THE ROLE OF CALCIUM/CALMODULIN-DEPENDENT KINASE IIα ON THE DEVELOPMENT AND PLASTICITY OF SENSORY PATHWAYS

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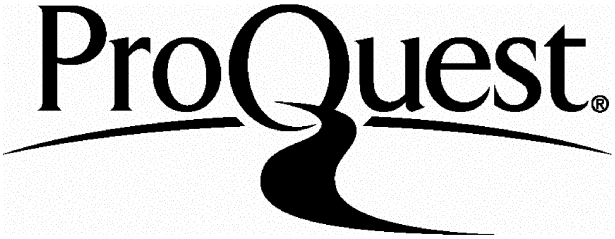

ABSTRACT

The dorsal horn of the spinal cord undergoes alterations during early postnatal life. For example, the primary afferent terminations from low-threshold Aβ fibres reorganise, and the interneurones increase their axodendritic elaborations. Many of these changes require activation of the NMDA receptor. The aim of this thesis was to examine the role of a major downstream signalling protein- calcium/calmodulin-dependent kinase II- in the activity-dependent development of the dorsal horn.

After confirming the presence of the kinase in the spinal cord from birth, and elucidating its localisation within this region, I obtained a transgenic mouse in which a point mutation of the T286A site (T286A) prevents the kinase from entering its autophosphorylated form, and thus from remaining active after dissipation of the calcium stimulus.

Using DiI labelling of Aβ fibres, it was shown the postnatal reorganisation that normally occurs during development, but is prevented by chronic NMDA receptor blockade, was not affected by the mutation, suggesting that CaMKII autophosphorylation is not required for normal A fibre development.

Electrophysiological analysis of the dorsal horn using *in vitro* whole-cell patch clamp and *in vivo* extracellular recordings revealed decreases in polysynaptic Aβ input to the superficial dorsal horn, combined with reduced C fibre input and increased neuronal receptive field sizes in the mutant compared with wild-type littermates. This suggests a role for CaMKII in the normal postnatal development of the dorsal horn interneuronal connectivity.

Finally, the pain-processing ability of the adult mutant mouse was examined using behavioural and electrophysiological techniques. It was found that, while the baseline sensory processing was unaffected in the mutant, there was a decrease in pain behaviour in response to intraplantar injection of formalin, accompanied by an increase in pain behaviour in response to nerve injury. These findings highlight the different mechanisms that control pain processing in the spinal cord, and suggest different roles for CaMKII in each of these mechanisms.

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LIST OF ABBREVIATIONS

AMPA	Amino-hydroxy-methyl-isoxalone propionic acid
ANOVA	Analysis of variance
BHRP	β subunit of cholera toxin conjugated to horseradish peroxidase
CaMKII	Calcium/calmodulin-dependent kinase II
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvent
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DiI	1,1(')-dioctadecyl-3,3,3('),3(')-tetramethylindocarbocyanine perchlorate
DLF	Dorsolateral funiculus
DRG	Dorsal root ganglion
E	Embryonic day
EF50	Force required to elicit a 50% response
GABA	γ amino butyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
HT	High-threshold
HTMR	High-threshold mechanoreceptor
IB4	Isolectin B4
KA	Kainate
LSN	Lateral spinal nucleus
LT	Low-threshold
LTD	Long-term depression
LTMR	Low-threshold mechanoreceptor
LTP	Long-term potentiation
MOR	Mu opioid receptor
mRNA	Messenger ribonucleic acid
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
OCD	Occular dominance column
P	Postnatal day
PAG	Periaqueductal grey
PKA	Cyclic AMP-dependent protein kinase
PKC	Calcium/diacyl-glycerol-dependent protein kinase
PSP	Postsynaptic potential
RT-PCR	Reverse transcriptase polymerase chain reaction
s/mEPSC	Spontaneous/miniature excitatory postsynaptic current
s/mIPSC	Spontaneous/miniature inhibitory postsynaptic current
SG	Substantia gelatinosa
SNI	Spared nerve injury
SNL	Spinal nerve ligation
STT	Spinothalamic tract
TrkA	Tryrosine receptor kinase A
TRPV1	Transient receptor potential 1 (also VR1)
TTX	Tetrodotoxin
WDR	Wide dynamic range

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Chapter 1

INTRODUCTION

1.1 Introduction

The immature nervous system is extremely adaptive. At birth, neurones have limited dendritic arbours, short axonal projections and immature synaptic transmission. During early postnatal life, as the variety of sensory experience increases, the system adapts accordingly, developing complex neuronal morphologies and forming new synapses to encode the incoming information. The sensory input in infancy and childhood shape our final nervous system, defining our ability to perceive the world around us. However, the extent of this developmental plasticity leaves the nervous system vulnerable to long-term deficiencies if normal sensory inputs are disrupted at a critical postnatal period.

While developmental plasticity is well known in the visual and auditory system, the somatosensory system is also vulnerable to malfunction following disruption early in postnatal life. Early pain and trauma can lead to sensory disturbances and pain perceptions that last well beyond the infant period (Porter *et al.*, 1999; Taddio *et al.*, 1995). It is therefore of great importance to ascertain the mechanisms by which the nervous system normally adapts to external stimulation, and so have a better understanding of how to treat young infants should anything go wrong.

At the spinal cord level, the neonatal somatosensory nervous system is highly excitable. The flexion withdrawal reflex- an indicator of noxious sensitivity in adults- can be induced in neonates by low intensity mechanical stimulation of the foot (Fitzgerald *et al.*, 1988a; Fitzgerald *et al.*, 1989; Andrews & Fitzgerald, 1994). Similar findings have been observed in the rat, with the hyperexcitability observed early in postnatal life corresponding to that seen in the human at the third trimester (Fitzgerald *et al.*, 1988b). The threshold of the reflex in human infants rises with postnatal age, reflecting a decrease in excitability of the spinal cord (Andrews & Fitzgerald, 1994).

Because the thresholds and receptive field sizes of cutaneous mechanosensitive primary afferents are generally the same in the adult and the neonatal rodent (Fitzgerald, 1987; Koltzenburg *et al.*, 1997), the changes in excitability that occur during postnatal development are most likely to occur centrally. Indeed anatomical, pharmacological and electrophysiological alterations in the spinal cord neurone connectivity has been reported that may underlie the reflex maturations, and these are detailed below.

1.2 Anatomical changes during development

1.2.1 Primary afferent input

Primary sensory neurones are a functionally and morphologically heterogeneous population of cells that exhibit a wide variety of neurochemical, anatomical and physiological properties. In the periphery, they innervate a wide variety of sensory receptors, such as hair follicle receptors, Pacinian corpuscles and Merkel cells, and form central synapses in the spinal cord. The afferents are divided into a number of subpopulations, defined by their conduction velocities, mechanical thresholds and myelination. The groups, first described by Erlanger and Gasser in 1937, consist of A, B and C fibres. Since their original description, the B fibres have since been found to be derived from preganglionic sympathetic fibres, so that the primary afferent fibres are now classed as A fibres (fast-conducting, myelinated fibres) or C fibres (slow-conducting, unmyelinated fibres).

C fibres are twice as numerous as A fibres in the peripheral nerve and are much smaller in their axon and cell body diameter. In addition, they are unmyelinated, and therefore conduct at less than $1ms^{-1}$ (Lynn & Carpenter, 1982). These fibres primarily encode nociceptive information to the CNS, and are generally polymodal, responding to mechanical, thermal and chemical stimuli, although small subsets have been found that are purely thermosensitive (Hunt & Mantyh, 2001). The C fibre group can be further divided into two subcategories depending on their neurochemical properties. The first group express glial-derived neurotrophic factor (GDNF) and purinergic P2X3 receptors as well as binding sites for the IB4-lectin, and terminate in the deeper part of lamina II (known as lamina II_i; Nagy & Hunt, 1982; Hunt & Mantyh, 2001; Bennett *et al.*, 1998). The second group contain peptides such as substance P, calcitonin gene-related peptide (CGRP) and the nerve growth factor (NGF) receptor, TrkA. They terminate in superficial part of lamina II (lamina II_o; Averill *et al.*, 1995; Hunt & Mantyh, 2001; Figure 1.1). Both groups express the vanilloid receptor, TRPV1 (also known as VR1), which responds to noxious chemical and thermal stimulation (Guo *et al.*, 1999; Caterina *et al.*, 2000; Caterina *et al.*, 1997). Further subsets have been isolated that contain receptors that respond to cold and menthol stimulation (called TRPM8 receptors; Peier *et al.*, 2002; McKemy *et al.*, 2002) or to mustard oil (called ANKTM1; Jordt *et al.*, 2004).

C fibres are born slightly after the A fibres, with the isolectin B4 (IB4)-positive population being born at embryonic day (E) 13-16 and peaking in birth-rate at E14, compared to the A fibres which are born from E12-15 and peaking at E13 in rats (Altman & Bayer, 1984; Lawson *et al.*, 1974; Kitao *et al.*, 1996; Jackman & Fitzgerald, 2000). Peripherally, both groups of fibres can be seen in the hindlimb at early ages, although TrkA-positive C fibres appear to innervate the epidermis more slowly than do the RT97-positive A fibres (Jackman & Fitzgerald, 2000). Functionally, the types of fibre can be classified early in development by their differences in conduction velocity and mechanical thresholds (Koltzenburg & Lewin, 1997; Fitzgerald, 1987). As with the periphery, the central projections of C fibres appear to develop slightly later than the A fibres. They first start to grow into the spinal cord at E19, where they enter Lissauer's tract and send collaterals into the dorsal horn. Here they can be seen at birth, as identified by the presence of their neurochemical markers, TrkA and CGRP (Jackman & Fitzgerald, 2000). Neonatal sciatic nerve injection of horseradish peroxidase-conjugated wheat-germ agglutinin (WGA-HRP) produces the same U-shaped staining along the spinal cord as is seen in the adult, implying that the somatotopic maps from the primary afferents are in position by birth (Fitzgerald & Swett, 1983). The synaptic connectivity, however, develops slowly so that, while the C fibres are capable of glutamate release at dorsal horn neurone synapses at postnatal day (P) 0 (bath application of capsaicin produces increases in miniature excitatory postsynaptic current (mEPSC) frequency (Baccei *et al.*, 2003), this release is not sufficient to generate postsynaptic action potentials until P10 (Fitzgerald, 1988; Jennings & Fitzgerald, 1998; Fitzgerald & Jennings, 1999). In addition, long-latency ventral root potentials are not recorded until P10 (Fitzgerald *et al.*, 1987; Hori & Watanabe, 1987).

A fibres have also been divided into a number of subgroups: large Aα fibres, which are also known as 1a afferents, are proprioceptive and innervate muscle spindles and Golgi tendon organs; smaller Aβ fibres, which transmit non-noxious mechanical information, primarily from the skin; and the smallest Aδ fibres, some of which comprise hair afferents, others having nociceptive and thermoceptive properties (Willis & Coggeshall, 1991). Each subgroup shows strict termination patterns within the adult spinal cord. Aα fibres project collaterals into the motorneurone pool of the ventral horn, while Aδ fibres enter the cord more laterally and travel along Lissauer's tract, sending collaterals into laminae I and V (Light & Perl, 1979). A small population of Aδ fibres encoding

non-noxious information from down hair (D hair) follicles have additionally been identified terminating in laminae II and III of the cat and monkey (Light & Perl, 1979). Aβ fibres enter the cord medially and travel in the dorsal columns, projecting collaterals into laminae III and IV of the dorsal horn (Brown, 1981; Shortland *et al.*, 1989); Figure 1.1).

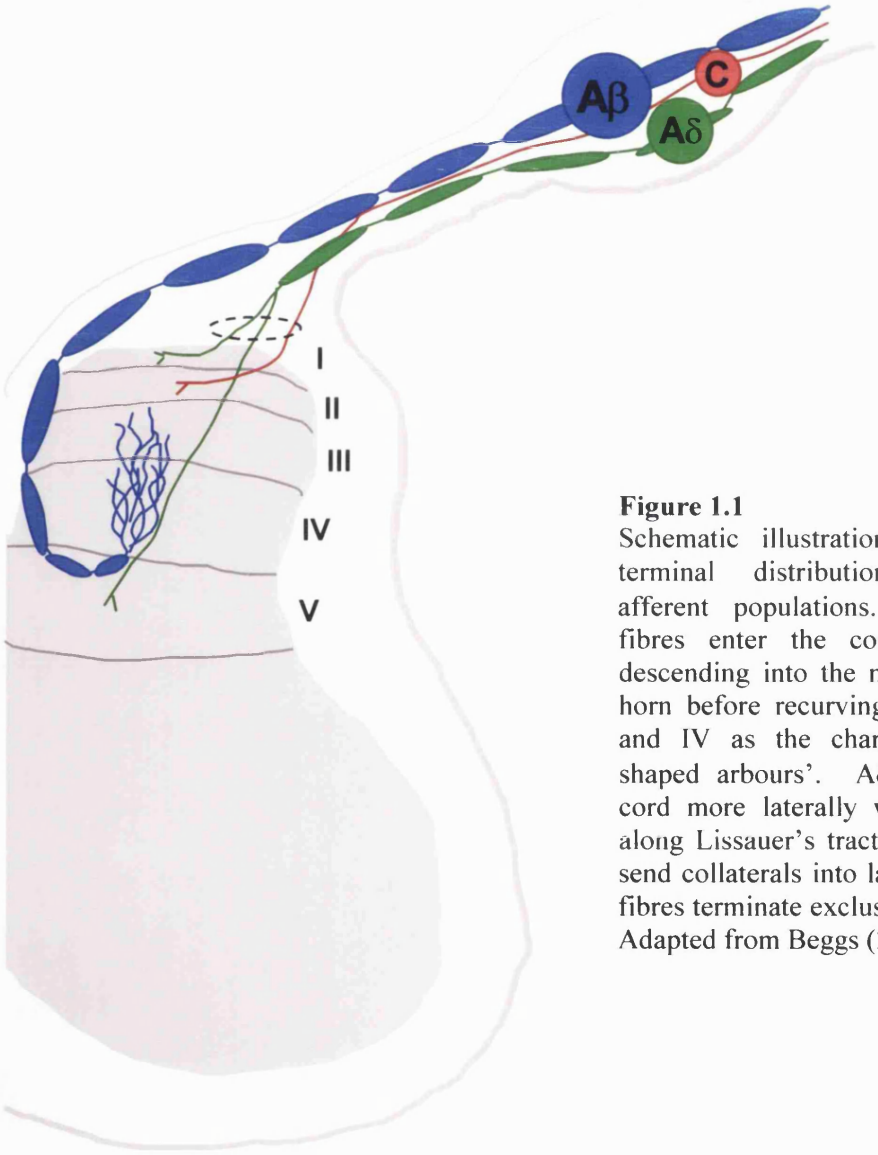

Figure 1.1
Schematic illustration of the central terminal distributions of primary afferent populations. The large Aβ fibres enter the cord medially and descending into the neck of the dorsal horn before recurving into laminae III and IV as the characteristic 'flame-shaped arbours'. Aδ fibres enter the cord more laterally where they travel along Lissauer's tract (dotted line) and send collaterals into lamina I and V. C fibres terminate exclusively in lamina II. Adapted from Beggs (2000).

It appears that the electrophysiological phenotype of A fibres is determined before birth. For example, Aβ fibres exhibit narrow, uninflected somal spikes and Aδ fibres exhibit broader, inflected spikes in neonates as well as in adults (Fitzgerald & Fulton, 1992; Woodbury *et al.*, 2001; Woodbury & Koerber, 2003). In addition, DRG neurones from E17 rats display narrow, uninflected spikes and TTX-sensitive and resistant sodium currents in a manner similar to the adults (Ogata & Tatebayashi, 1992; Mirnics & Koerber, 1997). Neonatal cutaneous fibres also display similar peripheral receptive properties to adults, as classified by the type of stimulation to which they respond, as well as their ability to adapt to this cutaneous stimulation (Fitzgerald, 1987). The categories include, (i) slowly-adapting mechanoreceptors, which display relatively low-threshold spike activity in both neonates and adults; (ii) rapidly-adapting mechanoreceptors, which respond to very low threshold stimulation at either Aβ or Aδ (D-hair receptors) conduction velocities in both neonate and adult; (iii) pressure receptors, which are only seen in the neonate; (iv) high threshold mechanoreceptors, which respond to noxious mechanical stimulation and have Aδ and C fibres in both adults and neonates; (v) polymodal receptors, which respond to noxious mechanical and thermal stimulation at all ages (Fitzgerald, 1987). This study demonstrates both the functional diversity of cutaneous primary afferents, and the similarities between these groups during development. The majority of changes that occur during development are therefore most likely to be in the central nervous system.

The developmental alterations in afferent input have been most clearly shown using *in vivo* electrophysiological recordings of dorsal horn neurones. This technique has revealed the predominance of low threshold input to the neonatal dorsal horn, with natural stimulation of dorsal horn cell receptive fields evoking long-lasting discharges, and repetitive low intensity A fibre stimulation leading to sensitisation of dorsal horn neurones (Jennings & Fitzgerald, 1998). Furthermore, receptive field sizes of dorsal horn cells are large in the neonate, and decrease in size during the first two postnatal weeks (Fitzgerald, 1985). As large receptive fields were not observed on analysis of DRG properties (Fitzgerald, 1987), the reduction in receptive field size is likely to be caused by changes in central connections of the dorsal horn cells. This could arise from changing primary afferent inputs such as through presynaptic controls or local circuit interneuronal activity.

Aβ fibres first grow into the rodent spinal cord at E15. Unlike the C fibres, however, which project directly to the discrete terminal fields found in the adult, Aβ fibres form aberrant terminations throughout the dorsal horn (Fitzgerald *et al.*, 1994; Mirnics & Koerber, 1995; Reynolds & Fitzgerald, 1992). While in the adult, the fibre collaterals enter the cord medially and curve through the grey matter to terminate in laminae III and IV, in the neonate, these collaterals extend throughout the superficial dorsal horn (Ramón y Cajal, 1909; Fitzgerald *et al.*, 1994). In addition, these aberrant collaterals have been shown to make synaptic connections with cells in lamina II (Coggeshall *et al.*, 1996). It appears that, during the first few weeks of postnatal life, the collaterals withdraw from the superficial dorsal horn, so as to display the adult-like profile by the end of the fourth postnatal week (Fitzgerald *et al.*, 1994; Mirnics & Koerber, 1995; Beggs *et al.*, 2002).

Because of the late postnatal reorganisation of Aβ fibres, there exists in early postnatal life a period where both C and A fibres are present in the superficial dorsal horn. This has led to the hypothesis that the two afferent inputs compete for synaptic sites during the first few postnatal weeks, and that this results in the withdrawal of the Aβ fibres into the deeper laminae. Evidence for such a hypothesis comes a study by Torsney *et al.* (2000), in which C fibres were ablated by neonatal injection of capsaicin. Such a procedure prevented the normal Aβ fibre withdrawal, so that terminals could still be seen in the adult SG, resulting in lower mechanical thresholds, seen both behaviourally and electrophysiologically, compared to vehicle-injected controls. However, the full extent of this competition is still not clear.

The withdrawal of Aβ fibres from the superficial dorsal horn has additionally been shown to be dependent on neuronal activity. Chronic blockade of NMDA receptors, which require correlated pre- and postsynaptic activity in order to function, prevents the withdrawal in a manner similar to C fibre ablation (Beggs *et al.*, 2002). This implies that the postnatal reorganisation does not occur from spontaneous activity, but rather requires evoked neuronal activity of a kind associated with tactile experience. This will be discussed in more detail later.

1.2.2 Dorsal horn

The dorsal horn of the spinal cord displays an extremely complex cellular organisation, with great diversity in cell type, dendritic branching and axonal projection. One form of classification is to divide the cells into those that project axons into the white matter (projection neurones) and those whose axons remain in the dorsal horn (interneurones). Both these groups can be further divided into subcategories depending on their dendritic morphology and physiological properties.

1.2.2.1 Projection neurones

Projection neurones make up a relatively small proportion of cells in the dorsal horn (Willis & Coggeshall, 1991), and of these, only about 2% project to supraspinal regions (Todd, 2002). They are identified by injection of tracers into specific brain regions, allowing for selective labelling of cell bodies in the dorsal horn (Spike *et al.*, 2003; Ikeda *et al.*, 2003). In addition, the proportion of projection neurones differs between laminae, so that lamina I contains between 5 and 11% (Spike *et al.*, 2003; Bice & Beal, 1997a; Bice & Beal, 1997b) and lamina III which contains 1% of the total neuronal population (Todd *et al.*, 2000). Some evidence exists for a very small population of projection neurones in lamina II (Willis *et al.*, 1978; Bice & Beal, 1997b), but this has yet to be confirmed (Todd, 2002) A larger proportion of neurones send axons and collaterals to other spinal segments, and are known as propriospinal neurones.

Lamina I contains neurones that project to the thalamus, periaqueductal grey (PAG), parabrachial area, nucleus of the solitary tract and medullary reticular formation (Lima & Coimbra, 1988; Todd *et al.*, 2000; Spike *et al.*, 2003). Approximately 80% of lamina I projection neurones are immunopositive for the substance P receptor, NK1 (Todd *et al.*, 2000). With regard to their morphology, lamina I projection neurones are generally believed to be fairly flat, with a horizontal dendritic arbour spreading in a rostrocaudal direction (Willis & Coggeshall, 1991; Lima & Coimbra, 1988; Woolf & Fitzgerald, 1983). There is also evidence that the arbours form some synaptic contact in lamina II (Woolf & Fitzgerald, 1983; Willis & Coggeshall, 1991). In keeping with such a finding, McLeod *et al.* (1998) have reported the presence of NK1-positive dendrites making synaptic contacts with substance P-containing primary afferents, which are therefore likely to be peptidergic C fibres (Todd, 2002).

In lamina II, only a very small proportion of cells, if any, project out of the cord. A larger proportion, however, are propriospinal (Willis & Coggeshall, 1991). Cajal, for example, first identified groups of neurones in SG that projected axons into the white matter. He divided them up into three main cell types: (1) 'les cellules limitrophes', or limiting cells which are found in the external region of SG; (2) 'les cellules centrales et anterieures', or central cells, which are seen in the central part of SG; (3) 'les cellules transversaux', or transverse cells, which have long dendritic projections that extend transversely across external SG (Ramón y Cajal, 1909). These studies were all performed on neonatal mammals so it is possible that they represent cell groups whose morphology is not yet mature (see below). The majority of these cells extend axons to other spinal segments, although there is some evidence that some project into the thalamus and brainstem (Willis *et al.*, 1978; Bice & Beal, 1997a; Bice & Beal, 1997b).

The majority of cells in the deeper laminae that project to the brain are spinothalamic (STT) neurones. These cells are small but have large dendritic arbours, sometimes extending as far as laminae II and IX (Willis & Coggeshall, 1991). An additional population of large, NK1-positive neurones have also been described in the deep dorsal horn. These cells have dendritic arbours that project dorsally into the superficial dorsal horn and are thus described as antenna-like neurones (Todd *et al.*, 2000; Willis & Coggeshall, 1991). This is intriguing as it implies that fine unmyelinated, in addition to the large myelinated, fibres can form synapses with these deep cells and, indeed, substance P-containing primary afferents have been shown making synaptic contacts with these cells (Naim *et al.*, 1997).

Developmentally, projection neurones are formed early in prenatal life (at E13-15- before the formation of interneurons; Beal & Bice, 1994). Golgi and Toludine blue staining has revealed the presence of each type of neurone described by Cajal by E19 (Bicknell & Beal, 1984). These neurones increase in size and exhibit further dendritic branching during the early postnatal period, so that an adult-like morphology is attained by approximately P10. This finding is in keeping with the reduction in number of growth cones, and in expression of the growth-associated protein, GAP-43, by this age (Fitzgerald *et al.*, 1991). In addition, it appears that neurones projecting along different tracts display strict developmental timeframes so that, for example, neurones sending axons to the thalamus (STT neurones) develop before those sending axons to the

cerebellum (spinocerebellar neurones; Beal & Bice, 1994). Interestingly, the increase in cell size of all dorsal horn neurones demonstrates a pattern very similar to the expression of many ionotropic glutamate receptors in the cord, with a substantial increase during the first two weeks of life, and a subsequent slight decrease into adulthood (Bicknell & Beal, 1984).

1.2.2.2 Intrinsic interneurones

Nonprojection neurones show different morphological and developmental profiles to projection/propriospinal neurones, and make up the larger proportion of the two groups. Classification of these interneurones is notoriously difficult, due to the huge diversity of dendritic morphology, axonal projections and physiology. Perhaps the most well described group is the islet cells (Gobel, 1975; Willis & Coggeshall, 1991; Grudt & Perl, 2002). These cells display a dense dendritic arbour that is elongated in the rostrocaudal direction but is limited in the dorsoventral and mediolateral directions (Gobel, 1978). Islet cell axons generally terminate in lamina II, although have been noted to branch extensively within this region, sometimes exceeding 1000μm in extent. Some axon branches have also been seen entering lamina III (Grudt & Perl, 2002). In addition, (Gobel, 1978) suggested that these cells could be inhibitory interneurones, and this was supported by findings by Todd & Sullivan (1990) that neurones with an islet cell morphology did indeed contain GABA and glycine. The finding that these interneurones were inhibitory is of particular interest since it has been demonstrated that some islet cell dendrites can be presynaptic and therefore can act on primary afferents as negative feedback loops. This finding demonstrates the complexity of dorsal horn circuitry, particularly with regard to inhibitory systems (Willis & Coggeshall, 1991). Physiologically, islet cells display resting transmembrane potentials that are nearly 10mV less than all other lamina II neurones, some inward rectification and evoked responses that are consistent with monosynaptic C fibre input (Grudt & Perl, 2002).

A second group of intrinsic interneurones have been classified as 'stalked' due to the presence of short, stalk-like dendritic spines (Gobel, 1978; Bicknell & Beal, 1984; Willis & Coggeshall, 1991). These cells have been likened to Cajal's limiting cells, although the similarities in terms of axonal projection are still unclear. Generally, stalked cells have dendritic arbours that extend into more ventral laminae, although some show dorsal-facing dendrites (sometimes known as inverted stalk-like cells;

Bicknell & Beal, 1984). Axonal projections of these cells vary greatly, with some sending collaterals to laminae I and III, others remaining solely in lamina II and still others projecting into the white matter (Figure 1.2). These cells could therefore be important for sending primary afferent information out of lamina II and into the projection neurone-rich regions of laminae I and III. Physiological characteristics of these neurones include high levels of spontaneous EPSCs and evoked currents consistent with input from C or Aδ fibres, in keeping with their dendritic arborisations into lamina I and II (Grudt & Perl, 2002).

Beyond these main two groups, categorisation of lamina II interneurones becomes increasingly difficult. Other categories include 'vertical neurones' (Bicknell & Beal, 1984), 'radial neurones' (Grudt & Perl, 2002) and 'arboreal cells' (Gobel, 1978), mainly due to their dendritic morphology, but it is clear that a more ubiquitous classification system is needed. Regardless, the morphology of both intrinsic interneurones and projection/propriospinal neurones indicates an extremely complex system of neuronal circuitry in the dorsal horn. While the primary afferent input implies a strict division between the laminae, the extensive dendritic morphology of dorsal horn cells demonstrate that afferents can terminate on cells from a variety of laminae (Figure 1.2). Such findings have been supported physiologically, with the description of superficial dorsal horn cells displaying synaptic input from low threshold Aβ fibres (Woolf & Fitzgerald, 1983; Baba *et al.*, 1999) and deep dorsal horn cells receiving synaptic input from substance P-containing nociceptors (Naim *et al.*, 1997; Naim *et al.*, 1998; Todd, 1989). Additionally, anatomical analysis has revealed NK1-positive lamina I neurones that project axons to laminae II-IV (Cheunsuang & Morris, 2000), and that these projections are capable of depolarising cells in deeper laminae in response to substance P application (Cheunsuang *et al.*, 2002).

In terms of development, dorsal horn interneurones display very different characteristics to projection/propriospinal neurones. In their report from 1984, Bicknell and Beal described how Golgi stained interneurones in the dorsal horn did not start axonal and dendritic development until birth. The authors described a population of neurones that started life as round cells that enlarge and sprout an axon in addition to many short, beadlike dendrites during the postnatal period. These cells then differentiate into the various categories of interneurones by approximately P20. The developmental profile

varies between neurones, with some reaching an adult-like morphology by P5 and others not doing so until the end of the third postnatal week (Bicknell & Beal, 1984). While this work raises the intriguing possibility that there are two separate groups of neurones in the dorsal horn that can be categorised by their axonal projection and developmental profile, Grudt and Perl (2002) found a similar morphology of star-shaped neurones in the spinal cord of older hamsters, implying that these cells, rather than being progenitor cells for all interneurones, were actually a subcategory in themselves. The youngest animals used by this group were, however, still young enough to display Bicknell and Beal's immature state, but more work is clearly required to find whether interneurones do indeed display a late developmental profile. Such a finding would be interesting with regard to the excitability seen in the neonatal cord: the lack of inhibitory interneurones, and additionally the negative feedback loops described in islet cells would clearly contribute to the lack of fine control that exists in the immature somatosensory system.

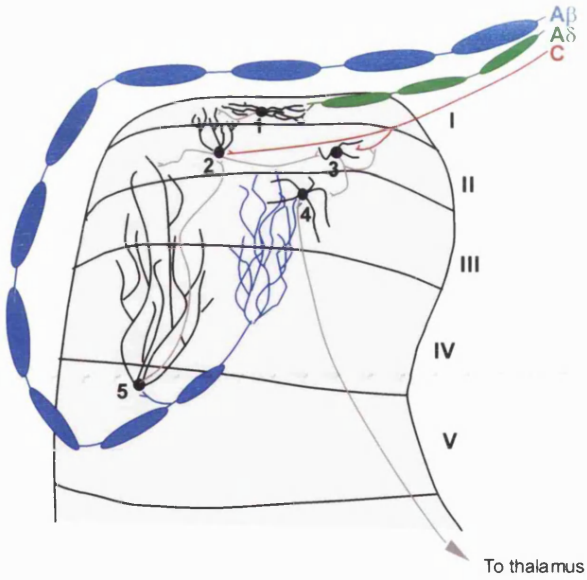

Figure 1.2
Schematic diagram demonstrating some types of cell seen in the dorsal horn. In this example, cell 1 is a marginal cell, which receives monosynaptic Aδ fibre input and sends axonal projections to the dendrites of a stalked cell (cell 2). This neurone lies in lamina II and projects axons with this lamina. It receives a monosynaptic input from the C fibre, as well as polysynaptic Aδ and Aβ input. Cell 3 is an inhibitory islet cell, which has short axodendritic projections in the mediolateral and dorsoventral plains, but long projections in the rostrocaudal plain. It receives C fibre input both from the primary afferent, which it can inhibit through its feedback loop, and from the stalked cell. Cell 4 is a spinothalamic projection neurone that receives monosynaptic input from the Aβ fibre, as well as inhibitory input from the islet cell. Cell 5 is an antenna-like cell that receives both low threshold input through the Aβ fibre, as well as high threshold input from the excitatory stalked cell.

1.3 Pharmacological changes during development

The neuropharmacology of the spinal cord consists of a vast array of neurotransmitters, trophic factors and neuromodulators. The majority of these show some form of developmental regulation, and thus contribute to the overall changes that occur in excitability during early life. Of particular relevance to activity-dependent alterations in the cord are the ionotropic, ligand-gated ion channels that respond to glutamate. These channels, and their roles in development, will be discussed here.

Most fast excitatory transmission in the spinal cord dorsal horn is mediated by the transmitter glutamate acting on postsynaptic ionotropic or channel forming receptors (iGluRs). There are three types of ionotropic receptor sensitive to glutamate: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate (KA) receptors. Glutamatergic synaptic currents are clearly evident in the superficial dorsal horn around birth in rats (Bardoni *et al.*, 1998) including those synapses due specifically to glutamate release from nociceptive primary afferent terminals (Baccei *et al.*, 2003). The frequency of both background and nociceptive afferent evoked EPSCs increases with postnatal age, particularly between P5 and P10, probably because of an increase in the number of synaptic release sites or age dependent changes in the probability of transmitter release.

1.3.1 AMPA receptors and the developing dorsal horn

Postsynaptic AMPA receptors are the main contributors of fast excitatory transmission in the dorsal horn. They are made up of a number of subunits, GluR1-4, arranged in a heterotetrameric fashion, each of which is expressed as at least 2 splice variants. The inclusion of particular subunits can drastically alter the functional properties of the receptor, for example by allowing the channel to be permeable to calcium as well as sodium ions (Hollmann *et al.*, 1991; and see below) or by altering its ability to open at certain membrane potentials (Michaelis, 1998; Dingledine *et al.*, 1999).

1.3.1.1 Developmental regulation of expression of AMPA subunits

There is a high level of AMPA receptor expression in the newborn dorsal horn followed by a downregulation in the total number of AMPA receptors from birth to adulthood. (Table 1.1). Autoradiographic studies have demonstrated that total [^{3}H]-AMPA and [^{3}H]-CNQX binding is reduced to approximately one fifth in the adult dorsal horn with

respect to the neonate (Jakowec *et al.*, 1995a). In addition, the expression of AMPA receptor subunits changes during development of the dorsal horn. Studies of messenger RNA (mRNA) expression using *in situ* hybridisation show that, at birth, the most prominent subunits in the dorsal horn are GluR1, 2 and 4 (Jakowec *et al.*, 1995b). During development, the levels of GluR1 and GluR4 mRNA decrease by approximately 50%, while the levels of GluR2 decrease by 30%, such that in the adult, the GluR2:GluR1 and GluR2:GluR4 ratios are significantly higher. However, immunohistochemical data show a lack of GluR4 in the dorsal horn, suggesting that posttranslational mechanisms may regulate this subunit during development (Jakowec *et al.*, 1995a).

Similar findings have been obtained using western blot analysis of protein content, demonstrating higher levels of AMPA receptor expression at P0 than in the adult (Brown *et al.*, 2002). Specifically, GluR1 and 2 are both highly expressed in the cord at P0, remain high for the first two weeks of postnatal life, and then decline to approximately one fifth of P0 levels by P35. GluR4 starts at relatively low levels, increases to a peak at around P14, before declining to P0 levels by P35 (Brown *et al.*, 2002).

1.3.1.2 Functional aspects of AMPA receptor development

Overexpression of AMPA receptors during early development suggests increased excitatory drive at these ages which is consistent with the high level of dorsal horn neuronal excitability in the postnatal period (Fitzgerald & Beggs, 2001). There is no change in glutamate mEPSC amplitudes in postnatal dorsal horn lamina II neurones over the postnatal period (Baccei *et al.*, 2003) but this may not be true of all neuronal types.

An important functional aspect of AMPA receptor development is the changing ion permeability that accompanies alterations in subunit composition. Unlike, NMDA receptors, AMPA receptors are not usually permeable to Ca^{2+} due to incorporation of at least one edited GluR2 subunit in the receptor tetramer (Dingledine *et al.*, 1999). However, receptors lacking the GluR2 subunit allow Ca^{2+} ions to enter the cell at normal resting potentials (rather than the depolarised potentials required for entry through NMDA receptors and voltage-gated calcium channels). The importance of such calcium permeability is that enough calcium may enter the cell through some AMPA

receptors to drive downstream changes associated with growth and synaptic plasticity (Gu *et al.*, 1996).

The increased ratio of GluR2 to GluR1/4 that occurs over postnatal development indicates a relatively greater number of calcium permeable AMPA receptors in young dorsal horn and an increase in the number of calcium-impermeable AMPA receptors as the animal matures. In many brain areas there is indeed a dramatic decrease in the number of calcium-*permeable* AMPA receptors over this period (Kumar *et al.*, 2002; Allcorn *et al.*, 1996), but studies in cultured embryonic spinal neurones suggest that, under these conditions at least, calcium-permeable receptor expression in dorsal horn cells *increases* during development (Albuquerque *et al.*, 1999). Calcium-permeable AMPA receptors with no GluR2 subunit are found in neurones of lamina I and II_o and to a lesser extent II_i in neonatal spinal cord (Engelman *et al.*, 1999) and may well be important for transient changes in synaptic strength and strongly influence projection neurone firing in the neonate. However, the degree to which a cell cultured for 3 weeks is similar to a cell in the 3 week-old cord is still unclear and the developmental regulation of this important group of receptors.

The functional effects of changing AMPA receptor subunit expression will depend upon where the receptors are located. In the adult cord, cells immunoreactive for GluR1 but not GluR2 are found primarily on inhibitory GABAergic neurones (Kerr *et al.*, 1998). Indeed, 78% of GluR1 positive neurones are GABAergic, while 97% of GluR2/3 positive, and therefore calcium-impermeable, cells are non-GABAergic. Similar findings are also obtained using embryonic spinal neuronees cultured for up to five weeks (Albuquerque *et al.*, 1999). However, calcium-permeable AMPA receptors, although involved in modulation of inhibitory transmission, are also expressed on excitatory projecting neurokinin-1 (NK1) neurones in young rats (P6-14) (Engelman *et al.*, 1999).

AMPA receptors therefore appear to be under tight developmental control in the spinal cord. In addition to a postnatal decline in AMPA receptor expression, it appears that changes also occur to the stoichiometry, and therefore the functional characteristics, of the receptor during postnatal life (Figure 1.3).

1.3.2 Kainate receptors and the developing dorsal horn

Kainate (KA) receptors act alongside AMPA receptors in mediating rapid excitatory neurotransmission in the CNS. The role of postsynaptic KA receptors in mediating synaptic transmission in the neonatal spinal cord was first demonstrated by Li *et al.* (1999), who showed that presynaptic stimulation combined with blockade of NMDA and AMPA responses still elicited a small evoked potential in dorsal horn neurones of young rats. This response was only seen when the stimulation strength was high enough to activate C fibres, leading them to conclude that these KA receptors were only present at C fibre synapses. The fact that little or no KA receptor expression is seen in the adult cord implies that these postsynaptic responses are of particular importance in developing pain pathways.

