 1994). The foetal dorsal horn expresses high levels of the NR2D subunit and low levels of the NR2B subunit. The NR2B subunit, when it combines with the NR1 subunit, forms NMDA receptors with fast kinetics and high $Mg^{2+}$ sensitivity (Monyer et al., 1994). More recent single channel recordings from dorsal horn neurones have demonstrated that channels with different conductance levels are present. The proposed subunit construction of these channels includes units NR2A/B (higher conductance) and NR2D (lower conductance) (Momiyama et al., 1996). Other brain areas and developing neurones, that include the NR2A subunit, have fast kinetics (Flint et al., 1997; Monyer et al., 1994)

This lower sensitivity to the voltage sensitive $Mg^{2+}$ block in the embryonic dorsal horn, appears to be particular to this part of the CNS. Not all investigators find this result. In a study examining dorsal root evoked spontaneous activity in the dorsal horn, a dramatic decrease in this activity was noted in younger preparations after the addition of 1mM $MgCl_2$. (Abdul-Razzak et al., 1994). A 92% reduction was reported at P6, 68% at P10, 48% at P16 and 13% at P28, there was not effect in the adult.

*AMPA:*

The expression of the subunits making up the AMPA receptor is developmentally regulated (Jakowec et al., 1995a). GluR1 and GluR2/3 stain strongly in the SG, but also in cells throughout the grey matter, in young postnatal animals. GluR4 is seen in large multipolar cells throughout the grey matter. GluR1 reaches a maximum value at P7, and GluR2/3 between P7 and P14, after which levels decrease to the adult values (Jakowec et al., 1995b). Quantitative autoradiography, shows an overshoot, in expression, of 140-170% of adult levels, in several brain structures (Standley et al., 1995). Different combinations of the subunits infer different functional properties to the receptor. Absence of the edited form of GluR2, in particular, makes the receptor more permeable to $Ca^{2+}$ and monovalent cations, and this subunit decreases during the first postnatal month, suggesting that older cells have less protection from the neurotoxic effects of $Ca^{2+}$ influx (Standley et al., 1995).

*Other Channels:*

Early in development $Ca^{2+}$ channels change from predominantly slow, low voltage activated, T type to predominantly N and L type, which transmit faster, but have a higher activation threshold (Barish, 1986). The precise characteristics of $Ca^{2+}$ channel in the presynaptic membrane of most vertebrate neurones is not known, as they do not correspond to either the L or T type (Sargent, 1992).

From E17, both tetrodotoxin sensitive and resistant $Na^{+}$ channels are present in the DRG. Their kinetics do not change considerably during development (Ogata and Tatebayashi, 1992).

### 3.32 Maturation of receptive field properties: *convergence and receptive fields.*

*CONVERGENCE OF AFFERENT INPUT IN POSTNATAL DORSAL HORN CELLS:*

When examining the responses to natural stimulation low threshold inputs are dominant in the very young dorsal horn. In young animals, the percentage of cells with convergent input was only 6% but had increased to 57% by P21. This result agrees with a trend reported in an earlier study (Fitzgerald, 1985a). The small number of convergent neurones in the first two weeks probably reflects the maturation of synapses discussed above.

In light of the reported lack of C fibre synaptic connectivity before P5 (Pignatelli et al., 1989), and the reports that C fibre evoked responses cannot be recorded before the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987; Jennings and Fitzgerald, 1998). Furthermore, reports of the presence of silent synapses in the dorsal horn first two postnatal weeks (Bardoni et al., 1998; Li and Zhuo, 1998), and that the peak of synaptogenesis in the dorsal horn is postnatal (Cabalka et al., 1990), suggest that this time is one of intense activity dependent plasticity, where appropriate synaptic connections between incoming afferent collaterals and dorsal horn cells, are the eventual survivors.

*RECEPTIVE FIELDS OF POSTNATAL DORSAL HORN CELLS.*

One of the most striking results from this study is the decrease in size of peripheral receptive field with increasing age *FIGS 3.3 & 3.4*. This trend has been reported in a previous study (Fitzgerald, 1985a). The central terminal fields of primary afferents in the newborn rat dorsal horn, occupy the same medial/lateral, and rostro/caudal area as they do in the adult (Fitzgerald and Swett, 1983; Smith, 1983). The underlying cause of this phenomenon is likely to be in the maturation of the afferent input to individual dorsal horn cells.

*Maturation of the dorsal horn:*

Axodendritic growth in superficial dorsal horn cells only begins in the postnatal period, in contrast to motoneurones, intermediate cells and deep dorsal horn projection neurones where maturation is well under way (Bicknell and Beal, 1984). This reflects the reported ventro dorsal gradient (Altman and Bayer, 1984; Nornes and Das, 1974). Synaptogenesis is also reported to follow this gradient with the

peak of synaptogenesis occurring at P4-5, in the deep dorsal horn, and at P7-9 in the substantia gelatinosa (Cabalka et al., 1990). These studies highlight the immaturity of the superficial dorsal horn from an anatomical standpoint.

Cells in the deep dorsal horn have a higher cell resistance and action potential half width in the first postnatal week (P3-6 age group) than they do in the second (P9-16) (Hochman et al., 1997). Neurones were split into four groups based on their responses to current injection. There was no difference in the responses between the two groups. The subdivision of deep dorsal horn neurones into these groups has been used previously (Lopez Garcia and King, 1994), and the groups correlated with the primary afferent synaptic input, suggesting that membrane properties of deep dorsal horn neurones are functionally differentiated, from an early age (Hochman et al., 1997; Lopez Garcia and King, 1994).

*Changes in receptive field size in the adult dorsal horn:*

Receptive field sizes have been shown to increase again in the adult following a peripheral injury or inflammation (Calvino et al., 1987; Cook et al., 1987; Hylden et al., 1989; McMahon and Wall, 1984; Woolf and King, 1990). Furthermore animals that were treated with capsaicin, as neonates, have expanded receptive fields once they have grown up (Cervero and Plenderleith, 1987; Wall et al., 1982a; Wall et al., 1982b). These changes in receptive field properties appear to be confined to WDR cells, and there is no change in the receptive fields of cells with an A fibre only input (Cervero and Plenderleith, 1987). In the neonate these large receptive fields are a property of dorsal horn cells of all modalities.

Additionally, following axotomy A fibres invade the superficial lamina in a process called collateral sprouting, thereby mimicking the ontogeny of A fibre development where A fibres are found in the superficial laminae for the first three weeks of rat life (Fitzgerald et al., 1994; Mirnics and Koerber, 1995).

The capsaicin experiments point to an important role for C fibres in the maturation of appropriate dorsal horn connectivity. Along with the expanded peripheral receptive fields in the spinal cord, expanded receptive fields of barrel cortex cells have been reported, following treatment with capsaicin. Although cortical anatomy appeared to be the same following treatment, cells responded to more than one whisker (2-7), in

contrast to the precise mapping seen in naive animals (Nussbaumer and Wall, 1985; Wall et al., 1982a).

### 3.33 Role of C fibres in the dorsal horn maturation

Although the C fibres enter the dorsal horn at E18-19 (Fitzgerald et al., 1991; Fitzgerald, 1987b; Jackman, 1997; Konstantinidou et al., 1995; Mirnics and Koerber, 1995; Plenderleith et al., 1992; Smith, 1983), the results here confirm that the functional maturation of C fibre evoked activity takes places over an extended postnatal period(Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987). The neurones in SG, which are the target neurones of the C fibre afferents, are the last to differentiate and mature in the spinal cord (Altman and Bayer, 1984; Nornes and Das, 1974), and it appears that the axodendritic growth in the region only begins postnatally (Bicknell and Beal, 1984). Furthermore, C afferent type terminals are not observed within the synaptic glomeruli until P5 (Pignatelli et al., 1989)

Administration of capsaicin at birth results in the death of the majority of C fibres, and this can be seen in the loss of the late wave from the cutaneous afferent volley, from P3 onwards (Fitzgerald, 1988). Despite this, evoked activity, from peripheral electrical stimulation in dorsal horn cells remains unchanged. This suggests that although both early and late (corresponding to A & C fibre) components of the afferent volley are present in the dorsal root of the neonatal rat, only the fastest, capsaicin resistant, large diameter, A fibre component is able to evoke dorsal horn activity (Fitzgerald, 1988).

Responses to noxious mechanical stimulation or chemical irritants, such as formalin, carageenan, CFA (complete Freund's adjuvent), produce clear reflex activity and c-fos induction from birth although both of these stimuli also activate A fibres (Fitzgerald and Gibson, 1984; Jennings and Fitzgerald, 1996; Williams et al., 1990a). But the C fibre specific irritant, mustard oil, does not evoke the flexor reflex before P10-11 (Fitzgerald and Gibson, 1984), suggesting that responses to pure C fibre inputs remain subthreshold until the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1988). Other studies in the first postnatal week, examining the ventral root reflex (Fitzgerald et al., 1987), and the flexor reflex (*in vitro*) (Hori and Watanabe, 1987), fail to show long latency C fibre evoked responses.

This does not mean that there is no C fibre effect in the first postnatal week, but rather that there is no suprathreshold C fibre evoked activity. Further intracellular

experiments are required to determine the exact nature of the subthreshold C fibre effect. Alternatively experiments in which C-fibre conditioning stimuli are administered, could lower the activation threshold, and allow the previously subthreshold responses to become suprathreshold.

It has been demonstrated that the slow component of the ventral root potential (VRP) is lost in neonates (aged P4) if they are given capsaicin 2 days previously. This treatment with capsaicin has no effect on the fast component of the VRP (Akagi et al., 1985). This slow component has been shown to depolarise motoneurones in the first postnatal week (Brugger et al., 1990). The slower latency of this component when coupled the fact that it is lost after treatment with capsaicin, which causes the selective degeneration of small diameter primary afferents (Nagy and Hunt, 1982), suggests that it is evoked by C fibres. These studies have demonstrated depolarising C-fibre activity in the first postnatal week, from which we must surmise that there is subthreshold C fibre activity in the dorsal horn during this time.

Subthreshold C fibre responses would explain the potentiation of pinch responses seen following mustard oil (Fitzgerald, 1991b). Potentiated, EMG responses to electrical stimulation, after the application of peripheral mustard oil have been reported in neonatal rats from the age of P3 (Jiang et al., 1994). The authors conclude that since the hyperalgesia that they report, is most likely to be mediated by C-fibres, that there is some C- fibre functionality within the first postnatal week. They also report that the hyperalgesia increases as the rat matures confirming that the C-fibre input is immature in the first week. That theses experiments were performed on spinalised animals would suggest that one possible difference between these results, and those reported by Fitzgerald & Gibson (1984), is some form of descending influence onto this system that, in Fitzgerald's experiments, masks the C-fibre activity in the first postnatal week. Fitzgerald & Koltzenburg (1986), have demonstrated that descending inhibition via the dorsal lateral funiculus (DLF), is not functionally mature until the third postnatal week, this study has not exhausted all the possibilities of descending influence, but does account for the main source of descending modulation of afferent input.

The cells of the substantia gelatinosa are the last in the dorsal horn to mature, with the axodendritic growth of the majority , the non projection neurones, taking place postnatally (Bicknell and Beal, 1984). The formation of synapses by primary afferents proceeds in a ventral to dorsal manner, with the terminations on the superficial cells being the last to develop (Smith, 1983).

In addition, synaptogenesis in the rat has been reported to peak at P4-5 in the deep dorsal horn and P7-9 in the SG (Cabalka et al., 1990). This postnatal maturation of the SG is likely to be the anatomical basis for the reports that C fibre activation cannot evoke spike activity in the dorsal horn until the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987; Jennings and Fitzgerald, 1998). In fact, formation of C fibre synapses appears to be an entirely postnatal event. Synaptic glomeruli are not observed on C fibre terminals in the SG until P5 (Pignatelli et al., 1989).

The studies presented here confirm this delayed maturation of the C afferent response and show that, even at P21, the percentage of dorsal horn cells that can be excited by peripheral C fibre activation is still relatively low. Further experiments using intracellular recording techniques are required to study subthreshold postsynaptic potentials in the first postnatal week.

When examining the functional connectivity of the dorsal horn one should also consider the transmitter/ receptor development. The presence and distribution of many neurochemical markers associated with C fibres, SP, CGRP, FRAP, and thiamine monophosphatase (Coimbra et al., 1986; Marti et al., 1987; Pignatelli et al., 1989) do not attain their adult conformation until the second or third postnatal week. Substance P receptors are widespread in the neonatal cord and only become localised to the SG in the second postnatal week (Charlton and Helke, 1986; Kar and Quirion, 1995). Fluoride resistant acid phosphatase does not appear in the cord until birth and then only reaches full intensity after one week (Coimbra et al., 1986; Fitzgerald and Gibson, 1984). $\mu$ and $\kappa$ opiate receptors are present at birth, whilst the receptor is only seen at P7 (Rahman et al., 1998). At birth the distribution of $\mu$ receptor is diffuse throughout the cord, binding peaks in the first postnatal week and then decreases to adult levels. The distribution of the receptor sites gradually regresses and mainly occupies the superficial dorsal horn (Kar and Quirion, 1995; Rahman et al., 1998).

Fitzgerald has shown that there is a critical period for C fibre sprouting of the central terminals of dorsal roots following neonatal crushing of adjacent roots, at older postnatal ages or in adults this sprouting doesn't occur (Fitzgerald and Vrbova, 1985; Fitzgerald et al., 1990; Fitzgerald, 1985b). This may be a marker of C fibre maturity, as the *critical period* for sprouting to occur is between birth and P10, which reflects the time for which there is no suprathreshold C fibre evoked activity. It has been

suggested that this critical period may be linked to the developmental levels of GAP-43 (Fitzgerald et al., 1991). At birth there are high levels of GAP-43 mRNA present in the majority of spinal cord neurones and in all DRG cells. This persists until P7 after which levels decline to adult levels which are reached at P21 (Fitzgerald et al., 1991).

### 3.34 Development of inhibitory controls

The dosal lateral funiculus (DLF), only becomes functional in the second postnatal week in the rat (Fitzgerald and Koltzenburg, 1986), and the action of serotonin injected intrathecally has little antinociceptive effect until around the same age (Giordano, 1997). (See 4.33). In the adult rat model, expansion of the peripheral receptive fields of lumbar dorsal horn cells is seen following a lidocain block of the DLF (Ren and Dubner, 1996). This suggests that the large receptive fields seen in the neonate may be as a result of the lack of descending control in the early postnatal rat.

GABA is the most precocious neurotransmitter in the developing rat brain and is known to be released from developing GABA neurones (Lauder et al., 1986; Ma and Barker, 1995; Ma et al., 1993). Transient patterns of $GABA_A$ receptor subunit expression have been found in developing rat and primate brain (Ma et al., 1993; Ma et al., 1994). In the rat inhibitory neurotransmitters have also been shown to have a changing role with postnatal age, for example: Glycine depolarises rat hippocampal CA3 neurones at postnatal ages of less than four days old. In neonatal rats aged P5-7, glycine hyperpolarised these neurones, and in the adult it had no effect (Ito and Cherubini, 1991).

Iontophoresis of the $GABA_A$ antagonist, bicuclline, into the primary somatosensory cortex, results in the increase of receptive field size of the cells in layer IV of the cortex, where the barrel fields are usually seen, and recorded from (Fox et al., 1996). This suggests that both local inhibitory and descending control (Ren and Dubner, 1996), mechanisms may affect the receptive field size of dorsal horn cells.

Since the main excitatory and inhibitory systems are going through many changes over the neonatal period, they will exert considerable influence on the physiology of the system. One such manifestation will be discussed in much more detail in the following chapter.

### 3.35 Methodological Issues:

The technique used here did not allow precise location of recording sites. Cell populations were therefore segregated into superficial and deep groups, on the basis of depth, as recorded by the microdrive. There can be problems with this. Even though the dura had been removed, there were occasions when the electrode would "dimple" rather than penetrate the cord, presumably due to other layers of meninges, or relative bluntness of the electrode. If these meninges apply resistance to the barrel of the electrode, when it appears to be entering the dorsal horn normally, then the microdrive will be over-reading the depth of the electrode. It would be more satisfactory to be able to mark the area recorded from, using a glass recording electrode and biocytin, for example.

There is also a possibility that the recordings in the superficial dorsal horn, are actually from the dendrites of deep dorsal horn neurones, rather than from the SG neurones. Care was taken during recording to maximise the spike height, of a single cell, by tracking up and down with the electrode. Also during the first postnatal week the dendritic arborisation is poorly developed, making this unlikely (Altman and Bayer, 1984).

The A-fibre evoked responses that we record could contain an Aδ component, as well as involving Aβ fibres. The stimulation was at twice the threshold to evoke the first response from the cell. Dorsal root recordings in the first postnatal week show a single A fibre and a separate C fibre peak, the second (A) peak only being discerned at P6-8 (Fitzgerald, 1985a). The changes in diameter and myelination of the peripheral nerves in the first postnatal week are small (Friede and Samorajski, 1968; Webster, 1971), making it difficult to distinguish between the types of A fibre input during this time. There were cells at all ages that responded to either pinch or brush or both, (to either low or high threshold mechanoreceptor stimulation), suggesting that both Aβ and Aδ are active from P3. The total numbers of cells responding to high threshold mechoceptors very low at age P3 (9%), and P6 (16%), increasing to over 50% of cells at ages P10 and P21. This suggests that although the high threshold mechanoceptors and pathways are present from a young age, they are not fully functional until the second postnatal week.

Although the A fibres are shown to be anatomically present in the superficial dorsal horn for the first few postnatal weeks (Fitzgerald et al., 1994; Mirnics and Koerber, 1995), and there is ultrastructural evidence for synaptic formation whilst there

(Coggeshall et al., 1996), there is no direct physiological evidence that they are functional whilst in this temporary position. The A fibre responses that have been recorded here in the superficial dorsal horn, may all be polysynaptic. The immaturity of dendritic arborisation would argue against this in the first week postnatally (Altman and Bayer, 1984).

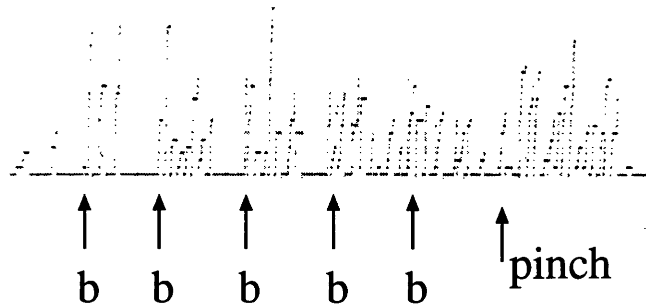
Cells were all recorded from the medial part of the lumbar spinal cord, as this area was easiest to access with the recording set-up as it was. As a result the majority of the peripheral receptive fields were in the tibial nerve dermatome, on the plantar surface of the hindpaw. These results therefore, are representative only of responses of neonatal cells in the medial half of the dorsal horn. The responses recorded in the lateral dorsal horn may be different.

The overall physiology of anaesthetised neonatal rats (and probably other mammals) is difficult to monitor and maintain effectively. Certainly strange responses were recorded from animals that died a few minutes later, although at the time the heart rate was within the normal parameters. These results were excluded from the study. Similar experiments occurring now in the lab use a mix of oxygen and air, and monitor $_{P}CO_2$. I monitored the general physiology of the neonate by recording heart rate alone.

Many anaesthetics appear to either depress or excite the neuronal responses of the spinal cord. Most depress the responses, *urethane* (which was the anaesthetic used in these experiments) is peculiar in that it can induce surgical anaesthesia with little effect on neurotransmission in subcortical areas and in the peripheral nervous system (Maggi and Meli, 1986).

## 3.4 Conclusion

This chapter focuses on the development of the afferent input to the spinal cord, and builds on earlier work by Fitzgerald (1985, 1988), and highlights some of the remaining unanswered questions.




scale bar=10secs

**Fig 3.1:** An example of a cell from animal aged P21, demonstrating a very clear brush (**b**) response and long lasting response to pinch with and after discharge. This cell wound up to repetitive C stimulation, but showed no sensitisation to A stimulation.

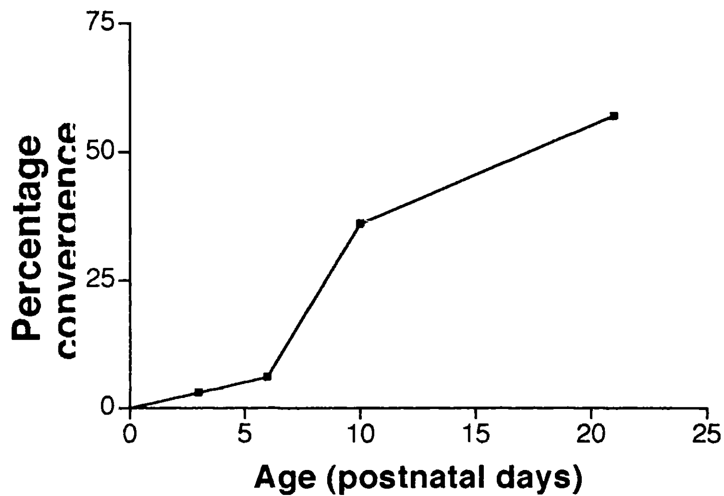
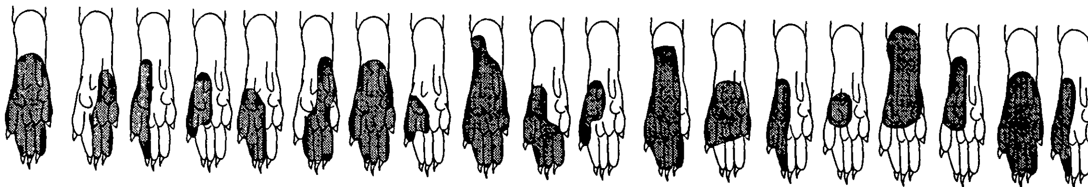
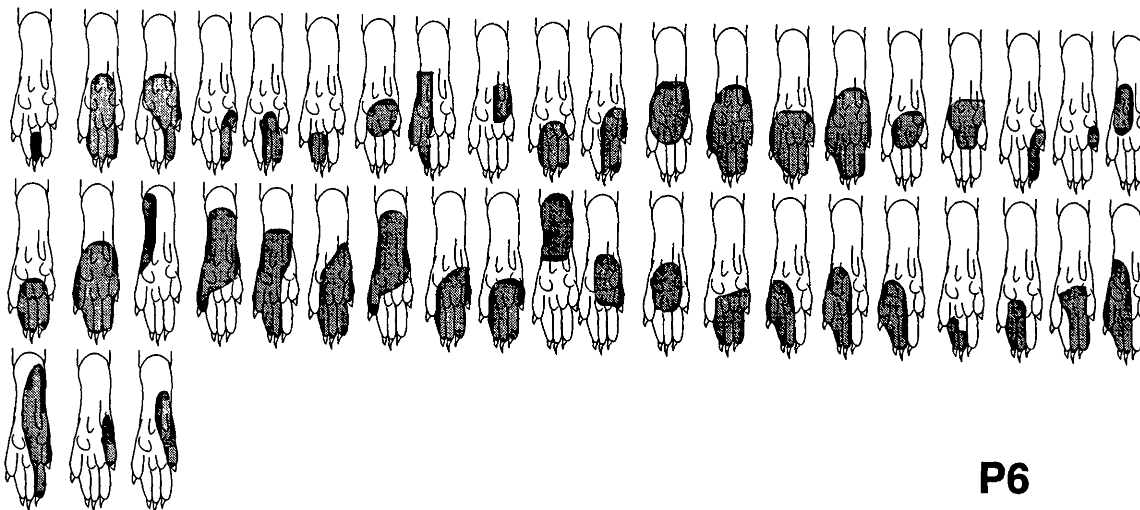



**Fig 3.2:**
Plot of the percentage of cells, at each age, responding to both pinch and brush, plotted against the age of the animal. This data is of the whole population of cells at these ages and includes cells in both superficial and deep laminae. Demonstratesthat there are many fewer cells with convergent input in the first postnatal week than later on in development. The percentage of convergent cells at P21is similar to that seen in the adult (Fitzgerald , 1982)

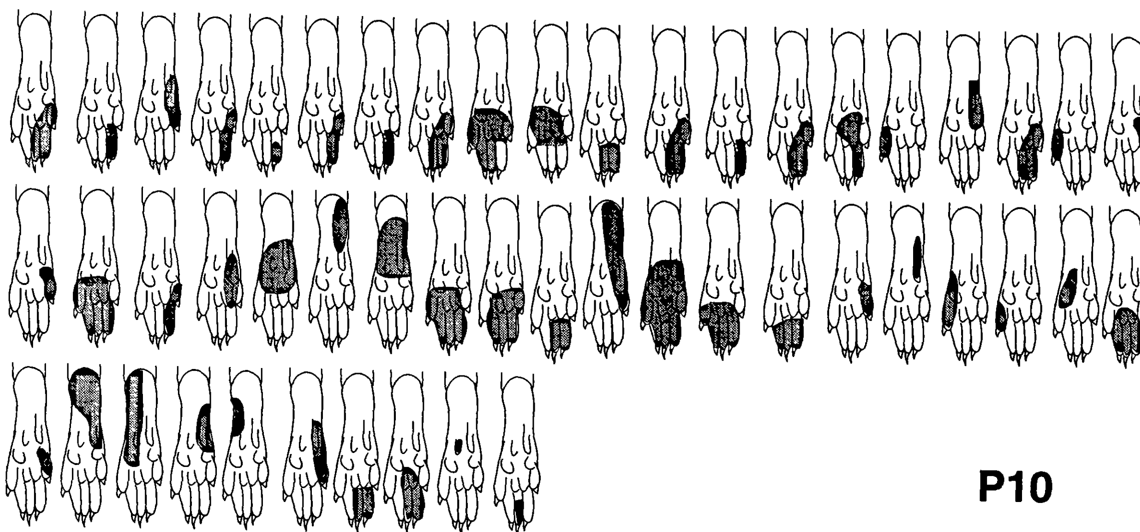
**Fig 3. 3:** Shows the peripheral receptive fields of cells recorded from, at different postnatal ages. See fig 3.4 & fig 3.5 for quantification of this data.



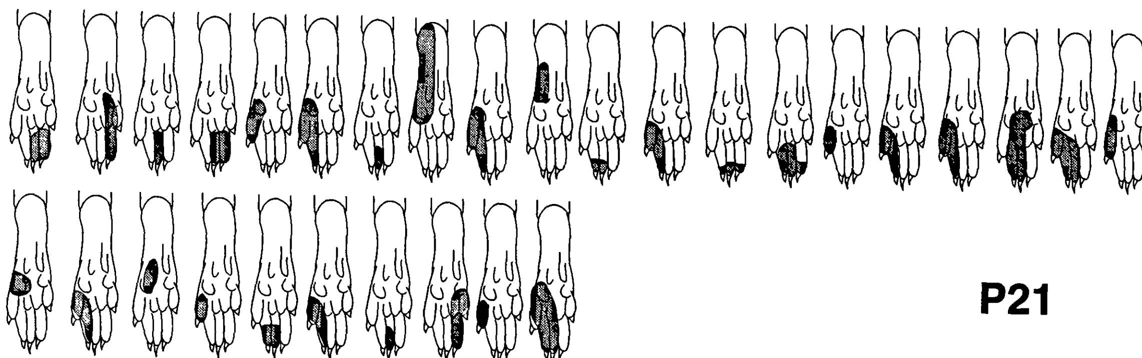
**P3**



**P6**



**P10**



**P21**

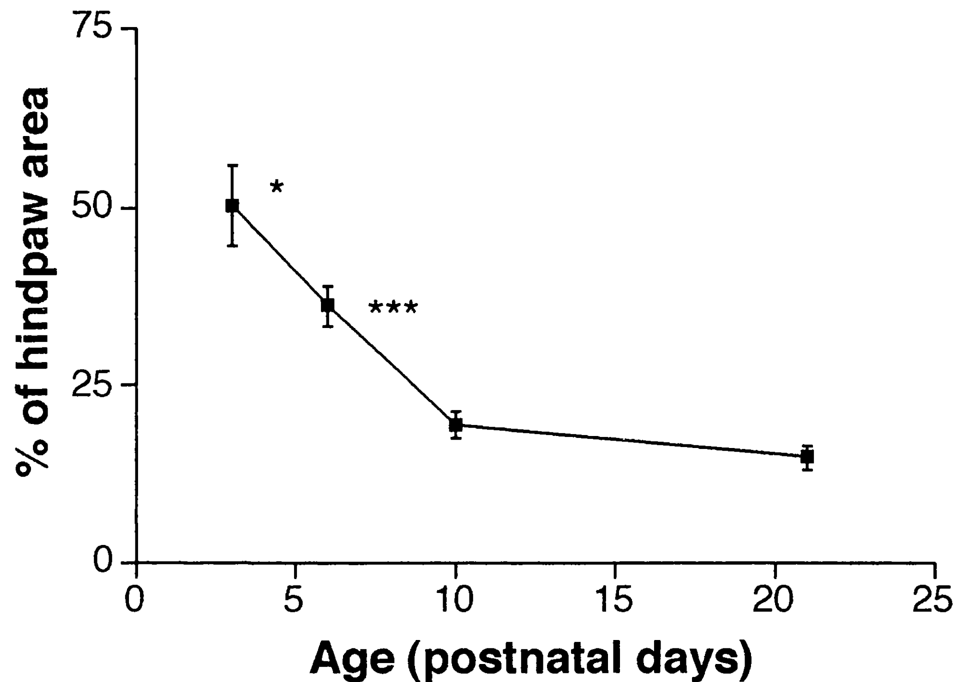
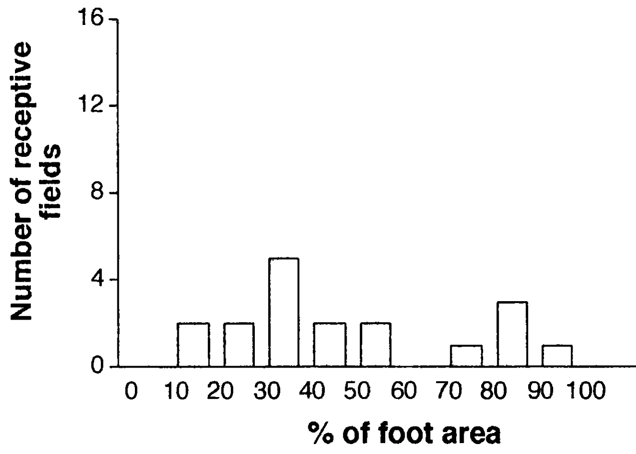
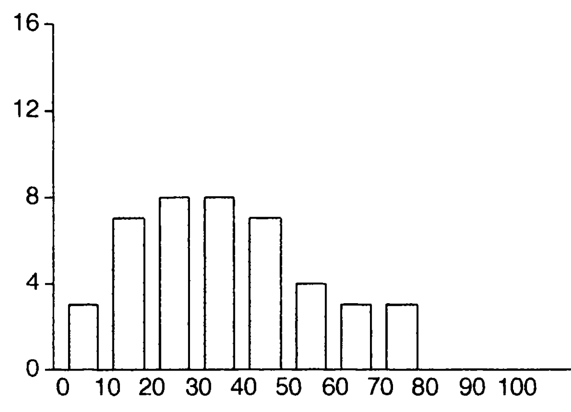
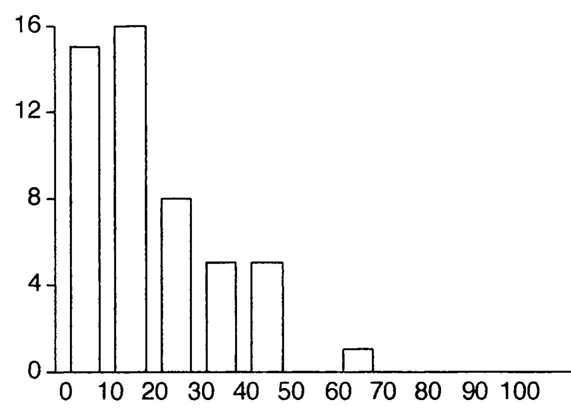



**Fig 3. 4:** Plot of the mean receptive field size ± SEM(as a percentage of the area of the plantar surface of the hindfoot), for each age investigated. There is significant reduction over the first 10 days of postnatal life. Comparisons of the means and SEM for each age, using the *student t test*, yields the following values of $p$. For P3/P6: $p=0.0397$, for P6/P10, $p<0.0001$ and for P10/P21, $p=0.093$.

**Fig 3.5:** Frequency distribution plots for receptive field sizes at the postnatal ages examined. Note the decrease in variability of the the field sizes and the decrease in size (as a percentage of the plantar surface of the hind foot).


P3
Number of receptive fields
16
12
8
4
0
0 10 20 30 40 50 60 70 80 90 100
% of foot area


P6
16
12
8
4
0
0 10 20 30 40 50 60 70 80 90 100


P10
16
12
8
4
0
0 10 20 30 40 50 60 70 80 90 100

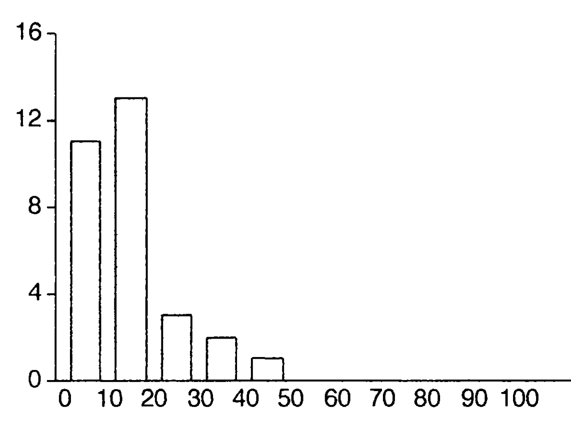
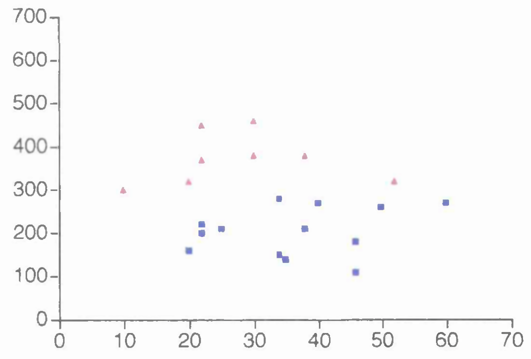
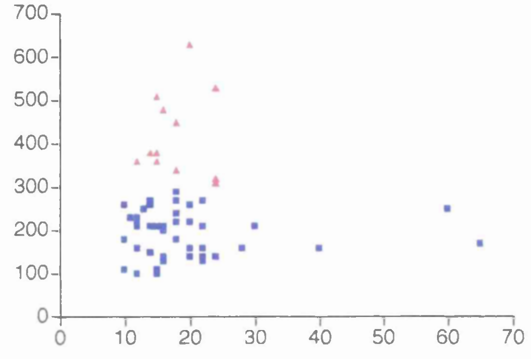
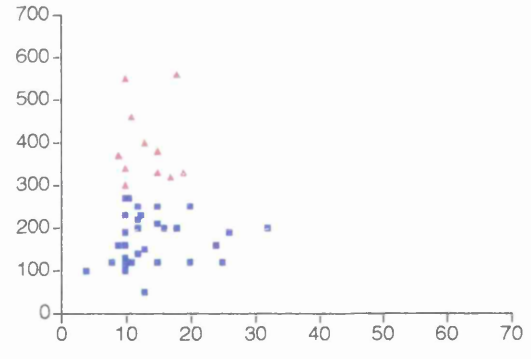

P21
16
12
8
4
0
0 10 20 30 40 50 60 70 80 90 100

**Figure 3.6: Postnatal latencies of A fibre evoked response.**
Scatter plots showing the latency of the A fibre evoked response plotted against depth of the cell from the cord surface, for the different postnatal ages examined. The red triangles (▲) represent deep cells and the blue squares (■) the cells in the superficial laminae. (The mean latencies ± SEM for the deep cells, at postnatal ages P3, P6, P10 and P21 respectively are: 28.0±4.5, 17.9±1.2, 13.1±1.0, and 6.45±0.4. The latencies for the superficial cells are: 36.3±3.4, 19.4±1.2, 13.7±0.9, and 7.8±0.5).