1.3.2.1 Developmental regulation of expression of kainate subunits

The KA receptor family is made up of 5 subunits, GluR5, GluR6, GluR7, KA1 and KA2, with each receptor consisting of four subunits arranged in a tetrameric fashion (Michaelis, 1998). *In situ* hybridisation studies have shown the presence of KA receptors in the spinal cord at embryonic day (E) 12 (Bahn *et al.*, 1994), although binding does not occur until E14. As with the other ionotropic glutamate receptors, KA receptors appear to follow strict developmental regulation. For example, GluR5 is expressed throughout the cord at P2, is seen only in the superficial dorsal horn by P10, and is not present at all by P21 (Stegenga & Kalb, 2001). A similar profile is seen with GluR6 and 7, although at generally lower levels, while KA1 is expressed at moderate levels in the dorsal half of the cord in the P2 rat, appears at low levels in the ventral horn at P10, and is not seen at all by P21. KA2 is expressed at high levels throughout the cord at P2 and stays at moderate levels throughout the first three weeks of life, before declining so that no signal can be seen in the adult (Stegenga & Kalb, 2001) (Table 1.1).

1.3.2.2 Functional aspects of kainate receptor development

The expression of postsynaptic KA receptors in the young, but not adult, dorsal horn highlights the importance of these receptors during development, but as yet little is known of their functional significance in developing pain pathways.

Presynaptically, KA receptors have been demonstrated to play a role in the developing thalamocortical connections of the rat whisker pathways. These synapses exhibit a short-term depression in response to a wide range of stimulus frequencies (Kidd *et al.*, 2002). During high-frequency stimulation (corresponding to the frequency elicited by whisker activation), this depression is dependent on presynaptic KA receptors, which produce a depolarisation of the presynaptic terminal and subsequent inactivation of P-type Ca^{2+} channels, but only up to postnatal day 7- the critical period in activity-dependent modifications in the barrel cortex. Because short-term depression may act to signal changes in presynaptic firing rates (Abbott *et al.*, 1997), this would imply an age-dependent, modulatory role for presynaptic kainate receptors (Kidd *et al.*, 2002).

Whether a similar system occurs in the immature dorsal horn, whereby presynaptic kainate receptors act to modulate glutamate release on nociceptive synapses is not known. Short-term depression of transmission has been demonstrated in the developing cord, with what appears to be a presynaptic locus (Li & Burke, 2002; Li & Burke, 2001). The presence of presynaptic KA receptors in the C fibres of the developing rat has been known for many years (Agrawal & Evans, 1986; Davies *et al.*, 1979; Evans *et al.*, 1987; Huettner, 1990), and the application of kainate to immature slices decreases AMPA and NMDA receptor mediated currents, thus showing that presynaptic kainate receptors can regulate glutamate release (Kerchner *et al.*, 2001). In early development, these presynaptic receptors are permeable to calcium, and switch to a Ca^{2+} impermeable form early in the first postnatal week. This process appears to be partially dependent on posttranslational modification of the GluR5 C terminus (editing of a Q to an R in the re-entrant M2 loop), and it has been proposed that these Ca^{2+} permeable receptors, which are found on small IB4-positive C fibres, could be important the in growth of C fibres into the dorsal horn (Lee *et al.*, 2001). Once the synapses are formed, the KA receptors could then switch to the Ca^{2+} independent form, and allow the receptors to act as presynaptic modulators of glutamate release.

KA receptor expression represents an intriguing and relatively unexplored area of developing nociceptive pathways. A postnatal decline in receptor expression, accompanied by a marked regulation of subunit composition suggests it has important role in the newborn (Figure 1.3).

1.3.3 NMDA receptors and the developing dorsal horn

While AMPA and KA receptors mediate fast excitatory transmission in the cord, NMDA receptors appear to contribute to slower synaptic processing, such as during repetitive stimulation. Extracellular Mg^{2+} blocks the channel at resting membrane potentials such that cations are only allowed through when there are a large number of excitatory inputs or by repetitive firing of the presynaptic input. When the postsynaptic cell is depolarised by, for example, repetitive C fibre activity, the Mg^{2+} block is shed, the channel can open in response to presynaptically released glutamate, and ions can enter the cell. Another important property is the receptor's high permeability to calcium, not normally a feature of most AMPA or KA receptors (see above). Once inside the cell, these Ca^{2+} ions acts as second messengers to activate intracellular signalling cascades leading to alterations in the synaptic strength. Because of this the NMDA receptor is sometimes described as a coincidence detector for pre- and postsynaptic activity, altering synaptic strength when such coincidence occurs (Woolf & Salter, 2000). NMDA receptors mediate long term changes in mono- and polysynaptic synapse strength in young rat dorsal horn neurones following high frequency dorsal root stimulation (Randic *et al.*, 1993).

1.3.3.1 Developmental regulation of expression of NMDA subunits

Like AMPA and KA receptors, the structure of the NMDA receptor consists of a number of subunits (NR1, NR2A-D and NR3A-B) arranged in a tetrameric fashion. All receptors contain two NR1 subunits, along with a combination of NR2 or 3 subunits. While NR1 is thought to be essential for channel function, the NR2/3 subunits appear to play a more regulatory role, allowing for variations in receptor kinetics and sensitivity to the Mg^{2+} block. Therefore, like the other iGluRs, the function of the NMDA receptor can greatly vary depending on its subunit expression. *In situ* hybridisation studies suggest that NR2D is the most highly expressed NMDA subunit in the rat embryonic spinal cord with NR2B also being present (Monyer *et al.*, 1994). This is of interest as the NR2D subunit, when recombinantly expressed with NR1, shows offset decays of between 10 and 40 times, slower than any other recombinant receptors (4.8 ± 0.9s for NR2D compared with 118 ± 11ms in NR2A). This could allow the receptor to detect coincidental firing of a much lower synchronicity than would be possible in the adult cord (Monyer *et al.*, 1994). In the mouse, ε2 (the murine equivalent of NR2B) mRNA expression is found throughout the cord at E13, but expression moves dorsally so that

by P21 it is restricted to laminae I and II (Table 1.1) (Watanabe *et al.*, 1994). The widespread expression in the neonatal period compared to older animals may be a reflection of the role of this subunit in synaptic growth and reorganisation in the dorsal spinal cord (Loftis & Janowsky, 2003). Electrophysiological recordings of single channels suggest more subtle differences between the neonatal and adult dorsal horn. The technique has revealed receptors that display a 69pS conductance level- one which appears unique to the developing cord- along with high sensitivity to Mg^{2+}, but low sensitivity to NR2B-specific antagonists, suggesting as yet unknown subunit compositions in the neonate (Green & Gibb, 2001).

Finally, calcium imaging studies show a decrease in calcium entry through NMDA receptors during development, implying changes in the receptor properties. Whether these changes are caused by alterations in subunit composition or by a general downregulation of receptor numbers is unclear, but interestingly, the decrease does not occur if the C fibres are ablated by neonatal capsaicin treatment. This implies that the changes are dependent on synaptic activity (Hori & Kanda, 1994).

1.3.3.2 Functional aspects of NMDA receptor development

Since NMDA receptors require depolarisation of the postsynaptic membrane to allow ion flow, their functional development is intimately related to that of fast transmission and AMPA receptors. While most glutamatergic synapses have both AMPA and NMDA receptors, in young animals, synapses appear to be present that express only NMDA receptors, effectively making them 'silent' at resting potentials.

Silent synapses were originally conceived by Pat Wall almost thirty years ago (Wall, 1977), when he suggested that many dorsal horn synapses may not function during baseline transmission, but are recruited during persistent pain. They were subsequently discovered in the dorsal horn by Li and Zhou (1998) and Bardoni *et al.* (1998), having been previously shown to exist in other parts of the developing brain such as the hippocampus, somatosensory cortex and visual cortex (Malenka & Nicoll, 1997). Since NMDA channels have a Mg^{2+} block at resting membrane potentials, synaptic release of glutamate at NMDA receptor-only synapses will not be readily detectable and the synapse will appear silent. Because they respond to activity by increases in expression of postsynaptic AMPA receptors, on repetitive stimulation these synapses become no longer silent. This occurs because high-frequency stimulation releases a large amount

of glutamate, which can continually bind to the NMDA receptor and cause a gradual increase in the membrane potential. This depolarisation causes the Mg^{2+} block to be removed from the NMDA channel, and for Na^{+} and Ca^{2+} ions to enter the cell (Li & Zhuo, 1998; Isaac *et al.*, 1995). The influx of Na^{+} ions can even be sufficient to produce firing of action potentials (Bardoni R *et al.*, 2000). The Ca^{2+} influx 'unmasks' the silent synapse by triggering secondary messengers systems that cause AMPA receptors to be inserted into the membrane, thus allowing the synapse to become functional (Isaac *et al.*, 1995; Durand *et al.*, 1996). Additional evidence suggests that, in the cord, postsynaptic depolarisation and subsequent unmasking of silent synapses is produced by the neurotransmitter, 5-HT (Li & Zhuo, 1998).

In the brain, silent synapses appear to be under tight developmental control. For example, in the somatosensory cortex, they disappear by postnatal day 8 - a noteworthy observation since this coincides with the end of the 'critical period' of synaptic plasticity in this area (Isaac *et al.*, 1997). However, whether such a disappearance occurs in the dorsal horn remains unclear. While original papers were able to detect NMDA-only synapses at the end of the second postnatal week (Bardoni *et al.*, 1998), other groups have been unable to find any such synapses after P14 (Baba *et al.*, 2000). In addition, a study performed on mice discovered the presence of NMDA-only synapses in the adult dorsal horn (Wang & Zhuo, 2002). Whether these discrepancies are caused by species differences or from the use of different electrophysiological techniques remains to be seen (Baba *et al.*, 2000).

The presence of silent synapses between primary afferent terminals and dorsal horn cells could allow for a certain amount of postnatal remodelling of the system. For example, if all afferents initially form silent synapses with their target neurone, inappropriate synapses would not become stable due to a lack of coincidental postsynaptic depolarisation from surrounding synapses. Those with correctly targeted inputs, however, could become stable by insertion of AMPA receptors to the postsynaptic density (Feldman & Knudsen, 1998). This will be discussed with regard to activity-dependent primary afferent input alterations below.

The interesting properties of the NMDA receptor make it especially important in development. Its voltage sensitivity makes it highly activity dependent and its role in allowing Ca^{2+} entry is particularly important for information storage at synapses.

Expression levels, subunit composition and colocalisation with AMPA receptors all appear to be under developmental control in the spinal cord (Figure 1.3).

	P0	P14	Adult
AMPA			
GluR1	+++	+++	++
GluR2	+++	+++	+
GluR3	+++	+++	+
GluR4	+	++	+
Kainate			
GluR5	++	+	-
GluR6	++	+	-
GluR7	++	+	-
KA1	+	+	-
KA2	+++	++	-
NMDA			
NR1	++	+++	+++
NR2A	+	++	++
NR2B	++	+++	++
NR2C	-	-	-
NR2D	++	+	-

Table 1.1 Comparison of expression levels of glutamatergic receptor subunits during postnatal development

1.3.4 The development of inhibitory synaptic activity in the dorsal horn

Although primary afferents release only excitatory transmitters, they can produce inhibition in the dorsal horn through inhibitory interneurones. As in the rest of the CNS, fast inhibitory transmission from these interneurones is mediated by $GABA_A$ and glycine receptors, and, in common with excitatory transmitter receptors, they are under considerable developmental control. In particular, the GABA receptor displays changing subunit expressions during development, allowing it to respond in different ways to presynaptic stimuli. In addition, changes in intracellular ion concentrations and neighbouring channel expression during development have recently been shown to have profound effects on the actions of these receptors (Reichling *et al.*, 1994).

1.3.4.1 GABA receptors in the developing dorsal horn

GABA plays important roles in many aspects of neuronal development- its receptors are, in fact, present at synapses before any other. In addition, the neurotransmitter itself

is present in much higher concentrations throughout the cord at birth, with approximately 50% of spinal neurones being positive for GABA, in comparison to ~15% in the adult (Schaffner *et al.*, 1993). The rate-limiting enzymes for the synthesis of GABA, glutamic acid decarboxylase (GAD; of which there are two forms, GAD65 and GAD67), are widely distributed in the spinal cord at birth, and, while initially thought to be downregulated in the ventral part of the cord during development (Ma *et al.*, 1994), are now thought the be simply redistributed away from the cell bodies to synaptic terminals (Tran *et al.*, 2003). Regardless of this, *in situ* hybridisation and immunohistochemical studies do show increases in reactivity from birth until P14, followed by a decrease in overall levels, and a relocation to the superficial dorsal horn (Table 1.2; Ma *et al.*, 1994; Tran *et al.*, 2003). In the adult dorsal horn, GABA is expressed in the islet cells (Todd & Sullivan, 1990).

1.3.4.1.1 Developmental regulation of expression of GABA subunits

Postsynaptically, the $GABA_A$ receptor is made up of combinations of the α, β, γ, δ, ε, π or θ subunits arranged in a heteropentameric formation (reviewed in Mehta & Ticku, 1999). In the neonatal dorsal horn, the most prominent subunits, found using *in situ* hybridisation and RT-PCR, are α2, α3, β3 and γ2. Reactivity for these subunits peaks during the first postnatal week and then declines to adult levels (Table 1.2; Ma *et al.*, 1993). The most highly expressed subunit is γ2- which is interesting, as this subunit appears to be essential in postsynaptic clustering of the $GABA_A$ receptor (Essrich *et al.*, 1998).

1.3.4.2 Glycine receptors in the developing dorsal horn

Glycine receptors are the most abundant inhibitory receptors in the spinal cord. They are composed of a number of subunits, α1-4 and β, which are arranged in a pentameric fashion (Rajendra *et al.*, 1997) and show clear developmental expression profiles. In the neonatal cord, the α2 subunit is expressed throughout the grey matter, forming heteromers with the β subunit (Malosio *et al.*, 1991; Watanabe & Akagi, 1995). While the expression of the β subunit remains high, α2 is strongly downregulated during the first three weeks of life (except in lamina I), to be replaced with the α1 subunit (Table 1.2) (Rajendra *et al.*, 1997; Watanabe & Akagi, 1995).

1.3.4.2.1 Developmental regulation of expression of glycine subunits

As with the glutamatergic receptor subunits, the glycine receptor subunits show extremely diverse kinetic properties. For example, the mean open time for α2 subunits expressed as functional channels in *Xenopus* oocytes is 174ms, while that of the α1 subunit is 2.38ms (Takahashi *et al.*, 1992). Accordingly, there is a developmental reduction in mean open times for glycine channels in spinal neurones, and a shortening of the decay time constant of inhibitory postsynaptic currents (IPSCs) in dorsal horn cells *in vitro*. This shortening is therefore likely to be caused by alterations in the subunit expression, whereby different ratios of fast and slow subunits would produce the intermediate channel kinetics observed during development (Rajendra *et al.*, 1997).

	P0	P14	Adult
$GABA_A$			
α2	++	++	++
α3	++	++	++
β3	++	++	++
γ2	+++	++	++
Glycine			
α1	+	+++	+++
α2	+++	+	-
β	+++	+++	+++

Table 1.2 Comparison of expression levels of inhibitory receptor subunits during postnatal development

1.3.4.3 Functional aspects of GABA and glycine receptor development

GABAergic and glycinergic synaptic activity in lamina I and II over the first two postnatal weeks characterized using whole-cell patch clamp recordings shows an age-dependent increase in the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs), while the sIPSC amplitudes were similar in the three age groups. GABAergic mechanisms appear to underlie the majority of spontaneous and evoked transmission at putative inhibitory synapses in the neonate with little contribution from glycine receptors (Baccei & Fitzgerald, 2003). In addition, a potent modulation of synaptic transmission by the $GABA_B$ receptor exists from the first days of postnatal life (Baccei & Fitzgerald, 2003).

An intriguing aspect of $GABA_A$ and glycine receptors is that in some regions of the developing CNS, they appear to act in an *excitatory* manner in early life, before switching to an inhibitory role during the postnatal period. This was first observed in chick embryonic spinal neurones in culture (Obata *et al.*, 1978), and has been subsequently confirmed in many other brain regions such as the hippocampus (Ben Ari *et al.*, 1989), cortex (Owens *et al.*, 1996) and hypothalamus (Ben Ari, 2002b; Chen *et al.*, 1996). The change in receptor function is not related to subunit composition, but to the concentration of intracellular chloride ions ($[Cl^-]_i$) (Ben Ari *et al.*, 1989; Owens *et al.*, 1996). During embryonic and early postnatal life, $[Cl^-]_i$ is high, which makes the reversal potential for chloride (E_{Cl}) less negative than the resting membrane potential (V_m), so that activation of the chloride-permeable $GABA_A$ and glycine receptors cause efflux of the ions, and subsequent depolarisation of the membrane. However, in the early postnatal period, a potassium-chloride cotransporter (KCC2) is inserted in the membrane, causing a decrease in $[Cl^-]_i$ (Hubner *et al.*, 2001; Rivera *et al.*, 1999). Now, E_{Cl} is more negative than V_m, so opening of the GABA and glycine receptors produces influx of Cl^- ions, and a subsequent hyperpolarisation of the membrane. Indeed, the E_{Cl} in neonatal hippocampal neurones is approximately –45mV compared with adult values of -75mV (Ben Ari, 2002c).

As with many postnatal synaptic changes, the conversion of GABA receptors from depolarising to hyperpolarizing forms is believed to be activity-dependent. The switch between excitation and inhibition appears to be brought about by GABA receptors themselves (Ganguly *et al.*, 2001) since chronic blockade of GABA receptors (but not glutamate receptors or sodium channels) on cultured hippocampal neurones prevents the switch. This implies that miniature postsynaptic currents (mPSPs) alone are capable of transforming the switch- an interesting finding, as spontaneous mPSPs are first seen at the same time as the switch in GABA signalling. The increase in expression of KCC2 mRNA is also under the control of GABA receptor activity (Ganguly *et al.*, 2001).

A similar situation appears to occur in the neonatal dorsal horn where, in 90% of E15-16 dorsal horn neurones cultured for more than a week, both GABA and glycine induced increased $[Ca^{2+}]_i$ and depolarisation (Reichling *et al.*, 1994). The depolarisation and entry of Ca^{2+} through voltage-gated channels by GABA receptors and glycine receptors decreases with age in culture and is gone by 30 days (Wang *et al.*,

1994). Ages in culture are hard to extrapolate to 'in vivo' development and the timing of the switch from hyperpolarizing to depolarising in neonatal intact dorsal horn is still unclear. By using the perforated patch-clamp technique, which allows for examination of intracellular ion concentrations by preventing the dialysis of cytoplasm that occurs with normal patch-clamping, Baccei & Fitzgerald (2004) have shown that GABA application does indeed depolarise cells in P0-2 rats, although the GABA reversal potential (E_{GABA}) is always more negative than the action potential threshold at this age (Baccei & Fitzgerald, 2004). Therefore, it appears that GABA cannot provide the main excitatory drive to the neonatal dorsal horn, but instead may contribute to synaptic refinement by allowing Ca^{2+} influx through voltage-gated calcium channels (Figure 1.3).

Inhibitory synapses in the dorsal horn undergo further alterations later in postnatal development, before becoming stable by the end of the third postnatal week. Although GABA and glycine are released from inhibitory dorsal horn interneurones simultaneously (Jonas *et al.*, 1998), a developmental shift occurs at the postsynaptic membrane. Therefore, while immature synapses (<P23) can co-detect both GABA and glycine, the synapses of older animals can detect only GABA *or* glycine, not both (Keller *et al.*, 2001). In addition, this developmental change appears to be region specific, so that in immature lamina I mIPSCs are either GlyR-only, GABAR-only or mixed, whereas in adult lamina I, mIPSCs are Gly-only. In lamina II, a similar mIPSC pattern is seen in the young animal, while mature cell responses are 52% GABAR-only and 48% are GlyR-only.

The development of excitatory and inhibitory neurotransmitters and receptors is highly regulated over the postnatal period, resulting in substantial functional changes at developing synapses over this time. These changes help explain the increased levels of excitability of the cord, and therefore impact the pain processing capabilities of the newborn. However, the neonatal pharmacology does allow for a great amount of plasticity, so that the animal may adapt in response to external stimuli.

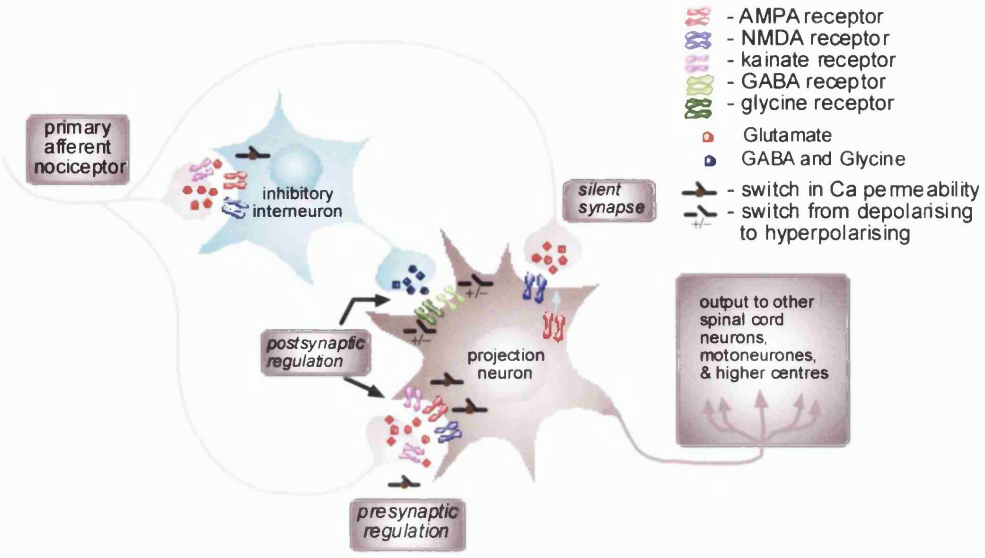

Figure 1.3
Diagram illustrating the synaptic basis for altered neurotransmission in the dorsal horn over the postnatal period. A simplified neuronal circuit involving a primary afferent nociceptor, an interneurone and a projection neurone is used as an example to show how glutamate release from primary afferent nociceptor terminals onto AMPA, NMDA and kainate receptors and GABA and glycine release from interneurone terminals are under developmental control. The change in receptor function with age will alter the output of the projection neurone and therefore the pain response.

1.4 Activity-dependent changes in the CNS

As previously noted, it has been recently established that the postnatal refinements that occur in the primary afferent input to the dorsal horn during development is dependent on activity (Beggs *et al.*, 2002). This is in keeping with structural alterations that occur in other parts of the sensory system, particularly in the afferent input to the neocortex. Two of the systems that have well described in displaying activity-dependent alterations during postnatal development are the visual system and the whisker barrel sensory system.

1.4.1 Postnatal refinement of connections in the visual cortex

The visual cortex displays a highly organised field of afferent input from the retino-geniculocortical pathway. The anatomical and physiological arrangement is characterised by domains that receive preferential input from one of the two eyes, known as 'ocular dominance columns' (OCDs; Hubel & Wiesel, 1969). The development of OCDs can be divided into two distinct phases: that which occurs before

eye-opening, where spontaneous electrical activity and signalling molecules contribute to axon guidance from the retina to the lateral geniculate nucleus (LGN; Penn *et al.*, 1998), and that which occurs after eye-opening, where visual experience produces neuronal firing and subsequent refinement of the afferent input (Katz & Crowley, 2002). While this work was originally undertaken in the cat, a similar developmental profile has now been shown to occur in the mouse (Gordon & Stryker, 1996).

The activity-dependence of the developing visual cortex was famously shown in the 1960s by Hubel and Wiesel who used elegant technique of depriving one eye, leaving the other open, and thus preventing the neuronal activity in one half of the ocular pathway. These studies revealed that the input from the deprived eye was almost entirely lost, so that the majority of cells in the OCDs responded only from stimulation of the non-deprived eye. If the eye deprivation was alternated during development, the cortical input could be reversed, implying that, rather than simply degenerating, the input was plastic and that the change was reversible. Finally, the authors demonstrated that the plasticity was limited to a 'critical period' of development, so that in older animals, ocular deprivation had no long-term effects on visual processing.

Binocular deprivation has the effect of equally depressing the whole geniculocortical pathway, so that retinotopy, ocular dominance and receptive field size are similar to non-deprived animals (Gordon & Stryker, 1996). These findings argue strongly that the effects of visual deprivation are caused by competition between inputs from the two eyes.

The cellular and molecular basis of this competition has become clearer in the last few years, due to the advancements in pharmacology and genetic manipulations. Of particular interest has been the NMDA receptor, on account of its ability to act as a coincidence-detector for pre- and postsynaptic activation. In support of such an involvement, it has been demonstrated that blockade of NMDA receptors produces disruptions to normal map refinement (Constantine-Paton *et al.*, 1990). This has led to the hypothesis that postnatal refinement is produced by some form of long-term potentiation (LTP) and long-term depression (LTD) in the retinogeniculocortical pathway, and physiological recordings have shown that such phenomena do occur at the level of both the cortex (Katz & Shatz, 1996) and the lateral geniculate nucleus (Cramer & Sur, 1995). According to this model, synapses where coincidence between pre- and

postsynaptic activity is high would become strengthened, while those that show little correlation would become weakened and would subsequently diminish, in accordance with Hebb's postulate (Hebb, 1949). Somewhat differently from the adult model of LTP, however, is the theory that much of the potentiation in the developing CNS is caused by unmasking of silent synapses. As previously discussed, silent synapses appear to be developmentally regulated, implying that they play a role in postnatal synaptic refinement. Therefore, synapses utilizing only NMDA receptors could be involved in early development of synaptic input, whereby ingrowing afferents form synapses with the postsynaptic cell which, having been depolarised, for example by GABA, insert AMPA receptors into the postsynaptic membrane. The system could then be refined by mechanisms in which tightly correlated pre- and postsynaptic activity produce LTP, combined with a heterosynaptic LTD of less correlated activity. Finally, the receptor subunit compositions shift, and the overall levels of iGluRs are downregulated, to allow for yet tighter control of coincidence detection (see above; (Constantine-Paton & Cline, 1998).

1.4.2 Postnatal refinement of connections in the somatosensory cortex

Tactile information from the rodent's whiskers is transmitted through the brain in a highly organised pattern. The afferents terminate in the trigeminal nucleus as 'barrelettes', which give rise to fibres that terminate in the ventroposterior thalamus as 'barelloids', which in turn send afferents to layer IV of the somatosensory cortex as 'barrels'. The nomenclature is derived from the columnar pattern of the cell groups, which are cylindrical in shape. Each group of cells, or barrel, describes an individual whisker, so that the 'barrel cortex' depicts a complete topographic map of the peripheral receptors sheet (O'Leary *et al.*, 1994).

As with the visual cortex, the development of afferent terminations to the barrel cortex occurs postnatally. At birth, the afferent input displays some aberrant connectivity, with some 30% of axons terminating in inappropriate barrels (Agmon *et al.*, 1995). By P4-8, the majority of these connections can no longer be seen, implying that they are reorganised postnatally. In addition, histochemical analysis shows an absence of the tightly segregated barrels at birth, and these only appear after the first few postnatal days. If evoked activity is limited to a single whisker by removal of all other vibrissae, the remaining whisker sends axons to not only its target barrel, but to the surrounding

barrels. In addition, the projection from layer 4 of the cortex to layers II/III show many aberrant terminations compared to undeprived animals. This suggests a competition for synaptic contacts between afferents from each whisker, in a manner similar to that seen in the visual system (Fox, 2002). The postnatal refinement of presynaptic input has been shown to be dependent on activity. By chronically blocking NMDA receptors during development with the slow release polymer, ELVAX, saturated with D-AP5, Fox *et al.* (1996) demonstrated that, while barrels were still formed in terms of histochemical arrangement, the physiological input to the cortex was grossly disrupted and the receptive field sizes were much larger compared to saline-injected controls. For example, while a cell in the C4 domain would normally display short-latency monosynaptic spikes in response to stimulation of the C4 whisker, in the AP5-treated group, the cell also responded to stimulation from eight surrounding whiskers (Fox *et al.*, 1996). Conversely, while in control animals, short-latency responses for each whisker were restricted to a single barrel, in the AP5-treated rats, monosynaptic responses to an individual vibrissa were also found in the surrounding barrels. This result implies that, while normal cellular development of cortical neurones can occur independently of activity, the primary afferent input to the barrel cortex does require activity and subsequent NMDA receptor activation.

As with the visual cortex, the model of developmental refinement of synaptic connections is believed to follow essentially Hebbian mechanisms (Fox *et al.*, 1996). Synchronous pre- and postsynaptic activity produce strengthening of connections in appropriate synapses, while asynchronous activity from inappropriate synapses are weakened and subsequently retract. In support of this theory, NMDA receptor-dependent LTP can be induced in the barrel cortex during early postnatal life (Crair & Malenka, 1995), and that this potentiation appears to be dependent on unmasking of silent synapses. Specifically, it has been shown that silent synapses are present in the barrel cortex during the first postnatal week (Isaac *et al.*, 1997). LTP-inducing protocols cause potentiation by seemingly unmasking these synapses and causing AMPA receptor insertion into the postsynaptic membrane. Therefore, in keeping with the findings in the visual cortex, it appears that activity-dependent refinement of cortical synaptic connections requires activation of NMDA receptors and unmasking of silent synapses.

1.4.3 Postnatal refinement of connections in the dorsal horn

As previously described, the spinal cord undergoes alterations in its afferent input during a critical period of postnatal life. This is best characterised by the withdrawal of Aβ fibre from lamina II during the first few weeks of life- a process that was recently shown to be dependent on NMDA receptors (Beggs *et al.*, 2002). In a manner similar to that used by Fox *et al.* (1996) in the barrel cortex, the authors implanted MK801-saturated ELVAX onto the dorsal horn of the spinal cord of neonatal rats and examined the structural, physiological and behavioural effects of such an intervention when the animal had grown up. They found that the receptive fields of MK801-treated animals had not refined in the normal manner, and that this was due to a lack of primary afferent reorganisation in the spinal cord. The authors proposed a mechanism of normal postnatal development whereby C fibre entry into the cord leads to competition between the two inputs. The synapses formed by these fibres would involve unmasking of silent synapses, which follow a developmental timecourse that closely matches that of C fibre synapse strengthening. Additionally, this unmasking could be produced by serotonergic input from the rostroventral medulla, whose development also matches that for C fibre strengthening (Bregman, 1987).

While NMDA receptor blockade can give insights into whether development is dependent on synaptic activity, it gives little insight into whether the postnatal changes actually require *experience*. In an attempt to answer this question, Waldenstrom *et al.* (2003) blocked either nociceptive or tactile input to the tail of neonates and examined the response properties in the adult. They found that refinement of nociceptive transmission was not dependent on early nociceptive experience, but rather by normal tactile input. This implies that the A fibre reorganisation is an active process, rather than a passive consequence of C fibre entry, and further supports the idea that postnatal refinement of sensory input requires activity.

As with the models of synaptic refinement in the brain, the alterations in the cord all appear to be due to alterations in afferent input. As the OCDs and barrels develop as normal histological structures, so does the dorsal horn (NeuN immunohistochemistry revealed no differences in cellular anatomy in the MK801-treated group compared to saline-treated controls; Beggs *et al.*, 2002).

If such a hypothesis is correct, and the mechanisms are similar to those described in other brain regions, one would expect a similar level of involvement by secondary messengers in the cord. While such messengers have been identified in the visual and barrel cortices, they have not yet been studied in the spinal cord. Here, we examine the role of perhaps the most important secondary messenger in developmental plasticity in the brain- the ubiquitous calcium/calmodulin-dependent protein kinase II (CaMKII).

1.5 Calcium/calmodulin-dependent kinase II

1.5.1 Structure and function of CaMKII

CaMKII is widely expressed in the CNS, and is the most highly expressed protein in the postsynaptic density. It is variably expressed in many of the 'plastic' areas of the brain, such as the hippocampus, cerebral cortex, cerebellum and spinal cord (Bruggemann *et al.*, 2000). Within the PSD, the kinase is localised distal to the central 'mesh' of the density, and some distance away from the postsynaptic membrane (Petersen *et al.*, 2003), but can translocate more centrally when activated (see below). Additionally, the kinase is present at the presynaptic terminal in some regions of the brain such as the hippocampus, thalamus and cortex (Nayak *et al.*, 1996; Liu & Jones, 1996).

The CaMKII family is made up of four isoforms (α, β, γ and δ), which display strong sequence homology (Braun & Schulman, 1995). Each of these isoforms consists of a catalytic domain, an autoinhibitory domain, a variable segment and a self-association domain (Lisman *et al.*, 2002). The molecular weight of the kinases varies between 54 (the α subunit) and 72 kilodaltons (the δ subunit). In the CNS, the most highly expressed subunits are α and β, while the γ and δ subunits are found in most types of tissue, including smooth muscle and epithelium. The brain-expressed isoforms are made up of twelve subunits, and are either homo- or heteromeric (consisting of both α and β subunits; Brocke *et al.*, 1999) in nature. Analysis of transmission electron microscopy images has revealed that the enzyme is hexagonal in shape, with two sets of six subunits stacked on top of one another (Woodgett *et al.*, 1983). Further analysis revealed that the core of the enzyme consisted of 6 struts, with foot-like processes attached to each end (Figure 1.4C). These processes were found to make up the catalytic/autoregulatory domains of the kinase, while the association domains were located in the hexagonal rings (Kolodziej *et al.*, 2000). The extended catalytic/autoregulatory domains would allow for the subunits to behave independently

of one another for catalytic activity but also in concert with one another for intracellular targeting, supramolecular self-assembly and autophosphorylation (Hudmon & Schulman, 2002).

The different domains of the CaMKII enzyme have well characterised functions. The catalytic domain, for example, produces the phosphorylation of receptor proteins due to the presence of ATP-binding sites. It is also the site responsible for interaction with anchoring proteins. Isolating a fragment containing the catalytic domain of the kinase (residues 1-271) produces a constitutively active monomer that can phosphorylate proteins without activation by Ca^{2+}/CaM. The reason that the kinase is not in this active confirmation at all times is due to the autoinhibitory domain (Hudmon & Schulman, 2002). This part of the protein lies proximal to the catalytic domain, and contains a region that resembles protein substrates, and which therefore binds to the catalytic domain under basal conditions and inhibits enzyme activity. Ca^{2+}/CaM binding causes the pseudosubstrate region to dissociate from the catalytic domain, allowing for future substrate binding and, more importantly, unmasking of the autophosphorylation site (Figure 1.4B).

Autophosphorylation of the α subunit of CaMKII occurs at the T286 residue on the autoinhibitory domain (Figure 1.4A). On the β subunit, autophosphorylation occurs at T287. These sites are phosphorylated by neighbouring subunits (Rich & Schulman, 1998) and produce a massive increase in the affinity of the kinase for calmodulin (from 45nM to 60pM; Meyer *et al.*, 1992), essentially trapping calmodulin onto the autophosphorylated subunit. The second function of the T286/287 autophosphorylation is to make the kinase persistently active, even after the Ca^{2+}/CaM signal has dissociated from the enzyme. This is possible because the region surrounding the T286/287 site binds to a neighbouring 'T' site, which allows for the alignment of the pseudosubstrate region to bind the substrate-binding site on the catalytic domain (the 'S' site). If T286/287 is phosphorylated, however, binding at the T site cannot occur, resulting in an inability of the autoinhibitory domain to bind the S site, and subsequent autonomous activity of the enzyme (Figure 1.4B).

The variable domain of the enzyme is the part that shows the most differences between isoforms. While the isoforms from all four genes have a conserved core structure, a number of inserted sequences into the variable domain allow for changes between

isoforms. Eleven different sequences have been observed to date, which are inserted at four different points along the variable domain, producing 30 different mammalian isoforms. Functionally, little is known about the effects of the inserts, although it appears that they could play roles in membrane targeting, nuclear localization signalling and sensitivity to Ca^{2+}/CaM. The α subunit is the smallest of the isoforms, containing no inserts at all (Hudmon & Schulman, 2002).

Finally, the association region allows for assembly of the kinase into its dodecameric structure. This region lies on the C terminus and makes up the 'hub' in the gear structure of the enzyme. Truncation of the association domain produces a monomeric kinase construct with similar activity and substrate specificity to the holoenzyme (Braun & Schulman, 1995).

1.5.2 Activation of CaMKII

A major reason for the interest in αCaMKII[1] with regard to synaptic plasticity is its ability to remain active after the calcium stimulus has disappeared. This is due to its ability to enter an autophosphorylated state. As mentioned previously, this autophosphorylation occurs at T286 on the autoinhibitory domain. After calcium enters the cell, it binds calmodulin, which in turn binds to the CaMKII protein at the 296-311 (Figure 1.4; Braun & Schulman, 1995). If one calmodulin molecule binds to one subunit, the autoinhibitory drive of that subunit is lifted, allowing the T286 residue (the 'T' site, Figure 1.4) to be revealed. If, in addition, a second calmodulin molecule binds to a neighbouring subunit, this is also activated and can in turn phosphorylate the revealed T286 site. The autophosphorylation is then propagated around the hexagonal ring, producing phosphorylation of the holoenzyme. (Lisman *et al.*, 2002). Phosphorylation at T286 results in the pseudosubstrate region (the 'S' site) being exposed, allowing the kinase to become active (Figure 1.4). The enzyme appears to maintain its phosphorylated form until it is deactivated by protein phosphatases (in particular PP1). The ability for the enzyme to remain active after the calcium has been dissociated allows it to act as a molecular switch, producing long-term changes in the cell after only a brief stimulus. With such a switch, the NMDA receptor becomes not merely a coincidence detector, but a gateway for synaptic memory.

[1] From here on, αCaMKII will just be called CaMKII, unless otherwise stated.