Fig 3.6



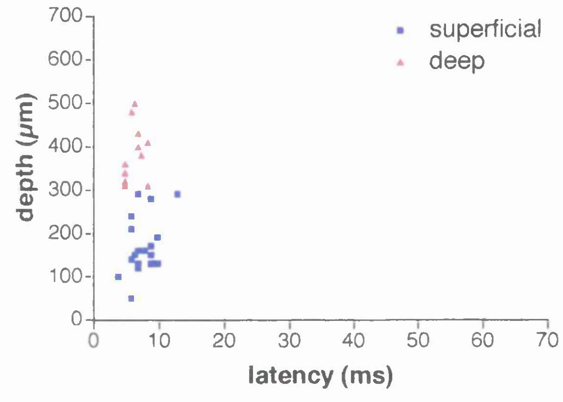
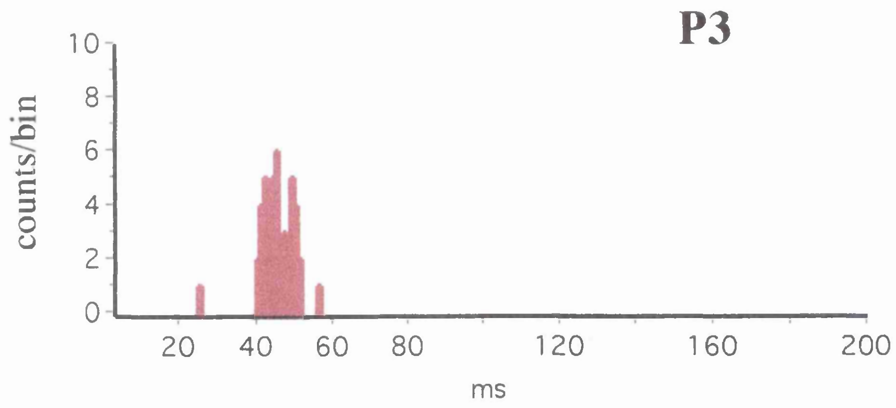
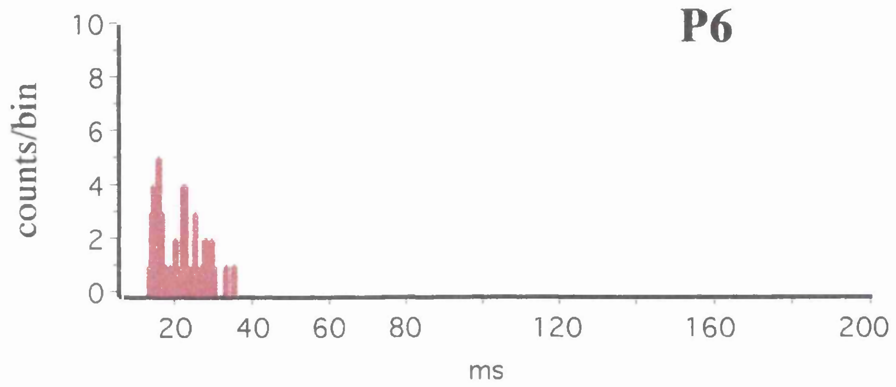
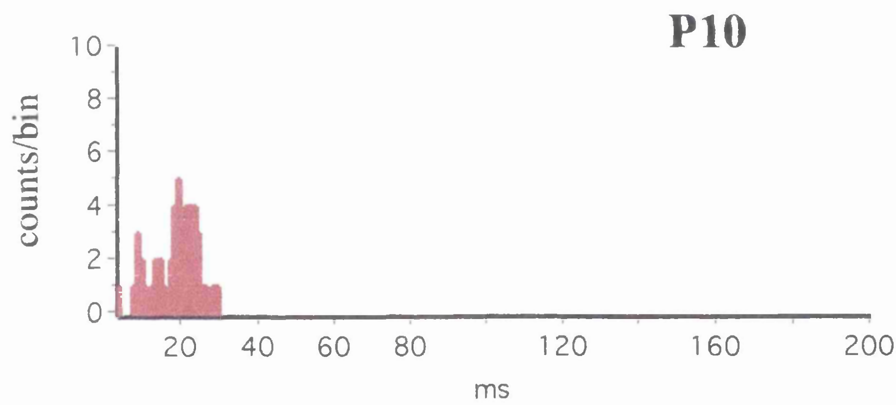



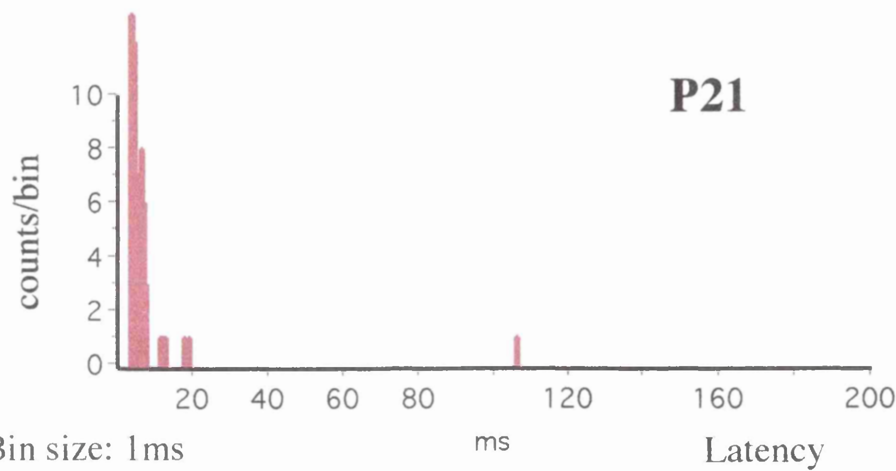
**Fig 3.7:** Typical examples of post stimulus time histograms (PSTH), showing the responses to a train of sixteen stimuli, at a frequency of 0.5Hz. Note the decrease in latency with age.

Fig 3.7



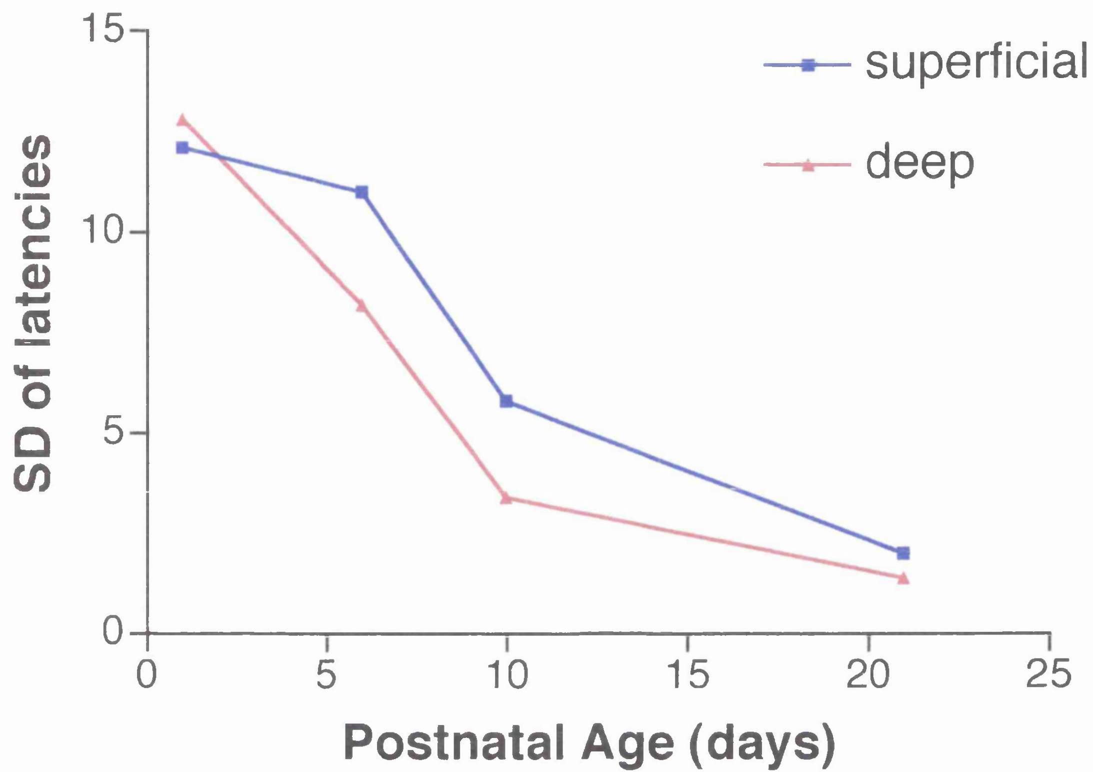
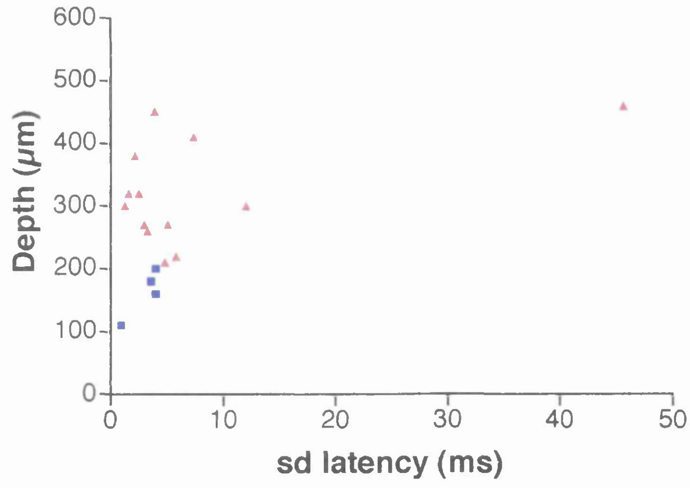
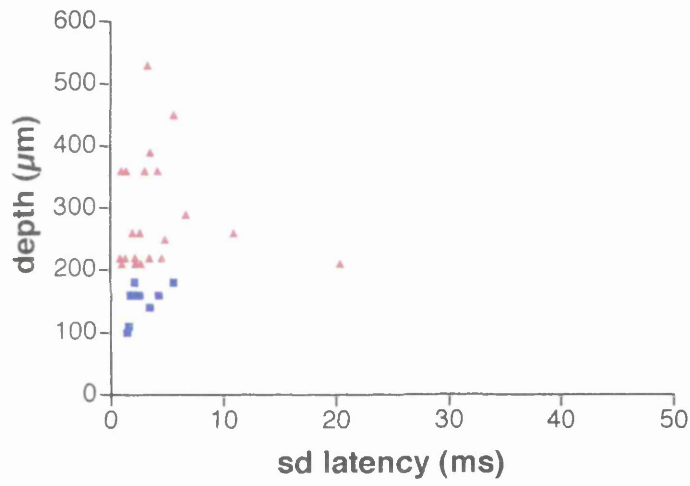



**Fig 3.8:** Postnatal variation in evoked latencies (sd).
The change in variation of response latency in individual cells on repeated A fibre stimulation with postnatal age. The variation is expressed as the standard deviation of the mean latency for the population of cells at each age.

**Fig 3.9: Stability of evoked latency on repeated stimulation.**
The "latency jitter" of dorsal horn cells at P3, P6 and P10. Latency jitter is the variation the response latency recorded from an *individual* cell, when comparing the evoked response to each stimulus in the train (16 stimuli at 0.5Hz). This is different to the variation in latency of the whole cell population at a specific age (Fig 3.8). Cells in the superficial dorsal horn are represented with the symbol (■), and those in the deep dorsal horn with (▲) .


P3
Depth (µm)
600
500
400
300
200
100
0
0
10
20
30
40
50
sd latency (ms)


P6
depth (µm)
600
500
400
300
200
100
0
0
10
20
30
40
50
sd latency (ms)

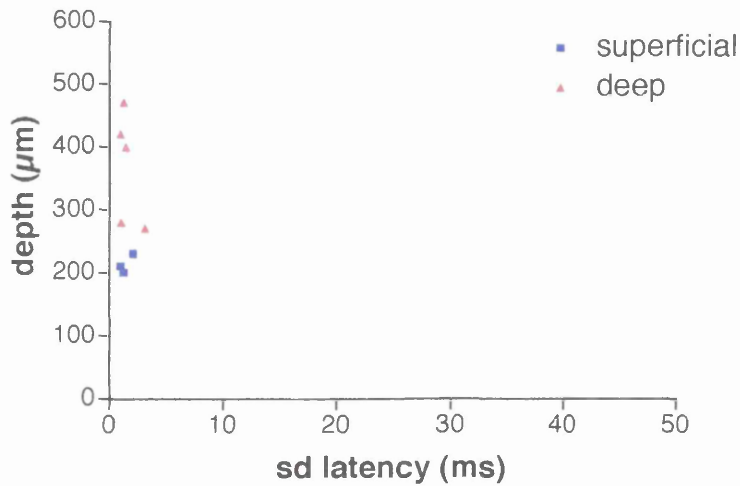
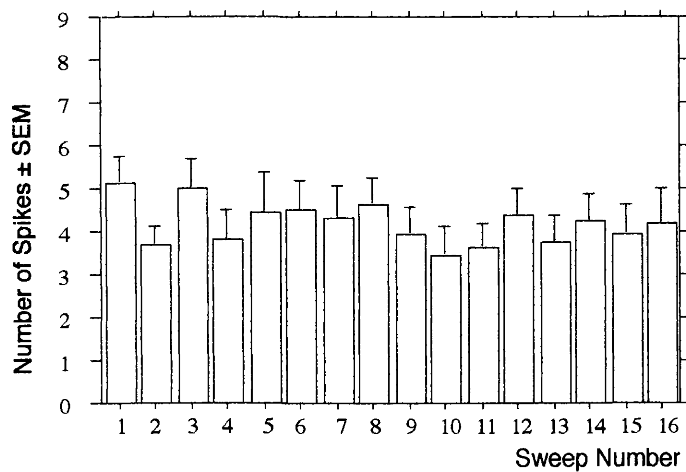
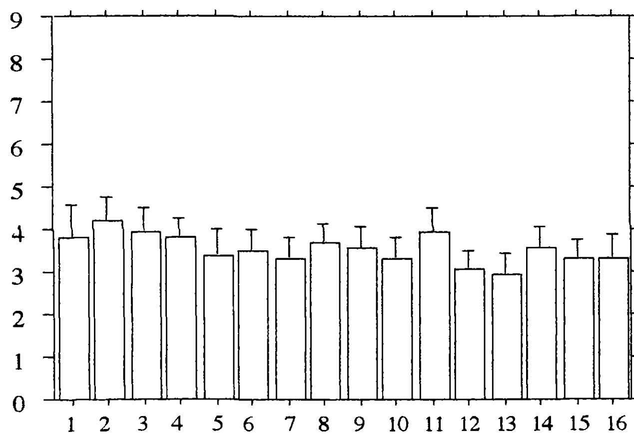
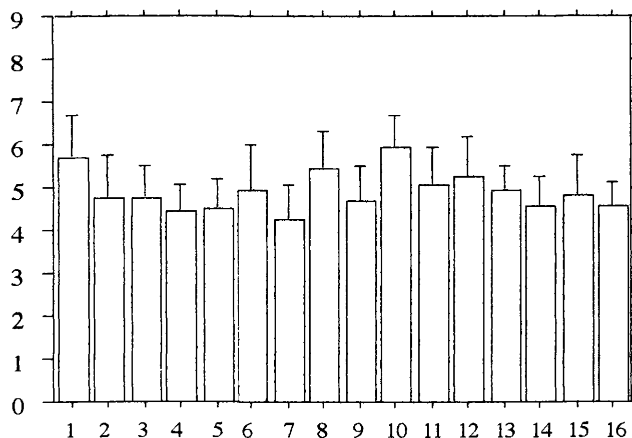

P10
superficial
deep
depth (µm)
600
500
400
300
200
100
0
0
10
20
30
40
50
sd latency (ms)

**Fig 3.10: Magnitude of the evoked response.**
Bar charts of the mean±SEM number of spikes evoked by A fibre electrical stimulation A train of sixteen stimuli was used (twice threshold, 0.5Hz). The P3 data is from 16 cells, the P6 is a mean from 24 cells, and the P10 data is from 12 cells.

**Fig 3.10**



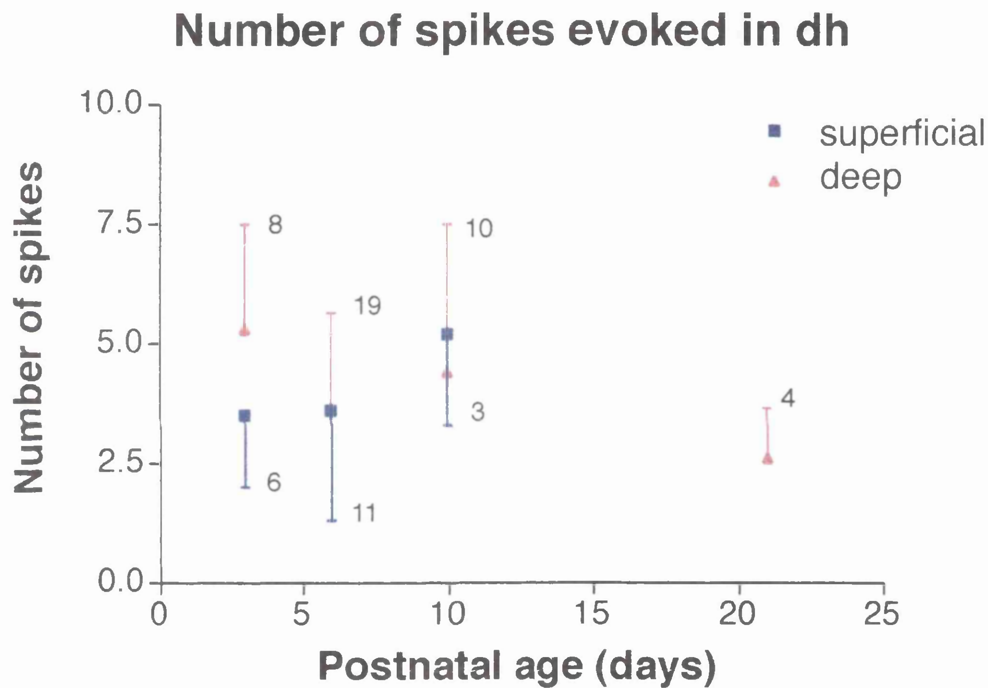



**Fig 3.11:** Mean ±SD number of spikes following train of sixteen stimuli (magnitude of the response) plotted against age. There appears to be no significant trend. The numbers beside each error bar represent the number of cells in each sample.

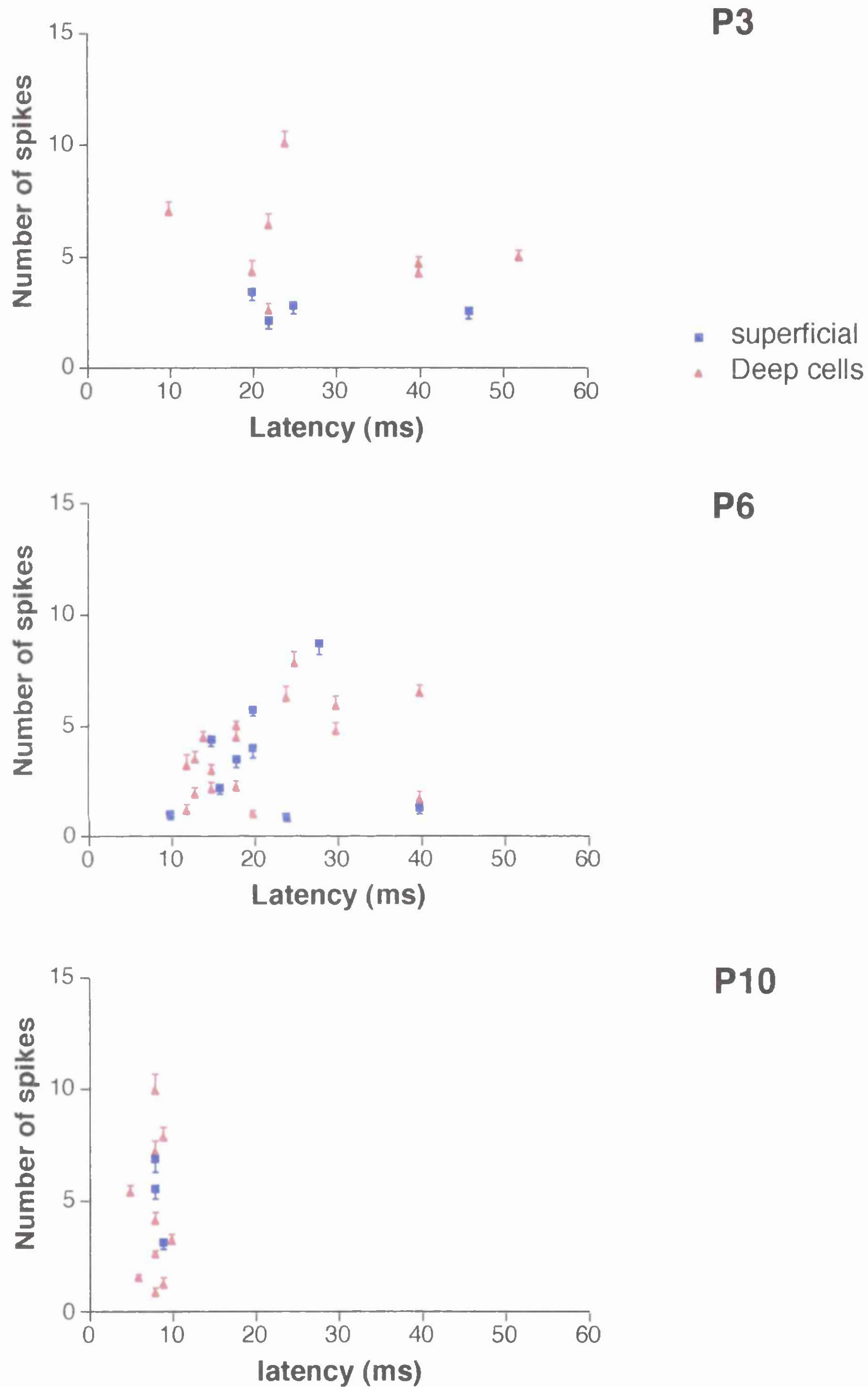



**Fig 3.12:** The mean ± SEM number of spikes plotted against latency for each age. There appears to be some correlation at P6 between the two parameters. At this age, those cells which fire more spikes also have a longer latency. The number of spikes (magnitude of the response) does not seem to be dependent on the depth of the at any age examined. (see fig 3.spikes)

# Chapter 4

## A fibre induced sensitisation

### 4.1 Introduction

The post-injury pain hypersensitivity of the flexion withdrawal reflex in animals (Woolf, 1983; Woolf, 1984), and pain sensations in man (LaMotte et al., 1983), are thought to relate to the increase in sensitivity of the nociceptors in the region of the injury (peripheral sensitisation) (Bessou and Perl, 1969; Campbell et al., 1979) and the increase of excitability of cells in the dorsal horn (central sensitisation) (Woolf, 1983). "Wind-up" is one form of central sensitisation. This was first described by Mendell & Wall in 1965, who demonstrated that when a peripheral nerve was stimulated at sufficient intensity to evoke C fibre action potentials, then repeating the stimulus at low frequencies results in a progressive build up in the amplitude of the central evoked response. They termed this phenomenon "wind-up", because the C evoked response appeared to do just that, with progressive afferent input volleys. (Mendell and Wall, 1965; Mendell, 1966).

Single unit dorsal horn studies have shown that wind-up is associated with an increase in the ongoing activity, expansion of the central (dorsal horn) receptive fields and a lowering of threshold of dorsal horn cells (Cook et al., 1987; McMahon and Wall, 1984). Wind-up has been observed in the dorsal horn cells of cat, rat and primate, and in ventral horn, trigeminal and thalamic cells (Chung et al., 1979; Kawakita et al., 1993; Mendell and Wall, 1965; Price et al., 1971; Woolf and King, 1987; Woolf and Swett, 1984). It has also been reported in humans (pain sensations appear to wind-up on repeated noxious stimulation) (Kristensen et al., 1992; Price et al., 1994).

Many investigators have developed animal models of central sensitisation to peripheral stimuli, induced by chemical substances or lesions to soft tissue or peripheral nerves (reviewed by: Coderre et al., 1993; McMahon et al., 1993). The results from these experiments indicate that central sensitisation, in the spinal cord, is different from other forms of synaptic plasticity such as that reported in the hippocampus. Long term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973), requires a brief high frequency input, and gives a potentiated response for a

long period of time. Central sensitisation, however, is induced by low frequency (<2Hz) high intensity stimuli, and is manifest following a train of repetitive stimuli (Woolf et al., 1988; Woolf, 1996).

In the hippocampus, a second form of potentiation, short term potentiation (STP) has been reported (Bliss and Collingridge, 1993). This type of potentiation decays within and hour, whilst LTP can be sustained for much longer periods(Bliss and Collingridge, 1993). STP can be induced by lower frequency tetanus, whilst LTP requires a much higher tetanus (typically a train of 50-100 stimuli given at 100-400Hz). Both STP and LTP have also been reported in the spinal cord (Liu and Sandkuhler, 1995; Pockett and Figurov 1993; Randic et al., 1993; Schouenborg, 1984; Svendsen et al., 1997).

Despite their differences, there are many close parallels between central sensitisation and LTP (Willis, 1997). They are both activated in neuronal circuits, by glutamate acting at either ionotropic or metabotropic receptors, and the associated influx of $Ca^{2+}$ appears to be important for both mechanisms.

*A brief account of the mechanisms of wind-up and central sensitisation:*

At resting membrane potentials the NMDA receptor ion channel is blocked by a $Mg^{2+}$ which prevents the influx of $Na^{+}$ and $Ca^{2+}$ ions (Mayer et al., 1984), so there is no current flow even if glutamate binds to the receptor. If the membrane is depolarised however, the $Mg^{2+}$ ion is removed from the channel and allows the influx of $Ca^{2+}$ and $Na^{+}$, which further depolarise the cell.

In the spinal cord wind-up requires a train of low frequency stimuli. The long duration of the synaptic potentials, generated by C fibres (up to 20s) and low repetition rates mean that marked temporal summation can occur, leading to cumulative depolarisation, and this removes the $Mg^{2+}$ ion block from the NMDA receptor ion channels (Thompson et al., 1990). The summation increases with each successive input, and generates a progressive increase in action potential discharge (wind-up) (Sivilotti et al., 1993). Wind-up has been shown to be dependent on NMDA receptor activation, with antagonists blocking it both *in vivo* and *in vitro* (Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Thompson et al., 1990; Woolf and Thompson, 1991). Wind-up has also been shown to be blocked strongly by antagonists to $NK_2$ (neurokinin A (NKA) receptor), and weakly by antagonists to $NK_1$ (the substance P receptor) (Nagy et al., 1993).

Wind-up is just one manifestation of central sensitisation. Central sensitisation results in changes in receptive field properties: there is a reduction in threshold, an increase in responsiveness and spatial extent and the recruitment of naive inputs (Cook et al., 1987; Woolf, 1983; 1996). Central sensitisation is like wind-up, prevented by NMDA receptor antagonists (Woolf and Thompson, 1991; Ren et al., 1992; Coderre & Melzack, 1992).

Cumulative depolarisation produces a large increase in intracellular $Ca^{2+}$ directly through the unblocked NMDA channel (MacDermott et al., 1986), and indirectly through G protein coupled neurokinin receptor activation, and the subsequent release of intracellular $Ca^{2+}$ stores (Heath et al. 1994). Increase in the intracellular $Ca^{2+}$ leads to the activation of protein kinase C (PKC) and other kinases, and PKC acts on the NMDA receptor by phosphorylating it and partially removing the $Mg^{2+}$ block (Chen and Huang, 1992). At normal resting potentials then, glutamate binding to the NMDA receptor will activate it. It is this increase in glutamate sensitivity that is thought to be responsible for the electrophysiological changes observed in central sensitisation, where inputs that were previously subthreshold begin to generate action potential discharges, altering the receptive field properties and making the whole system hypersensitive (Woolf and King, 1990)

In the adult spinal cord, and *in vitro*, wind-up cannot be elicited by the short duration A stimuli, as temporal summation cannot occur (Sivilotti et al., 1993). In the neonatal spinal cord, however this may not be true, since A fibre inputs terminate more superficially than they do in the adult {Fitzgerald et al., 1994; Mirnics and Koerber, 1995}. In the neonatal rat, cat and human, the cutaneous flexion withdrawal reflex can be evoked by low threshold mechanical stimuli (Andrews and Fitzgerald, 1994; Ekholm, 1967; Fitzgerald and Gibson, 1984; Fitzgerald et al., 1988), although the reflex has been shown to be purely nociceptive in the adult (Sherrington, 1910; Woolf, 1983).

In addition, c-fos can be induced by innocuous stimulation in the neonatal dorsal horn (Jennings and Fitzgerald, 1996). In the adult this requires noxious stimulation (Bullitt, 1990; Herdegen et al., 1991; Honore et al., 1995; Hunt et al., 1987; Lima et al., 1993; Menetrey et al., 1989; Williams et al., 1990b), except in cases of injury and inflammation, presumably once central sensitisation has been induced, where it can be induced by innocuous stimulation (Ma and Woolf, 1996).

The study reported in this chapter examines the effects of repeated A stimulation upon the responses of dorsal horn cells in the neonatal rat.

Some of the data presented in this chapter has been published elsewhere (Jennings and Fitzgerald, 1998).

## 4.2 Results

The following results were obtained from the same population of dorsal horn cells as described in chapter 3. Following initial localisation of each cell, and testing its modality to natural stimulation, mapping its receptive field and recording threshold and latency, the cells were then stimulated with a train of sixteen stimuli, at twice threshold (2T), at a frequency of 0.5Hz. Stimuli were delivered through pin electrodes placed in the centre of the peripheral receptive field on the hindlimb.

This stimulus paradigm could produce considerable sensitisation of the dorsal horn cells in the neonate. This sensitisation is illustrated in *FIG 4.1.* and took the form of a build up of background activity in the cells during repetitive stimulation that outlasted the stimulation period thereby producing a prolonged afterdischarge of up to 138 s. It was particularly apparent in younger animals and at P6, 19 out of 57 cells (33%) displayed background firing during, and a prolonged afterdischarge of 70.6±18 s following repetitive A fibre stimulation. At P10, 3 out of 48 cells showed this type of sensitisation (6%) with an after discharge of 63 s whereas at P21, it was not seen in any cells (n=31).

A fibre induced sensitisation was not accompanied by an increase in the direct A fibre evoked spike discharge (see *FIG.4.3*). However, it can be seen in *FIG. 4.2* that, during the stimulation period, the sensitised units showed a significant increase in activity outside of this short latency evoked burst. The mean background activity during the stimulation period, measured in the 40-2000ms period between stimuli, was 2.6±0.16 spikes in P6 sensitising cells, significantly greater ($p<0.0001$) than the 0.4±0.04 spikes in non-sensitising cells. At P10 there is a similar pattern, the mean background activity for sensitising cells was 15.7±0.84 spikes while that of non-sensitised cells was 1.3±0.13 spikes, another significant difference ($p<0.0001$).

Repetitive C fibre stimulation at three times the C fibre threshold produced a classical 'wind-up' as reported in the adult dorsal horn (Mendell and Wall, 1965; Mendell, 1966; Woolf and Thompson, 1991; Woolf et al., 1988). This C fibre-induced central sensitisation was observed in 3 out of 17 cells at P10 (18%), and 4 out of 10 cells at P21 (40%). An example of "wind-up" recorded from a dorsal horn cell in an animal aged P21 is shown in *FIG 4.4*.

### 4.21 Receptive field size.

In the adult, spinal cord cells that become wound up, following repetitive C fibre stimulation, have larger peripheral receptive fields than they did before becoming sensitised (Cook et al., 1987). The sizes of the peripheral receptive fields in this study, were not mapped out after the cells had displayed central sensitisation.

The initial receptive fields were measured, however, and showed no significant difference from their counterparts that displayed no central sensitisation. At P6 the mean (±SEM) peripheral receptive field size as a percentage of the plantar surface of the hindpaw, was 41.9±8.1 for those that became sensitised and 35.5±3.2 for those cells that did not. Comparing this data with an unpaired student t test reveals $p=0.4548$, indicating no significant difference between these two results. At P10, there is also no significant difference between the size of peripheral receptive fields for those cells that showed sensitisation and those that did not. The mean (±SEM) receptive field size for cells which became sensitised at this age is 32.4±8.4 (n=2), the mean receptive field size cells which did not is 19.1±2.0 (n=48). The student t test gives $p=0.182$.

## 4.3 Discussion

The major finding here is that, in contrast to the normal adult spinal cord, central sensitisation occurs in the immature spinal cord in response to electrical stimulation of large diameter A primary afferents and this disappears with postnatal maturation. The ability of some cells in the newborn to display long-lasting excitation to natural stimulation of receptive fields and for repeated natural stimuli to result in the build up of considerable background activity was reported in an earlier study (Fitzgerald, 1985a), but this was not analysed quantitatively nor was the afferent component identified. This type of central sensitisation in the adult spinal cord is normally evoked by high intensity C fibre input.

### 4.31 "Wind-up" versus central sensitisation.

Low frequency, repetitive stimulation of peripheral nerves at C fibre strength results in wind-up, a progressive increase in the amplitude of the C-fibre evoked response and a build-up of background activity (Mendell and Wall, 1965; Mendell, 1966;). This is due to a progressive depolarisation of the postsynaptic membrane with each incoming C fibre stimulus and is *not* produced by A fibre stimulation in either adult cord *in vivo* or young P12-14 cords *in vitro* (Thompson et al., 1990; Woolf and Thompson, 1991; Woolf et al., 1988). In adults, A fibre stimulation only produces such central excitability in pathological states, such as neuropathy or chronic inflammation (Neumann et al., 1996; Thompson, et al., 1994; Woolf and Doubell, 1994). The results presented here indicate that in young neonates, where all C fibre evoked activity is subthreshold, repetitive A fibre stimulation can induce central sensitisation but that this is qualitatively different from wind-up. The magnitude of the evoked response remains unchanged with each progressive stimulus, only the background discharge increases followed by a prolonged afterdischarge that outlasts the stimulus. Wind-up is, however, only one manifestation of central sensitisation, which can be more generally described as an expression of increased excitability of dorsal horn neurones (Woolf, 1983).

### 4.32 The contribution of EAA and their receptors

If the A fibre induced sensitisation reported here is NMDA dependent, as has been reported for the C-fibre induced wind-up of dorsal horn cells (Davies & Lodge 1987; Dickenson and Sullivan, 1990; Woolf and Thompson, 1991), and the central

excitability of the flexion reflex (Woolf and Thompson, 1991), these results may be a reflection of the ability of A fibre stimulation to activate NMDA receptors in the neonatal cord. The properties of the NMDA receptor in the postnatal spinal cord differ from those in the adult.

NMDA binding studies in the mouse lumbar spinal cord show that NMDA sensitive [$^3$H] glutamate binding peaks around postnatal days 6-10 and is labelled fairly evenly throughout the grey matter until P10-P12 when labelling begins to increase in substantia gelatinosa (SG). The labelling then decreases in other areas so that the adult pattern is visible by P30 (Gonzalez et al., 1993). The affinity of receptors for NMDA and NMDA-induced elevations of $[Ca^{2+}]_i$ are also markedly elevated in SG neurones in the first postnatal week while neither the AMPA response nor the resting $[Ca^{2+}]_i$ show these developmental changes (Hori and Kanda, 1994).