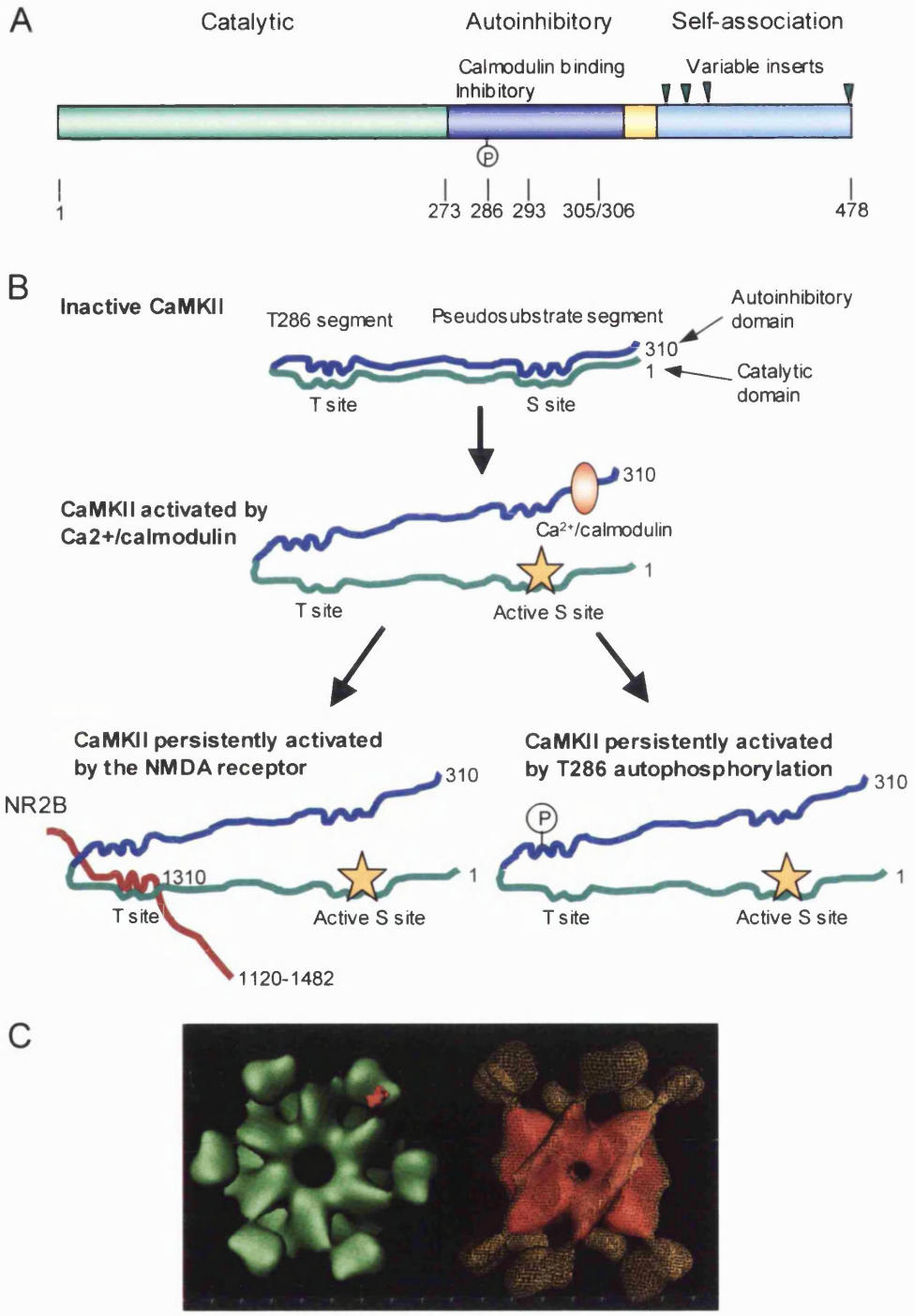

Figure 1.4
Structure and function of αCaMKII. (A) Functional domains on the primary CaMKII enzyme. (B) The autoinhibitory and catalytic domains form a gate that regulates activity. Activation of CaMKII by Ca2+/calmodulin leads to opening an unfolding of the structure, thus leading to activation at the S site. When open, the kinase can be persistently activated by either the NR2B subunit of the NMDA receptor or by autophosphorylation of T286. (C) 3D reconstructions of the enzyme showing the core hexagonal rings formed by the association domains of six subunits (left) and, at a perpendicular angle, the foot-like processes formed by the catalytic/autoregulatory domains (right). Adapted from Lisman *et al.* (2002).

Once in its autophosphorylated form, CaMKII acts in a number of different ways in order to affect the strength of the synapse. These include:

- Phosphorylation of ionotropic receptors
- Interaction with NMDA receptors
- Insertion and removal of AMPA receptors from the synaptic membrane

The majority of these mechanisms have been elucidated using biochemical and electrophysiological methods in hippocampal preparations, particularly by using protocols that induce LTP at the CA1 synapse.

1.5.2.1 CaMKII and ionotropic receptor phosphorylation

Some early studies of LTP in the hippocampus revealed that there were changes in the single-channel conductance levels of AMPA receptors after LTP induction (Benke *et al.*, 1998). This involved a 184% increase in single channel conductance in approximately two thirds of receptors. Concurrently, work by Tom Soderling and his colleagues demonstrated that conductance levels of AMPA receptors could be altered by phosphorylating the serine 831 site of the AMPA receptor subunit, GluR1 (Derkach *et al.*, 1999). CaMKII is known to phosphorylate this site after induction of LTP, as there is an increase in ^{32}P incorporation into GluR1 after theta-burst stimulation (a protocol known to induce LTP in hippocampal slices)- a process that is blocked by the CaMKII inhibitor, KN62 (Lee *et al.*, 2000). These findings indicate that CaMKII directly phosphorylates AMPA receptors during LTP, resulting in increases in single-channel conductance and therefore synaptic efficacy.

In addition to its action at AMPA receptors, CaMKII is also believed to act on inhibitory receptors. Potentiation of GABA currents by CaMKII was first seen in dissociated spinal cord dorsal horn neurones, where application of a constitutively active form of the kinase produced increases in the amplitudes of $GABA_A$ receptor-mediated, (Wang *et al.*, 1995) and glycine-mediated (Wang & Randic, 1996) IPSCs. A similar finding has also been observed in cerebellar granule cells (Houston *et al.*, 2003). The modulation of GABA receptors is probably due to increases in binding of allosteric modulators in response to phosphorylation (Churn *et al.*, 2002), probably at the serine 383 and 409 sites on the beta subunit (McDonald & Moss, 1997).

1.5.2.2 CaMKII and NMDA receptor interaction

More recent work on hippocampal LTP has studied the role of CaMKII in NMDA receptor modulation. The idea was initially proposed as a way of explaining how CaMKII was upregulated in the PSD after LTP induction. Under basal conditions, CaMKII can be seen in the cytosol, where it binds to F-actin. Once activated by calcium, it can be seen translocating to the synaptic membrane (Shen & Meyer, 1999). This translocation seems to be caused by the kinase dissociating from F-actin and binding to the NR2B subunit of the synaptic NMDA receptor (Strack & Colbran, 1998; Gardoni *et al.*, 1998) In addition, there also appears to be an interaction with the NR1 subunit in a manner dependent on autophosphorylation of CaMKII (Bayer *et al.*, 2001). This interaction appears to lock CaMKII in an active formation, allowing it to phosphorylate and insert AMPA receptors on the postsynaptic membrane (Figure 1.4). In addition to enhancing the activity of the kinase, the NMDA-CaMKII interaction may also play a role in increasing the structural stability of synaptic connections (see below).

1.5.2.3 CaMKII and AMPA receptor insertion

Of particular interest to the role of CaMKII in developmental alterations in structural reorganisation of primary afferents is its ability to cause AMPA receptor insertion into the postsynaptic membrane. If, as has been hypothesised, the refinement were caused by unmasking of silent synapses, the kinase would be expected to play a key role in this process. Direct evidence for a role for CaMKII in AMPA receptor insertion into silent synapses comes from a study in which recombinant, constitutively active green fluorescent protein (GFP)-tagged CaMKII was expressed in hippocampal CA1 neurones (Poncer *et al.*, 2002). This resulted in potentiation of AMPA receptor EPSCs that was associated with a reduction in the number of silent synapses. In addition, GFP-tagged AMPA receptors are inserted into the plasma membrane by either pairing presynaptic stimulation with postsynaptic depolarisation (a common LTP induction protocol) or by injecting active CaMKII into the cell (Hayashi *et al.*, 2000). These data imply that CaMKII plays a key role in inserting AMPA receptors into previously silent synapses.

In addition to AMPA receptor insertion, a new model by John Lisman and colleagues (Lisman & Zhabotinsky, 2001) suggests that, through its interactions with actinin, actin and protein 4.1/SAP97, CaMKII is able to bind to the NMDA receptor and mediate AMPA receptor anchoring to the postsynaptic membrane. Here, the CaMKII-NMDA

receptor interaction would allow the kinase to bind α-actinin, which would bind a series of other binding molecules and, in turn, form sites on the plasma membrane to which AMPA receptors could anchor (Lisman & Zhabotinsky, 2001). This model implies a role for CaMKII beyond its enzymatic function, whereby the kinase acts as a scaffolding protein and subsequently increases synaptic structural stability. This structural role has been described *in vivo* (see below).

1.5.3 Functions of CaMKII

1.5.3.1 The role of CaMKII in synaptic refinement of the sensory system

The advancement of transgenic technology has allowed for closer inspection of the molecular workings of synaptic plasticity. Such technology has been used to produce a multitude of mice that have disruptions to the CaMKII gene family, from complete deletion of the protein, to sensitive point mutations that disrupt individual functions of the enzyme. Of these, the most commonly studied have been the CaMKII knock-out mouse ($CaMKII^{-/-}$; Silva *et al.*, 1992b; Silva *et al.*, 1992a) and the $CaMKII^{T286A}$ mouse, which contains a point mutation at site T286 (a threonine for an alanine) and can therefore not enter the autophosphorylated state (Giese *et al.*, 1998). These mutants have greatly increased our understanding of the functions of CaMKII, with particular regard to activity-dependent plasticity.

The first study to use mutagenesis in examining the role of CaMKII in developmental plasticity was from Gordon *et al.* (1996), who examined the $CaMKII^{-/-}$ mouse with regard to visual cortex plasticity. The study revealed that, while the formation of OCDs appeared to be normal, severe plasticity deficits were seen after monocular deprivation. While this intervention usually produces increased drive in the non-deprived eye (see above), no such increase was seen in the knock-out animal. This suggests a role for CaMKII in ocular dominance plasticity (Gordon *et al.*, 1996). A subsequent study used the more sensitive $CaMKII^{T286A}$ mutant to show that ocular dominance plasticity was indeed dependent on CaMKII autophosphorylation, as these mice showed no shifts in the contralateral bias index (CBI; a scoring system used to ascertain the relative input strength from the contralateral eye's cortical drive) after monocular deprivation (Taha *et al.*, 2002). The results from these two studies indicate a role for CaMKII in the secondary input to the supragranular layer of the cortex- a pathway that also displays clear activity-dependent plasticity.

Similar findings have been obtained in the barrel cortex. Instead of the gross deficits observed after chronic NMDA receptor blockade, the CaMKII mutants display more subtle deficiencies in synaptic plasticity. Ablation of the CaMKII gene results in normal formation of barrels and receptive fields, as well as normal afferent growth into layer IV of the cortex (Glazewski *et al.*, 1996). However, plasticity in layers II/III of whisker-deprived mice does not occur in either the $CaMKII^{-/-}$ or the $CaMKII^{T286A}$ mouse (Glazewski *et al.*, 2000; Glazewski *et al.*, 1996). In addition, LTP could not be induced in layers II/III of the barrel cortex using three different protocols that are all known to produce potentiation in wild-type mice (Hardingham *et al.*, 2003). This again implies that, while afferent input may be able to develop normally, the plasticity associated with sensory deprivation is highly dependent on autophosphorylation of CaMKII.

1.5.4 The role of CaMKII in synaptogenesis and dendritic refinement

A second, less studied role for CaMKII involves its ability to stabilise dendritic growth and to regulate the formation and stability of synaptic connections. Studies in the *Xenopus* tadpole retinotectal system have demonstrated that CaMKII plays a major part in both dendritic arborisation and in axonal growth. By injecting a truncated form of CaMKII (tCaMKII), which is constitutively active, into the tectal cells, Zou and Cline (1996) demonstrated that axonal growth from the retinal cells was greatly inhibited by the kinase. This was found to be caused by an increase in the number of axonal branch retractions. The authors proposed a mechanism of active branch withdrawal, whereby the presynaptic axon would normally produce elevations in CaMKII activity if there was correlated presynaptic firing and postsynaptic activation. If CaMKII activity was high in the absence of synaptic activity, the synapse would not form and the axon would retract.

A later study demonstrated that tCaMKII also acted to decrease the amount of dendritic exuberance seem in the tectal cells themselves (Wu & Cline, 1998). The authors proposed a three-stage course of development in these cells: firstly, when CaMKII levels are very low, there is some axogenesis and limited dendritic arborisation; secondly the dendrites would grow rapidly, although CaMKII levels were still insufficient to produce structural stabilisation; and a third stage where CaMKII levels are high, meaning that calcium entry through NMDA receptors causes insertion of

AMPA receptors into the postsynaptic membrane, causing synaptic stabilisation. If pre- and postsynaptic activity are not correlated, NMDA receptors are not activated, and neither is CaMKII, resulting in the increased local branch additions as the dendrite searches for a suitable presynaptic partner. The converse of these results is seen if CaMKII inhibitors are injected into the cell, so that both the tectal dendrites and the retinal axons grow even more exuberantly due to their inability to from stable synapses in a CaMKII-dependent manner (Zou & Cline, 1999).

More recent evidence has demonstrated a more selective role for the enzyme in structural refinement of synaptic contacts in cortical neurones. By transfecting the cells with either a constitutively active ($CaMKII^{T286D}$) or an inactive ($CaMKII^{T286A}$) form of the kinase into cultured pyramidal neurones, Pratt *et al.* (2003) have demonstrated that CaMKII can act to selectively strengthen contacts with some presynaptic partners, while eliminating contacts with others. The authors found that transfection with the active T286D produced an increase in EPSC amplitude, accompanied by a reduction in the number of synaptic contacts. This appeared to be due to a selective elimination of some presynaptic partners (a process that was dependent on CaMKII activation but independent of the presynaptic activity), and an increase in the number of synaptic contacts from other neighbouring cells (a process that required both CaMKII activation and presynaptic activity). These findings are in keeping with others that report a competitive system of synaptic refinement, with simultaneous enhancement of some synaptic partners and elimination of others (Colman *et al.*, 1997; Lichtman & Colman, 2000). Whether this system involves a heterosynaptic 'punishment signal' from the postsynaptic neurone in response to correlation of activity with other cells (Lichtman & Colman, 2000), or by an NMDA receptor-dependent depression of uncorrelated inputs (Constantine-Paton & Cline, 1998) is not clear. However, the data do strongly suggest activity-dependent alterations of synaptic input that is dependent on CaMKII (Pratt *et al.*, 2003).

1.6 Summary and aims of this thesis

The neonatal nervous system is highly plastic and can adapt to experience through alterations at the molecular, synaptic and structural levels. In the spinal cord, alterations in primary afferent synaptic input are driven by calcium entry through NMDA channels, resulting in postnatal refinement and a decrease in general excitability. The way in

which calcium acts to produce such changes is still unknown in this system, but a prime candidate for mediating plasticity is the multifunctional holoenzymes, CaMKII. This kinase has been shown to be involved in postnatal refinement of other sensory systems in the brain through its ability to autophosphorylate, and therefore act as a molecular switch, in response to calcium influx.

The aim of this thesis is to examine the role of CaMKII in the development of the spinal cord using a transgenic mouse in which CaMKII cannot enter its autophosphorylated state, and therefore cannot remain active after calcium diffusion (the $CaMKII^{T286A}$ mouse; donated by K.P. Giese). The somatosensory phenotype are examined using anatomical tracing techniques to label A fibres, electrophysiological analysis using both *in vivo* extracellular and *in vitro* whole-cell patch clamp recordings, and behavioural testing to elucidate mechanical and heat thresholds in the awake animal. Finally, the effects of the mutation on the adult pain pathways are studied using a series of interventions to produce acute and chronic pain states.

Chapter 2

DEVELOPMENTAL EXPRESSION OF CaMKII IN THE SPINAL CORD

2.1 Introduction

CaMKII plays a major role in the development of synaptic connections in the CNS. It can act to strengthen synapses by phosphorylating glutamate receptors (Lisman *et al.*, 2002) and has also been implicated in altering axodendritic structure during synaptic modelling (Zou & Cline, 1999; Pratt *et al.*, 2003). It is also believed to be a major downstream signalling protein in NMDA receptor-mediated events, such as LTP induction. Because primary afferent development is disrupted in the spinal cord after NMDA receptor blockade (Beggs *et al.*, 2002), we hypothesised that a similar disruption may occur by blockade of CaMKII autophosphorylation.

The onset and pattern of expression of CaMKII in the spinal cord during development is still unclear. A number of reports have identified the presence of the kinase in the dorsal horn of the adult rat (Bruggemann *et al.*, 2000; Lund & McQuarrie, 1997; Terashima *et al.*, 1994) and mouse (Liang *et al.*, 2004a), but these studies have not extended to developmental expression.

Developmental expression of CaMKII has been studied more extensively in the forebrain. Northern blot analysis of the mouse brain revealed the presence of CaMKIIα mRNA at P1, with a sharp increase in expression levels at P5- a time when synaptic formation is peaking (Bayer *et al.*, 1999). In support of this, Herms *et al.* (1993) demonstrated, using *in situ* hybridisation, that CaMKII was expressed by P1, primarily in the CA3 region. During development, the expression was seen to extend to the CA1 and dentate gyrus regions, and to increase dramatically postnatally to peak at P15, before reducing to adult levels. This expression profile corresponds to that of some ionotropic glutamate receptors throughout the brain (Pattinson & Fitzgerald, 2004). In the cat visual cortex, CaMKII protein can be seen at P1 in the deep layers, but this immunoreactivity shifts to more superficial layers during the first few weeks of life (Jia *et al.*, 1992). Interestingly, this pattern of expression matches the timeframe of both synaptic maturation (Blue & Parnavelas, 1983) and of silent synapse expression (Rumpel *et al.*, 2004) in the visual cortex. Finally, northern blot analysis of foetal and adult human brains reveals a low level expression in late foetal brain tissue, which is upregulated in the adult (Li *et al.*, 2001a). These data suggest that CaMKII is expressed in the brain from birth, and that this expression is developmentally regulated, but the expression profile in the spinal cord over this period was not investigated.

Due to its multifunctional nature, CaMKII is expressed both in the presynaptic terminals of a number of excitatory synapses, such as thalamocortical projections (Liu & Jones, 1996), and at the postsynaptic densities of some excitatory (for example in the cortex (Liu & Jones, 1996) and hippocampus (Barria *et al.*, 1997)) and inhibitory (McDonald *et al.*, 2002) synapses. Presynaptically, the kinase acts as modulator of neurotransmitter release in response to presynaptic calcium influx (Nichols *et al.*, 1990). Postsynaptically, the kinase plays a role in regulating AMPA (and perhaps GABA) receptors- a function that requires autophosphorylation at T286. There is also evidence that CaMKII can regulate retrograde signalling across the synapse, allowing for additional control of synaptic strength (Haghighi *et al.*, 2003). The elucidation of the precise locus of expression, therefore, can give important insights into how the kinase is affecting synaptic transmission.

In the adult spinal cord, CaMKII has been shown to be present in a significant proportion of DRG neurones in the rat (Bruggemann *et al.*, 2000). Stereological analysis has demonstrated that CaMKII is expressed in 40% of L4 and L5 neurones, primarily in small diameter afferents (Carlton & Hargett, 2002). Of the different subpopulations of DRG neurones, 38% of IB4-positive, 23% of CGRP-positive and 35% of TRPV1-positive neurones express CaMKII. These data suggest that a significant proportion of dorsal horn CaMKII protein could be from the presynaptic primary afferent input, but the relative proportions of pre- and postsynaptic CaMKII in the spinal cord is not known. Because primary afferent inputs provide up to 50% of presynaptic terminals (Willis & Coggeshall, 1991; Chung *et al.*, 1989), we attempted to analyse the presynaptic expression of CaMKII by removing the afferent input using dorsal rhizotomy.

The dorsal horn is made up of a heterogeneous population of neurones, displaying differences in morphology, projection patterns and neurotransmitter content. In other parts of the brain, CaMKII is often expressed in specific populations of cells, which are either glutamatergic or GABAergic. For example, in the rat basolateral amygdala CaMKII expression is restricted to the cell bodies and proximal dendrites of the glutamatergic pyramidal neurones, with no expression in the GABAergic nonpyramidal neurones (McDonald *et al.*, 2002). Electron microscopy demonstrated that 60% of these GABAergic neurones formed synapses with the CaMKII-positive pyramidal

neurones, although whether the postsynaptic densities of these synapses contained the kinase was not clear. The olfactory bulb shows a quite different expression pattern. Here, the majority of GABAergic granule cells expressed CaMKII, while the glutamatergic mitral/tufted cells were CaMKII-negative. The piriform cortex, as with most of the cortex, displayed CaMKII expression in glutamatergic, but not GABAergic cells (Zou *et al.*, 2002). Finally, an electron microscopic study in the thalamus and cortex found CaMKII expression restricted to asymmetrical, glutamatergic synapses. Synapses with presynaptic GABA input never showed CaMKII immunoreactivity, even when the target cell was CaMKII-positive (Liu & Jones, 1996). The expression of CaMKII in different populations of dorsal horn neurones, such as GABAergic interneurones, is not well understood, so we performed double-labelling experiments with this neurotransmitter in order to ascertain in which cells the kinase was localised.

Finally, we examined the expression of CaMKII in the T286A mutant mouse compared with wild-type littermates. Although the original article in which the mutant was described showed similar total CaMKII levels between mutant and wild-type (Giese *et al.*, 1998), the assay was performed on whole-brain extracts. It has recently been demonstrated that the expression of total protein in the mutant was actually region-specific, so that the while the levels in the hippocampus were unaffected by the mutation (Hardingham *et al.*, 2003), the visual cortex showed a 40% decrease (Taha *et al.*, 2002) and the somatosensory cortex showed a 25% decrease (Hardingham *et al.*, 2003) in the mutant compared to the wild-type. In order to ascertain whether a similar decrease was observed in the spinal cord, we performed western blot analysis of total CaMKII expression in the lumbar spinal cord.

This chapter examines spinal CaMKII expression, with regard to its general localisation in the adult, its developmental profile, its synaptic locus and its coexpression with spinal neurotransmitters.

2.2 Methods

2.2.1 Animals

All experiments described in this thesis, except for the developmental study described in Chapter Three, were performed on mice. Both the rats and mice were housed in Biological Services, UCL, London, in cages containing between 1 and 5 animals. Environmental conditions were maintained at 21°C and 50% humidity, with tap water and food (Harlan Teklad TRM Rat/Mouse Diet; Harlan, Bicester, UK) *ad libitum*. Lights were programmed on a 12:12h cycle (lights on at 8:00am).

2.2.1.1 CaMKII T286A mice

The T286A mice used were a kind donation from K. P. Giese from the Wolfson Institute for Biomedical Research, UCL, London, and derived from those described previously (Giese *et al.*, 1998). Two point mutations (ACCGTGGAC was changed to GCCGTCGAC) were introduced by polymerase chain reaction (PCR) mutagenesis into the 866-base pair Hind III fragment containing the exon encoding Thr^{286}. The wild-type Hind III fragment of the 6.1-kb Pvu II genomic clone was substituted by the mutagenised Hind III fragment. A PGKneo cassette flanked by loxP sites was inserted into the Bgl II site (Figure 2.1). After transfection of R1 embryonic stem cells and selection with G418, targeted clones were identified by Southern (DNA) blot analyses. Because the PGKneo cassette could interfere with the expression of neighbouring genes, it was removed by transient transfection with pBS185, a plasmid containing Cre recombinase DNA. Chimeras, generated by injection of proper cells into blastocysts, were mated with C57BL/6J mice, and crosses of F1 heterozygotes yielded a Mendelian distribution of F2 offspring (Giese *et al.*, 1998).

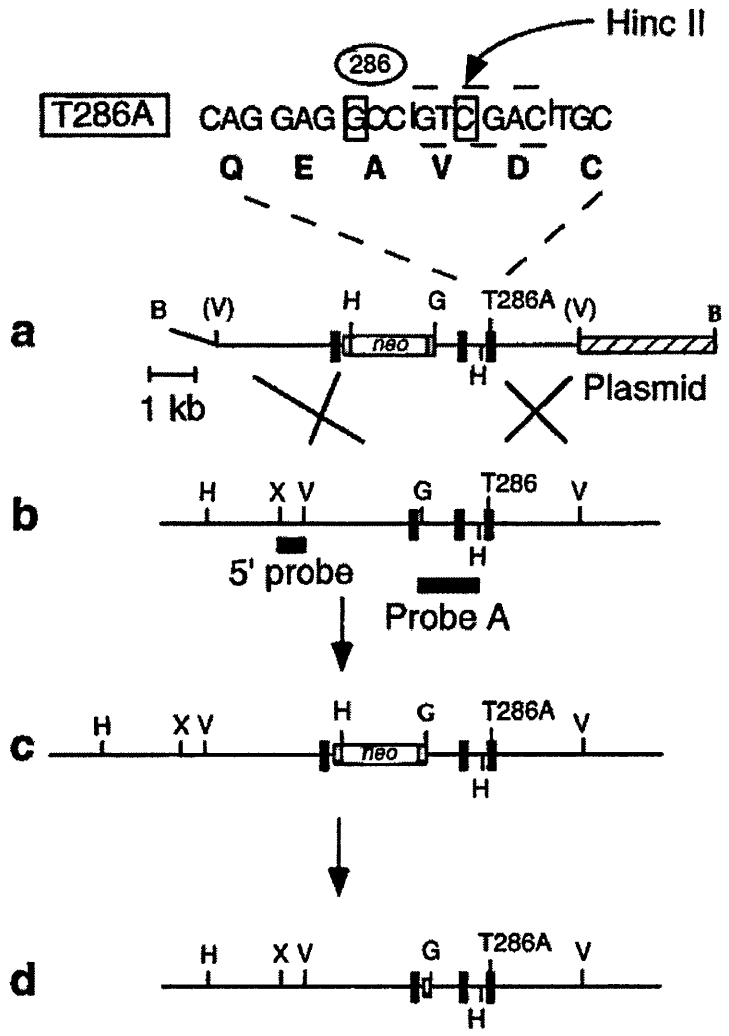

Figure 2.1
Generation of the CaMKII T286A mouse using the Pointlox procedure. The targeting construct (a), a partial map of the αCaMKII gene (b), the resulting targeted allele (c), and the targeted allele after Cre recombination (d) are illustrated. B, Bam HI; G, Bgl II; H, Hind III; V, Pvu II; X, Xba I. *From* Giese *et al.*, 1998).

2.2.1.1.1 Breeding

Due to the fact that homozygous animals did not mate, all mice were bred from heterozygous pairs. In addition, cardboard houses were placed in the cages to encourage mating. Litters were weaned at three weeks, and tail tips were taken for genotyping in animals over four weeks old.

2.2.1.1.2 Verification of genotype

The genotype of all mice used in experiments involving T286A mice and wild-type controls was determined or verified by performing the polymerase chain reaction (PCR)

on DNA extracted from tail tip samples. A 5mm piece of tail was removed and digested in tail lysis buffer (50mM Tris pH 8 (Sigma), 100mM ethylenediaminetetraacetic acid (EDTA; pH 8; Sigma), 10mM NaCl (BDH), 1% SDS (Sigma)) containing proteinase K ($20mg.ml^{-1}$; Sigma) overnight at 55°C. The DNA was purified with protein precipitation solution (Puregene, Minneapolis, USA), isopropyl alcohol (Sigma) and 70% ethanol, before being dissolved in TE buffer (10mM Tris pH 8, 1mM EDTA pH 8; Sigma).

PCR used two primers, Lox-1 (5′-CTGTACCAGCAGATCAAAGC-3′) and Lox-2 (5′-ATCACTAGCACCATGTGGTC-3′), which flank and amplify the inserted lox site. Resolution of these PRC products on a 2% agarose gel gave a single band at 267 bases for the T286A mutant, bands at 267 and 194 bases for heterozygous mice, and a single 194-base band for wild-type mice.

2.2.1.2 Culling

Because heterozygous mice were rarely used in the experiments described in this thesis, many of them were culled in accordance with Home Office guidelines. Mice were placed in a CO_2 chamber into which a slow, steady flow of gas was released, allowing CO_2 concentrations to rise slowly and prevent the animals becoming alarmed. When the mice had stopped breathing death was confirmed by physical cervical dislocation.

2.2.2 Western blot

For the developmental study, C57BL/6J mice aged P0, 3, 8, 14, 21 and 96 were sacrificed by intraperitoneal injection of 0.2ml sodium pentobarbitone (200mg/ml; Euthatal; Merial, UK) and decapitated. Dorsal laminectomies were performed and the whole lumbar enlargement of the spinal cord quickly removed, frozen on dry ice and stored at -80°C until ready for use. Although it would have been preferable to have dissected the dorsal horns from the rest of the cord, we found that the cords of young animals were too small to perform such a task adequately using low-power dissection microscopy, and we therefore used whole-cord samples.

For the comparison of expression levels in the mutant, T286A mutant mice and their wild-type littermates were sacrificed in the same way as described above, laminectomies performed and the L4-6 region of the lumbar spinal cord (as identified by tracing of the dorsal roots) removed and frozen as above.

Samples were individually weighed and transferred to a 1ml homogeniser containing ice-cold lysis buffer (0.625M Tris-Cl (pH 6.8; BDH), 2% SDS (Sigma), 0.1% bromophenol blue (Sigma), 50% glycerol (Sigma). Once homogenised, samples were heated to 100°C for 5 min. Genomic DNA / RNA was sheared by passing the lysate several times through a narrow gauge hypodermic needle and syringe prior to centrifugation at 13,000 RPM for 10 min at room temperature. The supernatant was transferred to fresh microcentrifuge tubes and normalised using a BCA plate reader (Biotrak II, Amersham Biosciences, UK), which uses chromatography to calculate total protein content, before being analysed by gel electrophoresis.

Polyacrylamide gel electrophoresis was performed using the Tris-glycine discontinuous buffer system for protein separation. 10-15 well 10% pre-cast gels (10% resolving gel, 4% stacking gel; Bio-Rad, UK) were used for the experiments shown. The gel tank was filled with electrophoresis running buffer (1M Tris base, 0.1M glycine (+SDS)). Samples and molecular weight marker proteins were loaded into the wells of the stacking gel and electrophoresed at a constant voltage of 100V, until the samples passed into the resolving gel. At this point the voltage was raised to 150V, and run for approximately 70 minutes until the dye front had run off the end of the resolving gel.

Polyvinylidenfluoride (PVDF) membranes (Bio-Rad, UK) were soaked in methanol for 1 minute. Following sodium dodecyl sulphate- polyacrylamide gel electrophorysis (SDS-PAGE), the gel, PVDF, pre-cut filter paper, and fibre pads were soaked in SDS buffer for 2 minutes. The Bio-Rad Protein II gel transfer apparatus was assembled, ensuring no air bubbles were present. The gel, membrane and filter paper sandwich was then placed in the electrophoresis buffer chamber, along with an ice pack, and filled with transfer buffer (0.1M Tris base, 0.5M glycine, 0.01% SDS). A constant voltage of 100V was applied for 45 min.

Following electrophoretic transfer, the PVDF membrane was rinsed briefly in phosphate-buffered saline and tween (PBS-T; Sigma). Blocking of non-specific antigens was performed in 4% Marvel dried skimmed milk in PBS-T for either 60 minutes at room temperature or overnight at 4°C on a rocking platform. Following blocking the membrane was briefly rinsed with PBS-T and incubated with the CaMKIIα primary antibody (Clone 6G9, Chemicon, 1:10000), in PBS-T with 4% Marvel at 4°C overnight. The specificity of this antibody, which recognizes both

phosphorylated and nonphosphorylated forms of the kinase, has been documented in previous studies (Erondu & Kennedy, 1985; McDonald *et al.*, 2002). After six X 5 minute washes in PBS-T at room temperature, the PVDF was incubated with Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody (1:2000; Santa Cruz, CA, USC) in PBS-T with 4% Marvel for 40 minutes. Finally the PVDF was washed as above with the addition of a final rinse in PBS only.

To detect bound antibody, membranes were incubated with a minimal volume of enhanced chemiluminescence (ECL) detection reagent for 1 minute at room temperature as described in the manufacturers protocol. PVDF was placed between two transparency sheets before exposure to Hyperfilm ECL autoradiography film. Several exposures were carried out to ensure linear exposure for direct comparison between bands.

In order to normalise the protein content for loading errors, the membranes were placed in a stripping buffer (1.5% glycine, 0.1% SDS, 0.1% Tween; pH 2), washed in PBS and then incubated in anti-GAPDH (1:750; Santa Cruz)- a glycolytic protein which is not developmentally regulated, and can therefore indicate whether the same amount of protein has been loaded in each lane- for 90 minutes at room temperature. They were then processed in the HRP secondary antibody and developed in the same manner as described above.

For quantitative analysis, the films were analysed densitometrically using MCID Basic imaging software. The greyscale density readings were calibrated using a transmission step-wedge standard. The relative optical density (ROD) of each band was multiplied by the area of the band to give an integrated density. This was normalised to its respective GAPDH level.

2.2.3 Immunohistochemistry

Animals aged P1, P8, P14 and adult were anaesthetized with sodium pentobarbitone (see above) and perfused intracardially with heparinised saline (0.9% NaCl (Baxter, Belgium) containing 5 units.ml^{-1} heparin (Monoparin, CP Pharmaceuticals, UK)) followed by 4.0% paraformaldehyde (BDH) in 0.1M PB at pH 7.4 (200mls for adult, 100mls for P14, 50mls for P8 and 10mls for P1). After perfusion, lumbar spinal cords were removed, postfixed for 1 hour and then transferred to a 0.1M PB solution

containing 30% sucrose and 0.02% sodium azide for storage overnight at 4°C. The cords were then sectioned on a freezing microtome (Leitz Wezlar, Germany) at a thickness of 50μm in the transverse plane. Sections were stored in a 0.1M PB solution containing 5% sucrose and 0.02% sodium azide.

Sections were processed for immunohistochemistry using the monoclonal antibody to the alpha subunit of CaMKII (Clone 6G9, Chemicon). They were washed in 0.1M PB, then incubated for 30 minutes in a blocking solution containing 0.1M PB with 4% normal goat serum (NGS, Vector), 0.4% Triton-X (BDH) and 0.02% sodium azide (Sigma), followed by an overnight incubation in the blocking solution containing the primary antibody (1:1000) at 4°C.

Sections were washed in 0.1M PB three times for ten minutes each, and the antigen was then visualised by incubation in 0.1M PB containing the fluorescent Alexa 488 goat anti-mouse secondary antibody (1:500, 1 hour at room temperature; Molecular Probes, USA). After immunofluorescence processing, sections were mounted on gelatinised slides, dried for 15 minutes and coverslipped with Gelmount (Sigma).

Sections were examined with a confocal laser-scanning microscope (BioRad MRC-600) equipped with an argon-krypton laser attached to a Nikon microscope. Brightness and contrast digital immunofluorescence images were adjusted in Adobe Photoshop.

For dual localisation of CaMKII with GABA, the polyclonal rabbit anti-GABA (1:500; Sigma) primary antibody was added to the blocking solution along with the anti-CaMKII antibody and incubated overnight at 4°C. Sections were washed in three changes of 0.1M PB for ten minutes each, and incubated in Alexa 488 goat anti-mouse (1:500) and Alexa 594 goat anti-rabbit (1:500) in 0.1M PB for 1 hour at room temperature. After another three rinses in PB, the sections were mounted and coverslipped as above. Sections were examined using a newly-obtained Leica SP1 confocal microscope and Leica confocal software.

2.2.4 Dorsal rhizotomies

In order to remove the primary afferent input to the dorsal horn, dorsal rhizotomies were performed on adult mice. Animals were anaesthetised with halothane (3% in 100% O_2) and a longitudinal skin incision approximately 2cm in length was made on the back.

Deep back muscles were retracted, and a laminectomy was performed. After incision of the dura mater, the left dorsal roots of L4-6 were identified under the operating microscope and cut. Back muscles and skin were then sutured with 5-0 Mersilk (Ethicon, USA) and the animal given analgesia (2mg/kg Buprenorphine; Temgesic, Reckitt & Colman, UK). The mice were allowed to survive for 3 days before being sacrificed. The L5 spinal cord was removed following perfusion fixation and processed for immunohistochemistry, as described above.

2.3 Results

2.3.1 CaMKII is strongly expressed in the adult dorsal horn

In the brain, CaMKII constitutes 1-2% of total neuronal protein (Lisman *et al.*, 2002). To characterise normal expression in the spinal cord, we used immunohistochemical and western blot analysis of protein content in the cord. Immunohistochemistry was performed on three wild-type adult C57BL/6J mice and analysis performed on between 10 and 20 lumbosacral sections from each animal. CaMKII immunoreactivity displayed distinct laminar specificity in the lumbosacral spinal cord, with dense staining seen in laminae I and II, and more moderate staining seen in lamina III (Figure 2.2A and C). Little labelling was observed in the deeper laminae of the dorsal horn, and little to no staining was detected in the ventral horn. In addition, dense labelling was observed in regions lateral to the dorsal horn grey matter. The fan-shaped organisation of this immunofluorescence is consistent with expression in the dorsolateral funiculus, although neurones from the lateral spinal nucleus could also be contributing to the immunoreactivity (Figure 2.2C). In some sections, very weak labelling could be observed in the corticospinal tract.

High magnification analysis of superficial dorsal horn showed that staining was concentrated in a ring of cytoplasm around the nucleus (Figure 2.2D). In laminae I and II, distinct labelling was observed in the surrounding neuropil, as identified by diffuse staining around the cell bodies and throughout the laminae. This diffuse labelling was not seen surrounding the population of immunopositive neurones in lamina III. It was also clear that not all cells in the superficial dorsal horn were immunopositive for CaMKII, as clear, cell-shaped gaps could be seen in the labelling (indicated by + signs; Figure 2.2D).

2.3.2 CaMKII is expressed in the spinal cord from birth

Western blots were performed on whole-cord preparations taken at a number of time points during postnatal development. One whole spinal cord (segments L3-6) was used for P3, P8, P14 and P96, and two P0 cords were pooled in order to obtain enough protein to be able to compare with the older animals (Figure 2.3). An equal amount of total protein from homogenates at various ages was loaded, as normalised with the glycolytic enzyme, GAPDH. Levels of CaMKII were determined by probing with the

αCaMKII-specific antibody as used in the immunohistochemical studies. A band was detected with a molecular mass of 50kDa, which corresponds to the size of αCaMKII.

Comparison of CaMKII expression levels during development revealed low levels at P0, followed by an upregulation towards P14, and a slight subsequent decline in protein levels into adulthood (Figure 2.3).