NMDA receptors, in their resting state, have their ion channels blocked by $Mg^{2+}$ in a voltage dependent manner (Mayer et al., 1984). In a study examining the spontaneous activity in the dorsal horn (extracellular recordings from an *in vitro* hamster lumbar spinal cord) Abdul-Razzak and colleagues (1994), have demonstrated a dramatic decrease in the spontaneously evoked activity in the younger preparations when 1mM $MgCl_2$ was added to the perfusate. At P6 MgCl2 reduced the spontaneous activity by 92%, the effect of the $MgCl_2$ was less in the older animals in an age dependent manner, and there was no effect in the adult. (P10 a decrease of 68%, P16 a decrease of 48%, and at P28 the decrease was 13%). When this result is paired with the observation that D-AP5 (a potent, specific NMDA antagonist) also significantly decreases this dorsal root spontaneous activity, in the same preparations, the authors conclude that this spontaneous activity, which is controlled by non-NMDA receptors in the mature cord, is under the control of NMDA receptors in the neonate. At older ages too, the addition of D-AP5 to the bath, causes a marked depression in the spontaneous activity in the cord, however, at younger ages, there is an initial short burst of increased activity followed by the marked depression (Abdul-Razzak et al., 1994).

In support of this, a recent paper examining the properties of cultured spinal cord neurones from P0 rats, defines a cascade of events that sustains spontaneous activity in these cells (Robert et al., 1998). The steps are: activation of NMDA receptors,

activation of AMPA receptors and Na+ channel spiking. Blocking any of these steps results in a rapid decrease in the cells activity (Robert et al., 1998).

These reports of an enhanced role for NMDA in the postnatal spinal cord offer some evidence that this receptor may be the key to the form of central sensitisation reported here.

### 4.33 Silent synapses

In the last few years there have been a few reports concerning the role of "silent synapses" in activity dependent plasticity seen in the neonatal hippocampus and cortex (Durand et al., 1996; Isaac et al., 1995; Liao et al., 1995). Recently there have been some reports on these synapses in the dorsal horn (Bardoni et al., 1998; Li and Zhuo, 1998).

The pure NMDA EPSC has been proposed to be developmentally important (Isaac et al., 1995; Isaac et al., 1995; Liao et al., 1995). These can be recorded at sites where there are only NMDA receptors present. These sites are called *silent synapses*, as they are inefficient, and due to the voltage dependent $Mg^{2+}$ block, do not appear to be fully functional at resting membrane potentials, although morphologically they appear normal (Malenka and Nicoll, 1997). This could be either as a result of a small postsynaptic current that fails to depolarise the postsynaptic cell to action potential, or from a cell with a normal postsynaptic current but with an increased threshold for action potentials (Li and Zhuo, 1998). To record from these cells investigators depolarise the membrane to +40 or +50 mV (Bardoni et al., 1998; Li and Zhuo, 1998). Silent synapses have been described in the hippocampus, NMJ, and cortex (Bardoni et al., 1998; Durand et al., 1996; Isaac et al., 1995; Isaac et al., 1997; Li and Zhuo, 1998; Liao et al., 1995; Malenka and Nicoll, 1997; Wu and Dun, 1997).

In the newborn rat dorsal horn, in vitro electrophysiological studies report that silent synapses are more abundant in the P2-10 age group than in the P11-17 group (Li and Zhuo, 1998). This trend has also been reported in the hippocampus (Durand et al., 1996), and in the cortex (Isaac et al., 1997; Wu and Dun, 1997). Another electrophysiological study reports that, there is no significant change in the number of neurones expressing pure NMDA synapses in lamina II over the first two postnatal weeks (Bardoni et al., 1998), although the first two postnatal weeks may

fall into the first age group in the Li & Zhuo study. By P8-9 silent synapses have disappeared from the somatosensory cortex (Isaac et al., 1997).

Low intensity stimulation was used to evoke synaptic responses from silent synapses in the superficial dorsal horn cells held at +40mV. The authors report that it is *likely* that only low threshold afferent fibres are activated, although they also find that these cells received inputs from high threshold afferent fibres (Li and Zhuo, 1998). This is interesting for a number of reasons. This could suggest that superficial dorsal horn cells receive convergent inputs in the first two weeks of postnatal life, although there are polysynaptic A fibre inputs to the superficial dorsal horn in the adult (Willis and Coggeshall, 1991). More importantly, this paper suggests that the low threshold fibres in the superficial dorsal horn can be functional under some conditions (Li and Zhuo, 1998). Bardoni and colleagues report that the all pure NMDA EPSCs in their study had lower threshold for activation than those of mixed receptor EPSCs (Bardoni et al., 1998).

The presence of the synapses between superficial dorsal horn cells and the A fibres that transiently terminate in the superficial horn has been demonstrated in an ultrastructure study (Coggeshall et al., 1996), but these synapses had not been directly shown to have functional potential. The maturation of silent synapses (pure NMDA receptors) into fully functional mixed NMDA and AMPA receptors requires the addition of 5-HT, which the authors suggest would arrive from the rostroventral medulla (Li and Zhuo, 1998). The descending pathways of the dorsal lateral funiculus, which contain serotonergic fibres, only reaches the lumbar dorsal horn towards the end of the second postnatal week (Fitzgerald and Koltzenburg, 1986), so the silent synapses in the superficial dorsal horn are unlikely to be changed into functional forms by serotonin, in the early postnatal period.

Other groups working in the hippocampus and cortex, have reported that the change from silent NMDA receptors, to functional NMDA receptors is activity dependent, (Durand et al., 1996; Liao et al., 1995). Furthermore there appears to be a degree autoregulation (Rao and Craig, 1997). This is not entirely surprising, considering the very large role that NMDA receptor mediated spontaneous activity is reported to play in the development of many CNS regions (reviewed by (Constantine-Paton et al., 1990; Hockfield and Kalb, 1993).

Silent synapses in these experiments can be converted into functional NMDA receptors after a brief, high frequency stimulus, which is sufficient to cause LTP. These changes occur in the first postnatal week in the neonatal hippocampus (Durand et al., 1996). At P0 there are no synaptic contacts in the neonatal hippocampus, by P2 82% of the EPSCs are mediated by pure NMDA receptors, and by P6 the authors often noted synaptic transmission at resting potentials, indicating that both AMPA and NMDA receptors present (Durand et al., 1996). Others report the presence of silent synapses in the second and third postnatal weeks in the hippocampus (Liao et al., 1995). In the young hippocampus Induction of LTP, does not necessarily cause the induction of AMPA. In one study 75% of cells stimulated showed both LTP and the induction of functional synapses, whilst the remaining 25% of cells showed LTP only (Liao et al., 1995).

The induction of functional synapses and LTP are mechanistically similar. Once established the conducting synapse becomes a persistent element in the neuronal circuit and the newly established AMPA induced EPSC was constant throughout the remaining time of recording (Durand et al., 1996). The NMDA receptor mediated component was not affected by the synapse induction, the amplitude of the EPSC was similar before and after induction (Durand et al., 1996). This suggests that during synapse induction, the changes are entirely postsynaptic. Synapse induction could be blocked by buffering the intracellular $Ca^{2+}$ or by perfusing the slice with D-AP5 (Durand et al., 1996). Synapse induction requires coincident activity at the pre- and post-synaptic sites, in line with Hebb's rule of association (Hebb, 1949).

The theory is that in the neonatal hippocampus pure NMDA receptors are the only ones functional, at least at birth. It follows that a large proportion of transmission occurs at theses synapses. These synapses only transmit information when the post-synaptic membrane is depolarised, as the voltage gated $Mg^{2+}$ is present from birth (Bardoni et al., 1998). Perhaps the post-synaptic membrane is depolarised, by other coincident inputs acting on nearby synapses with functional AMPA receptors (Liao et al., 1995). During development, newly formed, randomly connected synapses may only have pure NMDA receptors as a means of reducing the noise that would result from activity at the randomly placed synapses. Thus, information will only be transmitted at these synapses if the presynaptic activity coincides with other, presumably functionally relevant, information (Liao et al., 1995).

Recent exciting work in the young postnatal hippocampus affords an alternative method of removing the $Mg^{2+}$ block from silent synapses. In the early postnatal hippocampus $GABA_A$ receptor agonists have been shown to remove the voltage dependent $Mg^{2+}$ block of the NMDA receptor, suggesting that the combined action of depolarising GABA and the subsequent opening of the NMDA channel will cause a huge $[Ca^{2+}]_i$ (Ben Ari et al., 1997; Leinekugel et al., 1997).

*HETEROSYNAPTIC FACILITATION:*

Work in the spinal cord suggests a further alternative; that the summation of synaptic potentials may occur at a rate below that necessary to change the evoked response of the cells (Thompson et al., 1993) but sufficient to unblock NMDA receptor ion channels and allow direct calcium influx. Heterosynaptic facilitation is where a modification is produced in one synapse by as a result of inputs to other synapses. This requires the activitation of high threshold primary afferents in the form of a conditioning stimulus (Thompson et al., 1993) which is not present in this study, as the stimuli delivered were only twice the A-fibre threshold and the width of the stimulus pulse was only 50µs. Facilitaion produced by subthreshold C fibre action potentials or from A-fibres in the superficail dorsal horn of the neonate, cannot be ruled out, with out further experiments.

In neonates, where the rate of rise of EPSPs is already low (Seebach and Mendell, 1996), summation is more likely to occur. Certainly, the rate of rise of EPSCs in the pure NMDA synapses is longer than in the functional, mixed NMDA and AMPA receptors in the neonatal dorsal horn (Bardoni et al., 1998). This could be as a result of the subunit composition of the NMDA receptors (Monyer et al., 1994), or even that glutamate might be more likely to spill over at pure NMDA sites than at mixed sites (Kullmann et al., 1996), and that the slow rate of rise is due to diffusion times to pure NMDA receptors (Bardoni et al., 1998).

It has been suggested that the expression of NMDA receptors is mainly at non-synaptic sites in developing neurones. A study of synaptic connectivity in cultured ventral spinal neurones (E18-19) reports that between 2 and 4 days in culture, the pattern of staining of the GluR1 AMPA receptor subunits changes from diffuse to highly clustered. These clusters are coincident with the onset of miniature EPSCs and associated with presynaptic terminals in the majority of cases after 6 days in culture (O'Brien et al., 1997). In cultured neurones the distribution of the NR1

NMDA receptor subunit was diffuse, even after three weeks. There appeared to be no co-localisation of the NR1 subunit and GluR1 clusters (O'Brien et al., 1997). Another study reports that NMDA receptors are expressed as clusters in cultured hippocampal neurones, but are mostly *not* associated with presynaptic terminals (Rao and Craig, 1997). Chronic treatment of the preparation with either APV or TTX, dramatically increases the number of postsynaptic clusters, indicating that spontaneous activity mediated by NMDA receptors can block synaptic expression of NMDA receptor subunits (Rao and Craig, 1997). One possible mechanism for this could be through NMDA receptors located close to the presynaptic terminal (Aoki et al., 1994).

The sensitivity of the Ia motoneurone synapse to external $Ca^{2+}$, in a low $Ca^{2+}$ bath solution, decreases with postnatal age during the first 10 days after birth (Seebach and Mendell, 1996). If this sensitivity to $Ca^{2+}$ changes with age in all spinal neurones rather than just the motoneurones as reported, then this would certainly contribute to the sensitisation seen in the early postnatal dorsal horn, since the key to central sensitisation is the an increase in intracellular $Ca^{2+}$ (Woolf, 1996). Furthermore, studies in *Aplysia* indicate that excitation during, and after stimulation can be differentially regulated, in that the two phases of response have differential $Ca^{2+}$ dependence (Fischer et al., 1997).

In other brain areas, silent synapses have been shown to be transformed into functional NMDA receptors by a mechanism dependent on activity, the mechanics of this are not known (Durand et al., 1996; Isaac et al., 1995; Isaac et al., 1997; Liao et al., 1995). In the hippocampus it has been proposed that in LTP, the changes in synaptic strength may be underpinned by activity-dependent changes in the numbers of functional synapses (Malenka and Nicoll, 1997). Unlike the somatosensory cortex where silent synapses disappear at the end of the first postnatal week, these silent synapses are still present in the lumbar spinal cord, at least until P17, although the numbers appear to be developmentally regulated in many parts of the brain. Using whole cell patch clamp, silent synapses are only found in the early postnatal, spinal cord. Attempts to find them in the adult rat spinal cord, have failed (Woolf, *personal communication*), suggesting that pure NMDA synapses and their activity dependent regulation are purely developmental phenomenon, in the spinal cord.

*Nitros Oxide:*

Injecting NMDA intrathecally, induces a short term hyperalgesia, which can be blocked by either systemic or intrathecal nitric oxide synthase (NOS) inhibitors (Kitto et al., 1992). Wind-up of dorsal horn neurones has also been shown to be blocked by NOS inhibitors (Semos and Headley, 1994). Pre-emptive, systemic, administration of NOS inhibitors significantly reduces the magnitude of the response in the second phase of the formalin response (Haley et al., 1992). Furthermore, intrathecal injection of sodium nitroprusside, (a NO donor) causes a reversible hyperalgesia (Kitto et al., 1992). These data suggest that the underlying molecular changes during central sensitisation, include a role for nitric oxide (NO), as well as the NMDA receptor activation and subsequent influx of $Ca^{2+}$.

NOS is not found in primary afferent terminals, indeed it appears to be localised with GABA containing interneurones in lamina II of the rat spinal cord (Valtschanoff et al., 1992). There is a developmental progression in the expression of NOS. At P2 there is none in the SG, faint staining starts to appear by P5, although it remains immature until after P11. By P20 the overall staining in the dorsal horn resembles that of the adult (Soyguder et al., 1994). This data suggests that there is unlikely to be a role for NO in the superficial dorsal horn during the first postnatal week.

### 4.33 The development of inhibitory control

Other maturational changes in the postnatal spinal cord include the onset of descending inhibition, via the dorsolateral funiculus, which only begins to function in the second postnatal week (Fitzgerald and Koltzenburg, 1986), and presumably dampen this central excitability. This pathway was only fully functional by P22-24. Until P9 there was no inhibition, by P12 half the cells were inhibited and by P18 most cells were inhibited, although this required a higher stimulation intensity (Fitzgerald and Koltzenburg, 1986). There is a minimal pharmacological role for 5-HT before P10, and only at high doses at this age. Better antinociception from intrathecally injected 5-$HT_3$ agonists is seen at lesser doses in older animals (P14 and P21) (Giordano, 1997).

Functional $GABA_A$ synapses are present from before birth. In the hippocampus these GABA receptors depolarises neurones, so accounting for a large part of excitatory neurotransmission at this time (Ben Ari et al., 1997; Reichling et al., 1994). These early networks generate spontaneous giant depolarising potentials (GDP),

caused by simultaneous activation of $GABA_A$ synapses (Ben Ari et al., 1989; Leinekugel et al., 1997). GDPs disappear in the presence of TTX and APV and progressively diminish by P12. GDP activation depends on both NMDA receptor activity and $Na^+$ channel activity, which is similar to the AMPA receptor mediated EPSCs in cultured spinal neurones (Robert et al., 1998). In neonatal spinal cord cultured neurones, activation of $GABA_A$ receptors is inhibitory (Robert et al., 1998).

**Conclusion:**

There are elements in both the development of excitatory and inhibitory systems in the neonatal spinal cord that could account for the A fibre induced central sensitisation, shown here. What is needed now are further experiments, particularly focusing on the roles of NMDA and GABA in the developing spinal cord dorsal horn.

If the central sensitisation due to functional A fibres in the superficial dorsal horn, whilst the C fibres are immature, then it is possible that they fulfil a role in generating the activity required for the formation of the pathway at higher levels. There is strong evidence that in the neonatal period the basic neuronal pathways that have developed following genetic patterns are fine tuned by other factors, such as cellular interactions, hormones, neurotransmitters and electrical activity (Fields and Nelson, 1992).

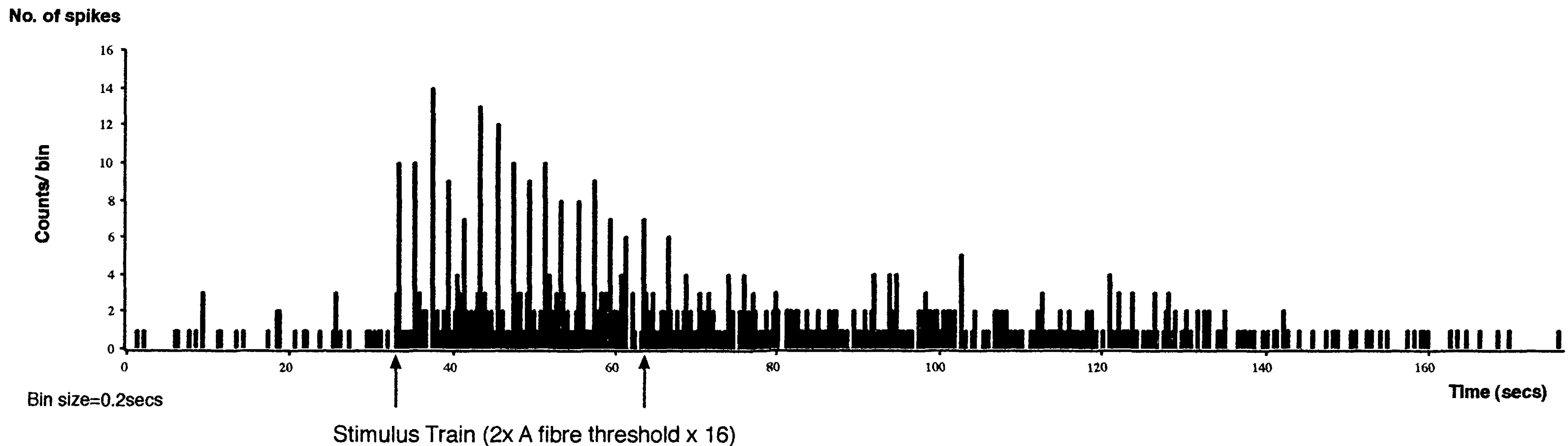
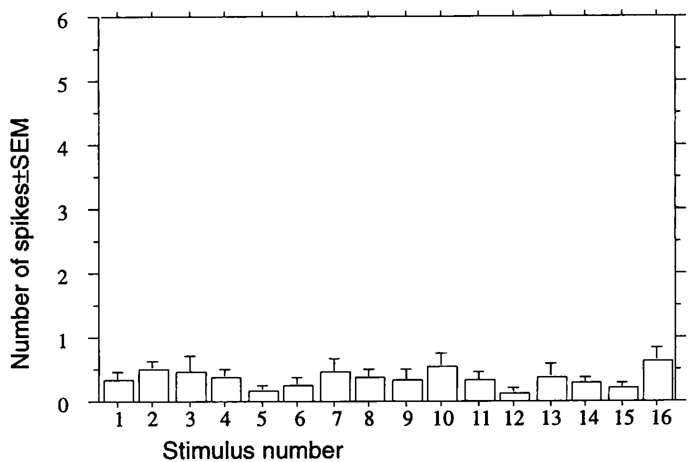



**Figure 4.1: Time histogram of a sensitised cell.**
A ratemeter record of the firing of a single P6 dorsal horn cell illustrating the sensitisation seen following a train of sixteen stimuli (0.5Hz) at twice the A fibre threshold. Note the low background rate before the stimulus, compared to the greatly increased afterdischarge rate after the stimulus train, which continues for more than a minute.

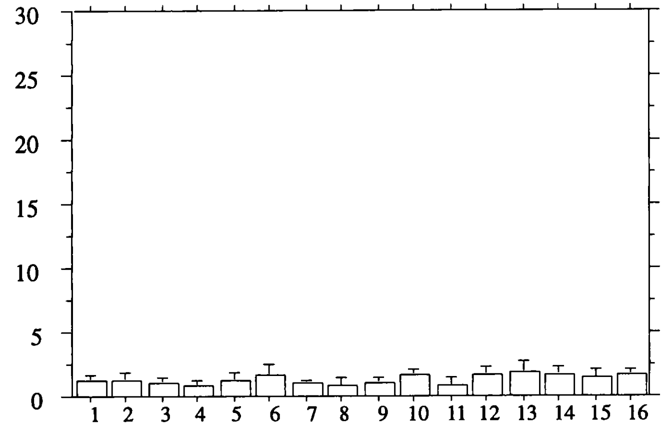
**Figure 4.2: Magnitude of the after discharge**
Bar charts representing the mean number of spikes ± SEM in the 40-2000ms range after each stimulus, in a train of 16 stimuli (twice threshold, 0.5HZ). This illustrates sensitisation of dorsal horn cells at P6 and P10 which is not reflected in the direct evoked responses occurring within a 40ms latency (Fig 4.3). At P6 the mean spike count is from 8 cells that sensitised and 21 that did not. At P10 the mean count is from 3 cells that sensitised and 12 that did not.

**Fig 4.2**

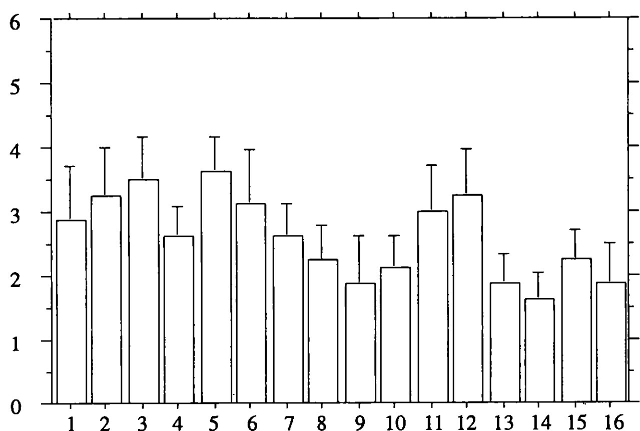
**P6 Not sensitised**




**P10 Not sensitised**




**P6 Sensitised**




**P10 Sensitised**

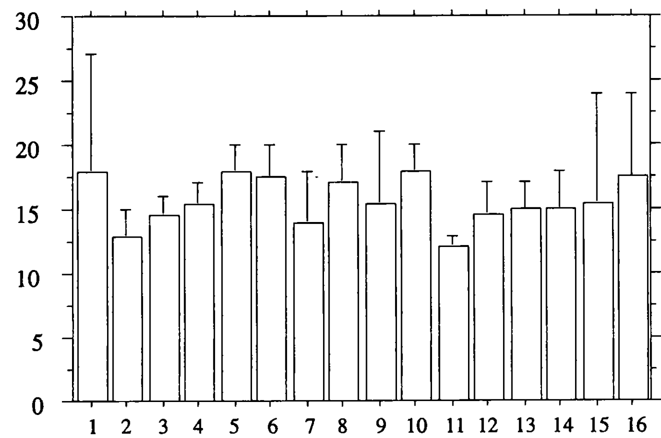
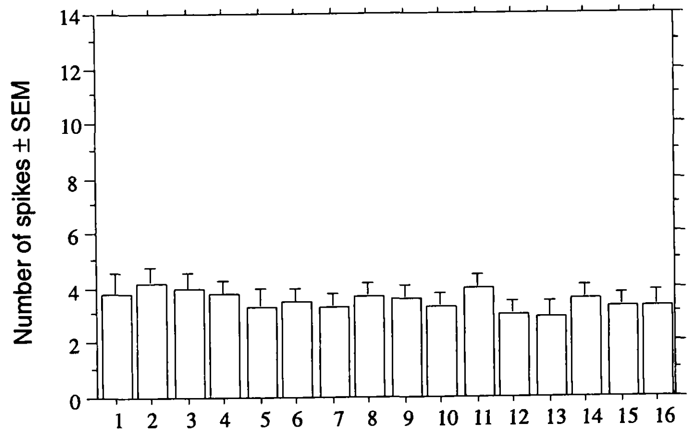


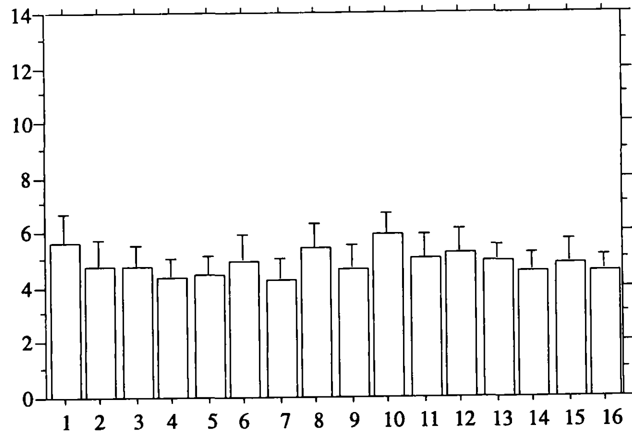
**Figure 4.3: Magnitude of evoked response.**
Bar charts representing the mean number of spikes SEM evoked by each stimulus in a train of 16 A fibre stimuli (twice threshold; 0.5Hz). Ages P6 and P10 cells are divided into those that sensitised on repeated A fibre stimulation and those that did not.

**Fig 4.3**

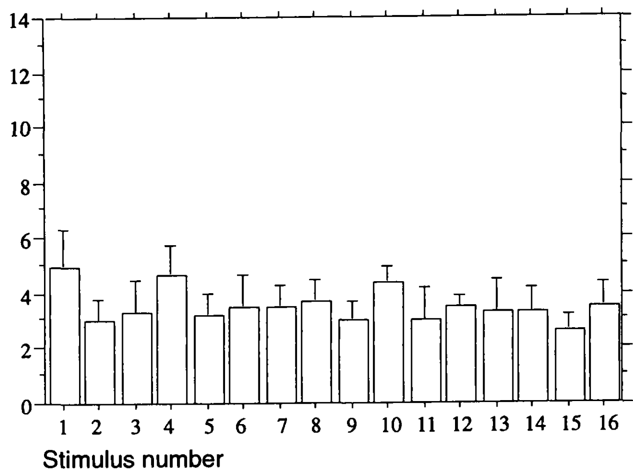
**P6 Not Sensitised**




**P10 Not Sensitised**




**P6 Sensitised**




**P10 Sensitised**

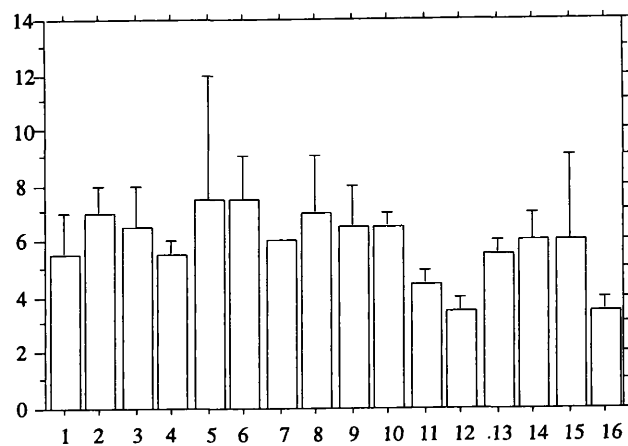
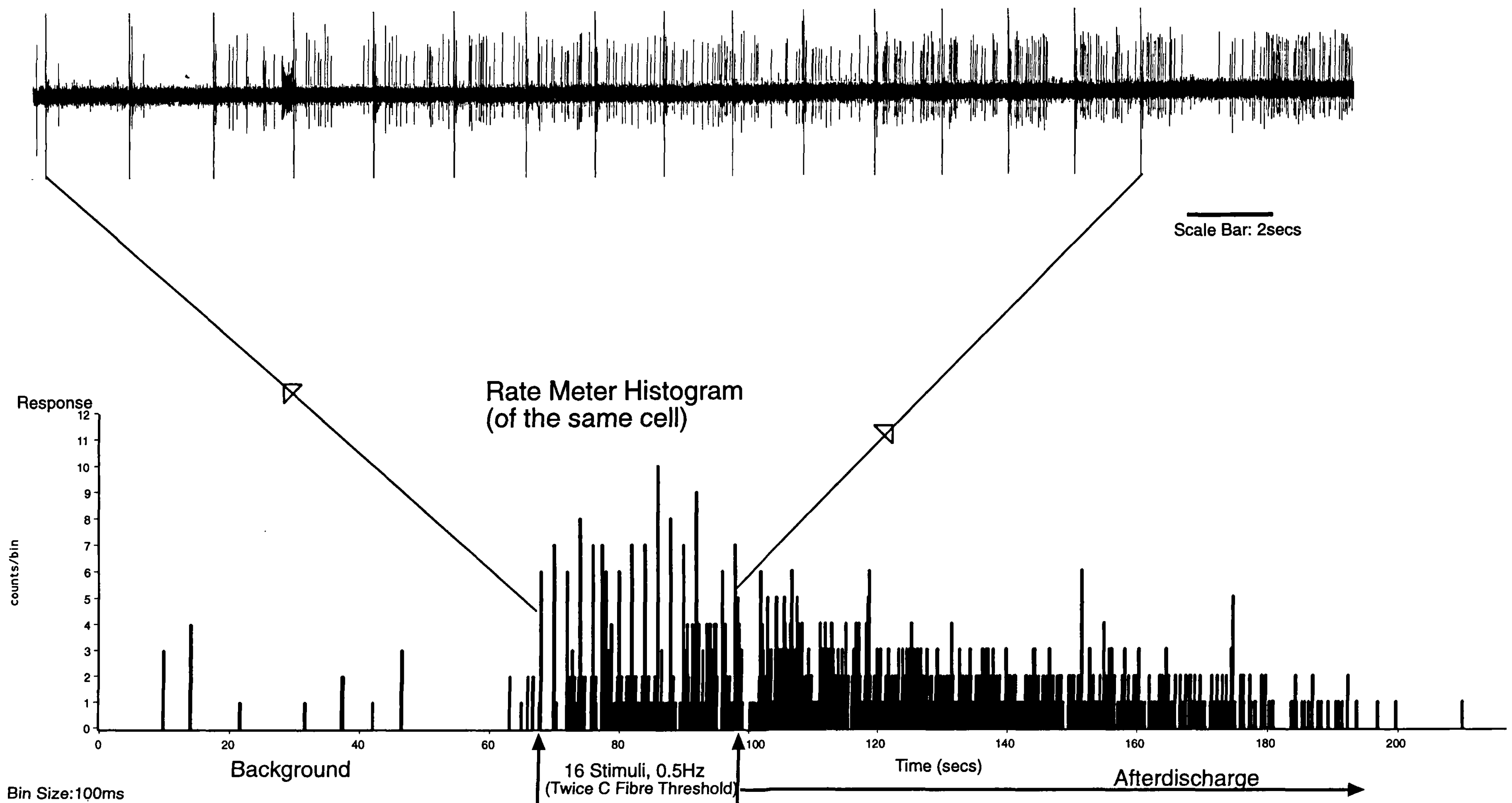


**Fig 4.4: C fibre evoked "wind-up" of a dorsal horn cell at P21.**
This figure shows the raw trace and rate meter histogram of a dorsal horn cell responding to electrical stimulation at twice the C fibre threshold (train of 16 stimuli) This cell wound up to the stimulus train.

Fig 4.4



# Chapter 5:

## Changes in evoked response in the neonatal dorsal horn following inflammation.

### 5.1 Introduction

The data presented in previous chapters related to dorsal horn responses evoked in the immediate (ms-second) time frame, following peripheral stimulation. In this chapter evoked responses in the longer (minutes-hours) time frame following stimulation are examined. The results are preliminary but give an indication of prolonged changes in activity that can be produced in neonatal dorsal horn cells following tissue injury.

Inflammation is the response to tissue injury or infection and leads to increased neuronal activity. Increased neuronal activity leads, in turn, to central changes in the spinal cord and brain, possibly in the form of immediate early gene expression mediated by the release of neurotransmitters, which ultimately lead to the clinical signs of inflammation, namely hyperalgersia and allodynia. Inflammatory models rely on the injection or of an irritant chemical; such as carrageenan, complete Freund's adjuvant (CFA), formalin, turpentine, the topical application of mustard oil or UV irradiation (reviewed by: Reeh and Sauer, 1997) The resulting inflammation is characterised by swelling, redness, and hyperalgesia, which are the peripheral signs of inflammation. The main differences between inflammatory models id the time course over which they act. Mustard oil typically produces an acute reaction, formalin has a biphasic action with a short acute phase followed by a longer second phase, and following carrageenan, activity usually peaks 2-4 hrsafter application. UV irradiation, turpentine and CFA, typically last days to weeks (reviewed by: Reeh and Sauer, 1997).

I have used carrageenan in these studies which is reported to be a more reliable agent in modelling inflammation than formalin (Xu et al., 1995). Carrageenan induced inflammation was first described in 1962 (Winter et al., 1962), and has since been used widely to produce a unilateral model of inflammation (Hargreaves et al., 1988; Hylden et al., 1991; Kayser and Guilbaud, 1987; Stanfa et al., 1992; Stanfa et al., 1996). After the injection of carageenan , oedema rapidly develops, followed by hyperalgesia, which peaks at 3-4hrs and decreases to baseline by 24-72hrs

(Hargreaves et al., 1988; Hylden et al., 1991; Kayser and Guilbaud, 1987). In some cases the period of hyperalgesia can last 10-14 days (Kayser and Guilbaud, 1987).

The molecular mechanisms of the centrally produced changes leading to hyperalgesia and allodynia are are the same as those in central sensitization (described in chapters 1 and 4). Indeed, inflammation causes central sensitisation.

*RECEPTIVE FIELDS*

Electrophysiological evidence has shown that the receptive field of dorsal horn neurons increases following inflammation and the expansion of the receptive field parallels the development of hyperalgesia. The receptive fields of lamina I dorsal horn neurons were observed between 4 and 8.5 hrs following injection of CFA and, on average, expanded to 2.4 times their original size (Hylden et al., 1989). It has been suggested that the increase of the receptive field may be responsible for the hyperalgesia. Since the receptive fields are larger there is a greater degree of overlap and so a single stimulus would activate many more neurons than in the control state, a summative effect (Dubner and Ruda, 1992). Although, since expansion of receptive fields is one of the classic signs of central sensitisation (Cook et al., 1987), it would appear that central sensitisation is required for expansion of receptive fields.

A behavioural response to inflammation is seen from P1 in rats, although the biphasic response to formalin is not seen until P15 (Guy and Abbott, 1992). Decreased flexor reflex thresholds have also been reported in both the rat and human, following skin wounding or inflammation (Andrews and Fitzgerald, 1994; Fitzgerald et al., 1987; Fitzgerald et al., 1988).

The responses of immature dorsal horn cells have not been investigated previously, and this study sets out to rectify this. Preliminary results are presented here.

## 5.2 Results

These are preliminary data. Data is presented from two different types of experiment:

1. Responses were recorded from dorsal horn cells in the same way as described in the methods and used in the previous chapters. In this study however, the inflammatory agent, carageenan, was injected in to the plantar hindpaw, prior to recording. Cells with receptive fields on the plantar hindpaw only, were included in this study. At P21 recordings were made from 4 cells, three of these were situated in the deep dorsal horn and the remaining one in the superficial dorsal horn (using the criteria defined in chapter 3 (≥300μm from the dorsal surface of the cord)). At P10 recordings were made from 15 cells, two of these were in the superficial lamina and the remaining 13 were in the deep laminae (≥250μm). Between one and three cells were recorded from each pup.

2. A single dorsal horn cell with stable responses was isolated, and the evoked response measured. After three stable evoked responses at 10 minute intervals, carageenan was injected into the plantar hindpaw, into the centre of the receptive field, and the response to a train of stimuli (16) at twice the A fibre threshold, and three times the C fibre threshold (if C fibre input found), was recorded every 10 minutes. This paradigm enabled a map of the progressive changes to the dorsal horn responses to be constructed. Three cells at P10 were recorded in this way, each from a single rat pup.

In the first group the afferent input of 8 of the cells at P10 was activated by stimulation with pin electrodes (6mA, 500μs). For the remainder of the experiments the stimulation was delivered directly to the sciatic nerve at mid thigh level, through two silver wire hook electrodes. Since the pin electrodes were placed in the centre of the receptive field, and this area became inflamed after the injection of carrageenan, there was concern that the massively oedematous tissue might interfere with the transmission of the stimulus, between the electrodes and the receptors, or free nerve endings.