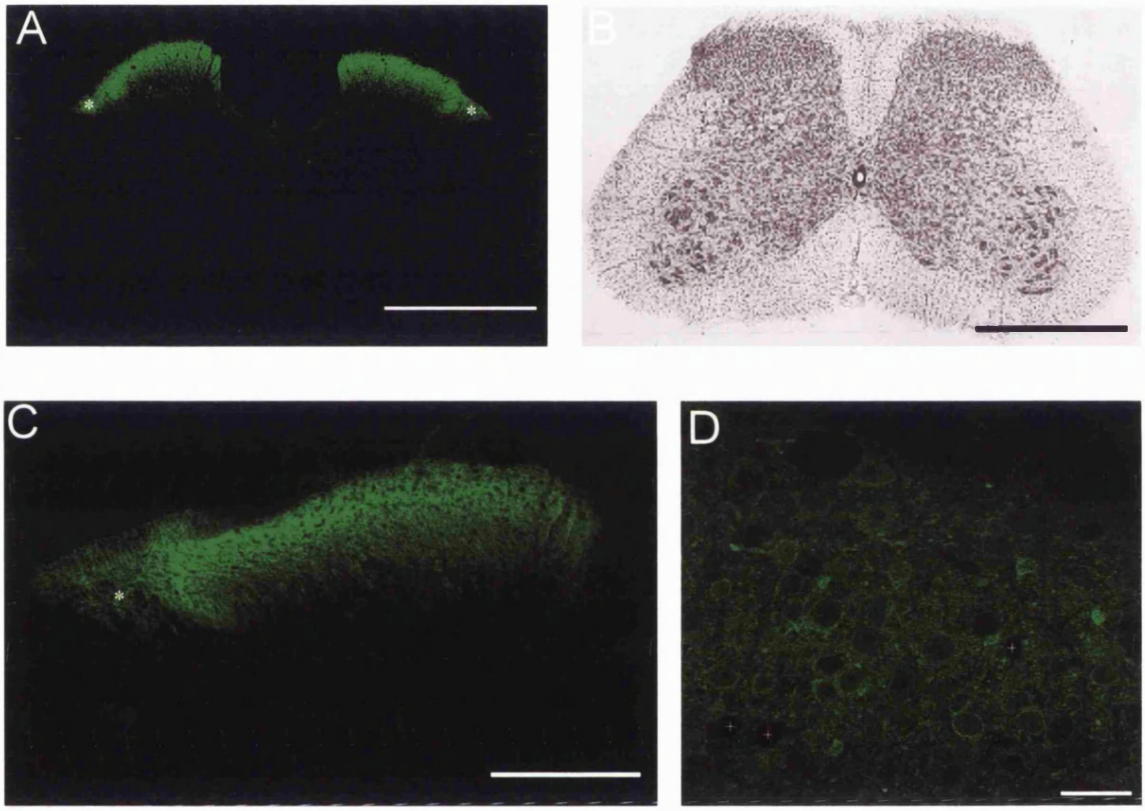

Figure 2.2
CaMKII immunoreactivity in the mouse lumbar spinal cord as detected with confocal microscopy. (A) Fluorescent detection of the antigen revealed dense labelling of the kinase in the superficial dorsal horn and dorsolateral funiculi/lateral spinal nuclei (*asterisks*). (B) Light microscopic image of a neighbouring section processed with a Nissl stain demonstrating the location of cell bodies within the grey matter of the cord. (C) Higher magnification of the dorsal horn shown in (A). Dense immunofluorescence can be detected in laminae I and II, with additional staining of a population of cells in lamina III. The dorsolateral funiculus/lateral spinal nucleus can also be detected with its characteristic fan-shaped morphology (*asterisk*). (D) High-magnification image of the dorsal horn. Immunoreactivity can be detected in the cytoplasm of the cells and in the surrounding neuropil. Some gaps can be seen in the labelling (+ *signs*), implying not all cells are immunopositive. Scale bars = 500μm (A and B), 100μm (C) and 25μm (D).

Immunohistochemical localisation of protein during development revealed a similar pattern to that seen in the adult. At P1, labelling was seen throughout the superficial laminae of the cord, with no staining seen in the deeper dorsal horn or the ventral horn (Figure 2.4A; n=3). High-magnification microscopy revealed a high density of labelling in the neuropil with little expression in the cell soma. (Figure 2.4D). Additionally, some immunoreactivity was observed in the lateral spinal nucleus (Figure 2.4C). The laminar distribution appears to be similar to that seen in the adult, with dense staining in laminae I and II, and lighter staining observed in lamina III.

Expression at P8 is similar to that seen in the adult. At this age, staining can be seen in the cytoplasm of the soma, as well as in the dendrites of the superficial dorsal horn (Figure 2.4F), and immunoreactivity is also present in the lateral spinal nucleus (Figure 2.4E).

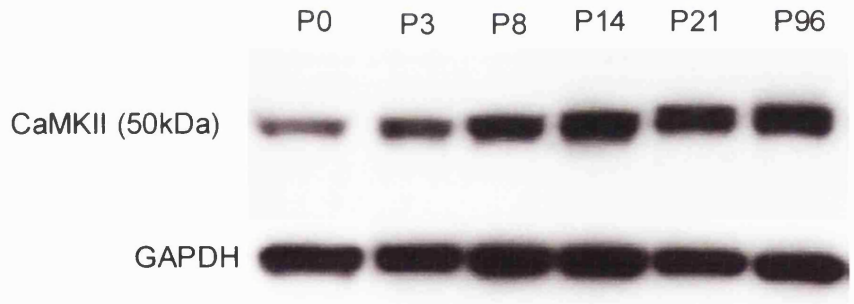

Figure 2.3
Western blot analysis of CaMKII expression levels in the spinal cord during development. The protein, as identified by a clear band at 50kDa, can be seen at low levels in the neonate, and is subsequently upregulated to peak at P14. The levels of protein in each lane were normalised using the housekeeper protein, GAPDH.

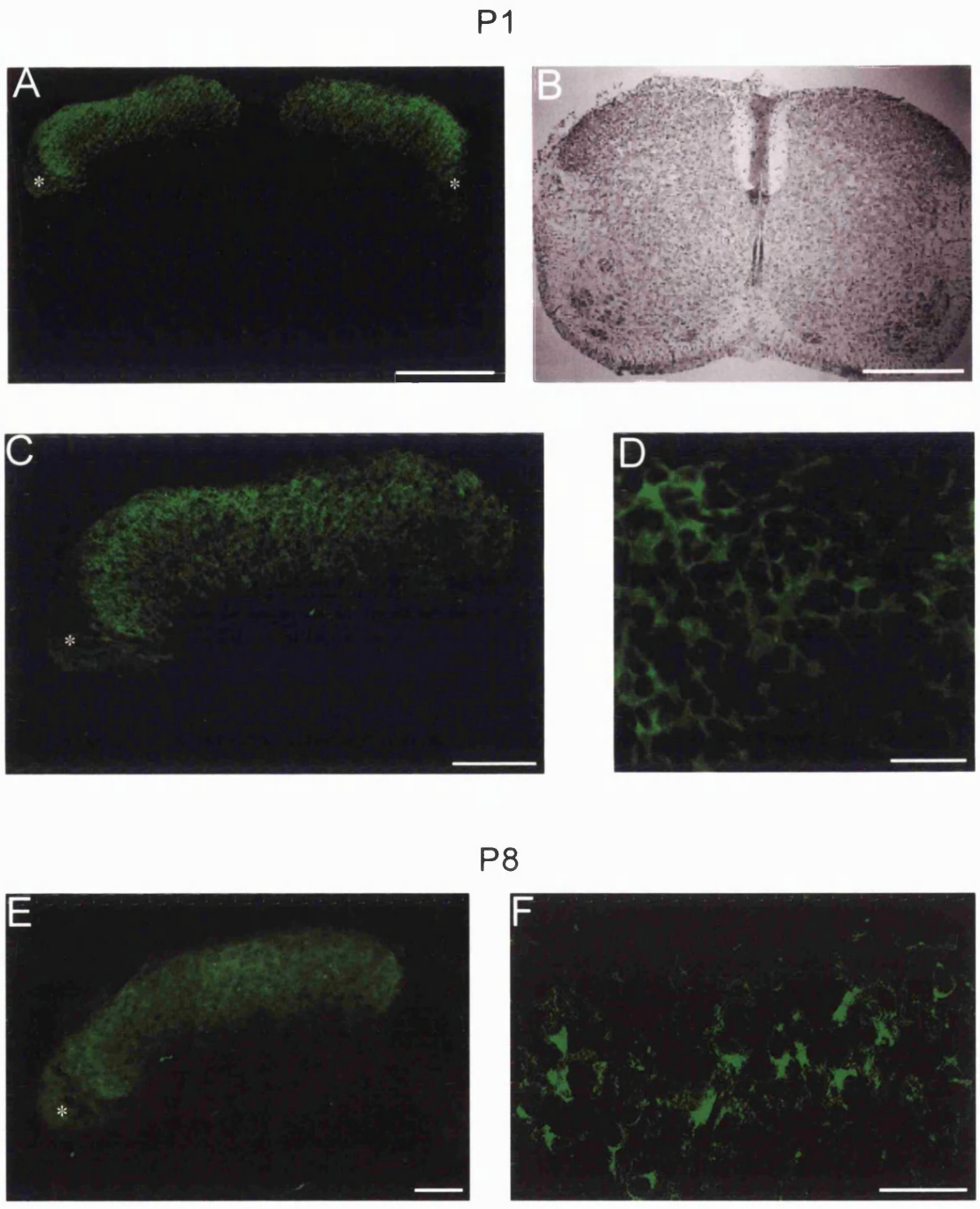

Figure 2.4
Developmental expression of CaMKII in the mouse lumbar spinal cord. (A-D) Immunofluorescence can be seen at P1 in a localisation similar to that of the adult. Dense staining is present in the superficial dorsal horn and dorsolateral funiculus/lateral spinal nucleus (*asterisks*). Additionally, high magnification imaging (D) shows an appearance of extrasomal protein, with no expression within the cell body. (E-F) Immunoreactivity in the P8 spinal cord is similar to that seen in that seen at P1, with staining in the superficial dorsal horn and dorsolateral funiculus/lateral spinal nucleus (*asterisks*). High magnification microscopy reveals a more adult-like pattern of expression within the cells at this age. Scale bars = 250μm (A and B), 50μm (C and E), 25μm (D and F).

2.3.3 Expression of CaMKII in dorsal rhizotomised mice

Since it has been previously reported that CaMKII is highly expressed in the dorsal root ganglia (Carlton & Hargett, 2002), it is likely that some of the immunoreactivity seen in the dorsal horn is localised in the primary afferent terminals in laminae I-III. In order to ascertain how much of the non-somal staining was from the afferent input, we performed dorsal rhizotomies on adult mice (n=3). Three days after section of L4-6 dorsal roots, CaMKII immunohistochemistry was performed on L5 spinal cord sections and the staining on the operated side compared to the contralateral, unoperated side. Contrary to expectation, the levels of CaMKII were not observably different on the two sides after this procedure (Figure 2.5). The lack of afferent input was confirmed by the absence of the isolectin, IB4, which labels a subgroup of C fibres (data not shown).

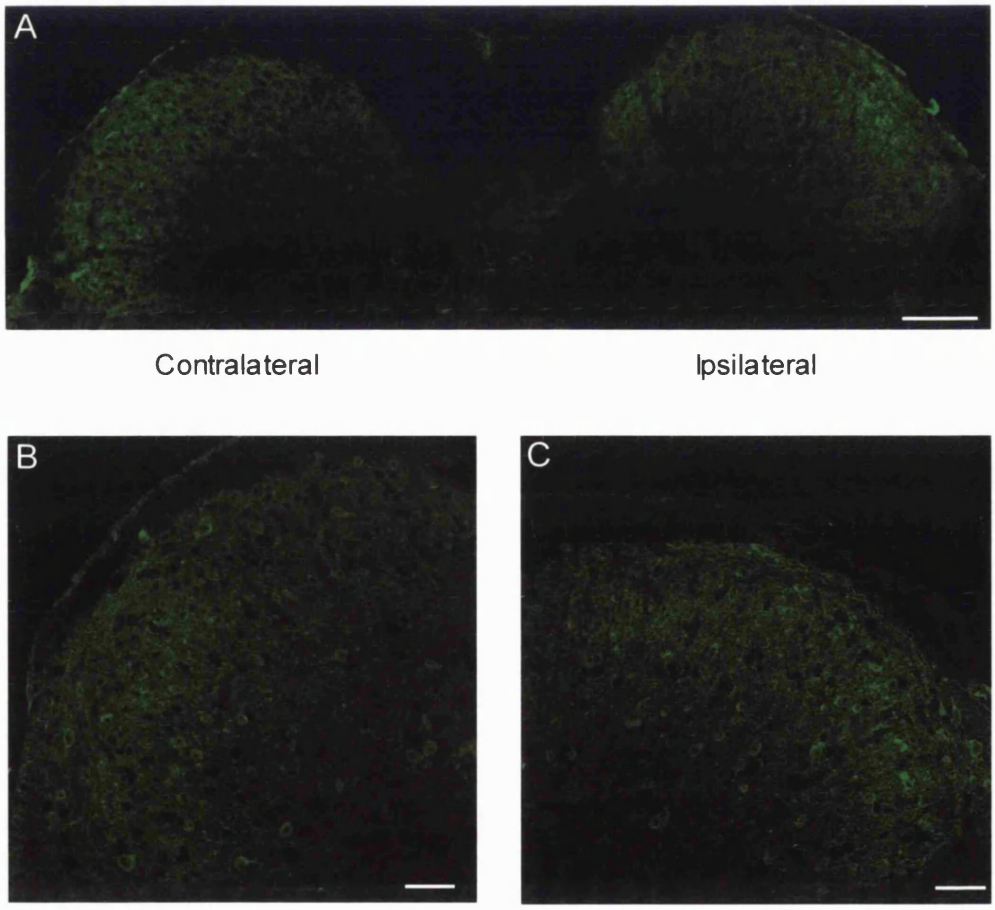

Figure 2.5
Expression of CaMKII in the adult mouse L5 spinal cord three days after dorsal rhizotomy. (**A**) shows a low-magnification image demonstrating similar levels of protein ipsi- and contralateral to the rhizotomy. (**B&C**) Higher magnification confocal microscopy reveals a similar pattern of expression on the ipsilateral side compared to contalateral, implying that the primary afferent terminals contribute only a small amount of CaMKII expression. Scale bars = 100μm.

2.3.4 Lack of coexpression between CaMKII and GABA

In order to establish whether CaMKII expression was restricted to particular subpopulations of adult dorsal horn neurones, we performed double-labelling experiments with the antibody to the inhibitory neurotransmitter, GABA (n=2 animals with 10 sections analysed from each). Expression of GABA in the dorsal horn was the same as that seen in previous studies (Tran *et al.*, 2003; Ibuki *et al.*, 1997). Cells expressing GABA were located throughout the grey matter, with a clear population concentrated in lamina II. The number of GABA-positive neurones (Figure 2.6B) was much lower than the number of CaMKII-positive cells (Figure 2.6A), and no noticeable colocalisation of CaMKII and GABA was seen in the dorsal horn (Figures 2.6C).

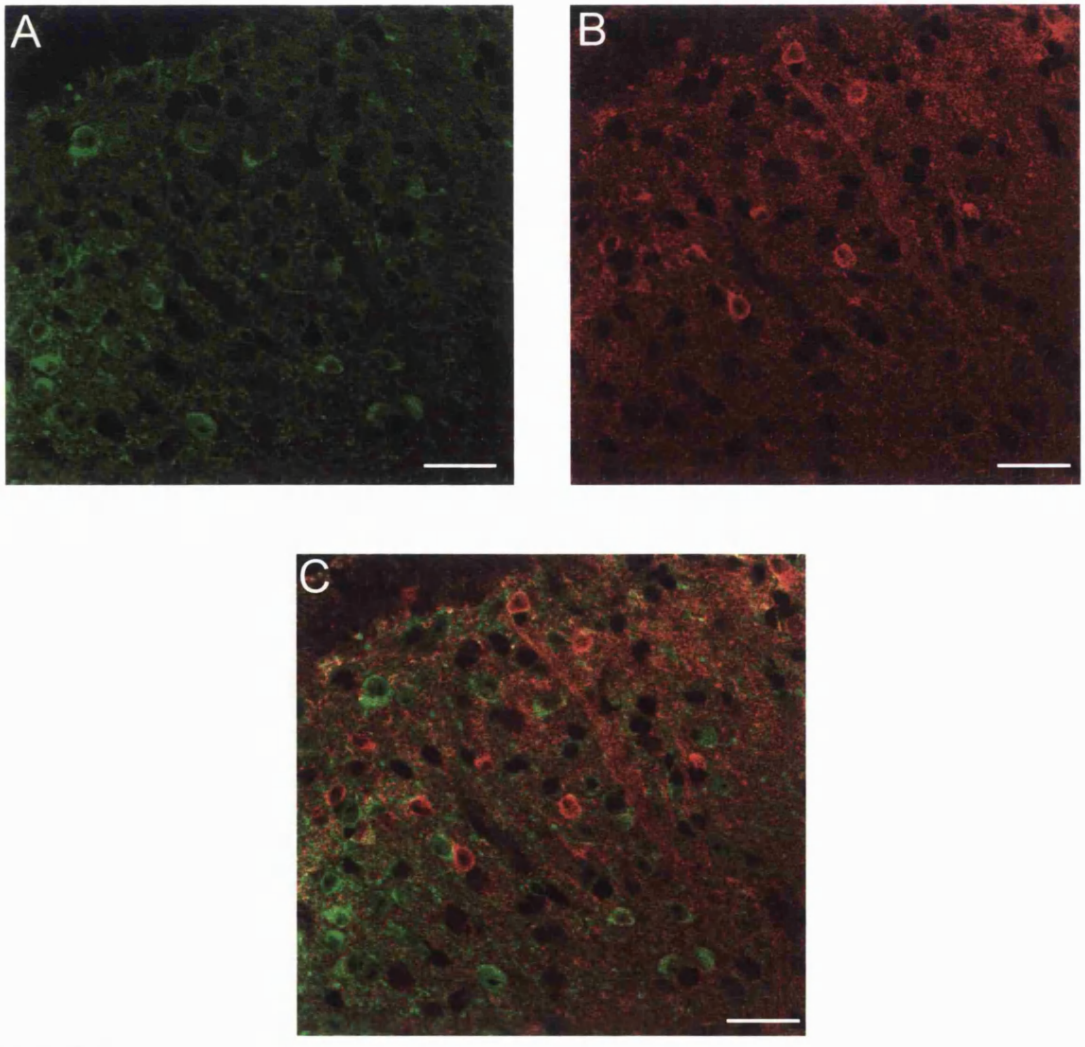

Figure 2.6
Coexpression of CaMKII and GABA in the dorsal horn of the adult mouse lumbar spinal cord. (**A,B**) Paired images from the same microscopic fields of sections stained for CaMKII (**A**, green) and GABA (**B**, red). (**C**) Merged image of **A** and **B** demonstrating the apparent lack of colocalisation of cytoplasmic CaMKII and GABA in the dorsal horn. Scale bars = 40μm.

2.3.5 Comparison of total CaMKII expression in the mutant and wild-type

Because previous studies in the cortex of the T286A mutant have shown a reduction in total CaMKII levels compared with wild-type (Hardingham *et al.*, 2003; Taha *et al.*, 2002), we examined the levels in the spinal cord, using the hippocampus- an area that shows unchanged levels between genotypes (Hardingham *et al.*, 2003)- as a positive control. Expression was normalised with the housekeeping protein, GAPDH. We confirmed the observation made by Hardingham *et al.*, finding no difference in expression between groups in the hippocampus ($p=0.8$, unpaired t test; $n=3$ in each group; Figure 2.7B). Additionally, we saw no change in total protein expression in the spinal cord between groups ($p=0.45$, unpaired t test; $n=6$ for wild-type and 5 for mutant; Figure 2.7A). Therefore, despite region specific alterations in expression levels in the mutant, no such change was observed in the cord.

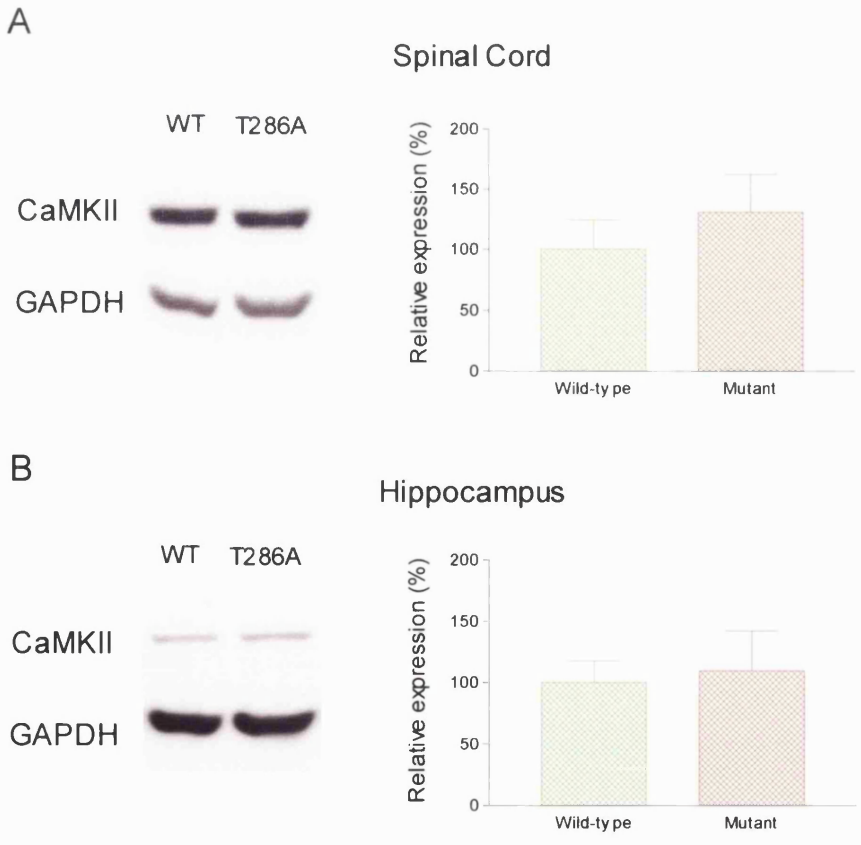

Figure 2.7
Comparison of total CaMKII expression in the T286A mutant and wild-type mouse. (A) shows western blot analysis of protein expression in the spinal cord. Examples of the bands obtained for CaMKII (top) and GAPDH (bottom) are shown in the left panel. The right panel shows the quanified data, expressed as a percentage change from wild-type. No significant differences were observed between the groups. (B) shows the expression in the hippocampus. Again, no significant difference was observed between genotypes.

2.4 Discussion

The results of this study reveal a number of aspects of CaMKII expression in the spinal cord:

- CaMKII is densely expressed in the substantia gelatinosa of the adult mouse
- It is expressed from birth and can be seen throughout development
- The expression in the adult is predominantly in dorsal horn neurones with minimal expression in the primary afferent terminals
- CaMKII does not appear to be expressed in the cytoplasm of adult GABAergic dorsal horn neurones
- The T286A mutation does not affect total CaMKII expression levels in the adult spinal cord

2.4.1 Expression of CaMKII in the adult mouse

We have found that CaMKII protein is present in the dorsal horn of the mouse spinal cord from P1 up until adulthood. In the adult, dense staining was observed in the superficial dorsal horn, mainly in laminae I and II, with some cell bodies being labelled in laminae III and IV. In addition, dense labelling could be seen in the dorsolateral funiculus and lateral spinal nucleus. Previous studies have demonstrated similar expression patterns in the rat spinal cord, with dense labelling in the superficial dorsal horn and lighter staining in the deep dorsal horn (Terashima *et al.*, 1994; Bruggemann *et al.*, 2000; Lund & McQuarrie, 1997). Additionally, one group found dense labelling in the corticospinal tract, whereas we saw only very light labelling in this area (Terashima *et al.*, 1994). In general, however, it appears that the kinase shows fairly close expression patterns between the species.

The localisation of CaMKII in the spinal cord is consistent with the kinase being involved with plasticity in this part of the CNS. The dorsal horn is known to be an area of great plasticity and neuromodulation, both in terms of activity-dependent development and synaptic alterations in the adult. The fact that the enzyme is indeed present from birth makes it an ideal candidate for a downstream effector of the NMDA receptor-dependent remodelling of axodendritic structure (Zou & Cline, 1999; Wu & Cline, 1998) and primary afferent input (Beggs *et al.*, 2002). In the adult, the presence

of the enzyme in the superficial dorsal horn implies a possible role as a mediator of plastic changes that occur after peripheral injury, such as central sensitisation. Indeed, the kinase is localised in parts of the CNS that process noxious information (Bruggemann *et al.*, 2000). The role of the kinase in pain processing will be discussed in Chapter Five.

The lack of expression in the ventral horn again is also consistent with previous immunohistochemical reports, although an *in situ* hybridisation study did find light expression in the motorneurone pool (Lund & McQuarrie, 1997). The fact that we did not detect any immunostaining in motorneurones could be due to either low antibody sensitivity, or perhaps by alternative splicing of the mRNA. Alternatively, the protein could be turned over rapidly, or transferred away from the cell bodies and into the axons.

The presence of the kinase in the descending pathways is slightly more curious, as it implies a possible presynaptic role for the kinase in descending transmission. The dorsolateral funiculus arises from the raphe magnus nucleus in the rostral ventromedial medulla. The fibres in this tract contain serotonin, noradrenaline and glutamate and are strongly implicated in the production of analgesia due to their strong inhibitory effect on dorsal horn neurones (Fields *et al.*, 1977). The role of CaMKII in this pathway is as yet unknown, although evidence in the hippocampus suggests that the kinase may be involved in 'exocytototic-like release' of serotonin, as addition of the CaMKII inhibitor, KN-62, to the superfusion medium of synaptosomes inhibits depolarisation-induced [^{3}H]5-HT release (Cinquanta *et al.*, 1997). Conversely, injection of activated CaMKII produces increases release of glutamate and noradrenaline (Nichols *et al.*, 1990).

It is now known that in addition to the fibre tracts of the dorsolateral funiculus, the region lateral to the dorsal horn also contains a nucleus called the lateral spinal nucleus (LSN). Although known about for some time (Gwyn & Waldron, 1968), the nucleus has only recently been described in detail with regard to its projections, input and cellular physiology. Morphological studies have found that this nucleus projects to a variety of supraspinal structures including the thalamus, PAG, hypothalamus, amygdala and parabrachial area (Ding *et al.*, 1995; Jiang *et al.*, 1999), and also to spinal laminae I, II, V and VII (Jansen & Loewy, 1997). The neurones also receive descending projections from the raphe nuclei, reticular formation and PAG (Masson *et al.*, 1991), as

well as local connections from peptidergic spinal cord neurones. They express a number of peptides such as CGRP and VIP, as well as receptors for substance P (Ding *et al.*, 1995). With regard to afferent input, the majority of cells appear to receive polysynaptic inputs from dorsal root stimulation, although a small proportion have been shown to have monosynaptic connectivity (Jiang *et al.*, 1999). The neurones could also be depolarised by substance P application (Jiang *et al.*, 1999). It therefore appears that cells within the LSN are important for processing of noxious information, and so the presence of CaMKII protein could imply a role in downstream signalling of this function. Furthermore, a possible disruption of axodendritic formation as a result of the T286A mutation could perhaps result in alterations in transmission of nociceptive information to the brain.

Finally, it is worth noting that the β isoform of CaMKII has also been shown to be expressed in the adult rodent spinal cord (Terashima *et al.*, 1994; Terashima *et al.*, 1995; Lund & McQuarrie, 1997). The expression of this isoform is more widespread throughout the laminae than is the α isoform, with immunoreactivity being observed in the motorneurons as well as in the superficial dorsal horn. The fact that this isoform is present in similar areas of the cord as the α isoform perhaps suggest alternate functional properties of the kinase, and an ability for the two to interact either through homomeric or heteromeric compositions (Fink *et al.*, 2003).

2.4.2 Developmental profile of CaMKII expression

The developmental regulation of αCaMKII has been a subject of some controversy over recent years. Early reports found a lack of mRNA expression in the cortex until P4 (Burgin *et al.*, 1990), although more recent studies have demonstrated expression perinatally. In particular, *in situ* hybridisation has revealed the presence of low levels of the kinase at P1 in the hippocampus, with upregulation during the first two weeks of life, and a subsequent downregulation into adulthood (Herms *et al.*, 1993). This profile matches that seen in the present study in the spinal cord. Expression at P1 is low, and is upregulated to peak at around P14. This peak in protein expression parallels that seen for ionotropic glutamate receptors in the cord during development (see Chapter One), suggesting perhaps that the levels of the kinase are regulated by increased postsynaptic activity, or to increased levels of calcium entry (Hori & Kanda, 1994).

What could be the significance of such an overexpression? The first two weeks of life are a critically important time for both synapse formation and maturation. In particular, intrinsic interneurones extend axons and dendrites throughout the dorsal horn, forming new synapses with primary afferents and neighbouring interneurones. C fibre synapses with dorsal horn cells are strengthening during this period (Baccei *et al.*, 2003). CaMKII may be required for this maturation, allowing suitable synapses to strengthen and unsuitable ones to weaken. Evidence for this comes from a study in visual cortical pyramidal cells by Pratt *et al.* (2003), where addition of T286D CaMKII (a mutation that renders the protein permanently activated) caused increases in EPSC amplitude, associated with an increase in the number of excitatory synaptic inputs from some neighbouring neurones. Overall, however, there was a 33% decrease in the number of neurones that formed synapses with the transfected cell. The process is also dependent on NMDA receptors (another protein which is overexpressed in the dorsal horn during the second postnatal week; see Pattinson & Fitzgerald, 2004). CaMKII can therefore cause specific structural changes of presynaptic neurones, whereby certain cells can form a large number of synapses with the postsynaptic, CaMKII-expressing neurone, while other cells weaken their synaptic input and lose contact altogether with the postsynaptic neurone. This could be a mechanism underlying the observed NMDA receptor-dependent withdrawal of Aβ fibres from the substantia gelatinosa- with CaMKII mediating the increase in connectivity with the incoming C fibres, and weakening those from Aβ fibres (Beggs *et al.*, 2002). However, it is worth noting that the cortical study was performed on a homogeneous population of cells (cortical pyramidal cells were selectively cultured). Whether such a process could cause complete loss of synaptic connectivity from one modality of cells, while making another distinct set stronger, remains to be seen.

In order to ascertain the localisation of the kinase during development, we performed immunohistochemistry on lumbar spinal cord sections at various ages during development. In general, this technique revealed a developmental profile that was similar to the adult localisation, in as far as expression appeared to be confined to the superficial dorsal horn at all ages tested. This is in contrast to many of the glutamate receptor subunits, which often showed alterations in location of expression during development (see Chapter One). However, on closer examination, some subtle changes could be observed. At P1, for example, the dorsal horn expression appeared to be

extrasomal in nature, with dense expression in surrounding neuropil, but with an apparent lack of staining in the cell bodies. By P8, staining looked more adult-like in its topography, with cell bodies and dendrites now expressing the protein. This developmental profile has not been noted in other parts of the brain, and could perhaps reflect the kinase's role in dendritic refinement during this period (Wu & Cline, 1998; Pratt *et al.*, 2003). The increase in somal expression during development perhaps represents a central store of kinase that can be rapidly trafficked to the peripheral dendrites in response to changes in synaptic acitivity, or perhaps just an increase in axosomal synapses during development.

2.4.3 Pre- vs. postsynaptic expression

As previously stated, CaMKII is seen both in the DRG and in the dorsal horn (Bayer *et al.*, 1999; Bruggemann *et al.*, 2000). Therefore it is difficult to detect how much of the expression outside the soma is being caused by presynaptic, primary afferent input using light microscopy. In order to remove this factor, we performed dorsal rhizotomies on adult mice, a procedure which causes the primary afferent terminals to degenerate over a few days. Immunohistochemical analysis on these mice revealed no noticeable decrease in protein expression, implying that the majority of antigen is from dorsal horn neurones. The fact that approximately 50% of synapses in the dorsal horn are formed by primary afferents (Chung *et al.*, 1989; Willis & Coggeshall, 1991), and that one study found 23% of CGRP-positive and 38% of IB4-positive fibres expressed CaMKII (Carlton & Hargett, 2002), the lack of noticeable change in immunofluorescence after rhizotomy implies that CaMKII is heavily expressed in postsynaptic neurones. It is worth noting, however, that immunofluoresce is difficult to quantify as the fluorescence is not always uniform, and bleaching occurs rapidly upon examination, thus altering the signal. Even when comparing to an unaffected contralateral side, and thus using each section as its own control, only noticeable differences would lead to significant changes. The more quantifiable western blot technique is required in order to confirm the absence of change after dorsal rhizotomy.

While the postsynaptic actions of CaMKII have been well described, its function at the presynaptic bouton has less well described. In the primary afferents, the kinase could also be acting on NMDA and AMPA receptors both peripherally and on the central terminations. This is supported by the finding that ligating the sciatic nerve leads to a

build-up of CaMKII on the proximal side of the ligation, implying that the protein is transported distally (Carlton, 2002). In addition, CaMKII immunoreactivity can be seen in both the digital nerves and dorsal roots at the electron microscopic level, implying transport both centrally and distally. However, the study does not demonstrate whether or not the kinase actually reaches the central terminals.

The function of presynaptic CaMKII in central afferent terminals is less well documented, but it could be acting to alter transmitter release by clustering the presynaptic proteins, synaptophysin and synapsin I, and subsequently unmasking 'silent' presynaptic terminals (Ninan & Arancio, 2004; Nayak *et al.*, 1996). Recently, the CaMKII gene was deleted in the adult CA3 pyramidal cell, thus allowing analysis of the presynaptic mechanisms at the CA3-CA1 synapse (Hinds *et al.*, 2003). These mice displayed enhanced facilitation of the EPSC amplitude during repetitive stimulation, while basal probability of release (P_r) was unaffected, suggesting that CaMKII exerts an inhibitory constraint on presynaptic neurotransmitter release but only during high-frequency activity. The authors proposed that the kinase was acting on the N-type calcium channel- a protein found in the presynaptic terminal where it mediates evoked neurotransmitter release. Phosphorylation at a protein interaction site causes the channel to disconnect from syntaxin 1A and SNAP-25, thus moving the synaptic vesicles further from the calcium influx, or alternatively releasing the channels themselves from the active zone, and causing a decrease in P_r (Yokoyama *et al.*, 1997). The presynaptic effects of CaMKII are believed to be independent of autophosphorylation, as both the T286A mutant and its wild-type littermate show similar biphasic responses to 10Hz tetanic stimulation (Giese *et al.*, 1998). Additionally, P_r is not affected by T286A or T286D mutations, even after repetitive stimulation (Y. Elgersma, personal communication).

As with most neurones, calcium signalling plays a vital role in neurotransmission in the primary afferents. Activation of receptors such as TRPV1 (Bleakman *et al.*, 1990), P2X (Xu & Huang, 2002) and mu opioid receptor (MOR; Bruggemann *et al.*, 2000) all produce changes in intracellular calcium concentration, be it through calcium entry or release from intracellular stores. While the downstream effects of such changes are not clearly established, some evidence does suggest that CaMKII could play a role. For example, Carlton (2002) has demonstrated that CaMKII expression is upregulated in

both myelinated and unmyelinated fibres of the digital nerves following injection of CFA into the hindpaw.

Despite the fact that CaMKII is present in a population of primary afferents, and that it can mediate transmitter release through functions at the presynaptic terminal in other parts of the brain, the absence of a downregulation after rhizotomy strongly suggests that its most important function in the dorsal horn is at the postsynaptic density. This role could be in strengthening excitatory synapses through phosphorylation and insertion of AMPA receptors to the cell membrane, and also by increasing inhibitory control via phosphorylation of GABA and glycine receptors.

2.4.4 Colocalisation with GABA

Unlike some regions of the brain, inhibitory and excitatory neurones in the dorsal horn cannot be readily distinguished by morphological structure or laminar organisation (Grudt & Perl, 2002). In order to ascertain whether the kinase was expressed in a single subpopulation of neurones, we performed double-labelling immunofluorescent experiments with the inhibitory neurotransmitter, GABA.

Analysis of double-labelled sections revealed that CaMKII did not appear to colocalise with GABA in the superficial dorsal horn. It appears that cells that display a CaMKII-positive cytoplasm do not express GABA, although the confocal microscopy used in this study was all of fairly low magnification, and so it is possible that CaMKII is in fact present at individual synapses in these GABA-positive neurones, but just absent from the cytoplasm.

In order to locate the kinase precisely within the cell, electron microscopy is needed. Such work has recently been undertaken in some parts of the brain, with one group finding CaMKII at synapses containing glutamate receptors only (Liu & Jones, 1996). However, CaMKII has been shown to be present at inhibitory synapses in the olfactory system (Zou *et al.*, 2002), implying possible functional differences across systems. The localisation in the spinal cord has yet to be studied, although recent work has indicated the presence of CaMKII at synapses responding to substance P (Larsson & Broman, 2003), consistent with a localisation at excitatory synapses.

A lack of colocalisation with GABA is consistent with findings in the amygdala, where CaMKII immunopositive cells are completely separate from those expressing GABA, and are selectively expressed on cells containing the AMPA receptors subunits, GluR2/3 (McDonald *et al.*, 2002). Expression studies in the olfactory bulb, however, demonstrate a selective colocalisation with GABAergic neurones, and an absence from glutamatergic cells (Zou *et al.*, 2002).

CaMKII-positive cells are likely to be glutamate-positive, but this is hard to demonstrate as glutamate plays roles in various metabolic events not connected to neurotransmitter release, so that an antibody raised against glutamate would give rise to many false-positive results (Ottersen *et al.*, 1992; McDonald *et al.*, 2002). Additionally, not all cells that are GABA-negative (and therefore glycine-negative- the two are always coreleased in the spinal cord; Jonas *et al.*, 1998) are immunolabelled for CaMKII, implying that the kinase is present only in a subset of glutamatergic neurones.

While it is yet unclear what group of cells contain the CaMKII enzyme, one group of cells that appears to show total coexpression is those that stain positively for the mu opioid receptor (MOR; Bruggemann *et al.*, 2000). These authors found that, while not all CaMKII-positive neurones expressed MOR, all MOR-positive neurones expressed CaMKII in the dorsal horn, and the majority did in the DRG. They proposed that CaMKII is could play a critical role in phosphorylating the MOR, and thus be important in the development of tolerance to morphine. MOR receptor-positive neurones are only seen in lamina II, where they make up approximately 10% of the total cell population. In concordance with the results presented here, they are not immunopositive for GABA, and respond to noxious heat stimulation, suggesting an input from C fibres. Furthermore, the strict colocalisation of MORs with CaMKII is seen not seen in all pain-processing parts of the brain, adding support to a dichotomy within the CNS, whereby CaMKII can have different effects in differing parts of the brain (Bruggemann *et al.*, 2000).

2.4.5 Comparison of CaMKII expression in wild-type and mutant mice

We have shown that expression of total CaMKII levels in the spinal cord is not affected by the T286A mutation. This is in contrast to other parts of the brain, for example the cortex, where it was shown that the mutant mouse displayed significantly lower levels of total protein compared to the wild-type (Taha *et al.*, 2002; Hardingham *et al.*, 2003).

However, the hippocampus displayed similar levels between groups (Figure 2.7B; Hardingham *et al.*, 2003), suggesting that the changes are specific to particular regions of the brain. The fact that the spinal cord does not show any changes suggests that there are no compensatory mechanisms occurring in response to the loss of CaMKII function, and so any alterations in somatosensory system are likely to be a direct result of the disruption to CaMKII autophosphorylation.

In conclusion, we have shown that CaMKII is expressed in the mouse spinal cord from birth, and is developmentally regulated. The expression is limited to the dorsal horn, and so could play a role in the development and plasticity of the somatosensory system. The majority of the kinase seems to be restricted to dorsal horn neurones, as removal of the primary afferent input did not alter expression levels. We have also demonstrated a strict cellular specificity of the kinase, in that it is not expressed in GABAergic neurones. Further coexpression studies, such as with the glutamate transporter proteins VGLUT1-3, are required to further understand how the role of the kinase in sensory processing. Finally, we have shown that the T286A mutant expresses similar levels of the kinase as the wild-type littermates, suggesting that the disruption in function is not compensated by increased/decreased total protein levels.

A circuit demonstrating the position of CaMKII in the dorsal horn is shown in Figure 2.8. With regard to the activity-dependent changes that occur in the dorsal horn, the kinase appears to be ideally placed to mediate the downstream cascade of events that lead to axodendritic alterations during development. As autophosphorylation appears only to be important for postsynaptic processing (see above), the T286A mutant will allow for a detailed analysis of how dorsal horn neurones affect afferent input and synaptic maturation during development.

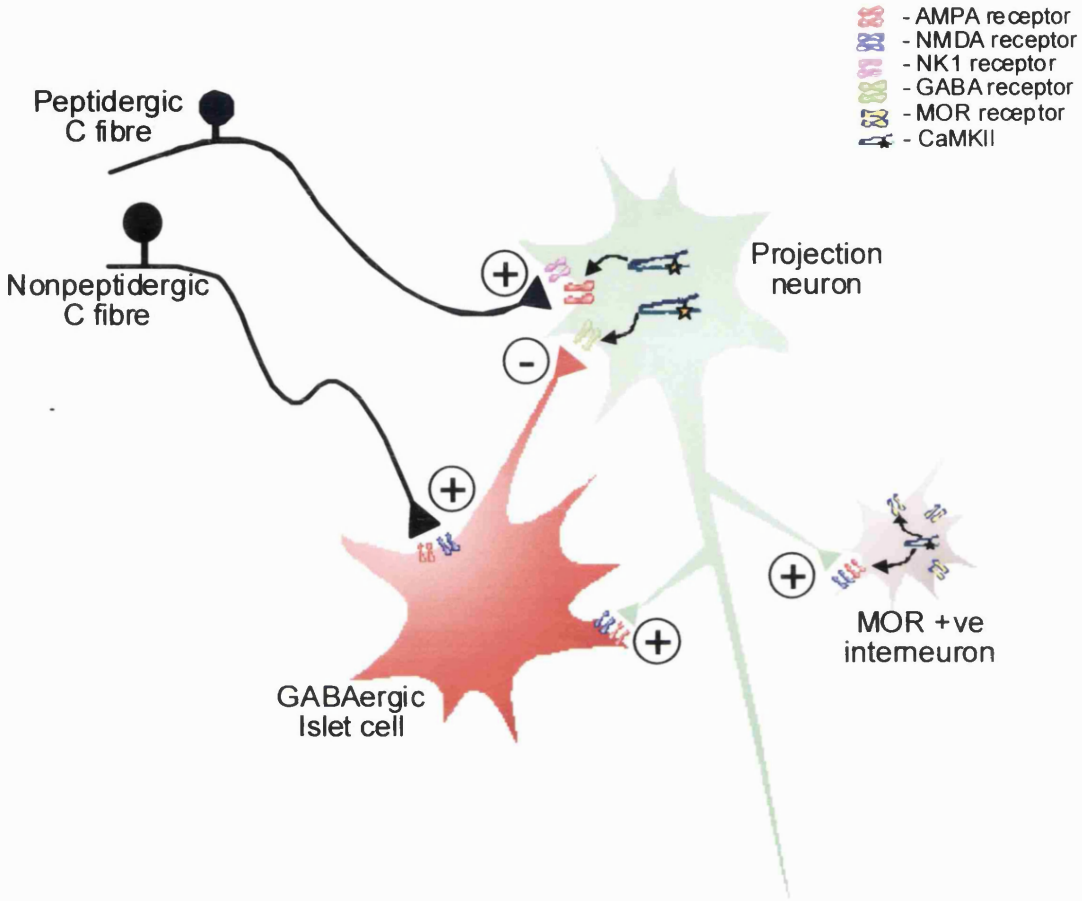

Figure 2.8
Diagram illustrating the involvement of CaMKII in a nociceptive circuit in the dorsal horn. The inhibitory islet cell and projection neurones both receive monosynaptic input from C fibres, but can also modulate one another. CaMKII acts on the projection neurone to enhance synaptic strength at both excitatory (via phosphorylation of AMPA receptors) and inhibitory (via GABA receptor phosphorylation) synapses. The mu-opioid (MOR)-positive interneurone contains CaMKII, which could phosphorylate both AMPA receptors and MORs themselves, but does not express NK1 receptors.

Chapter 3

Aβ FIBRE INPUT TO THE DEVELOPING DORSAL HORN

3.1 Introduction

The developing spinal cord undergoes structural alterations during postnatal life. These include maturation of dorsal horn neurones, which increase in size and expand their axodendritic arbours during the first few postnatal weeks (Bicknell & Beal, 1984), and reorganisation of the primary afferent input, most notably the withdrawal of Aβ fibres from the superficial dorsal horn (Fitzgerald *et al.*, 1994). Many of these changes are activity-dependent and may therefore require activation of CaMKII in order to develop normally.

The presence of Aβ afferent fibres in the superficial dorsal horn of the neonatal spinal cord was first described by Cajal in 1909 (Ramón y Cajal, 1909), who used Golgi stains to study the morphology of the neonatal spinal cord. He noted that the fibres entered the dorsal horn medially and curved upwards to the dorsal-most aspect of the grey matter (Figure 1.1). Later work in adult cats demonstrated a lack of Aβ fibres in this part of the cord (Brown, 1981), and similarly very few such fibres were seen in the adult rat SG (Shortland *et al.*, 1989), leading to the hypothesis that they withdrew into deeper laminae during postnatal development.

The withdrawal hypothesis was confirmed by Fitzgerald *et al.* (1994), and later by Beggs *et al.* (2002), who used the B subunit of the cholera toxin conjugated with horseradish peroxidase (B-HRP) to selectively label the myelinated fibres. In order to address whether the changes in termination pattern was caused simply by non-selective labelling of unmyelinated C fibres, the DRGs were also examined, and in all cases, the labelling was shown to be specific to A fibres. A more specific technique, whereby DRG cells were recorded and filled with neurobiotin so as to allow for identification of central processes, found a similar profile of Aβ fibres in the neonatal rat spinal cord (Mirnics & Koerber, 1997). Therefore, it appears that Aβ fibres are present in SG at birth, but withdraw into the deeper laminae during the first few weeks of postnatal life.

Later studies using an *ex vivo* preparation, whereby the skin, nerve, DRG and spinal cord were removed and individual DRG cells recorded and filled, questioned whether low-threshold mechanoreceptors (LTMRs) did indeed enter lamina II in the neonate (Woodbury *et al.*, 2000; Woodbury *et al.*, 2001). They proposed that the appearance of BHRP-labelled fibres in SG was due to labelling of high-threshold mechanoreceptors

(HTMRs), which they did see crossing the lamina II border (Woodbury & Koerber, 2003). Some fibres, however, could still be seen at P21, implying they do not all withdraw. The afferent fibres labelled with BHRP in SG during development remain an as yet undefined population of A fibres that are, nevertheless, not observed in the adult SG.

The question of Aβ fibre withdrawal was addressed here using a novel technique that takes advantage of the fact that these fibres enter the cord more medially than do the other fibre types, which enter laterally and run in the Lissauer's tract. (Figure 1.1). By injecting small quantities of the lipophilic tracer, DiI, into the dorsal columns of the spinal cord, we were able to label small groups of Aβ fibres and to track their termination profile during development. This technique has the advantage of only labelling small numbers of fibres, so that individual terminals can be seen, as opposed to the BHRP (or 'bulk-labelling') studies, in which all fibres are labelled giving a diffuse appearance of staining in the cord.

As previously described, BHRP labelling was also used to map primary afferent terminations after chronic NMDA receptor blockade (Beggs *et al.*, 2002). Such an intervention prevents the normal withdrawal from SG, and is accompanied by physiological and behavioural changes (see Chapter One). It was suggested that the mechanism underlying such a process could involve an LTP-like process occurring at the newly-formed C fibre synapses, accompanied by a heterosynaptic LTD of the A fibre synapses (Beggs, 2000; see Chapter One). Such a process is believed to cause the activity-dependent reorganisation of afferent input to the somatosensory (Crair & Malenka, 1995; Fox, 1995) and visual (Katz & Shatz, 1996; Kirkwood *et al.*, 1995) cortices.

CaMKII is known to play a vital role in this synaptic plasticity due to its ability to enter a state of autophosphorylation in response to calcium entry through NMDA channels. Once phosphorylated, kinase acts to both phosphorylate AMPA receptors and to increase their insertion into the postsynaptic membrane. Therefore, inactivation of CaMKII by preventing autophosphorylation could prevent the activity-dependent plasticity, and subsequently the withdrawal of Aβ fibres from SG. Disruption of

CaMKII has been previously shown to cause deficits in developmental plasticity in the barrel cortex (Glazewski *et al.*, 2000; Glazewski *et al.*, 2001; Hardingham *et al.*, 2003).

A second, more intriguing role of the kinase in synaptic development has been recently shown in cultured visual cortex pyramidal neurones (Pratt *et al.*, 2003). These cells were injected with an activated form of CaMKII (in which autophosphorylation was mimicked by insertion of asparagine at site T286), and the synaptic input measured. The authors found that such a technique produced an increase in both EPSC and mEPSC amplitude, caused by a modest increase in quantal amplitude, and a large increase in the number of synaptic contacts made by the presynaptic partner. In addition, there was an active elimination of synapses from neighbouring cells, producing a net loss of excitatory synapses. Again, this study implies that blockade of CaMKII autophosphorylation could have profound effects on the postnatal reorganisation of dorsal horn primary afferents, preventing activity-dependent synapse elimination. Finally, CaMKII has been shown to play an important role in dendritic morphogenesis through its phosphorylation of MAP2 (Vaillant *et al.*, 2002) and the transcription factor, Neuro-D (Gaudilliere *et al.*, 2004).

In order to examine the role of CaMKII autophosphorylation on Aβ fibre withdrawal, we used the DiI tracing method on mice containing the T286A mutation and their wild-type littermates.

3.2 Methods

3.2.1 Iontophoretic injection of DiI

For the developmental study, Sprague-Dawley rats aged P0, P21 and adult were used. Animals were anaesthetised with pentobarbitone (Euthatal, Merial) and perfused intracardially with 0.9% heparinised saline, followed by 4.0% paraformaldehyde in 0.1M PB at pH 7.4. After perfusion, the spinal cords were removed and pinned onto a cork board inside a petri dish, which contained 0.1M PB, with the dorsal horns facing upwards. Cords were then trimmed of roots and the dura removed. A glass microelectrode (tip diameter=1μm) containing a DiI solution (3mg/ml in 100% ethanol (Molecular Probes, USA) was lowered onto the surface of the medial dorsal column, then inserted 100-300μm into the column itself using a micromanipulator. Current was passed through the electrode from a 9V battery, connected to one silver wire in contact with the DiI solution (+ve), and another in contact with the cord nearby (-ve). The DiI was thus iontophoretically injected into the dorsal column of the cord for between 15 and 60 seconds (Figure 3.1; Birgbauer *et al.*, 1995). This was performed 3-4 times down the length of the lumbar enlargement. Cords were then transferred to a 4% PFA solution and stored at 37°C, which increases the uptake and transport along the axons (Godement *et al.*, 1987; Snider & Palavali, 1990), for 2-4 days.

The application of the tracer into the dorsal columns reduces the problem of diffusion out of the neurones and into the surrounding tissue. Because DiI moves by passive diffusion, it begins to leak through the axonal membrane and out of the cell. By placing the tracer in the dorsal columns, the distance it must travel, and therefore the amount of time it takes to the reach the fibre termination is greatly reduced. The technique therefore reduces the lateral diffusion associated with more distal injections of the tracer.

3.2.2 Preparation of sections

Cords were removed from the fixative solution and transferred to wells containing liquid low melting point agarose (3% in PB; Invitrogen, UK). The gel was then allowed to set, with the cords being held in an upright position. After the gel had set, it was removed from the well, glued onto a platform and placed in a Vibroslice chamber (Campden Instruments, UK) containing 0.1M PB. Sections were cut as thinly as possible (~70μm) and either immediately mounted on gelatinised slides, or transferred

to wells containing 0.1M PB for histochemical processing. Because DiI is soluble in glycerol, a normal mounting medium could not be used in these experiments. Instead, a small number of sections were placed on each slide and then coverslipped with PB before the sections were allowed to dry. As each slide was produced, it was immediately examined under fluorescent illumination (594 nm bandwidth). If sufficient labelling was apparent, the section was photographed using a Roper digital camera with MCID software or a Nikon analogue camera. In some cases, the sections were dehydrated with ethanol after photographing and were counter-stained with cresyl violet for 10 minutes. They were then re-coverslipped with DPX and photographed.

3.2.3 IB4 histochemistry

Those sections that were not immediately mounted were collected in wells containing 0.1M PB and processed for labelling of the non-peptidergic C fibres with the isolectin, IB4. Because DiI rapidly diffuses out of the axons after sections have been cut, and this is exacerbated by addition of detergents that perforate the membrane (such as Triton-X), a modified histochemical reaction was used, making use of the rapidity with which IB4 binds to its target. Here, sections were incubated in 0.1M PB containing IB4 conjugated to Fluorescein Isothiocyanate (IB4-FITC; 1:500) for 1 hour at room temperature. They were then washed 3 times (5 minutes per wash) in 0.1M PB, mounted on gelatinised slides and coverslipped with 0.1M PB. The sections were viewed and photographed immediately.

3.2.4 Measurement of the SG border

Those sections that were processed for IB4 histochemistry were used to calculate the distance from the dorsal aspect of the grey matter to the lamina II_o border. Images were captured on a Roper Digital Camera with MCID software and the ventral aspect of the IB4-positive region was measured to the surface of the grey matter (distance *x* in Figure 3.1) using the calibrated MCID software. In order to compare the different sizes of SG as a proportion of the total grey matter, the entire dorsoventral extent was also measured (distance *y* in Figure 3.1), and a percentage calculated.

3.2.5 Analysis of afferent terminations

Aβ fibres that displayed dorsally directed terminals were measured from the dorsal-most aspect of the grey matter using calibrated measuring software (distance *z* in Figure

3.1). Those fibres whose termination distance was smaller than that of the mean SG distance were considered to terminate in the superficial dorsal horn. The number of fibres entering SG was taken as a proportion of total dorsally-directed fibres from each section analysed.

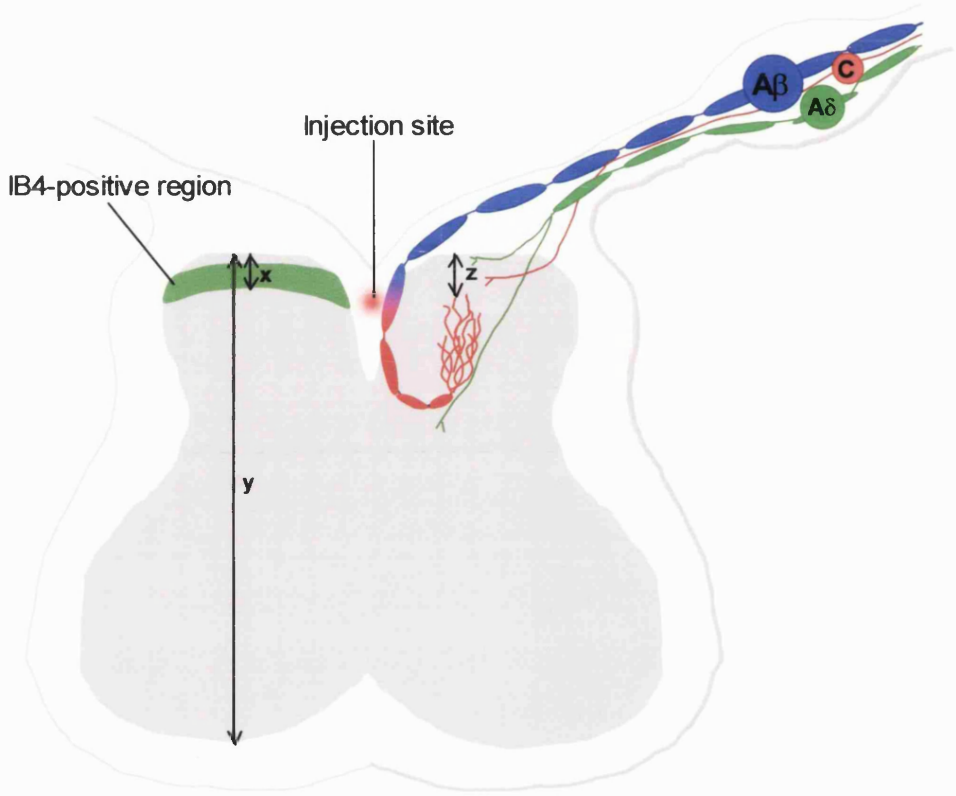

Figure 3.1
Diagram illustrating the location of the DiI injection. The microelectrode was situated slightly to one side of the dorsal midline and then lowered 100-300mm into the tissue, thus delivering a small quantity of DiI into the dorsal column where it selectively labelled the Aβ fibres. The distance from the surface of the grey matter to the dorsally-directed terminals of these fibres was measured (z). IB4 histochemistry revealed a band of labelling across lamina II_o (left-hand side) and the distance from its border to the surface of the grey matter (x) was taken as SG. This was compared with the total dorsoventral extent of the grey matter (y) at each age examined.

3.3 Results

3.3.1 Definition of the SG border

Of key importance to any study identifying the laminar organisation of primary afferent terminals is the definition of the laminar borders. While bright-field analysis can identify a clear translucent band corresponding to the substantia gelatinosa (SG) in the adult spinal cord, such a boundary cannot be seen in younger animals. Indeed, the absence of myelin in the neonatal cord makes it difficult to identify the grey matter at all (Figure 3.2A&B). While cresyl violet labelling did show translucent areas in the dorsal horn, this area in the neonate occupied a larger proportion of the dorsal horn, and therefore could not be used as a marker for the C fibre-innervating area (Figure 3.2C&D). To avoid this problem, we defined the ventral border of SG by the area occupied by IB4-positive C fibres (Figure 3.2E&F).

Sections of spinal cord, where Aβ fibres in the dorsal columns had been labelled with DiI, were incubated with IB4 and analysed using fluorescence microscopy. The distance from the dorsal aspect of the midline of the grey matter to the border of the IB4-positive area was taken as the border of SG (see Figure 3.1). Measurements at P0 (n=10 from 3 animals), P21 (n=8 sections from 3 animals) and P42 (n=10 from 3 animals) were then compared with the total dorsoventral extent of the grey matter (Table 3.1). The proportion of the total dorsoventral extent of the grey matter occupied by SG was significantly larger -in P0 than in either P21 or adult ($p<0.005$ for both, unpaired t test), but no difference was seen when comparing P21 and adult.

Age	Depth of SG (μm)	Total dorsoventral depth of grey matter (μm)	Proportion of total depth occupied by SG (%)
P0	100.3+5.6	828+8.6	12.09+0.64
P21	120.2±6.9	1447±21.8	8.35±0.37
P42	136.9±6.7	1609±17.8	8.82+0.47

Table 3.1
Comparison of SG boundaries in the rat at P0, P21 and P42, as defined by the extent of IB4 labelling.

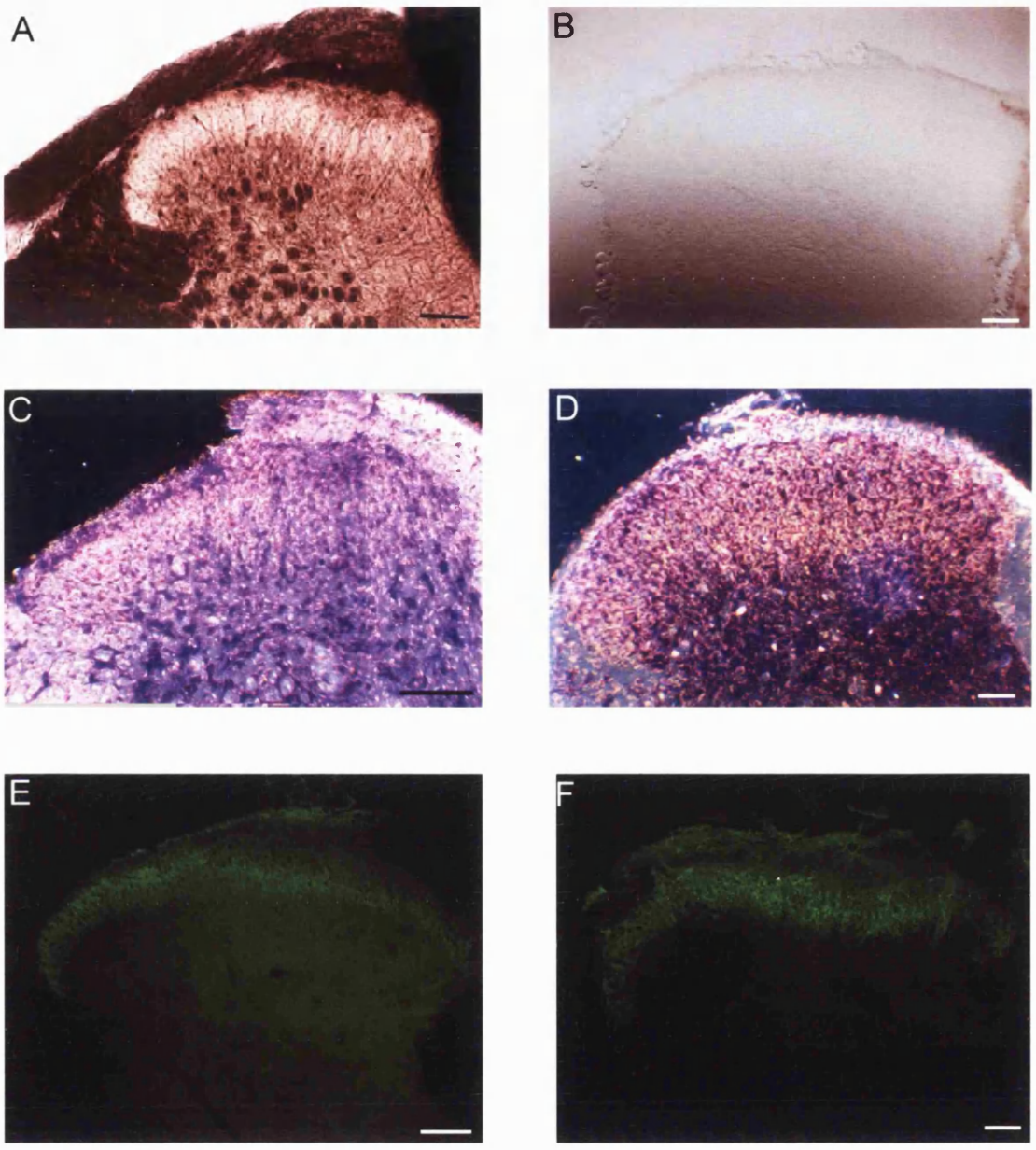

Figure 3.2
Methods of detecting the SG border in adult and neonatal rat sections. (A and B) show bright-field filtered microscopy of unprocessed tissue (both images were filtered in an identical fashion. While SG can be clearly be detected as a translucent band in the adult (A), no such band can be detected in the neonatal section (B). (C and D) Cresyl violet stain of nissl bodies. A lighter band caused by the densely packed cells of SG can be detected in the adult (C), but this band occupies a larger proportion of the dorsal horn in the neonate (D), and therefore does not correspond to the C fibre-innervating area. (E and F) IB4 labelling reveals a clear band corresponding to lamina II_i in the adult (E) and the neonate (F). The lectin appears to specifically bind to blood vessels in the neonatal section, which can thus be detected with fluorescent microscopy (F). Scale bars=100μm in A, C and E, and 50μm in B, D and F.

In the adult wild-type mouse, the border of SG, as defined by IB4 labelling, was located at 86.1±2.6μm ventral of the dorsal grey matter border (Figure 3.3A). For the mutant, this distance was 84.6±3.7μm, and was not significantly different from the wild-type (Figure 3.3B; $p>0.6$, unpaired t test). The total dorsolateral depth of the grey matter was 963±22.1μm for the wild-type and 988±17.9μm in the mutant. SG therefore occupies 8.96±0.3% of the total grey matter in the wild-type and 8.58±0.2% in the mutant.

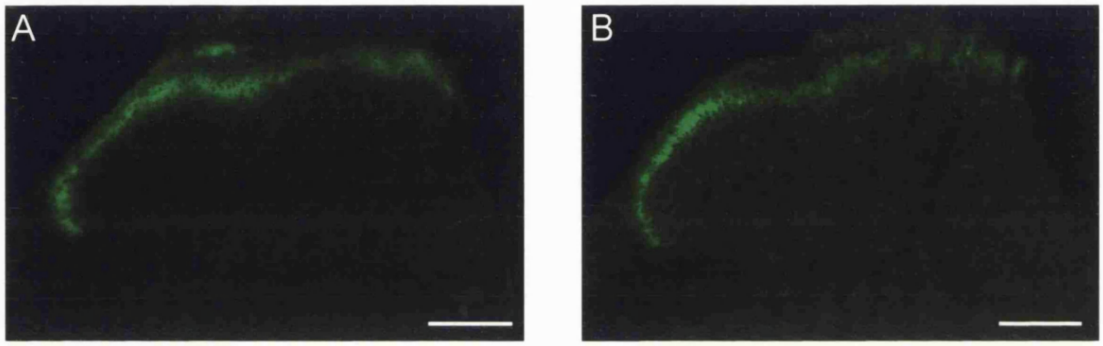

Figure 3.3
IB4 labelling in the wild-type (A) and mutant (B) mouse. In both groups, a clear band can be seen in lamina II_i. No significant differences were observed in the depth of the lamina II/III border. Scale bars=100μm.

3.3.2 Profile of Aβ fibre terminations in the P0 rat dorsal horn

At P0, A fibres can be seen arcing up into the most superficial regions of the rat spinal cord. Due to their unique pattern of entry into the cord, injection of DiI into the dorsal columns was able to selectively label the large Aβ fibres as they arced into the grey matter of the dorsal horn. These fibres gave rise to collaterals that coursed into the superficial dorsal horn. Many of these display the flame-shaped morphology described by Scheibel & Scheibel (1968) as being Aβ fibres (Figure 3.4A). In many cases, the arbour itself lies in the deeper laminae of the dorsal horn, but projects individual collaterals into the superficial laminae (Figure 3.4B and C). Again, this is in keeping with previous reports (Beggs, 2000). Of the 165 dorsally-directed collaterals counted from 38 spinal cord sections taken from 5 animals, the total proportion entering SG at P0 was 56.7±10.5%.

3.3.3 Profile of Aβ fibre terminations in the P21 rat dorsal horn

The total number of fibre terminals entering SG was less at P21 than at P0. Because of the greatly increased size of the cord, a larger quantity of tracer was injected into the dorsal columns (iontophoresis times were between 40 and 60 seconds). Once again, the A fibre could be seen coursing through the dorsal horn and projecting flame-shaped arbours superficially. These arbours generally terminated at the lamina II/III border (Figure 3.5A), although some could be seen entering lamina II (Figure 3.5B and C). In total, 114 collaterals were counted from 31 sections taken from 3 animals at this age, and the proportion seen entering SG was significantly less than at P0 (14.2±1.6%, $p<0.05$; Figure 3.6C).

3.3.4 Profile of Aβ fibre terminations of the P42 rat dorsal horn

In the P42 rat, the number of collaterals terminating in SG was even less than at younger ages. Of a total of 228 collaterals labelled from 59 sections from 4 animals, the proportion crossing the SG border was 5.17±0.84%. This is significantly less than seen at P0 and P21 ($p<0.01$ for both; Figure 3.6C). The majority of dorsally-directed fibres arced into the dorsal horn and terminated at the border of lamina II/III (Figure 3.6A). Those that crossed into SG displayed a fine, undivided morphology that sometimes reached the border of lamina II_o (Figure 3.6B)

3.3.5 Comparison of mutant and wild-type mice

The same procedure of DiI injection was performed on T286A mice and their wild-type littermates. For the wild-type group, a total of 198 collaterals were measured from 34 sections from 3 animals, and for the T286A group, 153 collaterals were measured from 29 sections from 3 animals. In both groups, Aβ fibre collaterals could be seen entering the cord medially before curving up into the dorsal horn of the grey matter. As with the adult rat, the majority of fibres terminated in laminae III and IV in the form of flame-shaped arbours (Figure 3.7A and 3.8B). In both cases, a small proportion of fibre collaterals could be seen terminating in the superficial dorsal horn (Figures 3.7B and 3.8A). The total proportion of fibres terminating in SG in the wild-type mouse was not significantly different from that of the adult rat (6.43±0.56%; $p=0.277$). In addition, there was no difference between the proportion of fibres terminating in SG in the wild-type and the mutant (6.67±1.7%; $p=0.9$).

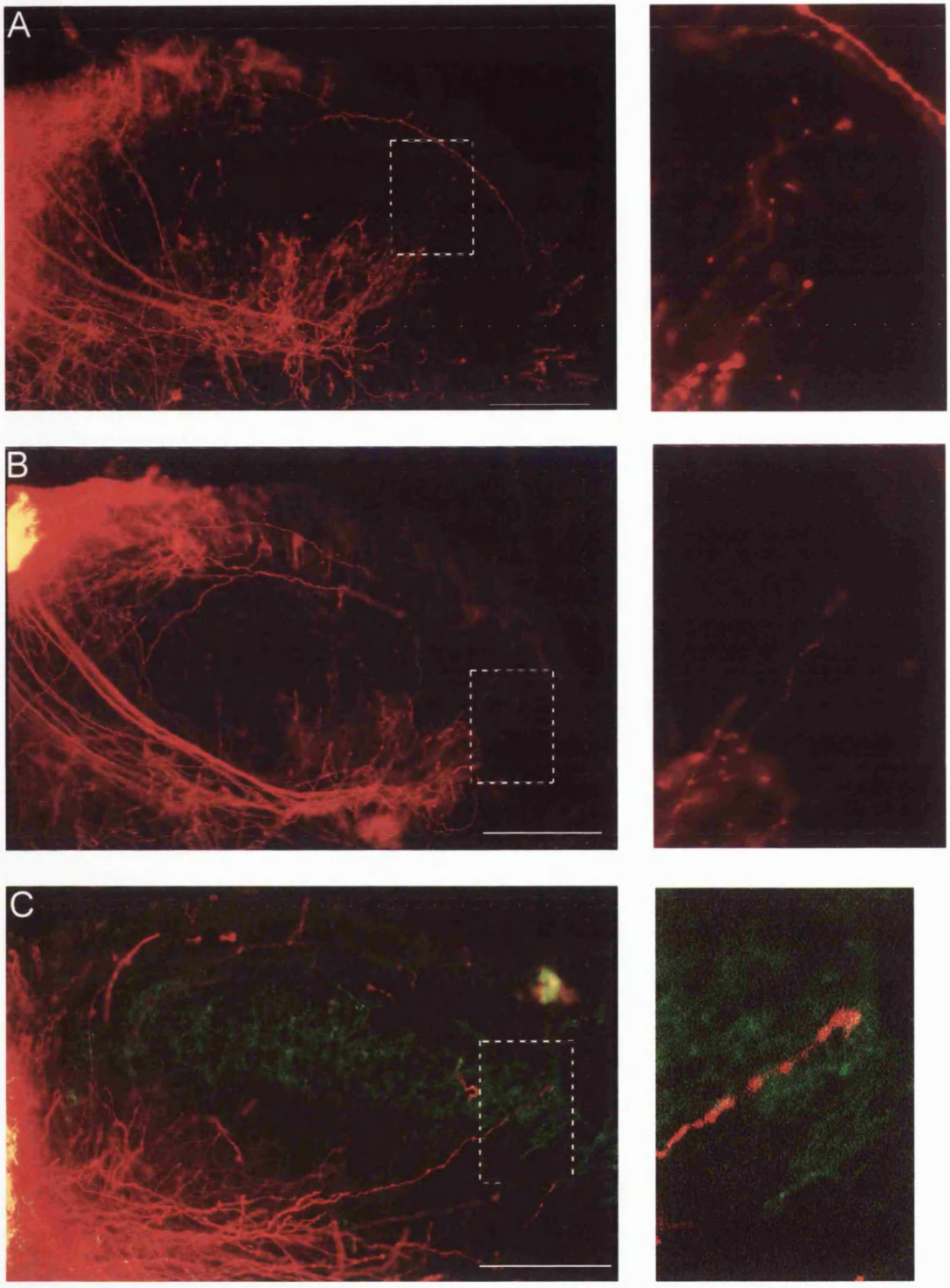

Figure 3.4
DiI-filled Aβ fibres in the P0 rat spinal cord. The injection sites can be seen as areas of bright fluorescence in the medial white matter of the dorsal columns, with large Aβ fibres arcing through the dorsal horn and sending aberrant collaterals into SG. Right-hand panels show higher magnification images of the boxed areas. (A) Example of a flame-shaped arbour terminating in SG. (B) Example of a single fibre projecting into SG from a flame-shaped arbour. (C) Double-labelled image of DiI-filled Aβ fibre collaterals entering SG using IB4 (green) as a marker for lamina II_i. Scale bars=100μm.

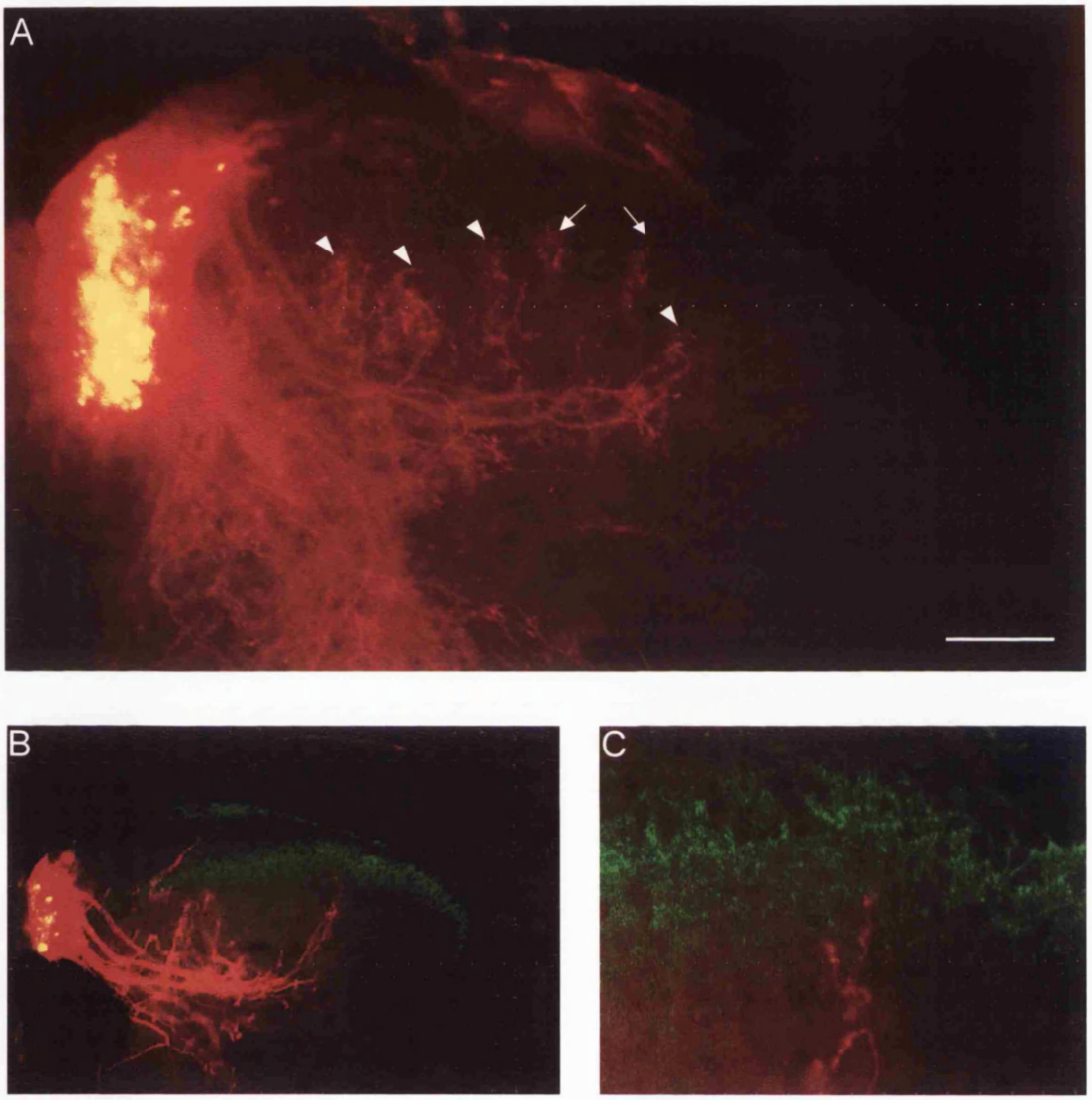

Figure 3.5
DiI-filled Aβ fibres in the P21 rat spinal cord. (A) shows the characteristic flame-shaped arbours projecting from the A fibre into either lamina III (arrowheads) or into lamina II (arrows). (B) Double-labelled section using IB4 (green) as a marker for lamina II_i. The DiI labelled A fibre projects an arbour into lamina II_i (C) Higher magnification image of the arbour seen in (B).