### 5.21 Time course of acute inflammation:

Results from the second experiments demonstrated the time course of the effects of peripheral inflammation on the dorsal horn cells in the P10 rat pup. The background activity, measured for one minute before the train of stimuli was delivered, and the after discharge rate (measured between 200ms and 2000ms after each stimulus in the train), indicate that the activity of these cells peaks within 100mins post inflammation. This is considerably less than the reported 3-4hrs in the adult (Hylden et al., 1991; Kayser and Guilbaud, 1987). Since only three cells were successfully recorded from in this set of experiments, further experiments are required to fully confirm these results. Recordings deteriorated beyond three hours after the injection of carrageenan, so activity after this time is not known. These results are presented graphically in *FIG. 5.1.*

### 5.22 Receptive field properties:

Receptive fields were measured in the same way as those in chapter 3, and expressed as a percentage of the plantar foot area. There was a significant increase in the size of peripheral receptive fields in animals in both the P10 and P21 age groups ($P<0.0001$ in both cases). At P10 the size of the peripheral receptive fields increased by 2.5 times, and at P21 the increase was 3.4. *FIG 5.2* shows these results. At P21 only 4 were tested, following inflammation, but they can be compared to 30 controls. At P10 there were 48 cells in the control group and 15 cells in the inflamed group. Mean size ± SEM (as a percentage of the plantar hindpaw area), of the peripheral receptive field in the inflamed group at P10 was 47.2±6.4%, and that of the control was 19.1±2.0%. At P21 the mean size of the receptive field in the inflamed group was 51.8±12.2% (large SEM), and that of the control group was 14.9±1.6%.

### 5.23 Threshold: A fibres

The A fibre stimulation thresholds at P10 in the presence of inflammation did not change significantly. The mean ± SEM threshold value on nerve stimulation was 1.0±0.27 mA for the control cells and 0.95±0.2mA for the inflamed group. When the stimulation was delivered through pin electrodes, the threshold values were higher, 2.0±0.5mA for the control and 2.78±0.5mA for the inflamed cells. The higher thresholds required for pin electrode stimulation was probably due to current leakage into the tissue, and the difficulty of stimulating terminal nerve branches.

At P21 the threshold changes were greater. The A threshold change was extremely significant ($p<0.0001$; Student's t test). The mean A fibre threshold for the control group was 2.0±0.32mA, and for the inflamed group it was 0.19±0.01mA. These results are represented in graphical form in *FIG. 5.3*.

### 5.24 Threshold: C fibres

Of the 15 cells in the inflamed group at P10, 4 had C fibre evoked responses with thresholds of 1.83±0.27mA. There were no cells with C fibre input in the control group where the nerve had been stimulated directly.

At P21 the mean threshold for four cells in the inflamed group was 1.76±0.23mA, compared to 3.0±0.59mA for four cells in the control group. This change in threshold is not quite significant ($p=0.0513$).

### 5.25 Magnitude of the response

The magnitude of the evoked response (number of spikes), following electrical stimulation directly to the nerve significantly increased following inflammation in P10 cells stimulated at twice A fibre threshold. Mean±SEM evoked response for the controls animals was 3.2±0.25 spikes and for the inflamed animals it was 7.6±0.21 spikes. The Student's t test gives a $p<0.0001$ when comparing these two groups, suggesting that the difference is extremely significant.

At P21, the mean magnitude of the evoked response for cells receiving an A afferent input was 6.8±0.32 spikes in the control group, and 6.6±0.21 spikes in the inflamed group. The difference between these two groups is not significant ($p=0.59$). For those cells responding to C afferent input the magnitude of response was 4.9±0.5 spikes for the control group, and 10.1±0.67 spikes for the inflamed group. These two results were significantly different, with a $p<0.0001$. These results are presented in graphical form *FIG. 5.4*.

## 5.3 Discussion

This is the first time that the effect of inflammation on dorsal horn cell activity has been investigated in the neonatal rat spinal cord. Although these results are preliminary, they provide a good insight into the basic responses of dorsal horn cells, in neonatal rats, to peripheral inflammation. The reported increase in receptive field size and lowering of afferent input thresholds from inflamed tissue that is reported in the adult, is also seen in neonatal cells.

### *5.31 The time course of acute inflammation in the neonate.*

The results presented above and in *FIG. 5.1* suggest that the time course of acute inflammation in neonates, following peripheral, subcutaneous injection of carrageenan is shorter than that reported in adults. This experiment was only done at one age,P10. Much of the data presented in the adult literature about time course is behavioural (Hylden et al., 1991; Kayser and Guilbaud, 1987), and some investigators see little in the way of background spontaneous activity in dorsal horn cells when recording extracellularly *in vivo*, although changes in the after discharge and pharmacological changes can be observed three hours after inflammatory insult (personal communication Louise Stanfa)(Stanfa et al., 1992; Stanfa et al., 1996; Stanfa et al., 1997). Other investigators report spontaneous background activity following peripheral inflammation, which increases substantially once the tonic descending inhibition is removed (Cervero et al., 1991; Ren and Dubner, 1996; Schaible et al., 1991).

Background activity and after discharge, were used by the above investigators in the adult rat and cat as a measure of the neuronal activity following peripheral inflammation. In the experiments reported here, the same parameters are examined, and the peak of excitation is around 60 mins in the after discharge and around 100 mins for the background activity. Two factors must be borne in mind when considering these results. Firstly this experiment has only been done in three cells, and secondly, the physiological condition of the animals began to deteriorate at around three hours after the injection of carrageenan, so activity after this time is unknown.

### *5.32 Change in receptive field size with inflammation.*

In the adult rat model, expansion of the peripheral receptive fields is seen following a lidocain block of the dorsal lateral funiculus (DLF), at thoracic level. Furthermore, this expansion is greater in animals with peripheral inflammation than in non-inflamed animals (Ren and Dubner, 1996). Therefore two contributing factors to receptive field expansion in the adult are:

1. C afferent input
2. Intact dorsal lateral funiculus

*C AFFERENT INPUT*

Following inflammation, the barrage of nerve impulses from the periphery into the dorsal horn lead to hyperexcitability. One measure of this hyperexcitability or central sensitisation is an enlargement of the peripheral receptive field of these neurones (Calvino et al., 1987; Cook et al., 1987; Hylden et al., 1989; McMahon and Wall, 1984; Woolf and King, 1990). Brief inputs from C fibres will also cause expanded receptive fields in the superficial dorsal horn (Cook et al., 1987).

In this study the C fibre input is still a little immature at P10, although it is possible to evoke suprathreshold responses they are not as frequent as at older ages (Fitzgerald, 1985a; Fitzgerald, 1987a; Jennings and Fitzgerald, 1998). At P21 the C fibre evoked response is much more mature. Despite the immaturity of the younger neurones investigated, their mean peripheral receptive fields were 2.5 times larger following inflammation than in a control situation. The increase at P10 is from a background mean control receptive size which is already larger than that of older animals (see chapter 3 (*FIG 3.3*)). Nevertheless, this value equates well with the reported 2.4 times enlargement of the peripheral receptive fields, following inflammation in the adult (Hylden et al., 1989; Hylden et al., 1991; Ren and Dubner, 1996; Schaible et al., 1991; Traub, 1997). At P21 the increase in size, measured from just four cells is 3.4 times the mean control value. There are too few data points at this age to make much of this result (note the large SEM). These results are presented in *FIG 5.2*.

*DORSAL LATERAL FUNICULUS*

Receptive fields of dorsal horn neurones have been shown to enlarge, and thresholds decrease after the inhibition of the dorsal lateral funiculus (DLF) (Wall, 1967). The DLF is also a source of excitatory input to some (mainly lamina I) dorsal horn

neurones (Dubuisson & Wall, 1980; McMahon & Wall, 1988}. Neurones in the deep dorsal horn were inhibited by stimulation of the DLF (McMahon and Wall, 1988). In the neonate, at lumbar levels of the spinal cord the DLF exerts no influence at P9, a very weak influence by P12 and only becomes fully functional, in the adult sense, at P22-24 (Fitzgerald and Koltzenburg, 1986). The functional maturation of this pathway lags behind the anatomical development significantly. The pathway appears to be anatomically present from P6. Not surprisingly, the functional maturation parallels the pharmacological maturation. Intrathecal administration of a 5-$HT_3$ agonist first produces antinociception against formalin induced pain at P10, but once again only at high doses. Lower doses of the agonist (MDL-72222), attenuated the nociceptive response at older ages (P14 and P21) (Giordano, 1997).

This lack of descending control might contribute to the increase in size of the peripheral receptive field, at P10, but by P21 other investigators report adequate function of this pathway (Fitzgerald and Koltzenburg, 1986; Giordano, 1997).

### 5.33 Reduced stimulation threshold

As in the adult, dorsal horn cells in neonates had reduced stimulation thresholds following acute inflammation. This is the hyperalgesia that is widely reported following peripheral tissue injury, and is the behavioural correlate of sensitisation. As such, it is not surprising that neonatal dorsal horn cells also have reduced thresholds. The reduction of flexor reflex thresholds has been reported in neonatal humans and rats, following inflammation or tissue damage (Andrews and Fitzgerald, 1994; Fitzgerald et al., 1989).

*PERIPHERAL VERSUS CENTRAL SENSITISATION:*

There are thought to be two main components to hyperalgesia. Sensitisation of the peripheral receptors (Bessou and Perl, 1969; Campbell et al., 1979; Raja et al., 1984), and central sensitisation of dorsal horn neurones (Woolf, 1983).

Following peripheral inflammation or tissue damage, the sensitivity of a proportion of sensory fibres has altered sensitivity to heat (Bessou and Perl, 1969). This is the phenomenon of peripheral sensitisation, and is caused by the release of endogenous chemicals in the area around the inflammation eg., bradykinin and cytokines (reviewed by Woolf, 1995). Central sensitisation on the other hand is manifest as secondary hyperalgesia, expansion of receptive fields, and prolonged after discharge, allodynia (reviewed by Woolf, 1995). The receptive field expansion seen in this

study is a secondary event, and the lowered threshold can be both peripheral and central.

Bilateral lesion of the DLF has little effect on the baseline nociceptive withdrawal reflexes, evoked by transient noxious stimulation (McMahon and Wall, 1988; Ren and Dubner, 1996). This suggests that these reflexes in the neonate should not be affected by the lack of functional DLF until the second postnatal week.

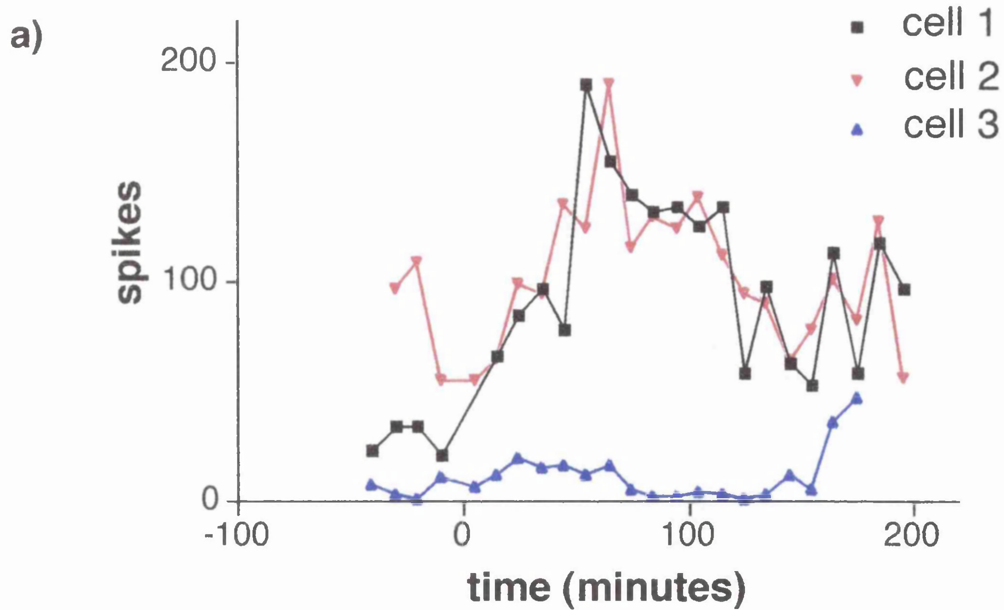
Carrageenan induced peripheral inflammation has been shown to causes a rise in GABA immunoreactive cells in the ipsilateral dorsal horn; reaching a value of 23.4% more immunostaining than the contralateral side four days post injection. This increase in GABAergic activity can be prevented by neonatal treatment with capsaicin or sciatic neurectomy, suggesting that GABA is upregulated in the dorsal horn by the increased C fibre afferent input, from the site of inflammation.(Castro Lopes et al., 1994).

Intrathecal injections of $GABA_A$ or glycine receptor antagonists significantly reduce the cutaneous mechanical thresholds, demonstrating significant segmental inhibitory mechanisms(Sivilotti and Woolf, 1994). A more recent study confirms that the $GABA_A$ antagonist, bicuculline, enhances pain behaviours following peripheral inflammation (John et al., 1998). These results are similar to those obtained from C fibre primary afferent input, in the same study. Therefore central sensitisation can be brought about by either enhanced excitatory input into the dorsal horn or by disinhibition (removal of the inhibitory influences) (Sivilotti and Woolf, 1994).

This is particulary important in the neonate where many of the pathways are still immature, especially considering the interactions in the early postnatal hippocampus between $GABA_A$ and NMDA receptor agonists. One group has reported that $GABA_A$ agonists, in the hippocampus, at this age are excitatory, and furthermore, can remove the voltage dependent $Mg^{2+}$ block on NMDA receptors, many of which are silent synapses during the first part of the postnatal week (Ben Ari et al., 1997; Durand et al., 1996; Leinekugel et al., 1997). Since silent synapses have also been shown to be present in the neonatal dorsal horn (Bardoni et al., 1998; Li and Zhuo, 1998), and more than 90% of dorsal horn neurones in culture respond to $GABA_A$ agonists and strychnine by a transient rise in intracellular $Ca^{2+}$ (Reichling et al., 1994; Wang et al., 1994), it is possible that similar interactions could occur in the young postnatal dorsal horn, although no evidence yet, in cultured neonatal spinal cord neurones $GABA_A$ receptor activation has an inhibtory effect (Robert et al., 1998).

The interaction of neonatal $GABA_A$ receptor agonists depolarising the post-synaptic membrane and the subsequent activation of NMDA receptors leads to a huge increase in the $[Ca^{2+}]_i$ (Ben Ari et al., 1997), and since $[Ca^{2+}]_i$ is the key to central sensitisation (Woolf, 1996), this neonatal interaction is likely to have a profound affect on sensitisation seen in the neonatal dorsal horn, including that seen following inflammation. Silent synapses are discussed in more detail in chapter 4.

a)




b)

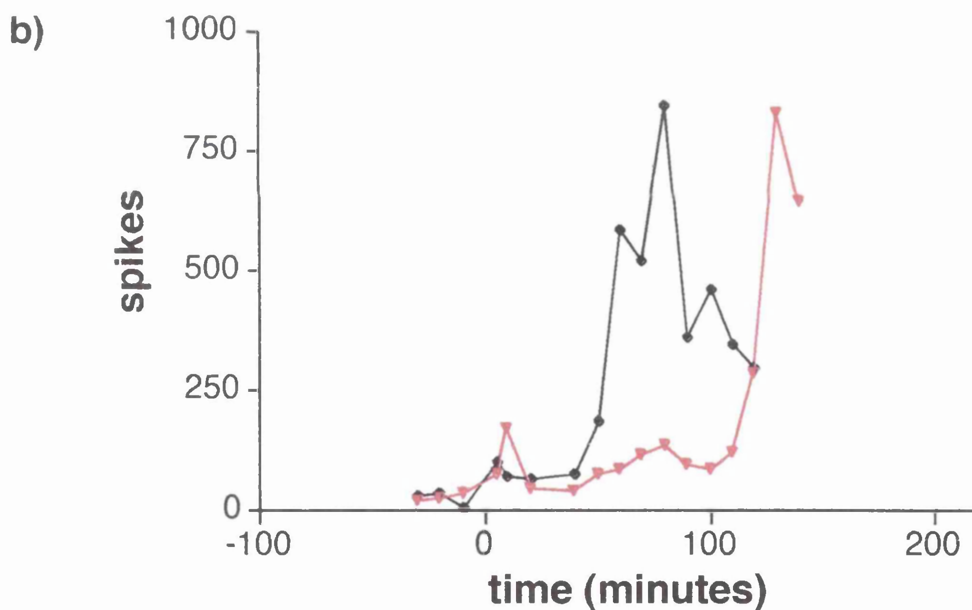
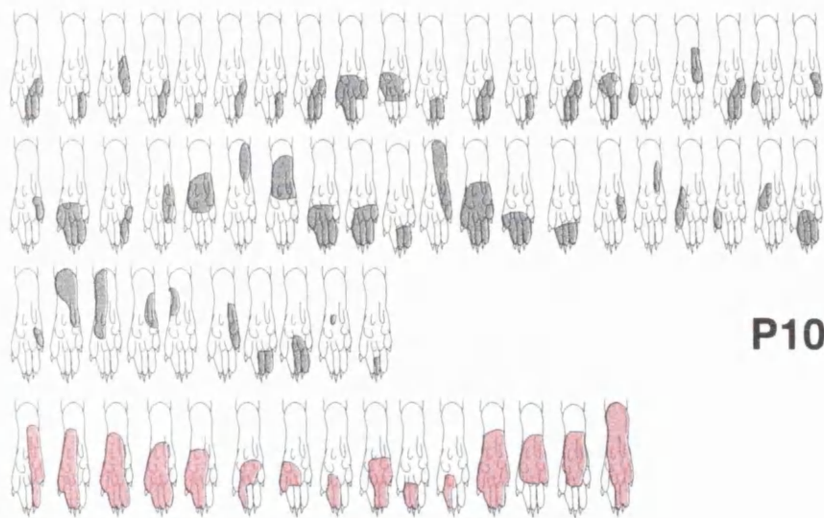
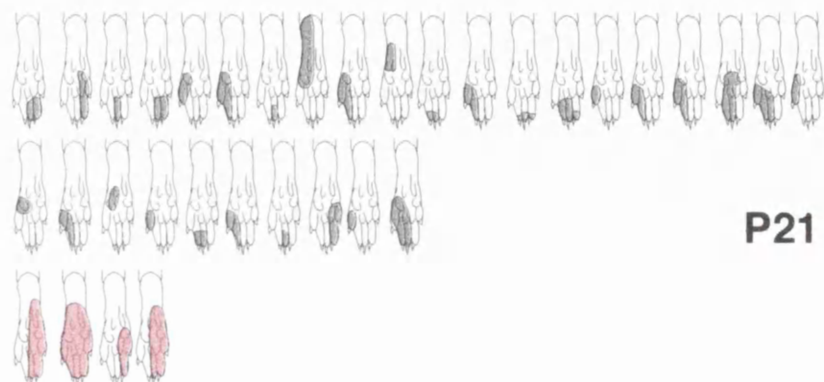



**Fig 5.1: Acute inflamation time course.**
The graphs above plot the after discharge activity (a) and background (b) activity of individual cells (P10) following carrageenan injected into the plantar hindpaw (at time 0). The peak activity in both cases can be seen at about 100 minutes after the injection. This is considerably earlier than that reported in adult models. There was no data for background activity in cell 3.