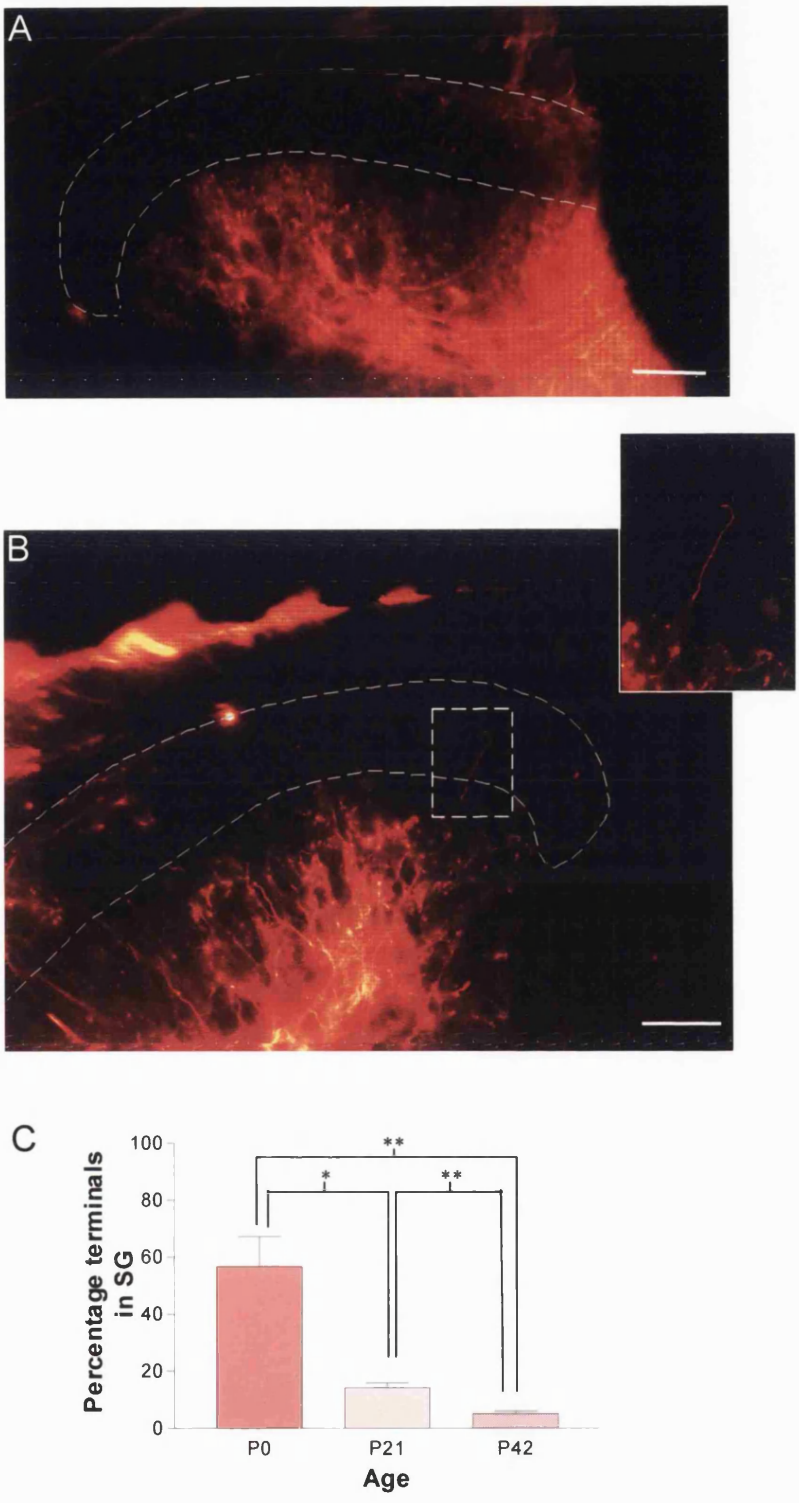

Figure 3.6
DiI-labelled Aβ fibres in the P42 rat spinal cord. Grey dotted lines indicate the boundaries of SG. (A) Example of flame-shaped arbours coursing through the dorsal horn and terminating at the lamina II/III border. (B) Example of a single fibre collateral terminating at the border of lamina II_i. The fibres on the medial side of SG are entering the grey matter, as opposed to entering from the deeper laminae. The inset shows a higher magnification image of the boxed area. (C) Graph showing the proportion of collaterals crossing the lamina II border at the different ages tested. All groups were significantly different from one another, implying an age-related decrease in the number of Aβ fibre collaterals that terminate in SG.

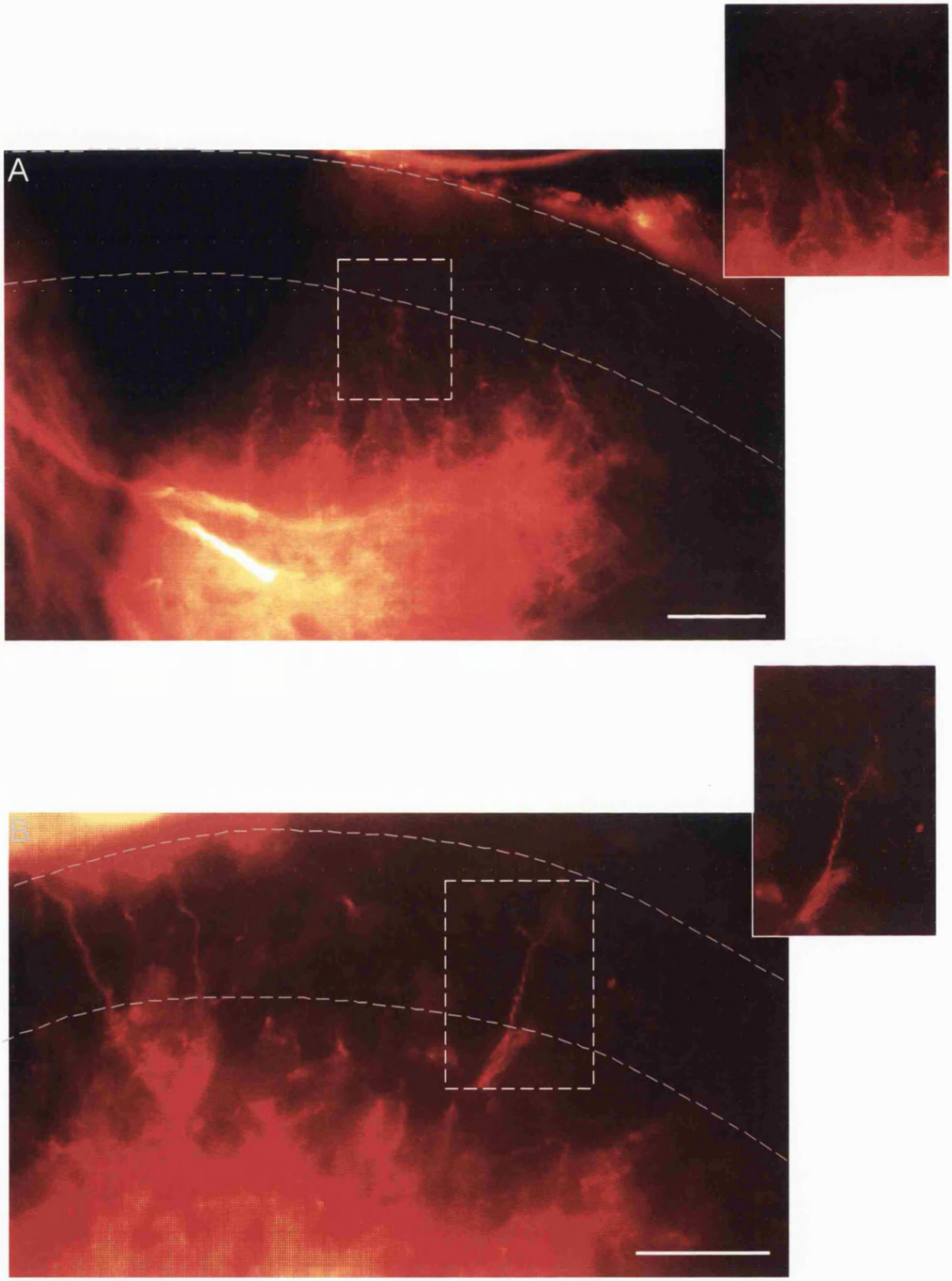

Figure 3.7
DiI-labelled Aβ fibres in the wild-type adult mouse. The grey dotted lines represent the boundaries of SG, as identified by bright-field analysis. Insets show enlarged images of the areas highlighted by white boxes. (A) Example of flame-shaped arbours projecting from the A fibres to the border of SG. (B) Example of a collateral crossing into the outer region of SG. Scale bars=100μm

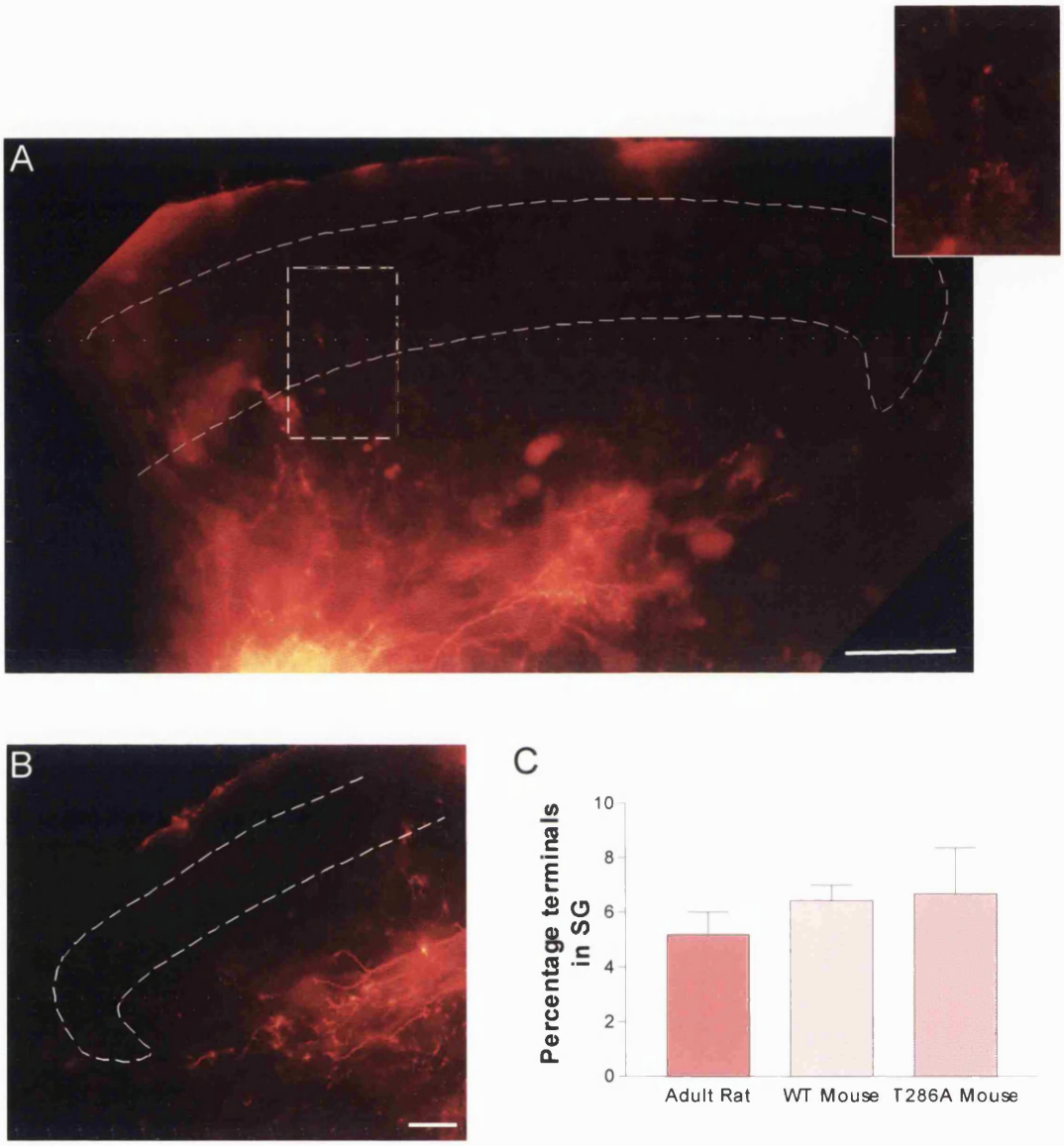

Figure 3.8
DiI-labelled Aβ fibres in the T286A mutant mouse. Grey dotted lines represent the boundaries of SG, as identified by bright-field analysis. (A) Example of an Aβ fibre collateral crossing the SG border and terminating in lamina II_o. The inset shows a high-magnification view of the fibre, as highlighted by the white box. (B) Example of flame-shaped arbours curving up to, but not crossing, the border of SG. (C) Graph comparing the proportions of total fibres crossing SG with the adult rat, and the mutant and wild-type mouse. There was no significant difference between groups ($p>0.05$; $n=3$). Scale bars = 100μm

3.4 Discussion

This chapter has shown that Aβ fibres send collaterals into the superficial dorsal horn in neonatal rats, and that these fibres then withdraw into the deeper laminae during the first few weeks of postnatal life. Secondly, it has been shown that the T286A mutation of the CaMKII gene does not affect this withdrawal, as both wild-type and mutant adult mice show a similar Aβ fibre termination profile.

3.4.1 Technical considerations

It was discovered early in this study that both the experimental protocols and the analysis of the data presented significant problems due to the instability of the tracer, the diversity of the labelling and the developmental alterations in laminar boundaries. This is the first time that DiI has been used to label Aβ fibres during development and I was therefore required to adapt protocols from a number of sources in order to achieve satisfactory results.

3.4.1.1 Treatment of the dye

While DiI has been used as a tracer of afferent fibres for many years, it has not before been used to selectively label Aβ fibres in the spinal cord, and technical problems associated with tracer are rarely reported. The majority of studies use insertion of DiI crystals into target areas of the brain (Godement *et al.*, 1987; Terashima *et al.*, 1995; Ikeda *et al.*, 2003) in order to trace axons originating in these areas, but we found that this method caused lateral diffusion into Lissauer's tract and subsequent labelling of C fibres, particularly in the younger animals. We therefore used iontophoretic injection of an ethanol solution that was saturated with DiI (Birgbauer *et al.*, 1995), as this allowed for highly accurate placement of very small quantities of the dye.

DiI is transported by passive diffusion, due to its two long fatty acyl chains, that can become inserted into the lipid fraction of the plasma membrane (Klausner & Wolf, 1980). This allows for transport even in fixed tissue, but also means that it can leak through the axonal membrane and out of the cell if left too long. The placement of the tracer in the dorsal columns, as opposed to in the dorsal roots, reduces the distance it must travel and the time it takes to reach the afferent terminals. The process can also be accelerated by keeping the tissue at 37°C, which allows the tracer to move at approximately 400μm per day (Godement *et al.*, 1987). In the young animals,

therefore, we found that leaving the cord for two days allowed for complete labelling of the primary afferent termination, while in the adult, we allowed up to five days for transport.

Once the fibres had been labelled, the methods with which to cut and view the sections were also problematical, with early attempts resulting in diffuse labelling of the cord in general rather than clear tracing of the A fibres. This was caused by a number of factors: (1) DiI appears to dissolve in glycerol, meaning that the section could not be mounted in the usual fluorescent mounting mediums (such as citifluor); (2) it severely degrades if the tissue is allowed to dry; (3) it dissolves in ethanol if the tissue is dehydrated; (4) it rapidly leaks from the cell membrane if the tissue is frozen and cut on a microtome. We therefore refined the tissue processing by cutting the sections on a Vibroslice and mounting and photographing immediately. Although extremely time-consuming, this method allowed for accurate analysis of the collateral terminals in the dorsal horn.

3.4.1.2 Analysis of the primary afferent terminations

Two points should be noted concerning our method of analysis in this study. Firstly, only the dorsally-directed afferents in the dorsal horn were selected for measurement. At all ages, a large population of collateral fibres can be seen entering the cord medially and descending into laminae IV and V. While the majority of these may well originate from Aβ fibres, they were not analysed. Therefore, the percentage of fibres crossing into SG will be a higher value compared to total number of Aβ fibre collaterals entering the cord. Secondly, because in many cases the DiI labelled large numbers of collaterals (particularly in the P0 group), an accurate count of the terminals was extremely difficult. Therefore, whole arbours were counted as single terminations, even though they may have arisen from more than one collateral, causing an artificial increase in the proportion of collaterals terminating in SG. While the study can therefore not be used as an exact count of the number of SG-terminating fibres as a proportion of total Aβ fibre numbers, comparisons between ages and genotypes can still be accurately be made.

3.4.1.3 Lamination

Of key importance to the termination pattern of Aβ fibres in the dorsal horn is how the laminae are identified. The definition of the substantia gelatinosa, in particular, needs to be clearly established with regard to its cellular make-up and primary afferent input in order to compare the properties of young and old animals. Historically, the SG was so called because of its gelatinous appearance, which can be seen with the naked eye (Figure 3.2A). The reason for such an appearance is the dense concentration of small cells, as well as an absence of myelinated axons in this regions (Willis & Coggeshall, 1991). However, physiological studies revealed that there was no clear boundary between lamina II and III implying that, although differences could be seen in the cytoarchitecture, the functional significance of this was limited (Wall, 1967). In addition, previous studies have demonstrated that SG, as defined by the cytoarchitecture of the laminae, appear to show the region occupying the majority of the dorsal horn in the neonate (Woodbury *et al.*, 2000). Therefore, it appears that a more useful definition of the lamina II/III boundary could be made through the properties of the afferent input. Small unmyelinated C fibres are believed to terminate exclusively in laminae I and II of the spinal cord, and so antibodies that selectively label this group of afferent can provide a useful marker for these laminae. As previously stated, the C fibre population can be subdivided by their peptide content, and these two populations terminate in different regions of the SG. Therefore, by selectively labelling the IB4-positive group of C fibres, we were able to identify lamina II_i, and define a clear boundary between this and lamina III.

In accordance with previous studies (Woodbury *et al.*, 2000), we have shown that SG, as characterised by its cytoarchitecture, is much larger in the neonate, in which it occupies the majority of the dorsal horn, than the adult. In addition, we have shown that IB4-positive C fibres also occupy a larger proportion of the grey matter when compared to older animals. This finding has been reported previously in a study that used labelling of fluoride-resistant acid phosphatase (FRAP) to study the same population of C fibres as examined here (Coimbra *et al.*, 1986). The authors suggested that the larger area of input was due to the fact that the C fibres invaded the cord before the dorsal horn neurones had matured, and before the A fibres had entered, so that an adult-like profile was achieved while the rest of the cord was immature.

3.4.2 Developmental profile of Aβ fibre terminations in the dorsal horn

In accordance with previous findings (Fitzgerald *et al.*, 1994; Coggeshall *et al.*, 1996; Beggs *et al.*, 2002), we have described a developmental alteration in the anatomical profile of primary afferent input into the dorsal horn. At birth, a large proportion of the flame-shaped arbours, as first described by Brown (1981) as originating from Aβ fibres, can be seen terminating in SG. In the older animal, the superficial terminations are greatly reduced. In contrast to previous studies in the cat (Light & Perl, 1979) and the rat (Woolf, 1987; Shortland *et al.*, 1989) we rarely saw the distal parts of the flame-shaped arbours extending into lamina II_i, but instead saw termination at the very border of lamina III. A small proportion of fibre collaterals were seen entering the SG in both the rat and mouse, although it was unclear whether or not they originated from flame-shaped arbours.

The A fibre collaterals that terminate in the dorsal horn as flame-shaped arbours were first detected by Ramón y Cajal (1909) in the neonatal cat using the Golgi staining technique. The technique allows for a small, random sample of fibres to be labelled. As he described,

'These collaterals give rise to an abundant arborisation with varicose, highly flexuous branches that stretch throughout the depth of SG and establish intimate contacts with the neurones of this region' (Ramón y Cajal, 1909).

The morphology of these fibres suggested that they comprised the major proportion of collaterals labelled with B-HRP, and were therefore thought to occupy SG only in the neonate, before retracting to the deeper laminae during postnatal development (Fitzgerald *et al.*, 1994; Beggs *et al.*, 2002). Further studies by Coggeshall *et al.* (1996), confirmed these fibres to be myelinated A fibres, when electron microscopy was used to detect BHRP-labelled fibres making synaptic contacts with superficial dorsal horn neurones. In addition, it was demonstrated that stimulation at A fibre intensities produced activation of c fos- an immediate early gene that is used as a marker for neuronal activity- in the superficial dorsal horn of neonatal, but not adult, rats implying that these A fibres were capable of causing neuronal firing (Jennings & Fitzgerald, 1996). These data support the theory that large myelinated fibre collaterals are present in SG in the neonate, and that they subsequently withdraw during development.

The majority of the studies performed on this system have employed the bulk-labelling of fibres with BHRP, and recently some doubt has been cast over its accuracy due to a number of experiments that question the selectivity of the toxin to large myelinated axons. Specifically, a number of groups have recently claimed that BHRP can be taken up by C fibres after peripheral axotomy (Bao *et al.*, 2002; Jancso *et al.*, 2002; Santha & Jancso, 2003; Shehab *et al.*, 2003). Therefore, it is possible that a similar phenomenon occurs during development, whereby neonatal C fibres are capable of taking up the cholera toxin, and lose this ability during development. This seems very unlikely, however, as the neonatal studies did analyse the DRGs at all ages examined, and found no alteration in size distribution of the labelled cells during development, implying that the toxin was selectively labelling the myelinated fibres (Fitzgerald *et al.*, 1994).

While the technique described here does not allow for functional classification of the afferents, recent work suggests that they are not the rapidly-adapting hair follicle afferents that they were originally assumed to be. Using an *ex vivo* setup, (Woodbury *et al.*, 2001) have developed a method of analysing the electrophysiological properties of individual primary afferent fibres, before filling them with neurobiotin in order to examine their central termination pattern. Such an approach has revealed an apparent lack of rapidly adapting hair follicle afferent inputs in the superficial dorsal horn at any age tested. This subset of mechanoreceptors was defined as those which show narrow uninflected somal action potentials. The study is the first of its kind to both describe physiologically and anatomically the profile of myelinated fibres from the skin to the dorsal horn, and raises questions as to whether those fibres that form aberrant connections in SG are low-threshold mechanoreceptors.

While it is possible that the authors simply missed the collaterals that projected into the superficial dorsal horn (only 19 afferents were adequately labelled, while the DiI technique allows for labelling of many hundred), it is more likely that those that form aberrant connections originate from an alternative source. These could include Pacinian corpuscle afferents, mechanoreceptors from glaborous skin or slowly-adapting mechanoreceptive afferents.

3.4.3 The origin of collaterals that do not withdraw

A small proportion of fibres appeared not to withdraw into the deeper laminae during development in neither rats nor mice. These have not been previously noted in bulk-

labelling studies, perhaps implying that they are not sensitive to the cholera toxin. A possible identity of such collaterals has recently been suggested by Woodbury & Koerber (2003), again by using the *ex vivo* preparation described above. In this study, the authors examined the properties of high-threshold mechanoreceptors (HTMRs), which displayed characteristics resembling both Aβ and Aδ fibres. The group was found to comprise approximately 30% of incipient skin sensory neurones in the neonate. Their somal spike shape, for example, was narrow implying an Aβ-like profile, but was inflected, in a manner usually associated with Aδ fibres. In addition the mechanical thresholds were lower than is usually seen with Aδ fibres and, although on average higher than Aβ fibres, there was substantial cross-over between the LTMRs and this population of HTMRs. Finally, the conduction velocities of this group showed distinct overlaps with those from LTMRs. These properties are similar to those first described by Fitzgerald (1987) as being high-threshold pressure receptors.

When filled with neurobiotin, this group of HTMRs displayed termination patterns that are associated with Aβ fibres. The central axons ascended and descended in the dorsal columns of the cord, and projected collaterals into the grey matter that terminated in the superficial laminae of the dorsal horn (Woodbury & Koerber, 2003).

While this study primarily examined the presence of HTMRs in the neonate, the authors found that such fibres were still found in the P21 rat. They proposed that the group would not be seen in BHRP studies, as they are very diffuse in nature and would subsequently not be picked up by the relatively crude bulk-labelling technique (Woodbury & Koerber, 2003). The presence of a group of fibres that pass through the dorsal columns and send collaterals into the superficial dorsal horn would explain the results reported here. As the authors reported that these HTMRs account for 30% of sensory neurones derived from the skin, some withdrawal must occur during development, as we saw only ~6% of collaterals terminating in SG.

In conclusion, we have shown that Aβ fibre collaterals can be selectively labelled by injection of tracers into the dorsal columns of the spinal cord. These collaterals take the form of the classically described flame-shaped arbours and can be seen penetrating the superficial dorsal horn in the neonate, but not in the adult. This would imply that some form of withdrawal occurs during postnatal development. A small population of

collaterals can be seen terminating in the SG of the adult, which we hypothesise as being a population of HTMRs that display properties previously associated with Aβ fibres.

A future means of studying the central characteristics of mechanoreceptors has come from a recent study that has found a calcium channel that is specifically expressed in a subtype of large myelinated Aβ fibre (Shin *et al.*, 2003). The study used a combination of mutagenesis, gene expression analysis using gene chips and whole-cell patch clamp recordings to isolate a gene encoding a T-type calcium channel ($Ca_v3.2$) that is expressed in DRG cells of D-hair receptor neurones only. This method of finding genes that are specifically expressed in subsets of mechanoreceptors could be combined with GFP tagging in order to label distinct populations of primary afferent. Such a technique could help elucidate the precise profile of mechanoreceptors in the spinal cord during development.

3.4.4 Comparison of mutant and wild-type

In order to ascertain whether the Aβ termination profile in the dorsal horn was affected by CaMKII autophosphorylation, we performed DiI tracing on adult T286A mice and their wild-type littermates. With regard to morphology of the collaterals, the termination pattern in the wild-types was the same as that seen in the rat- flame-shaped arbours can be seen arcing through the dorsal horn to terminate in lamina III/IV with only a small proportion of fibres remaining in SG. This would imply close homology between the species, as has been previously described (Woodbury *et al.*, 2000).

In addition, we performed IB4 labelling of non-peptidergic C fibres on adult spinal cord sections. No difference was seen between the adult wild-type mice and the adult rat with regard to the proportion of total grey matter occupied by SG, suggesting a similar profile of C fibre terminations between species. Additionally, the depth of IB4 labelling was not different between genotypes. This is in keeping with the findings of Beggs *et al.* (2002), who showed that, while A fibre reorganisation was compromised in the MK801-treated animals, C fibre input, at least with regard to IB4 and CGRP labelling, appeared normal. Therefore, IB4 labelling in the T286A mouse revealed a normal termination pattern of C fibres, in accordance with the blockade of NMDA receptors, but DiI labelling also showed a normal pattern of Aβ fibre terminations. Why, then,

would blockade of NMDA receptors during development produce such a clear phenotype, whilst disrupting the action of one its most important downstream effectors not have any effect?

The hypothesis originally proposed by Beggs *et al.* (2002), suggested that the activity-dependent withdrawal of A fibres from SG was due to competition for synaptic sites between A and C fibres, which the C fibres eventually win. In keeping with such an idea, the timeframe for which silent synapses are present in the dorsal horn matches that for the critical period of A fibre withdrawal (Li & Zhuo, 1998; Bardoni R *et al.*, 1998). Therefore, it is possible that, as the C fibres grow into the cord, they form synapses with postsynaptic dorsal horn neurones, which are then made functionally by unmasking of previously silent synapses. This in turn could lead to a heterosynaptic LTD at the A fibre synapses, causing these fibres to retract into the deeper laminae. Preventing the plasticity by chronic blockade of the NMDA receptor would therefore prevent the synaptic, and subsequently structural, alterations to take place.

Certainly, evidence supporting changes in structural organisation of primary afferents in response to alterations in postsynaptic excitability is substantial (Colman *et al.*, 1997; Cramer & Sur, 1995; Fox *et al.*, 1996; Li *et al.*, 1994). However, there are some important differences between those systems described in the cortex and hippocampus and those in the spinal cord. Firstly, the LTP/LTD phenomenon seems to be strictly selective to a particular group of afferents, so that the C fibre synapses potentiate, while those from A fibres depress. In the visual cortex, by contrast, the primary afferent input is essentially from one cell type (with respect to activation properties), so that postnatal reorganisation is dependent on the activity of one afferent, and on the inactivity of a neighbouring cell (Constantine-Paton *et al.*, 1990; Cramer & Sur, 1995). Secondly, while in the auditory, barrel and visual cortices the most active inputs become strengthened while the inactive ones become weakened, a very different pattern is seen in the cord. It has recently been demonstrated that normal development of nociceptive function is not dependent on nociceptive activity (Waldenstrom *et al.*, 2003). Blockade of nociception using local analgeisic creams during the critical period of sensory development did not affect the development of refined nociceptive behaviour, but blockade of tactile activity with local anaesthetics did. This implies a system in which the A fibres are more functionally important during development (as would be

expected), and can act to cause synaptic maturation of C fibre input. The authors proposed a mechanism whereby spontaneous muscle twitching produces high levels of activity in certain cells, which in turn produces synaptic depression at the dorsal horn interneurone, while those that produce low-level activity in response to spontaneous motor action are strengthened (Waldenstrom *et al.*, 2003). An alternative mechanism, however, could involve the convergent dorsal horn interneurone being depolarised by the highly active A fibre, and thus allowing potentiation of the C fibre input through unmasking of silent synapses. Blockade of A fibre activity, therefore, would affect the C fibre plasticity, producing the deficits in the nociceptive processing.

Assuming that the structural alterations in the cord are due to changes on a synaptic level, and particularly to the unmasking of silent synapses, one would indeed suspect a role for CaMKII- a protein that plays a key role in AMPA receptor insertion into the postsynaptic membrane (see Chapter One). However, findings in other parts of the brain imply a subtler role for the kinase during developmental plasticity. In the barrel cortex, for example, CaMKII does not appear to affect normal development of input to the barrels under normal conditions (Glazewski *et al.*, 2000; Glazewski *et al.*, 2001; Hardingham *et al.*, 2003). Similarly, CaMKII knock-out mice show normal synaptic development in the visual cortex (Gordon *et al.*, 1996). However, after an intervention such as whisker removal or blockade of an eye, the mutant mice show severe deficits in normal synaptic plasticity (Gordon *et al.*, 1996; Glazewski *et al.*, 2000; Glazewski *et al.*, 2001). This implies that mutating the CaMKII gene is not sufficient to eradicate plasticity in the cortex, and that other mechanisms must exist to allow for normal development to occur.

The lack of normal developmental deficits in this, or any other sensory model, could come from recent evidence suggesting that CaMKII is not the major downstream kinase involved in juvenile plasticity. (Yasuda *et al.*, 2003) have demonstrated that in young (P7-8) rats, the CaMKII inhibitor KN-93 does not block hippocampal LTP induction, although it does in the adult. Furthermore, they showed that LTP could be blocked by the PKA inhibitor, PKI, although this compound had no effect on adult plasticity. A second study also found that hippocampal LTP was not blocked by the CaMKII inhibitor, KN-62 in young (P12-16) rats (Wikstrom *et al.*, 2003). Here, however, they found that PKA inhibitors alone could not prevent induction either (a discrepancy they

put down to differences in LTP induction protocols). Inhibition of both kinases, or inhibition of CaMKII and PKC, however, produced complete blockade of LTP induction. They therefore proposed a mechanism by which two parallel cascades operated in young animals, one which involved CaMKII and the other involving PKA and PKC, so that blockade of single pathway was not sufficient to prevent LTP induction. These findings are interesting in phylogenetic terms- activity-dependent plasticity is vital for normal survival, and so for it to be dependent on a single kinase would be dangerous. By having at least two separate systems in place while the nervous system reorganises would allow for normal development even if one of the kinases are disrupted.

While such findings do seem somewhat distant from structural reorganisation of primary afferent input to the spinal cord, hippocampal LTP has been shown to closely resemble that seen in the cord, and if such mechanisms of plasticity are important for developmental alterations, then these alternate downstream pathways might explain the absence of gross anatomical deficits seen in the T286A mutant.

The study may also help explain the report by Malmberg *et al.* (1997), in which the other main calcium-dependent kinase known to be involved in plasticity in the cord- PKCγ- was knocked out. Behaviourally at least, these animals showed no developmental alterations in sensory plasticity. This could be due to the parallel cascades described above, whereby the CaMKII pathway would have remained intact. A double mutation of CaMKII and PKC would perhaps produce more profound developmental deficits. In addition, the parallel cascade theory would explain why Beggs *et al.* (2002) saw such deficits- blockade of the NMDA receptor would prevent the rapid influx of Ca^{2+} ions into the cell, thereby preventing activation of any of the downstream kinases, and thus preventing normal developmental plasticity.

If activity-dependent synapse *elimination* is occurring in the dorsal horn, as the work by Waldenstrom *et al.* (2003) suggests, then perhaps PKC is a more likely candidate for mediating these effects. Activation of the kinase at the neuromuscular junction (NMJ) produces a decrease in synaptic strength (Li *et al.*, 2001b), and blockade *in vivo* causes a loss in synapse elimination during the first two thirds of the 2-3 week period during which this process occurs (Lanuza *et al.*, 2002). Moreover, genetic deletion of the PKCθ gene causes a delay in the activity-dependent reduction of synaptic strength *in*

vitro and a delaying of the refinement from polyneuronal to mononeuronal neuromuscular synapses *in vivo*. Were a similar process to occur in the dorsal horn, whereby Aβ fibre terminals in the superficial dorsal horn are eliminated in an activity-dependent manner, then the process would presumably be diminished if the kinase activation was prevented by NMDA receptor blockade. Such an occurrence could explain a CaMKII-independent, NMDA receptor-dependent elimination of Aβ fibres from the superficial dorsal horn.

A final reason for the apparently normal somatosensory development of these animals could be that there has been developmental compensation by other downstream effectors, such as the MAP kinases. Perhaps more likely would be a compensatory action of an alternative CaMKII isoform, in particular the β isoform. It has been shown to be present in the superficial dorsal horn (Terashima *et al.*, 1994), and can be autophosphorylated in a manner similar to the a isoform (although at a different site). Therefore, a loss of function of one isoform could well result in an increase in function of a different one. As the mutation is performed in the blastocyst, so that the kinase is unable to autophosphorylate from conception, it is possible that other proteins would rectify any problems in converting the calcium signal. This is contrary to the ELVAX study, in which NMDA receptors were not blocked until after birth (Beggs *et al.*, 2002). It does seem somewhat unlikely that such a phenomenon would occur, primarily because the kinase is actually present in the mutant (see Chapter Two), and can be seen in the same locations as the wild-type (Giese *et al.*, 1998; Taha *et al.*, 2002), but just cannot function normally. However, some form of compensatory mechanism cannot be ruled out.

In conclusion, we have shown here that the CaMKII T286A mutation does not affect the gross structural changes that occur in the primary afferent input of the dorsal horn during development. This is in contrast to findings in rats that have received chronic spinal NMDA receptor blockade during development, and might suggest that afferent alterations occur via a CaMKII autophosphorylation-independent mechanism. Whether any changes occur at the synaptic level, however, cannot be elucidated using this technique. In the following chapter, therefore, we examine the dorsal horn physiology of these mice.

Chapter 4

PHYSIOLOGICAL PROFILE OF THE T286A DORSAL HORN

4.1 Introduction

In order to test whether the synaptic circuitry of the superficial dorsal horn is altered in the T286A mouse, we performed *in vitro* and *in vivo* electrophysiological recordings of dorsal horn cells. While tracing studies can give morphological information about afferent input to spinal cord laminae, they cannot be used to predict functional synaptic input to specific cell groups. For example, a proportion of laminae II cells project dendrites into deeper laminae, and thus can receive monosynaptic connections from afferents terminating in these areas (Woolf & Fitzgerald, 1983; Willis & Coggeshall, 1991; Park *et al.*, 1999; Grudt & Perl, 2002). Conversely, cells whose somata lie in lamina IV have been shown to project dendrites to the superficial dorsal horn (Miller & Woolf, 1996; Todd, 1989).

Thus, while the profile of Aβ fibre terminations in the dorsal horn is normal in the T286A mutant, alterations in dorsal horn synaptic organisation and postsynaptic dendritic growth may still have occurred. Chronic NMDA receptor blockade in the developing spinal cord did not only alter Aβ fibre terminal fields, but also changed the physiology of the spinal cord, leading to an increase in the number of spikes evoked by A fibre-intensity stimulation and an increase in receptive field size compared to vehicle-treated controls (Beggs *et al.*, 2002). Such enhanced afferent-evoked activity in dorsal horn neurones could occur independently of anatomical changes in afferent terminal fields, such as through reduced inhibitory controls. Such controls could be affected by the T286A mutation, even if Aβ fibre withdrawal is not.

Studies in the somatosensory and visual cortices of T286A mutant mice support this notion. While gross anatomical alterations do not appear to occur during postnatal development, the physiological profile of second order neurones display severe deficits. For example, disruptions in the visual cortex plasticity by the T286A mutation are observed in the supragranular layers (II-III) of the cortex, as opposed to the primary afferent-receiving layer IV cells (Taha *et al.*, 2002).

Beyond its role in activity-dependent synaptic plasticity, CaMKII has been shown to play roles in dendritic growth and synaptic elimination during postnatal development. In cortical neuronal cultures, the kinase has been implicated in causing active synaptic elimination from some presynaptic neighbouring cells, while increasing the number of

synaptic connections with others (Pratt *et al.*, 2003). This highly selective process could well occur in the cord as the intrinsic interneurones extend their axodendritic branches during the first few postnatal days, producing disruptions in the normal synaptic development.

To test whether such changes in synaptic physiology do indeed occur in the dorsal horn, we have used whole-cell patch clamp recordings of lamina II cells in spinal cord slices and *in vivo* extracellular recording of dorsal horn cells in intact spinal cord in wild-type and mutant mice to examine the effects of the mutation on synaptic responses to afferent stimulation. Intracellular recordings of spinal cord slices can be used to elucidate synaptic circuitry activated from both the primary afferents and intrinsic interneurones (Yoshimura & Jessell, 1989; Baba *et al.*, 1999; Li & Zhuo, 1998; Miller & Woolf, 1996; Bardoni R *et al.*, 1998). The use of transverse slices allows for stimulation of primary afferents through the dorsal root while recording the response properties of the postsynaptic neurone. Laminae can be easily identified in this preparation as a band of translucent grey matter, thus allowing for cellular recording of spatially identified neurones. Previous studies in rats have employed this technique to elucidate the primary afferent input to SG neurones in immature (Park *et al.*, 1999; Nakatsuka *et al.*, 2000) and inflamed (Baba *et al.*, 1999) animals. EPSC activity can be observed in response to evoked Aβ, Aδ and C fibre stimulation of the dorsal root and mono- and polysynaptic pathways computed from stimulus threshold, latency and frequency-following characteristics.

To complement the *in vitro* preparation, where natural skin stimulation is not possible, and where all descending input to the dorsal horn, as well as a large proportion of interneurones which project their dendritic arbours in a rostrocaudal direction, is destroyed, we have also recorded single dorsal horn spike activity *in vivo* in anaesthetised mice. In this preparation, cells can be recorded responding to innocuous brushing and noxious pinching, in addition to electrical stimulation of the skin, and receptive fields can be mapped. The use of both *in vivo* and *in vitro* recording techniques allows for a cross-disciplinary analysis of the sensory phenotype of the T286A mutant.

4.2 Methods

4.2.1 Whole-cell patch clamp recordings of dorsal horn cells *in vitro*

4.2.1.1 Preparation of spinal cord slices

Mice aged P17-22 were randomly selected from litters produced from heterozygous parents. They were anaesthetised with halothane (5% in 100% medical oxygen), decapitated and the spinal columns quickly removed. The tips of the tails were also removed for genotyping (see Chapter Three), meaning that all experiments were performed blind. Spinal columns were placed in an ice-cold dissection solution consisting of (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 6 $MgCl_2$, 0.5 $CaCl_2$, 25 glucose and continuously bubbled with 95 % O_2-5 % CO_2. A ventral laminectomy was performed and the cord removed with the dorsal roots still attached. After trimming the ventral roots and removing the dura mater, the cord was placed in low melting point agarose (3% in above dissection solution; GibcoBRL, Paisley, UK). After allowing to cool, the block was glued to the chamber of a Vibraslice tissue slicer (HA-752; Campden Instruments, Leicester, UK) with the caudal end of the cord facing downwards. This allowed for the blade to be positioned directly above the rostral end of the dorsal root entry zone and a 500-600μm transverse slice cut that included the attached dorsal root (which was approximately 5mm in length). The slices were transferred to a chamber filled with oxygenated dissection solution and allowed to recover for 1.5-2 hours at room temperature.

4.2.1.2 Patch clamp recordings of SG neurones

Following recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments) and mounted on the stage of an upright microscope (Zeiss Axioskop 2, Welwyn Garden City, UK). The slices were continually perfused at room temperature with oxygenated artificial cerebrospinal fluid (aCSF) solution containing 125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.0mM NaH_2PO_4, 1.0mM $MgCl_2$, 2.0mM $CaCl_2$, 25 mM glucose, at a rate of 1-3 $ml.min^{-1}$.

Patch electrodes were constructed from thin-walled single-filamented borosilicate glass (1.5mm outside diameter; World Precision Instruments, Hertfordshire, UK) using a two-stage vertical microelectrode puller (PP-830; Narishige, London, UK). Pipette resistances ranged from 5 to 7 MΩ. As only voltage clamp recordings were performed, electrodes were filled with a solution containing 130mM caesium gluconate (which

reduces the flow of K^+ ions through leak channels, thus increasing R_m and reducing noise), 10mM CsCl, 10mM Hepes, 11mM EGTA, 1.0mM $CaCl_2$, 2.0mM MgATP. Dorsal horn neurones were visualized with differential interference contrast (DIC) and whole-cell patch-clamp recordings were obtained using a Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA). EPSCs were recorded from a holding potential of -70 mV, thus minimizing the contribution of $GABA_A$- and glycine-mediated currents (Yoshimura & Nishi, 1993). Currents were filtered at 4-6 kHz through a -3 dB, 4-pole low-pass Bessell filter, then digitally sampled at 20 kHz and stored on a personal computer (Viglen, Middlesex, UK) using a commercially available data acquisition system (Digidata 1322A with pCLAMP8.0 software, Axon Instruments). This level of filtering allowed for elimination of the majority of high-frequency noise without greatly affecting the time course of the signal. Orthodromic stimulation of the dorsal root was performed with a suction electrode using a constant-current stimulator (Neurolog). The suction electrode was constructed in a manner similar to the patch electrodes, except that the pulling was interrupted after the first heat cycle and the capillary removed. It was then cut with a diamond-tipped glass cutter so as to form an hour-glass shape.

4.2.2 Compound action potential recordings

Because of the short length of dorsal roots in P21 mice, compound action potential recordings could not be obtained from the roots that were attached to the spinal cord slice. Therefore, separate experiments were performed to obtain thresholds and conduction velocities of fibres in the dorsal root. Here, the sciatic nerve, DRG and dorsal root were removed from the mice and placed in the recording chamber but not perfused (so as reduce noise). Suction electrodes were placed on each end of the nerve and root. Compound action potentials were evoked by stimulation of the nerve with a constant-current stimulator (Neurolog), set to stimulate at a fixed duration (either 50 or 500μs). Low-intensity stimulation was performed at 50μs from 0-500μA increasing in 20μA increments. High-intensity stimulation was at 500μs from 100μA-1mA in 100μA increments.

4.2.3 *In vivo* extracellular recordings

This work was performed in collaboration with Ms Ragnhildur Karadottir, Dr Carole Torsney and Prof Maria Fitzgerald. T286A mutant and wild-type adult (>6 weeks) mice were used in these experiments. All recordings were performed blind of genotype.

Mice were anaesthetised with 2.5g kg^{-1} intraperitoneal urethane (Sigma). This dose of urethane is sufficient to produce deep anaesthesia for the course of the experiment (up to 6 hours). Once the mice were areflexic, a tracheotomy was performed using a tapered plastic cannula (Argyle Medicut) and the animal placed in a small animal Kopf stereotaxic frame, with its head and pelvis firmly held with ear bars and hip bars. ECG electrodes were attached to each forepaw, and the heart rate monitored throughout the experiment as an indication of the health of the animal (normal heart rate: 540-660 beats min^{-1}). Rectal temperature was maintained with a heated blanket at 36-37°C.

The lumbar cord was exposed by laminectomy, the dura mater (and arachnoid) removed, and the surface of the cord bathed in mineral oil (Sigma) to prevent drying. A small clamp was positioned rostral to the laminectomy to stabilise the cord. Finally, the left hindlimb was supported with a suture through the Achilles tendon in order to reduce the movement artefact when applying brush and pinch stimuli to the hindfoot.

Extracellular recordings were made from cells in the dorsal horn of the L4-5 lumbar cord using glass-covered platinum-coated tungsten microelectrodes (tip diameter, 10μm). The electrodes were attached to a Neurolog (Digitimer, UK) headstage mounted on a microdrive (New Brain- Digitimer). The recording electrode was lowered onto the surface of the dorsal horn and then stepped down through the grey matter in 2μm or 10μm steps. The reference electrode was placed into muscle adjacent to the laminectomy. The depth of recorded cells from the surface was measured with the microdrive. Spike recordings were amplified 2000-fold in an AC preamplifier, and filtered (upper limit, 10kHz and the lower, 2kHz with an additional 50Hz notch filter) and visualised on a digital storage oscilloscope (Gould-4041). The signal was then passed through the spike trigger (NL200) and on to a MacLab/4s analogue/digital interface (AD Instruments).

Single cells with receptive fields on the hindpaw were mapped using natural mechanical stimuli (light brush, touch and pinch). Receptive field areas were drawn onto a template of the hindpaw and measured using a digital drawing pad (Wacom, Japan) and QWin Leica software. Areas are given in arbitrary units only for comparison between groups. Mechanical thresholds were determined by applying von Frey hairs to the centre of the receptive field. The mechanical threshold was defined as the lowest von Frey hair required to evoke spike activity. The magnitude of response to suprathreshold (three

von Frey hairs above threshold) mechanical stimulation applied for approximately 1 second was also assessed.

Electrical stimulation of the skin was applied through subcutaneous pin electrodes in the centre of the receptive field at stimulus intensities of 100μA-10mA, 100-500μs. Electrical stimuli were generated by Neurolog modules: pulse buffer (NL510), delay width (NL402) and stimulus isolator (NL800). A fibre threshold was defined as the minimum electrical stimulus (50μs, 100μA-1mA) required to produce a short-latency response from the dorsal horn cell. The latency of response to A fibre stimulation was defined as the latency to the first spike after a single stimulus at twice the threshold level. Evoked A fibre response amplitude was measured in the 70ms period following stimulation. All cells were also tested at higher stimulus intensities (500μs, 1-10mA) to test for longer-latency C fibre input evoked at a latency of 100-200ms.

4.2.4 Statistical analysis

Patch-clamp data were analysed with pClamp software (Clampfit, Axon Instruments) and Mini Analysis (v.5.4.1; Synaptosoft, Inc., Decatur, GA, USA) and GraphPad Prism (GraphPad Software, CA, USA). *In vivo* analysis was performed on MacLab (AD Instruments) and GraphPad Prism. Unless otherwise stated, independent two-tailed t-tests (when only two data sets were analysed) or ANOVAs (when more than two data sets were examined) were used to test for levels of significant difference between groups. Non-parametric tests were performed if the variances were significantly different between groups. Types of input and convergence were analysed using the Chi-square test of association. All graphical and stastical analysis of mechanical thresholds was performed by transforming the data into log(mg), so as to linearise the logarhythim scale of the Von Frey hairs. However, these data were expressed in the text as grams, in accordance with the related literature. Data are expressed as mean ± SEM.

4.3 Results

4.3.1 Membrane and synaptic properties of lamina II neurones

Whole-cell patch-clamp recordings were made in spinal cord slices from a total of 19 cells from wild-type mice, 28 cells from heterozygous mice and 24 cells from mutant mice (n=9 animals from each group). Mean input resistances of lamina II cells were 947±180MΩ for wild-type, 1116±297MΩ for heterozygote and 1076±104MΩ for mutant animals, suggesting that similar sized cells were patched in all three groups. Spontaneous EPSC frequencies were 1.5+0.3Hz for wild-type (n=14 cells), 1.8+0.4Hz for heterozygote (n=7) and 2.7+0.8 for mutant (n=14; $p>0.2$). Spontaneous IPSC frequencies were 0.7+0.4Hz for wild-type (n=5), 1.8+0.6Hz for heterozygote (n=11) and 0.9+0.3Hz for mutant (n=10). No significant differences were observed between any genotype, implying that passive and active membrane properties were similar between groups.

4.3.2 Primary afferent threshold and conduction velocity

Primary afferent input could be divided into three groups, Aβ, Aδ and C fibre, according to the electrical stimulation thresholds and conduction velocities of their compound action potentials. Figure 4.1A shows an example of a trace obtained by stimulating the isolated sciatic nerve of a wild-type mouse and recording from the dorsal root using suction electrodes. Low intensity, low duration (50μs) electrical simulation produced a fast, Aβ fibre-mediated wave that was joined by a slower Aδ-fibre mediated deflection as stimulation intensity was increased (Figure 4.1A, left panel). Stimulating for a longer duration (500μs) produces a C fibre-mediated wave, as identified by its long latency and duration (Figure 4.1A, right panel). Thresholds and conduction velocities were not different between wild-type, heterozygous or mutant animals (Figure 4.1B). However, there was a significant difference between the thresholds and conduction velocities of the different waves ($p>0.05$ between each group). It was therefore possible to obtain threshold values that could be used to stimulate distinct populations of fibres when recording from lamina II cells in spinal cord slices. These were 200μA (50μs pulse) for Aβ fibres, 300μA for Aδ fibres (50μs pulse) and 400μA (500μs pulse) for C fibres.

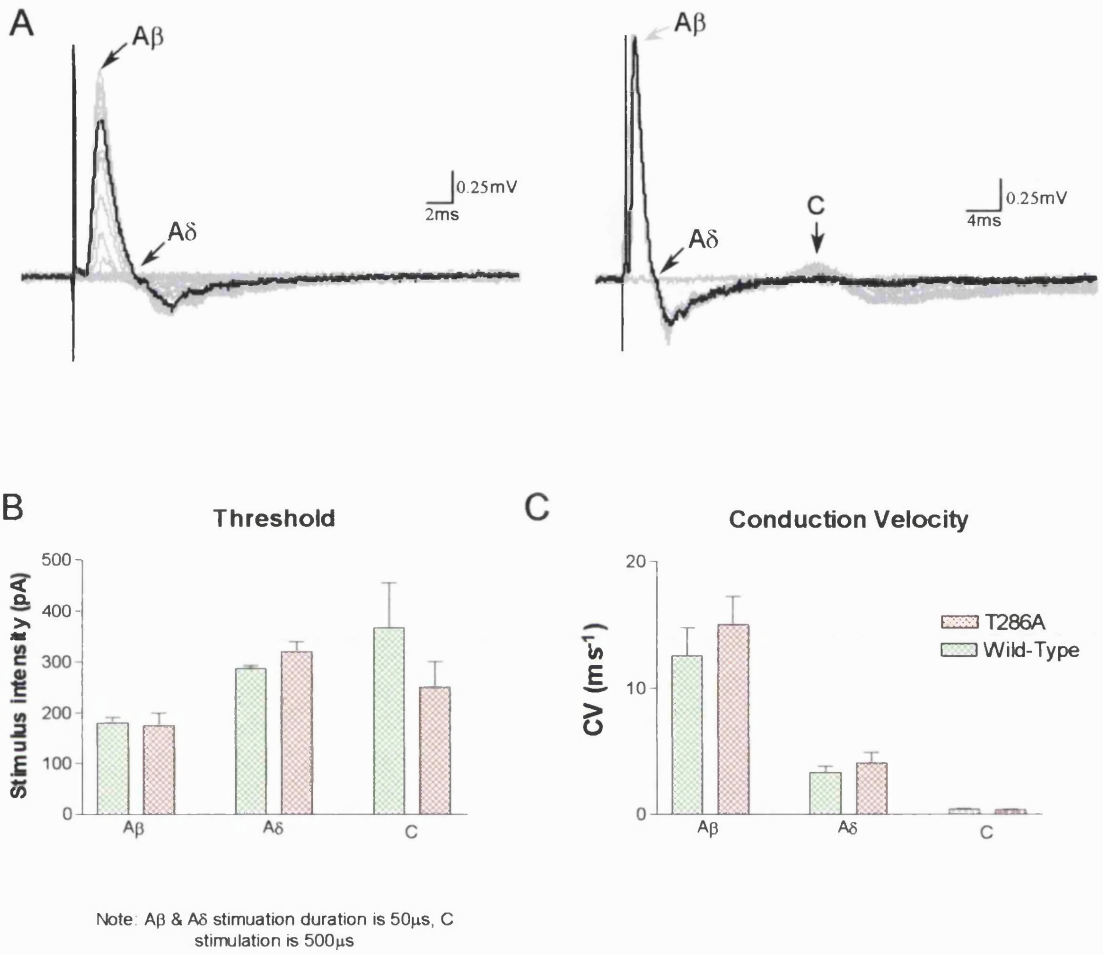

Figure 4.1
Compound action potentials in the wild-type and mutant sciatic nerve (A) Examples of extracellularly recorded compound action potentials evoked at graded stimulus intensities (*left,* 20-400μA, 50μs; *right,* 100-1000μA, 500μs). *Left*, low duration stimulation produces a fast wave which displays a slower, Aδ fibre-mediated deflection as the intensity increases (demonstrated by red line). *Right*, High duration stimulation again recruits the A fibres, but a slower C fibre-mediated wave can be recruited with increased stimulus intensity. The red line demonstrates an intensity at which the A wave has peaked but the C wave is not yet visible, allowing for differentiation between the groups in the dorsal horn preparation (see results). (B) Comparison of thresholds for Aβ, Aδ and C fibre activation between genotypes. While there is no significant difference between intensities for each genotype, there is a significant difference between fibre groups ($p<0.05$). (C) Comparison of conduction velocities shows no difference between genotypes, although significant differences are seen between the fibre groups ($p<0.05$).

4.3.3 Synaptic responses in SG neurones

Whole-cell patch clamp recordings were made from 19 lamina II neurones in slices with dorsal root attached prepared from wild-type mice (n=8 animals), 28 neurones from heterogeneous mice (n=9) and 24 neurones from T286A mutant mice (n=8). Of these, 14 from wild-type, 15from heterozygous and 14 from mutant mice responded to orthodromic dorsal root stimulation. Cells were classified in terms of their afferent input, and whether that input was monosynaptic or polysynaptic. The type of input was calculated by the cells' response to stimulation at thresholds known to activate Aβ, Aδ or C fibres (see above), and by conduction velocity of the fibre volley, as derived from the latency of the response. Classification of mono- or polysynaptic input involved analysis of response latency and of response to repetitive stimulation, as reported previously (Yoshimura & Jessell, 1989; Baba *et al.*, 1999; Nakatsuka *et al.*, 2000). Identification of A fibre-mediated EPSCs as monosynaptic was based on a constant latency and absence of failures at 10Hz. For C fibre input, those that could follow repetitive stimulation of 1Hz were classified as being monosynaptic in nature, in accordance with previous studies (Nakatsuka *et al.*, 2000). Those that did not follow repetitive stimulation were classified as being polysynaptic (Figure 4.3B).

Approximately half the cells in lamina II of all three genotypes responded to stimulation at A fibre (both Aβ and Aδ) intensity (57% of wild-type, 52% of heterozygous and 50% of mutant). In the wild-type and heterogeneous animals, this input was entirely polysynaptic in nature (Figure 4.2A). In the mutant, however, one cell was observed that received a monosynaptic Aδ fibre input (Figure 4.2B). The A fibre polysynaptic input could be divided into those activated by Aβ fibre stimulation and those that required a higher intensity stimulation to evoke synaptic activity that was, therefore, probably due to Aδ fibre activation. Those with Aβ polysynaptic input may also have recruited Aδ inputs as the stimulus intensity was increased, but these currents would have been masked by the low intensity stimulation-evoked EPSCs. In the wild-type and heterozygous animals, the input was predominantly mediated by Aβ fibres (43% and 40% respectively; Figure 4.2C), whereas in the mutant, the majority of cells appeared to require Aδ intensity stimulation to be activated (7% responding to Aβ stimulation compared to 37% responding to Aδ; $p<0.01$ χ^2 test; Table 4.1). Therefore, the majority of mutant lamina II neurones have no Aβ fibre-meditated polysynaptic input. While it

appears that the mutant shows increased Aδ polysynaptic-only input (Table 4.1), we propose that this is more likely to be caused by the reduced Aβ fibre-evoked responses allowing for visualisation of the previously masked Aδ fibre-evoked activity. However, such a hypothesis cannot be validated using the present selection criteria.

	Aβ poly	Aδ mono	Aδ poly *only*
+/+ (n=14)	6 (43%)	0 (0%)	1 (7%)
+/- (n=15)	6 (40%)	0 (0%)	1 (7%)
-/- (n=14)	1 (7%)	1 (7%)	5 (36%)

Table 4.1 Classification of synaptic responses

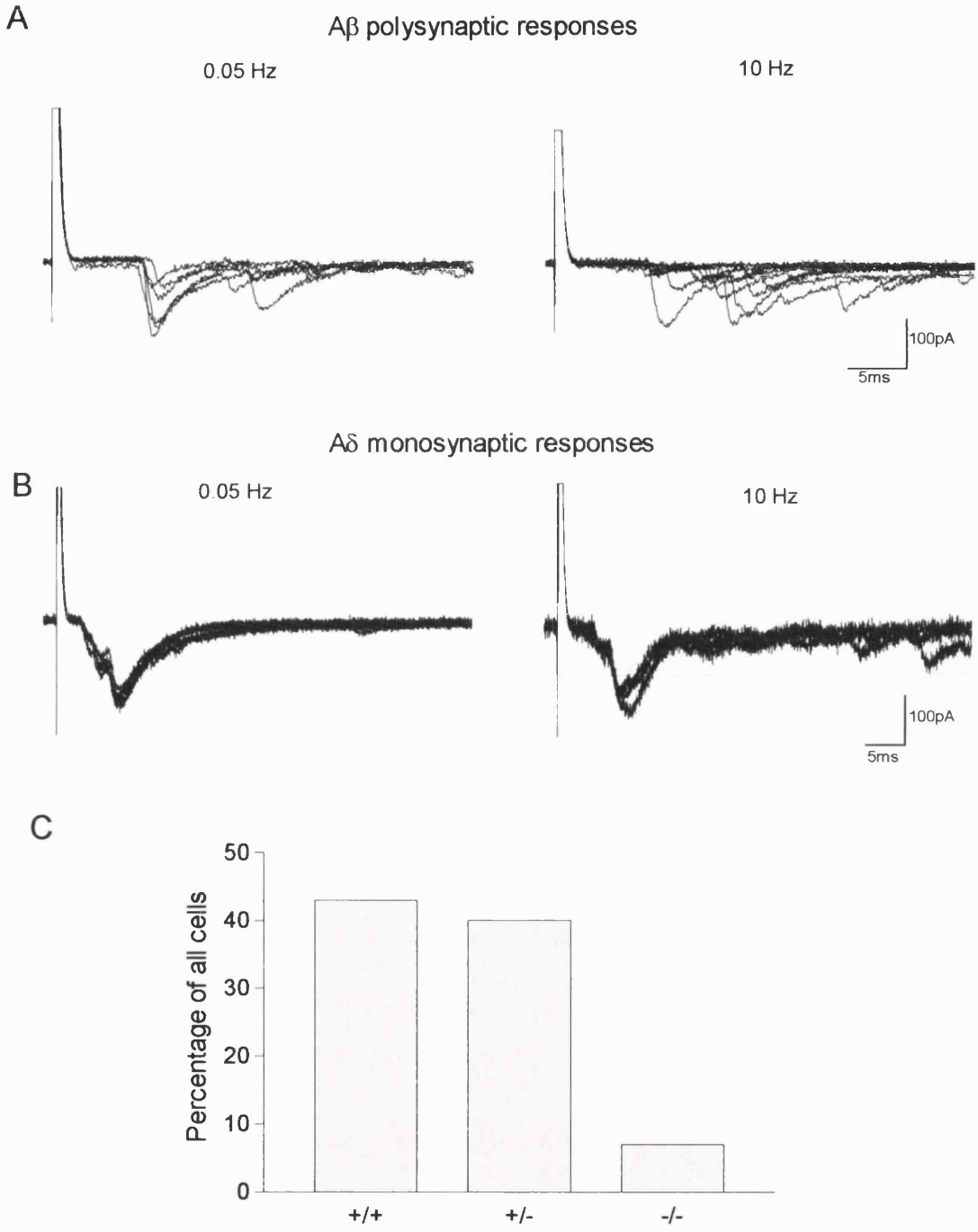

Figure 4.2
A fibre-mediated input to the dorsal horn. (A) Example of a wild-type cell displaying polysynaptic Aβ-fibre mediated input, as defined by its low threshold (200μA), variable latency EPSC to low frequency stimulation (*left panel*) and its inability follow 10Hz repetitive stimulation without failing (*right panel*). (B) One T286A cell displayed a monosynaptic Aδ fibre-mediated input, as defined by its high threshold (400uA), fixed latency EPSC (*left panel*) that followed repetitive stimulation at 10Hz (*right panel*). (C) Aβ polysynaptic input to lamina II is reduced in the mutant compared to the wild-type and heterozygote ($p<0.01$).

With regard to C fibre input, high intensity, high duration stimulation produced responses in approximately half of the neurones in all genotypes (58%, 53% and 64% in wild-type, heterozygote and mutant respectively). Many cells responded to both A and C fibre activity, and therefore a means of distinguishing one from the other was required. While on some occasions the C fibre component could be observed as a slow, long latency wave that was only seen with longer duration stimulation (Figure 4.3A), more often the C fibre-meditated current was incorporated in the A fibre-mediated EPSC (Figure 4.4A and B). Because the amplitude of A fibre-mediated compound action potentials reached a peak before any C fibre wave could be observed at longer duration stimulation (Figure 4.1A, right-hand panel), we assumed that any increase in size or decay time of the EPSC was caused by recruitment of C fibres that also synapsed on the neurone (Figure 4.4B). The proportion of neurones that received monosynaptic C fibre input was also similar between genotypes (29%, 20% and 21% for wild-type, heterozygote and mutant respectively; Figure 4.3C; $p>0.05$), as were the amplitudes of these EPSCs (351.3±254pA, 104.2±32pA and 147.1±8pA respectively; n=3 for each group; Figure 4.3D). The wild-type group displayed one cell that had an amplitude of 1118±17pA, thus greatly adjusting the mean amplitude for this group. However, there was no significant difference in mean amplitudes in any genotype ($p>0.05$).

Figure 4.4 shows the proportion of cells that had both A and C fibre input, as compared to those that just had one or the other. As demonstrated in Figure 4.4B, moving to a longer duration of stimulation resulted in some cells recruiting a slower wave, suggesting a C fibre input. It is unlikely that this slower wave resulted from the broader stimulus recruiting more A fibres as the compound action potential recordings showed the A fibre-mediated wave reaching a plateau before the C fibre-mediated wave became apparent (Figure 4.1, right panel). In all groups, the majority of cells displayed either A or C fibre input only, and a smaller proportion displayed both A and C fibre input (21%, 25% and 14% for wild-type, heterozygote and mutant respectively; $p>0.05$ χ^2 test; Figure 4.4).

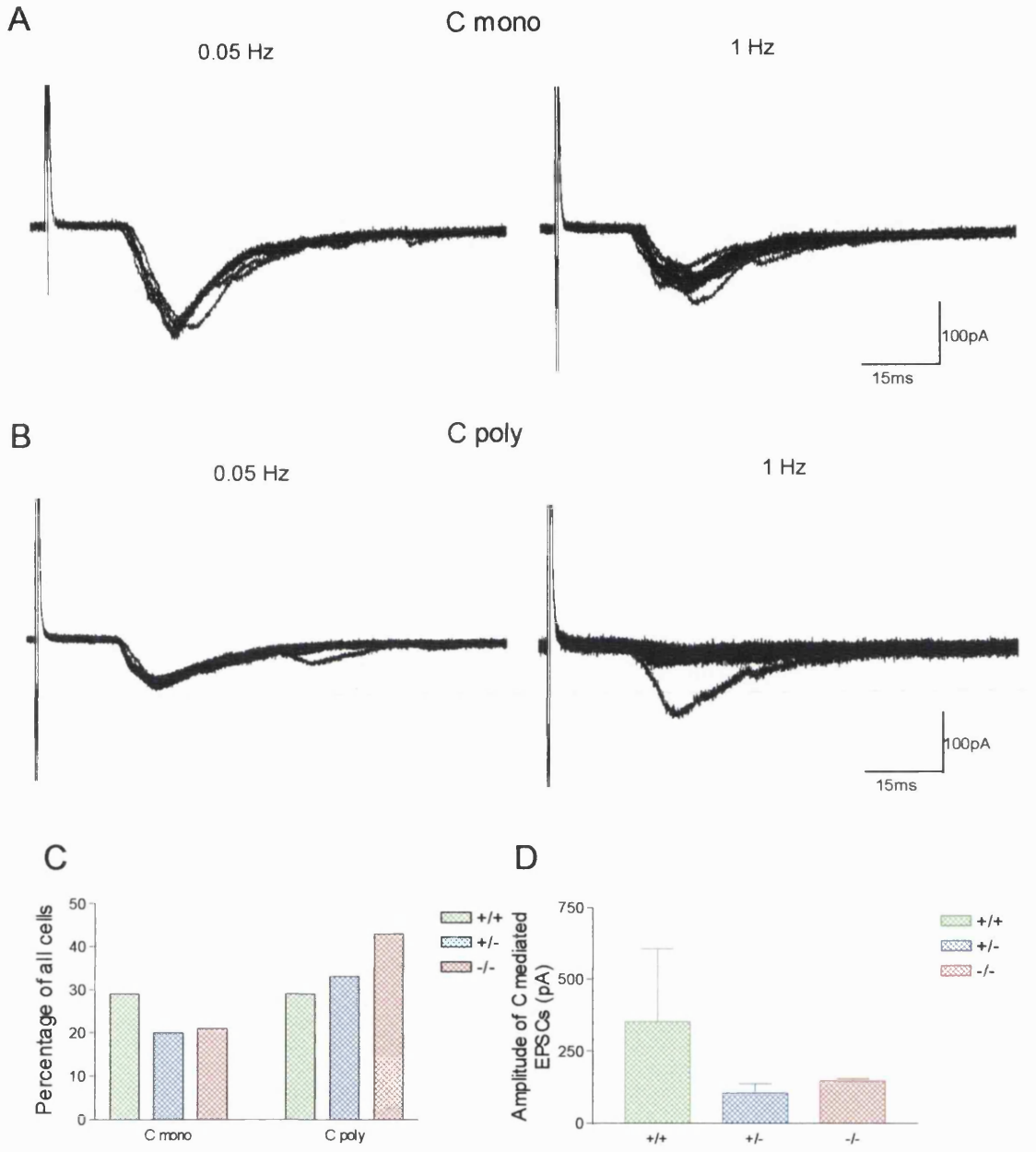

Figure 4.3
C fibre-mediated input in the dorsal horn. (A) Example of a cell displaying monosynaptic input. At low frequency stimulation, the cell displays an EPSC with a fixed latency and relatively fixed amplitude (*left panel)*. At repetitive 1Hz frequencies, the EPSC follows with a relatively fixed latency, although the amplitude is reduced, suggesting a polysynaptic component that did not follow the train *(right panel*). (B) Cell showing polysynaptic C fibre-mediated activity. While low frequency stimulation elicits a response similar to that seen in (A) (*left panel*), stimulation at 1Hz produces failures in the postsynaptic cell (*right panel*). (C) Comparison of C fibre-mediated input between genotypes shows similar proportions of both mono- and polysynaptic input between the groups (n=14,15 and 14 for wild-type, heterozygote and mutant respectively). (D) Mean amplitudes of monosynaptic C fibre-mediated EPSCs show no significant differences between the genotypes (n=3 for each group).

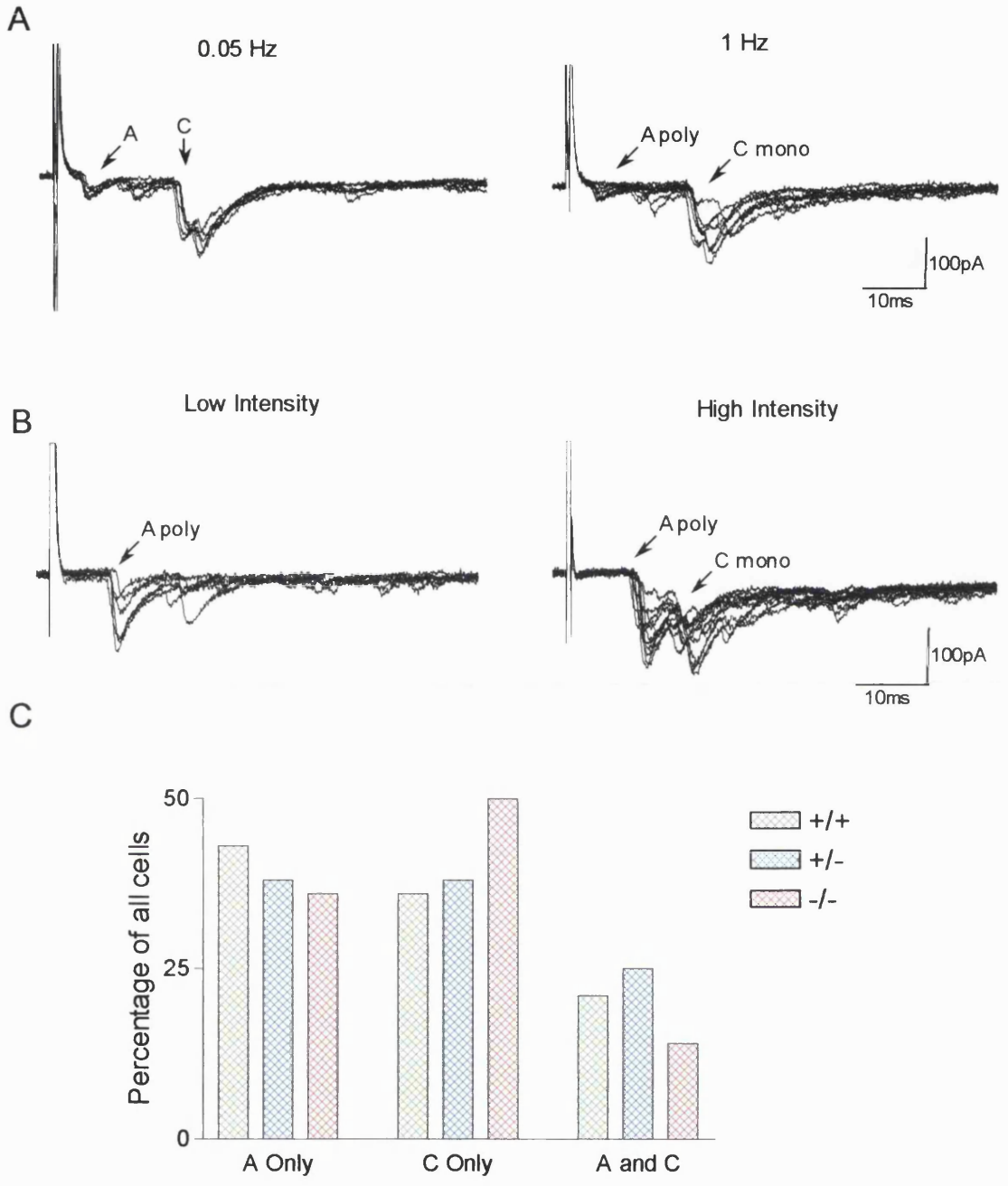

Figure 4.4
Convergence of A and C fibre-mediated input to the dorsal horn. (A) shows a cell displaying two clear waves at high intensity stimulation, the first being mediated by an A fibre input, the second from C fibre input *(left panel)*. During repetitive stimulation at 1Hz, the early wave begins to show failures implying a polysynaptic input, while the C wave follows the stimulation, implying a monosynaptic input *(right panel)*. (B), low intensity stimulation produces a fast wave with variable latency, implying polysynaptic A fibre input *(left panel)*. At high duration stimulation, the wave shows a more complex pattern and a slower decay time, implying the recruitment of C fibres. (C) Comparison of convergence of input between genotypes. No significant difference was observed between the groups.

4.3.4 Electrophysiological profile of dorsal horn cells *in vivo*

A total of 89 cells were recorded extracellularly from the L4/5 dorsal horn of anaesthetised intact mice, 46 of which were from mutant mice (n=7) and 43 from wild-type mice (n=8). The dorsal horn cells were analysed with respect to their responses to both natural and electrical stimulation.

4.3.4.1 Responses to natural stimulation

Cutaneous receptive fields were mapped as the area of the hindpaw skin that produced action potential spiking in response to either noxious pinching or innocuous brushing. We were unable to differentiate between noxious and innocuous receptive fields due to the fact that noxious pinching would also have activated non-noxious fibres. Noxious stimulation with radiant heat would have allowed us to perform such a comparison, but we did not have the necessary equipment. Only cells that had a receptive field on the paw itself were studied, and the data were stratified between the toe and the ankle of the paw, to ensure equal distributions between genotypes. In the wild-type, mean receptive field sizes were 2215±223 units (n=29), while in the mutant, the sizes were significantly larger (3593±615 units; n=16; $p<0.05$, unpaired t test with correction for unequal vairances; Figure 4.5). This corresponds to a 54±22% increase in the mutant compared to the wild-type.

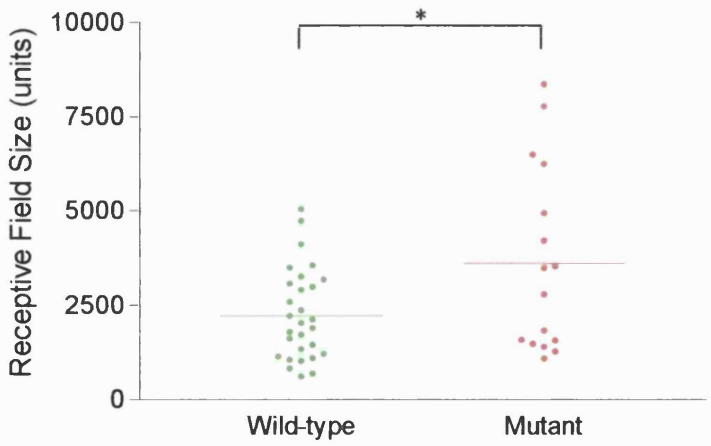

Figure 4.5
Receptive field sizes of neurones in the wild-type and T286A mutant mouse. The mutant displayed significantly larger receptive field sizes than the wild-type ($p<0.05$). Bars represent mean values.

The mechanical thresholds of individual dorsal horn neurones were measured by applying Von Frey hairs to the centre of dorsal horn neurone receptive fields. Dorsal horn cells responded to forces of 0.86±0.4g in the wild-type (n=21) and 0.56±0.1g in the mutant (n=13). The data when expressed in grams appear different to when expressed on a graph as log(mg) (Figure 4.6) because the means were taken after the data had been transformed. Regardless, the two groups were not significantly different from one another ($p>0.4$, unpaired t test; Figure 4.6).