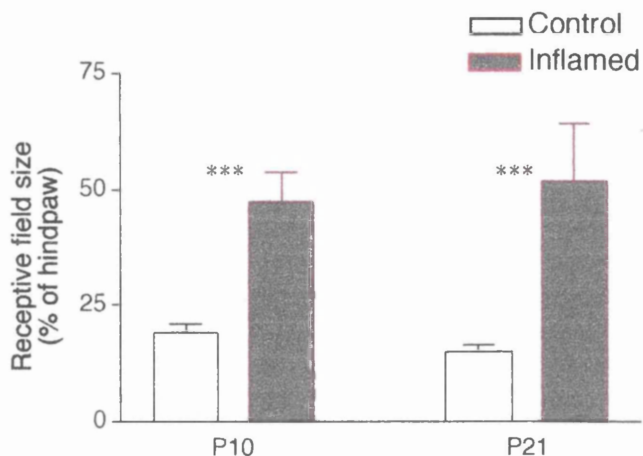




**Fig 5.2. Peripheral receptive field changes following inflammation.** Representations of the peripheral receptive fields. Those in the red represent cells receiving afferents from inflammed skin, whilst those in grey are the control. The mean receptive field size ± SEM (as a percentage of the total plantar foot area is, for P10: 19.13±1.97 (control), and 47.20±6.43 (inflamed). For P21 the figures are: 14.9±1.62 (control) and 51.83±12.19 (inflamed). These values are plotted on the bar chart.

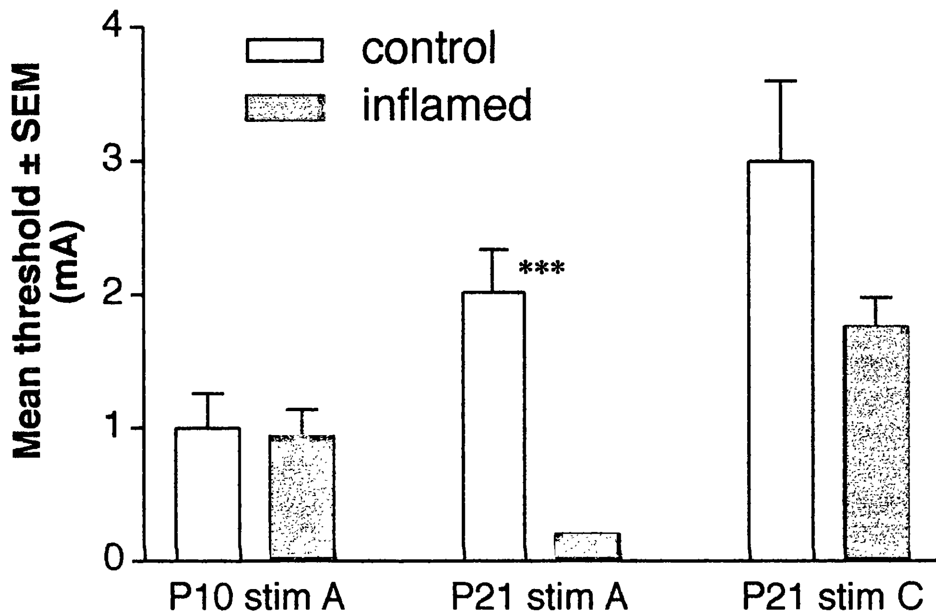



**Fig 5.3: Changes in stimulating threshold, following inflammation.**
Bart chart demonstrating these changes in threshold values (mA) for cells receiving afferents from either control or inflamed periphery. The width of the stimulus used to stimulate A fibres was $50\mu s$, and that to stimulate C fibres was $500\mu s$.
Student t tests, comparing stimulation thresholds in control and inflamed cells reveal a p value of 0.8728 for P10, $p<0.0001$ for P21 A threshold and $p=0.0513$ for P21C fibre threshold.

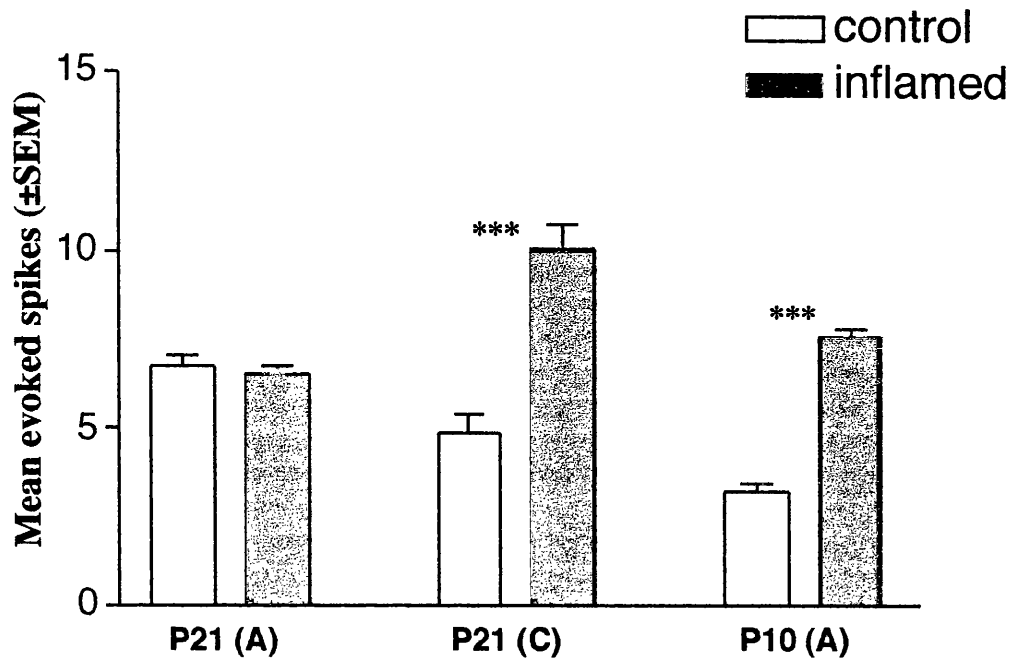



**Fig 5.4: Magnitude of the evoked response, following inflammation.**
Bar chart showing the mean±SEM evoked response (number of spikes) of cells with A and C afferent input at P21 and A afferent input at P10. Student t tests comparing control and inflamed data for the P21 C response and P10 A response give a p value of <0.0001 in both cases. The same comparison for P21 cells responding to an A fibre stimulus yields a p=0.5941.

# Concluding Remarks

The work presented in this thesis and the accompanying paper (Appendix 1: "C-fos can be induced in the neonatal rat spinal cord by both noxious and innocuous peripheral stimulation". Jennings, E and Fitzgerald, M, Pain, **38.** 301-306, 1996), strongly suggests that central processing of afferent input is different in the neonate when compared to the adult. This is not surprising, given that the neonatal system is maturing, and as such these results should be considered in this light. Much of what is known of the later mechanisms of neurodevelopment has been done in the visual system. (Goodman and Shatz, 1993; Katz and Shatz, 1996).

*VISUAL SYSTEM DEVELOPMENT*

Early neurodevelopment involves axon pathfinding, and growth to regionally localised targets. The axon terminals then retract or expand to select a specific subset of cells with which to form synapses. This remodelling is called "address selection", and is dependent on competition from the surrounding inputs. The whole process is capable of transforming a coarse grained and overlapping projection into a refined and highly tuned pattern of connections (Goodman and Shatz, 1993).

In the neonatal dorsal horn the basic pattern, and regional localisation of the afferent terminals has taken place by birth. A fibres enter the spinal cord dorsal horn, by E15-16, and the C fibres follow, entering the grey matter, by E20 (Fitzgerald et al., 1991; Fitzgerald, 1987b; Jackman, 1997; Koerber and Brown, 1980; Plenderleith et al., 1992; Smith, 1983). Synaptogenesis in the dorsal horn follows the ventro-dorsal pattern of development (Altman and Bayer, 1984; Nornes and Das, 1974), and peaks at P4-5 in the deep dorsal horn and P7-9 in SG (Cabalka et al., 1990). The somatotopic map of the central terminations of afferents is also established by birth (Fitzgerald and Swett, 1983; Smith, 1983).

Although the anatomy is in place, and A afferent responses can be recorded prenatally from both the dorsal horn and ventral root ( Fitzgerald et al., 1987; Fitzgerald, 1987c; Fitzgerald, 1991a), C fibre evoked afferent responses can not be recorded in the dorsal horn until the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1988; Hori and Watanabe, 1987; Jennings and Fitzgerald, 1998). Over the first three postnatal weeks C fibre afferent connectivity matures probably using a process similar to the "address selection", described in the visual system (Goodman and Shatz, 1993).

The developmental modulation of the central nervous system connections by peripheral activity was first shown in kittens by Hubel & Weisel (1970). They showed the columnar structure of the visual cortex called occular dominance columns (LeVay et al., 1980), and importantly that closure of one eyelid in young kittens or monkeys, resulted in the expansion of the territory occupied by the axon terminals of the open eye (in layer IV of the visual cortex), and a corresponding decrease in the territory occupied by the axons from the closed eye (Hubel et al., 1977; LeVay et al., 1980). Furthermore, they also showed that there is a "critical period" in neonatal life, when the columns are forming, that these changes can occur. Similar manipulations in the adult do not alter the structure of the columns in layer IV (Hubel and Wiesel, 1970; LeVay et al., 1980).

This was the beginning of a surge of experiments aiming to determine the mechanisms and players in activity dependent plasticity (ADP) during neurodevelopment. A graphic example is an experiment in which the visual activity from both eyes was completely blocked by intraoccular injections of TTX throughout neonatal life (Stryker and Harris, 1986). This treatment resulted in the prevention of the formation of occular dominance columns. Furthermore, experiments during the critical period, where the visual stimulation through one eye was paired with postsynaptic depolarisation produced by a cortical stimulating electrode enhanced the strength of inputs from the stimulated eye in some cells for hours (Shulz and Fregnac, 1992). These results suggest that Hebb synapses, possibly involving NMDA receptors may be necessary for occular dominance column formation. There is physiological evidence that NMDA receptor activation contributes to the visually driven responses of cortical neurones during the critical period (Fox et al., 1989), and there is a decline in NMDA receptor function in layers IV -VI which correlates with the end of the critical period in cats. Recordings from slices of rat visual cortex, show that cortical neurones can undergo LTP, dependent on the activation of NMDA receptors, following activation of the incoming axons from the lateral geniculate nucleus, although they require the disinhibition of $GABA_A$ receptors with bicuculline (low doses) to do this effectively, suggesting that LTP induction in the hippocampus and visual cortex are under slightly different controls (Artola and Singer, 1987).

*Barrel field cortical development (somatosensory).*

The majority of the work on somatosensory system development has been done in the whisker- barrelfield cortex pathway. Somatosensory information from the face and teeth, is carried to the trigeminal dorsal horn. Sensory information from the whiskers, is precisely mapped in a pathway relayed by three synapses (reviewed by: (Killackey et al., 1990)). The whiskers are represented by barrelettes in the brainstem trigeminal complex (BSTC), by the barreloid in the thalamus (VPM) and by barrelfield cortex in the primary somatosensory cortex (S1).

As in the visual system there is a critical period in the barrel cortex during which the barrel fields form (P0-3) (Belford and Killackey, 1980). Surprisingly, histochemical studies fail to show any changes in barrel field formation after either blocking vibrissae inputs (Henderson et al., 1992) or by directly blocking barrel cortex activity (Chiaia et al., 1992; Schlaggar et al., 1993). However, the plasticity associated with cautery of a row of whiskers, was absent after treatment with AP5 (Schlaggar et al., 1993). Physiological studies reveal that there is a disorganisation in the receptive fields of cells in the barrelfield cortex, after treatment with AP5 during the critical period (Fox et al., 1996).

*Primary afferent connectivity with the brainstem trigeminal nuclei.*

Knockout mice bred without the NR1 subunit die within a day of birth, although they can, with careful nurturing, live one more day (Forrest et al., 1994; Li et al., 1994). These animals lack the central somatotopic representation of the whisker receptive fields in the BSTC, called barrelettes, although the *gross* topographic organisation of the trigeminal afferents was normal (Li et al., 1994). In the same study, whole cells patch clamp recordings from neurones in the barrelettes regions of the BSTC show the absence of NMDA receptor mediated currents, although AMPA mediated currents are present (Li et al., 1994).

Further evidence that the NMDA receptor is important in the formation of barrelettes comes from a more recent study from the same group. They attempted to rescue the NR1 knockouts with high levels of NR1 transgene, and found that not only did the animals live to adulthood, but their somatotopic patterns were restored, including barrelette formation (Iwasato et al., 1997).

This fits in with the model of neurodevelopment, where axon pathfinding is independent of NMDA, but that the fine tuning of the connections or "address selection" in activity dependent, and that NMDA receptor mediated activity plays a major role in the refinement of axonal projections (Constantine-Paton et al., 1990; Goodman and Shatz, 1993). This is also direct evidence of the role of NMDA receptor-mediated activity, in connectivity between primary afferents and the first CNS relay.

The NR1 knockout animals had a reduction in the levels of the NR2B but not the NR2A subunit, which indicates some interdependence in subunit expression (Forrest et al., 1994). Mice with this NR2B subunit knocked out also lacked clearly defined barrelette formation, although as in the NR1 knockouts the gross somatotopy was present (Kutsuwada et al., 1996). This knockout also showed the absence of NMDA receptor mediated activity in physiological experiments in barrelfield cortex (Kutsuwada et al., 1996)

NMDA mediated activity is known to be involved in associative processes such as LTP or LTD (Bliss and Collingridge, 1993), and this is thought to be the basis of the specific connectivity in the visual system and barrelfields (Goodman and Shatz, 1993). The hypothesis is that the correlated activity in afferents converge on specific neurones which results in the depolarisation of the postsynaptic membrane via non-NMDA receptors and the removal of the voltage sensitive $Mg^{2+}$ block on the NMDA receptors. Subsequent action potentials release more glutamate which opens the NMDA receptor channels with the resulting significant $Ca^{2+}$ influx into the postsynaptic cell. Then follows a cascade of events (mechanisms not known) in which feedback is provided to the presynaptic fibres, leading to consolidation of the synaptic connections with fibres that fire in synchrony. Those connections made by axons that do not fire in synchrony are weakened, due to their inability to activate NMDA receptors and so trigger the subsequent cascade of events in postsynaptic cells (Constantine-Paton et al., 1990;Goodman and Shatz, 1993).

The recent reports (in the last four years) of silent synapses in neonatal hippocampus and dorsal horn (see 4.33), will change the above hypothesis a little. NMDA receptor activation is still paramount, but initially this is required to assist in the activation of AMPA receptors, so as to make a functional glutamatergic receptor at resting membrane potentials. This has been achieved in the neonatal hippocampus with a brief high frequency stimulus, sufficient to induce LTP (Durand et al., 1996). Since silent synapses are found predominantly in the neonatal CNS, and since their

conversion to functional synapses appears to be activity dependent, they are likely to play a major role in the "address selection" posited in the models described above. Silent synapses still require an initial activation as the voltage dependent $Mg^{2+}$ block on the NMDA receptor has been shown to be present from birth (Bardoni et al., 1998). One hypothesis is that the $Mg^{2+}$ block can be removed if the membrane nearby is depolarised by another axon releasing glutamate onto a functional synapse (Liao et al., 1995). The synchronous firing of this system fits with the developmental hypothesis outlined above.

The peripheral receptive fields of the neurones in the barrel cortex, can be disrupted following treatment with NMDA receptor antagonists (Fox et al., 1996), as they are following treatment with capsaicin (Nussbaumer and Wall, 1985; Wall et al., 1982a). Since C fibres are known to have a role in activating NMDA receptors in central sensitisation (Woolf and Thompson, 1991), the role of the NMDA receptor in the neonatal spinal cord deserves further investigation.

Studies of another transgenic mouse, the monoamine oxidase A deficient, show that barrels are absent in S1, although the somatotopy appears to be conserved in thalamus and BSTC nuclei (Cases et al., 1996). Administration of an inhibitor of serotonin synthesis restores the formation of the barrels in S1, suggesting that an excess of serotonin during the critical period is responsible for the loss of the barrels (Cases et al., 1996). This may be the reason that the spinal serotonergic system only becomes functional in the second postnatal week (Fitzgerald and Koltzenburg, 1986; Giordano, 1997), as it may be toxic to the somatosensory dystem in early stages of development.

Since A fibres are found in the superficial laminae of the dorsal horn for the first three postnatal weeks, at around the same time as the C fibres are forming appropriate connections (Fitzgerald et al., 1994; Fitzgerald, 1985a; Mirnics and Koerber, 1995), and that they appear to form synapses (Coggeshall et al., 1996) there is the possibility that they are functional. If this was the case then what would this function be? One possibility is that they drive neurones in the superficial laminae, so that these cells can make appropriate synaptic connections at higher levels in the CNS, during the *critical period*, since these neurones do not appear to receive afferent input from C fibres at this time.

## *Conclusion:*

Although a substantial body of work has been done on neurodevelopment, little has focused on the spinal cord. Since the spinal cord plays a major role in the integration and modulation of somatosensory input, there should be more work done in this area.

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