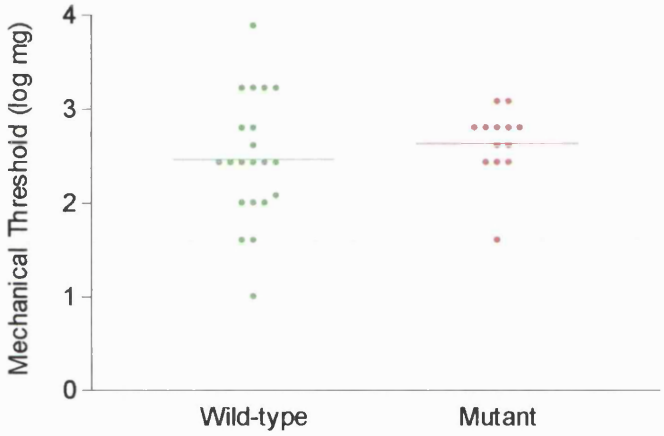

Figure 4.6
Mechanical thresholds of dorsal horn neurones in the wild-type and T286A mutant mouse. Thresholds are expressed in log (milligrams). No difference was seen between groups. Bars represent mean values.

The convergence of input differed between the two groups. While the wild-type displayed few cells that received only low or high threshold inputs (30% and 14% respectively) and a greater number of wide dynamic range neurones (WDR; those that respond to both brush and pinch), cells in the mutant dorsal horn received primarily low threshold input (those that responded only to brush; 61%). The remaining cells received either high threshold-only input, or were WDR neurones (11% and 27% respectively). This difference in input profile was significantly different between the genotypes ($p<0.001$, χ^2 test; Figure 4.7).

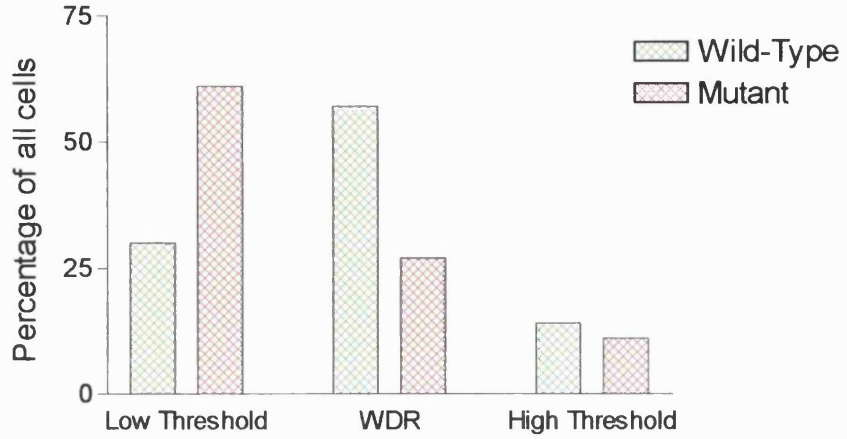

Figure 4.7
Convergence of high and low-threshold input in wild-type and T286A mutant mice. The mutant mouse shows a significantly higher proportion of cells responding to low intensity-only stimulation and a lower proportion responding to both high and low intensity stimulation ($p<0.0001$, χ^2 test).

4.3.4.2 Responses to electrical stimulation

The response of dorsal horn neurones to electrical stimulation, applied through subcutaneous pin electrodes in the centre of the receptive field, was also assessed. Cells responding to A fibre intensity stimulation at similar thresholds and with the same latencies in all groups (defined as the latency to the first spike after a single stimulus at twice the minimum threshold required to evoke a response). The mean threshold for wild-type was 1.06±0.04mA and 1.06±0.02mA for mutant ($p>0.9$; Figure 4.8A), while the mean latency was 3.85+0.2ms for wild-type and 4.00+0.1ms for mutant ($p>0.4$; Figure 4.8B).

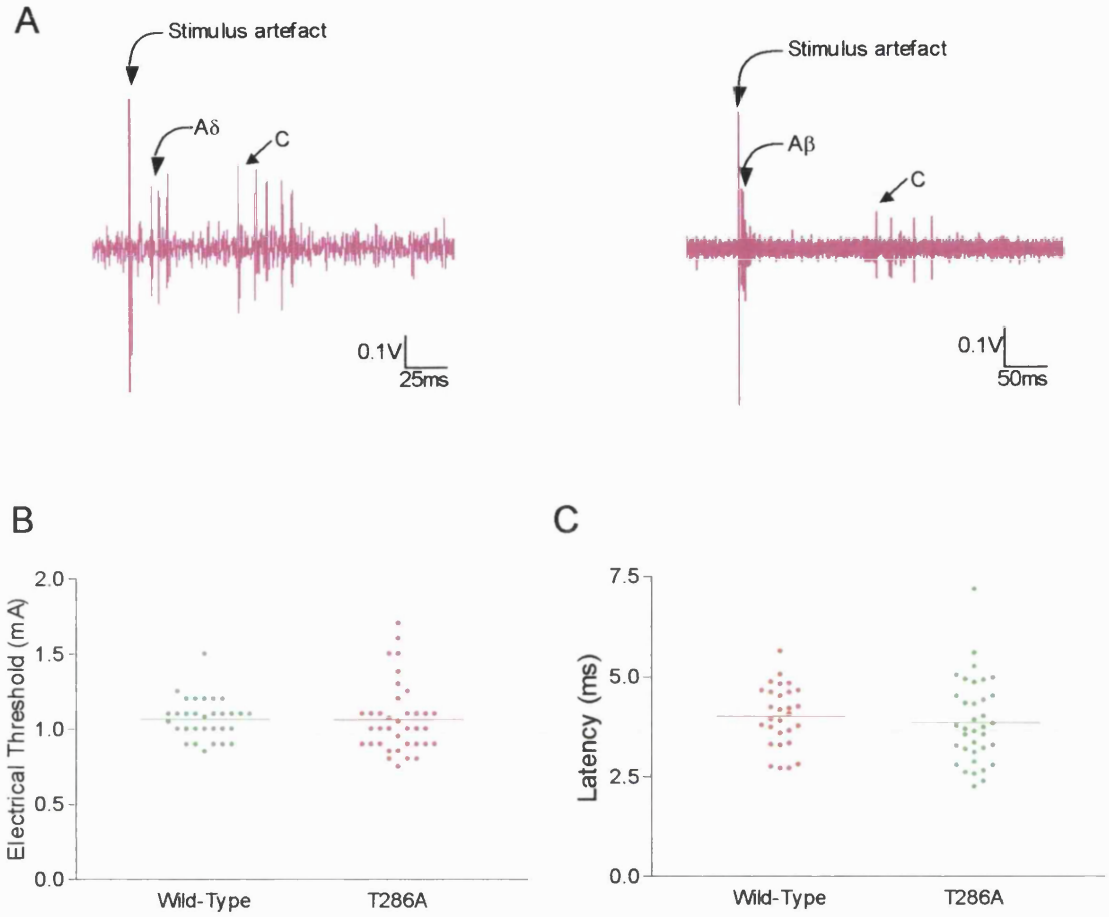

Figure 4.8
Responses of dorsal horn neurones to electrical stimulation of the hindpaw receptive field. (A) Examples of responses from wild-type dorsal horn neurones in response to high-intensity electrical stimulation. The left panel shows a cell that receives input from Aδ and C fibres. The right cell receives input from Aβ and C fibres. (B) Scatter plot of the electrical stimulation thresholds required to elicit an A fibre response. Thresholds are very similar between the groups. (C) Latency of the first spike after electrical stimulation at twice A fibre threshold. No difference was seen between genotypes. Both genotypes show similar responses. Bars represent the mean value.

The A fibre-evoked amplitude (as defined by the number of spikes fired within a 70ms time-period after electrical stimulation at twice A fibre threshold) was not different between groups. In the wild-type, the mean evoked response was 6.02±0.6 spikes (n=20), while in the mutant it was 4.6±0.9 (n=11). No significant difference was observed between genotypes ($p>0.05$, unpaired t test; Figure 4.9).

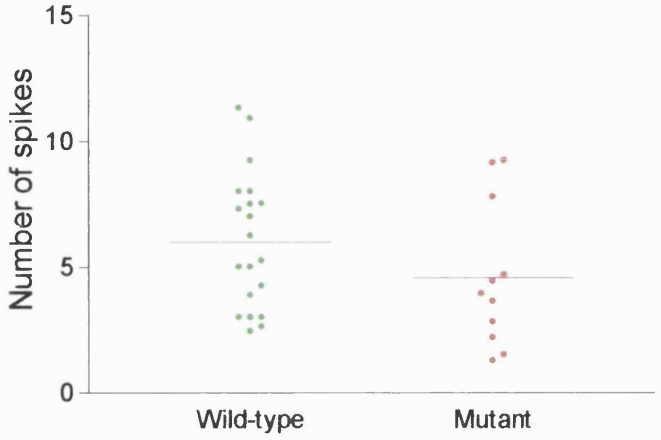

Figure 4.9
Number of spikes evoked by electrical stimulation at twice A fibre threshold in wild-type and mutant mice. Both genotypes show similar responses. Bars represent mean values.

Contrary to findings obtained in the slice, the response to C fibre stimulation in the periphery differed significantly between the wild-type and mutant. The proportion of cells responding to C fibre stimulation was greatly reduced in the mutant, with only 4 out of 37 cells (11%) receiving C fibre input, compared with 21 out of 48 (44%) in the wild-type. The four cells in the mutant were all in deep laminae, but the small number makes firm conclusions about lamina-specific changes difficult. With respect to those that did respond to C fibre-strength stimulation, no differences were seen in threshold or amplitude between these groups. The mean thresholds were 2.05±0.2 mA for wild-type and 2.08+0.7mA for mutant ($p>0.05$; Figure 4.10A) and the mean number of spikes were 6.2±0.9 spikes for wild-type and 10.5±0.2 in mutants ($p>0.05$; Figure 4.10B).

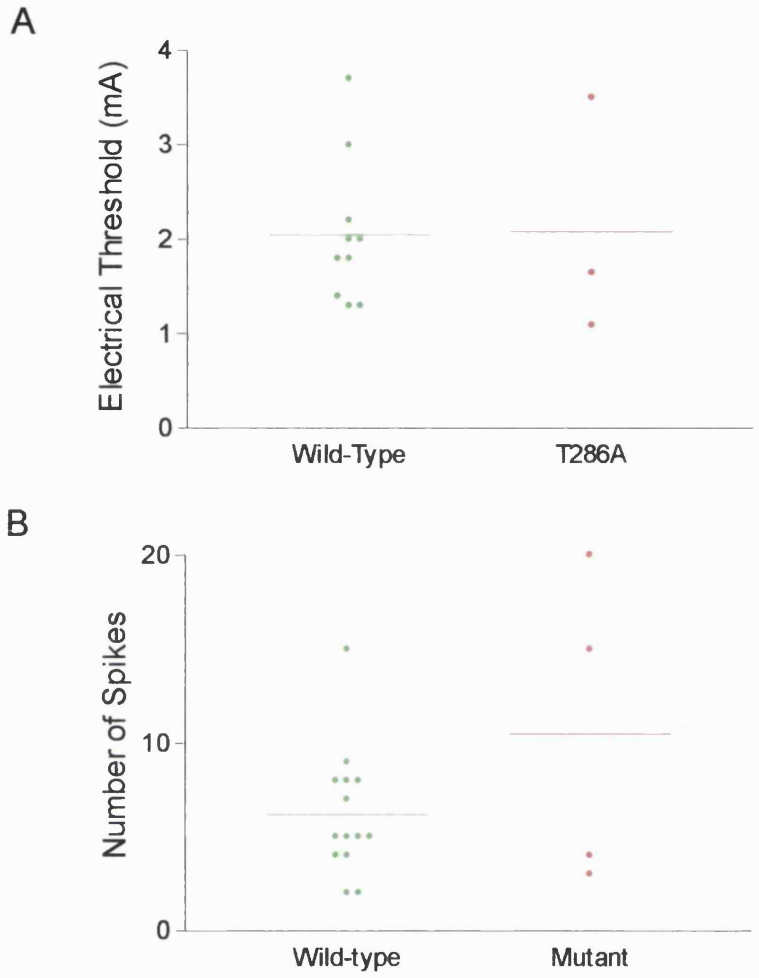

Figure 4.10
Responses of dorsal horn neurones to C fibre-intensity electrical stimulation. (A) Electrical thresholds for C-fibre mediated activity in the dorsal horn. No difference was seen between genotypes. (B) Number of spikes evoked by three-times C fibre-threshold stimulation. Results were not significant between groups. Bars represent means.

4.4 Discussion

The electrophysiological profile of the T286A dorsal horn provides evidence for a role for CaMKII in normal postnatal development of the somatosensory system. Combined with the findings from the A fibre-labelling study, the data suggest that autophosphorylation of CaMKII plays a subtle role in the normal postnatal synaptic alterations that occur during development. The evidence suggests that primary afferents themselves are not drastically affected by the mutation. The compound action potential recording of the sciatic nerve and dorsal root did not reveal any alterations in conduction velocity or activation threshold between the genotypes. Similarly, latencies of responses to mechanical and electrical stimulation were not different in the *in vivo* experiments, consistent with normal neural transmission in both groups.

The cellular properties were also unchanged, with passive membrane properties and spontaneous activity showing no alterations between the genotypes. This implies broadly similar properties in terms of cell size and excitability. The differences that were observed between the genotypes all imply a role for the kinase in synaptic connections within the cord itself. The mutant displayed four main alterations in dorsal horn synaptic circuitry:

- Receptive field sizes were larger in the T286A mutant compared to wild-type and heterozygous littermates.
- Convergence of input was altered in the mutant compared to wild-type and heterozygote with a reduction in the number of cells that responded to both high- and low-threshold natural stimulation.
- There was a reduction in the number of cells with C fibre-evoked spike activity in the dorsal horn overall but C fibre-evoked EPSCs in lamina II cells were normal.
- There was a reduced Aβ fibre polysynaptic input to the lamina II cells of the mutant compared to wild-type, but Aβ fibre-evoked spike activity in the dorsal horn overall was no altered.

The diversity of these alterations support the theory that the kinase acts in a number of different ways during postnatal life to refine the synaptic connectivity of the CNS.

4.4.1 Comparison of electrophysiological techniques

In some cases, for example with regard to the C fibre input, we obtained apparently contradictory results from the *in vivo* and *in vitro* preparations. As demonstrated in Table 4.2, and discussed below, there are many variables that affect the recordings obtained from the different techniques, in particular with regard to the connectivity within the dorsal horn. When combined, it is possible to obtain an accurate profile of the circuitry of the dorsal horn, and how it is affected by the T286A mutation.

	In vitro	*In vivo*
1	No anaesthetic	Anaesthetic
2	Disrupted rostrocaudal circuitry	Intact circuitry
3	No descending inhibition	Intact descending inhibition
4	Lamina II cells only	Large cells and dendrites from entire dorsal horn
5	Direct root stimulation	Diffuse skin stimulation
6	No natural stimulation	Natural stimulation
7	Accurate latency analysis	Less accurate latency
8	Subthreshold EPSCs	Suprathreshold spike activity only

Table 4.2
Comparison of variables affecting outcome of *in vitro* and *in vivo* electrophysiological recording techniques.

1. Anaesthetic

The *in vivo* recordings were obtained in urethane-anaesthetised animals, while the *in vitro* preparation used a transverse section of spinal cord. Therefore the anaesthetic, which may affect GABA receptors in the brain (De Lima, 2001), could be altering the excitability of the cells in the spinal cord.

2. Circuitry

As discussed in Chapter One, the spinal cord contains many interneurones that project dendrites rostrocaudally up to 1000μm away from the cell body. In addition, collaterals of Aδ and C fibres can travel large rostrocaudal distances in Lissauer's tract before terminating in the dorsal horn. These distal projections are all truncated in the *in vitro*

preparation, which uses transverse slices of a thickness no greater that 600μm. Therefore, the communication with synapses distal to the cell body are lost in the patch-clamp recordings, but are left intact in the *in vivo* preparation.

3. Descending inhibition

In addition to cutting distal dendrites of dorsal horn neurones, the *in vitro* preparation also severs the connections from supraspinal structures, so that the modulation from the brain is lost. Taking into account the fact that a significant amount of inhibitory drive to the dorsal horn is from these higher centres, it is likely that the spinal cord cells are more excitable in the *in vitro* preparation than would be seen either in the awake animal, or in the anaesthetised *in vivo* preaparation.

4. Selection of cells

A major advantage of the *in vitro* preparation is that the recorded cell can be visualised with DIC microscopy. We can therefore know for certain the location of the cell within the dorsal horn, allowing for specific recording of lamina II neurones. The *in vivo* preparation does not allow for such specificity and, despite the fact that the depth of the cell can be measured, one can never know whether the recording is being made from the cell body, or from a large dendrite. It is possible to mark the location of the electrode with a electro??? Lesion, but this results in the destruction of the electrode, and the same possibility of recording from a dendrite from a cell in a different lamina. Additionally, because the cell is discovered by lowering the electrode into the cord until response is seen, there will be a tendency to record from large cells and dendrites, and not from the small SG neurones. It is therefore difficult to ascertain precise circuits within the dorsal horn using this technique as one can never be sure of the laminar location of the recorded cell.

5-7. Stimulation

In vitro recordings allow for direct electrical stimulation of the dorsal root, meaning that precise current pulses can be applied to the nerves and the postsynaptic response measured. This technique allows for precise examination of latency, and therefore the ability to differentiate between mono- and polysynaptic connectivity. However, it is not possible to record the cell's responses to natural stimulation, such as brushing and pinching. One can therefore never be sure whether the stimulation protocols are physiologically relevant, and that similar EPSCs would be observed in response to

noxious and innocuous stimulation. The *in vivo* preparation does allow for such discrimination, and can therefore give interesting insights as to how a cell responds in response to physiological stimulation. This stimulation is generally of a more diffuse nature, and increases the likelihood of activating neighbouring nerves (for example the saphenous nerve). This exemplifies the high degree of accuracy that can be obtained when the two recording techniques are combined.

8. Thresholds

Of particular importance with regard to the differences between the electrophysiological techiniques is that fact that the *in vitro* preparation allows for analysis of *subthreshold* EPSCs, while the *in vivo* preparation only examines the spike activity of the cells. Therefore, the patch-clamp data can give insights into how a cell responds even if it does not fire action potentials. The specificity of the *in vitro* preparation has recently been demonstrated in the developing dorsal horn, which was previously thought to have no C fibre input until P10 (Jennings & Fitzgerald, 1998). Whole cell patch clamp recordings, however, have recently demonstrated, that activation of C fibres with capsaicin can indeed evoke activity in dorsal horn cells of neonatal animals, but that the majority of this activity is subthreshold (Baccei *et al.*, 2003). The significance of this with respect to the C fibre data obtained in the T286A mutant will be discussed below.

4.4.2 Receptive Fields

The T286A mice displayed enlarged cutaneous receptive field sizes compared to wild-type littermates. While such a phenomenon has not previously been reported in the spinal cord of naïve transgenic animals, receptive fields are known to be extremely plastic and therefore vulnerable to alter in response to pharmacological intervention, injury and development. For example, it has been shown that neonatal rats display larger cutaneous receptive fields compared to adults (Fitzgerald & Jennings, 1999; Fitzgerald, 1985; Torsney *et al.*, 2000). The reduction in size during development can be prevented by chronic application of NMDA receptor antagonists (Beggs *et al.*, 2002), or by neonatal skin wounding (Torsney & Fitzgerald, 2003), implying that it is an activity-dependent process. Furthermore, the size can be altered in the adult by interventions such as peripheral nerve section (Lewin *et al.*, 1994) and inflammation (Torsney & Fitzgerald, 2002), suggesting that the plasticity remains in mature animals.

At the supraspinal level, receptive field sizes of the whiskers and eyes have also been shown to be highly adaptable. The developmental reduction can be prevented by blockade of NMDA receptors in both the barrel cortex (Fox *et al.*, 1996) and superior colliculus (Huang & Pallas, 2001). The plasticity of receptive field sizes is again maintained into adulthood in the barrel cortex, with interventions such as whisker deprivation leading to increased sizes (Fox, 2002) and increased natural sensory input to the whiskers leading to decreased sizes (Polley *et al.*, 2004). It therefore appears that second and third order neurones can adapt to external stimulation by increasing or decreasing their receptive fields, thus allowing for tight control over sensory discrimination.

Functionally, large receptive field sizes are believed to contribute to hypersensitivity in models of tissue damage, such as inflammation (Woolf & King, 1990; Torsney & Fitzgerald, 2003), and in the neonate (Fitzgerald, 1985). A large receptive field will mean that more dorsal horn neurones are activated in response to skin stimulation, producing reduced spatial discrimination and increased input to the dorsal horn. Additionally, the threshold for spike activity in tertiary cells, such as thalamic and motor neurones, will be reduced (Figure 4.11). Expanded receptive fields can therefore contribute to lower behavioural thresholds, in that previously subthreshold input at a motorneurone or thalamic neurone may now be capable of evoking the withdrawal reflex or activating nociceptive neurones (Torsney & Fitzgerald, 2003).

How might a dorsal horn cell alter its receptive field properties? A number of mechanisms may account for the ways in which a cell responds to afferent input- either through alterations in the primary afferents themselves, or through centrally-mediated effects. Peripherally, the receptive field sizes of the primary afferents themselves may be larger in the T286A mutant, perhaps due to increased branching at the dermis so that each nerve innervates a larger area of the skin. While this possibility cannot be ruled out, it appears to be unlikely as the majority of other studies during development and after injury have not found such an effect. For example, the receptive field sizes of sensory neurones relative to body size are unchanged during development (Koltzenburg *et al.*, 1997; Fitzgerald, 1987), even though the dorsal horn receptive fields are refined during this period (Fitzgerald, 1985). For such a process to occur in the T286A mutant, the kinase would have to play a role in axon guidance to the skin, through some form of

growth factor-meditated event. There is no evidence to suggest such an involvement for CaMKII.

Alternatively, the large receptive field sizes could be caused by wider terminal fields of the primary afferents within the spinal cord. This is the model that was suggested to be affected in the chronic NMDA receptor-blockade study of Beggs *et al.* (2002), so that the Aβ fibres projected aberrant collaterals into the superficial dorsal horn and thus increased the receptive field sizes of the terminal neurones. However, analysis of the primary afferent terminations revealed a normal profile in the mutant (Chapter Three), suggesting that CaMKII does not play a role in the afferent refinement of afferent connectivity.

The most likely explanation for the increased sizes of receptive fields in the T286A mutant is therefore through central changes in inhibitory drive to the dorsal horn neurones. This hypothesis was suggested to cause the large sizes seen in young animals (Fitzgerald, 1985), whereby the central dorsal horn cells are hyperexcitable due to the lack of inhibition seen in young rats, and can thus fire action potentials in response to weak stimulation from afferent terminals. As discussed in Chapter One, dorsal horn inhibition develops late in postnatal development, with the axodendritic maturation of inhibitory interneurones (Bicknell & Beal, 1984) and maturation of descending inhibitory pathways from the brainstem (Fitzgerald & Koltzenburg, 1986). The inhibition acts both presynaptically, by hyperpolarising terminals and reducing the probability of release of neurotransmitters, and postsynaptically by hyperpolarising the dendritic spines. Because the CaMKII is expressed in both dorsal horn cells and in the dorsolateral funiculus, it is unclear which form of inhibition is disrupted by the mutation. The fact that GABAergic interneurons do not express the kinase might suggest that it is the descending inhibition that is disrupted, although synaptic connecitivy may still be affected in cells that do not express CaMKII by postsynaptic mechanisms, as will be discussed below.

The lack of inhibitory tone of neonatal animals is also thought to be caused by the depolarising nature of inhibitory neurotransmitters in very young animals (Owens *et al.*, 1996; Ben Ari, 2002a; Chen *et al.*, 1996; Ben Ari *et al.*, 1989) and see Chapter One). It is possible that the change in the reversal potential of chloride ions is somehow mediated by CaMKII autophosphorylation, although this seems unlikely: if GABA and

glycine had a depolarising effect in adult animals, the absence of inhibitory activity would have profound effects on all aspects of neuronal development, probably to the point of making the animals unviable. Indeed, prevention of the switch to a hyperpolarizing action of GABA by knocking out the chloride transporter, KCC2, produces animals that die at birth due to severe motor deficits and disrupted respiration (Hubner *et al.*, 2001). It is more likely that the kinase plays a role in the maturation of inhibitory interneurones, so as to prevent the axodendritic refinement that occurs postnatally (Figure 5.12). This mechanism will be discussed below.

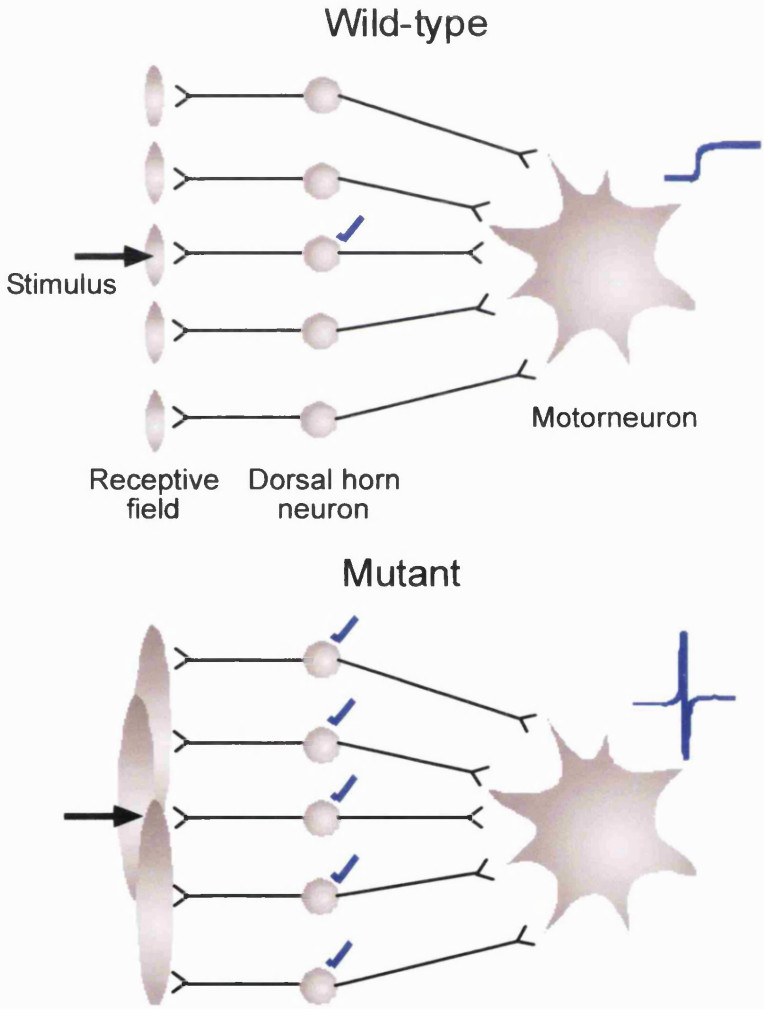

Figure 4.11
The effects of increased receptive field size in the T286A mutant. In the wild-type, receptive field sizes are small, and so skin stimulation produces firing in only a small group of neurones in the dorsal horn. This spiking is not sufficient to activate the tertiary motorneurone. In the mutant, however, the skin stimulation produces firing in a much larger population of dorsal horn neurones, allowing the motorneurone to reach threshold and fire action potentials, thus evoking the withdrawal reflex. Adapted from (Torsney & Fitzgerald, 2003).

4.4.3 C fibre input

We have shown that the T286A mutant displays reduced C fibre input and a subsequent alteration in the convergence of input to the dorsal horn. The convergence of input reflects whether a cell responds to high threshold, low threshold, or both high and low threshold stimulation. High threshold input is generally equated with noxious, and therefore C and Aδ fibre-mediated, stimulation. At birth, the input to the cord is dominated by low-threshold input by way of the high proportion of A fibres that innervate the superficial dorsal horn during this time (Fitzgerald *et al.*, 1994; Jennings & Fitzgerald, 1998). As the C fibres grow into the dorsal horn, they form synapses with cells that still receive some form of low-threshold input, and thus become wide-dynamic range (WDR) neurones. In the T286A mutant, however, the convergence of input appears to maintain a more neonatal phenotype, with *in vivo* extracellular recordings revealing a higher proportion of low threshold-only cells and a smaller proportion of WDR neurones.

The loss in high-threshold input was only seen in the *in vivo* experiments, with whole-cell patch clamp studies finding a similar proportion of cells responding to C fibre intensity stimulation between genotypes. This finding reflects the different types of information that can be obtained using *in vitro* and *in vivo* electrophysiological techniques. As discussed earlier, a major difference between the two techniques is the fact that *in vitro* recording allows for analysis of subthreshold EPSCs, while the *in vivo* preparation only detects suprathreshold EPSPs. It is therefore possible that the mutant does indeed posess a normal C fibre input, but that it is insufficient to produce action potential firing in the postsynaptic neurone. This would support the convergence data, so that noxious pinching would activate C fibres normally, but these fibres would not be able to depolarise the dorsal horn enough to reach threshold. Current clamp recordings from neurones recorded intracellularly or in whole cell mode would help to ascertain whether this is indeed the case.

Of particular importance to the analysis of dorsal horn connectivity is that fact that the patch recordings only examined cells in lamina II, while the extracellular recordings looked at cells throughout the dorsal horn. Therefore, it is possible that the loss of C fibre input was specific to the deeper laminae. This is interesting as C fibres are known to terminate in the superficial dorsal horn, implying that there is a loss of connectivity

of deep dorsal horn cells receiving C fibre input. In support of this, no alteration in C fibre termination was seen in the mutant using IB4 histochemistry (see Chapter Three). Therefore, the deficit could be caused by a reduction in the dendritic field of neurones in the deep laminae. It is known that cells in laminae IV and V do project dendrites into the superficial dorsal horn, and that they can therefore receive high-threshold input (Miller & Woolf, 1996; Woolf & King, 1987; Todd, 1989). In addition, lamina II cells are known to send axonal projections to the deeper laminae, allowing for C fibre input to deep cells via polysynaptic circuitry (vertical cells; Grudt & Perl, 2002). A large proportion of both categories of cells are interneurones, and therefore develop their dendritic and axonal projections postnatally (Bicknell & Beal, 1984). It is possible that the dendritic growth and refinement is dependent on CaMKII autophosphorylation, and disruption of this process produces cells with either limited dendritic fields, or inadequate synaptic contacts at these distal processes (Figure 4.12). Evidence for this hypothesis is discussed in section 4.4.4.

4.4.4 Polysynaptic input to lamina II

The whole-cell patch clamp studies here show that almost all low-threshold input to lamina II involves pathways containing multiple relays. This is in contrast to studies performed in rats, which have shown large proportions of lamina II exhibiting monosynaptic input from the A fibres. For example, Baba *et al.* (1999) showed that 26% of rat SG neurones received monosynaptic Aδ input, while Nakatsuka *et al.* (2000) put this figure at 65%. A study by Yoshimura & Jessell (1989) found that 70% of SG neurones received monosynaptic A fibre input. Classification of mono/polysynaptic connectivity is extremely difficult when electrical stimulation of an unknown number of fibres is performed. The discrepancy between studies could be explained by the use of different species of animal; although this seems unlikely- mice have been shown to display similar sensory profiles in the majority of studies (Woodbury *et al.*, 2001; Cain *et al.*, 2001). Additionally, there appeared to be no differences between the species in the A fibre tracing study described in Chapter Three. A more likely explanation concerns the criteria for selection of monosynaptic pathways, which may have differed between studies. A major method of identifying such a pathway comes from the hypothesis that repetitive stimulation will produce monosynaptic responses with a fixed latency. Stimulation of a 5mm dorsal root would be expected to produce monosynaptic Aδ responses with a latency of approximately 1-2ms. When such a response was seen,

the variability of latency after repetitive stimulation was, at best, 0.5ms. This variability was unlikely to be caused by some form of postsynaptic receptor desensitisation because there was no rightward shift in the latency, just an increased variability. We therefore assumed that even those cells responses that would follow 10Hz without failing were generally polysynaptic. The only monosynaptic response that was seen displayed no shift whatsoever in latency in response to 10Hz repetitive stimulation. However it is important to note that the interpretation of mono- and polysynaptic input is extremely difficult when large numbers of fibres are stimulated, as each one might be forming both direct and indirect connections with the recorded cell.

Whole-cell patch recordings revealed a significantly smaller population of cells receiving polysynaptic Aβ input in the mutant compared with the wild-type and heterozygote. This deficit leads to an apparent increase in the number of Aδ fibre inputs to SG, although this finding is almost certainly artefactual- a large proportion of cells receiving Aβ fibre input would also receive higher threshold input but this would be masked due to the similarity of their latencies. A reduction in Aβ polysynaptic input would unmask the higher threhold evoked EPSCs, producing an apparent increase in A fibre-mediated currents.

The deficit in Aβ fibre-mediated currents again suggests a disruption in the maturation of interneurones in the superficial dorsal horn. As these fibres terminate in the deeper laminae, they require interneuronal signalling to transmit to the SG, and so disruptions in the axodendritic elaborations of these cells would impair the low threshold input to the superficial laminae (Figure 4.12).

4.4.5 The role of CaMKII in interneuronal development

The primary deficits in synaptic circuitry of the dorsal horn of T286A mutants can be explained by disruptions to local interneuronal circuitry. Taking the tracing and physiological studies together gives a picture of a fairly normal developmental profile of primary afferent termination- C fibres appear to grow into the cord and form connections with superficial dorsal horn cells, and Aβ fibres appear to withdraw in a normal manner. Physiological changes are seen in polysynaptic A fibre input, receptive field size and C fibre input to deep cells- phenomena that are dependent on postnatal maturation of interneurones. Therefore, CaMKII could be acting in a number of ways.

Firstly, cells within SG that are CaMKII-positive may not be developing normal interlaminar axons, so that cells within the deep dorsal horn do not receive adequate polysynaptic C fibre input (Figure 4.12C), and superficial dorsal horn neurones would not receive adequate polysynaptic Aβ fibre input (Figure 4.12B). Disruption in axonal growth has been observed in tectal cells injected with CaMKII inhibitors (Zou & Cline, 1999). These cells display elongated axons with exuberant collateral branching, implying that CaMKII somehow acts to inhibit axonal growth, probably by increasing synaptic stabilisation. Blockade of this stabilisation would cause the axon to continue to elaborate in order to find more suitable synaptic partners. This suggests that the T286A mutant may not display a neonatal pattern of interneuronal morphology- that is, one of limited axonal and dendritic projection (Bicknell & Beal, 1984), but rather one of exuberant process spreading due to the axons' inability to form stable synapses. Morphological analysis of dorsal horn neurones using cell filling techniques would be required to confirm this hypothesis.

Secondly, CaMKII-positive cells might not receive refined synaptic input from neighbouring cells, which could or could not express the kinase. Therefore despite the fact that CaMKII is not expressed in GABAergic cells (see Chapter Two), their input could still be disrupted by actions at the postsynaptic cell. Similarly, disruption of synaptic contacts from deep dorsal horn dendrites could still occur regardless of the fact that little kinase expression is seen in this part of the cord (see Chapter Two). This action of CaMKII has been shown in cortical neurones. Expression of a constitutively active form of the enzyme causes increases in spontaneous and evoked EPSC amplitude by a mechanism of increased synaptic contact with certain neighbouring cells and elimination of synapses from other cells (Pratt *et al.*, 2003). Disruption of the kinase would presumably prevent this delicate refinement from occurring, although it is worth noting that in other parts of the brain, for example the cortex, pyramidal cells that are immunopostivie for CaMKII still do not appear to express the kinase at inhibitory PSDs (Liu & Jones, 1996).

Finally, the most established mechanism of action of the kinase concerns its ability to alter synaptic strength through phosphorylation of both excitatory and inhibitory ionotropic receptors (Kolaj *et al.*, 1994; Wang *et al.*, 1995) While this action could be tied in with the morphological changes described above, the two mechanisms are not

the same. For example, inhibition of CaMKII in adult slices causes disruptions in synaptic plasticity (Lisman *et al.* 2001), whereas the changes in axodendritic morphology are only believed to occur during early postnatal life (Wu & Cline, 1998). Therefore even after the growth of the neuronal processes has stopped, the T286A mutant would still be experiencing deficits in synaptic refinement through its inability to alter synaptic strength. Even if, for example, inhibitory interneurones do develop normal axodendritic branches, the inhibitory control may still be unrefined as the synapses cannot be strengthened and weakened in normal manner. Therefore, the kinase can affect local circuitry by altering the actual morphological profile of axons and dendrites and by influencing the sensitivity of the synaptic receptors to their ligands.

These data suggest that the SG of T286A mutants is more of a closed system than is seen in wild-type animals. This concept was originally proposed by Szentagothiai in 1964 (see Willis & Coggeshall, 1991), who suggested that all axons from SG that entered the white matter returned to the SG, and did not project beyond it. By this hypothesis, SG neurones could only act by influencing dorsal dendrites that arise from neurones in the deeper laminae. Since then, it has been shown that SG neurones do in fact send axons to the projection neurones of lamina I, as well as to deeper laminae (eg Grudt & Perl, 2002), suggesting that the neurones do in fact modulate information flow beyond lamina II. If disruptions were occurring in the axodendritic elaborations of the T286A mutant, such communication may be limited, producing the 'closed system' of Szentagothiai (1964).

4.4.6 Conclusions

Combined with the tracing experiments, this study reveals a subtle role for CaMKII in the synaptic development of neurones in the spinal cord. Primary afferent input appears to be relatively unaffected by theT286A mutation, despite reports that some cells in the DRG express the kinase. In addition, the dorsal horn cells do not display any gross malfunction, with mutants displaying similar levels of spontaneous activity and responsiveness to afferent stimulation. These results differ from those found after chronic blockade of NMDA receptors during development, implying that these receptors can function in manners that do not involve CaMKII.

The main differences appear to involve the interneuronal circuitry, in keeping with the fact that these neurones develop postnatally- the time when the kinase is first expressed. Our findings can be explained by these neurones' inability to form the stable connections that normally occur in response to activity, thus disrupting the delicate refinement that allows for the exquisite control of sensory processing.

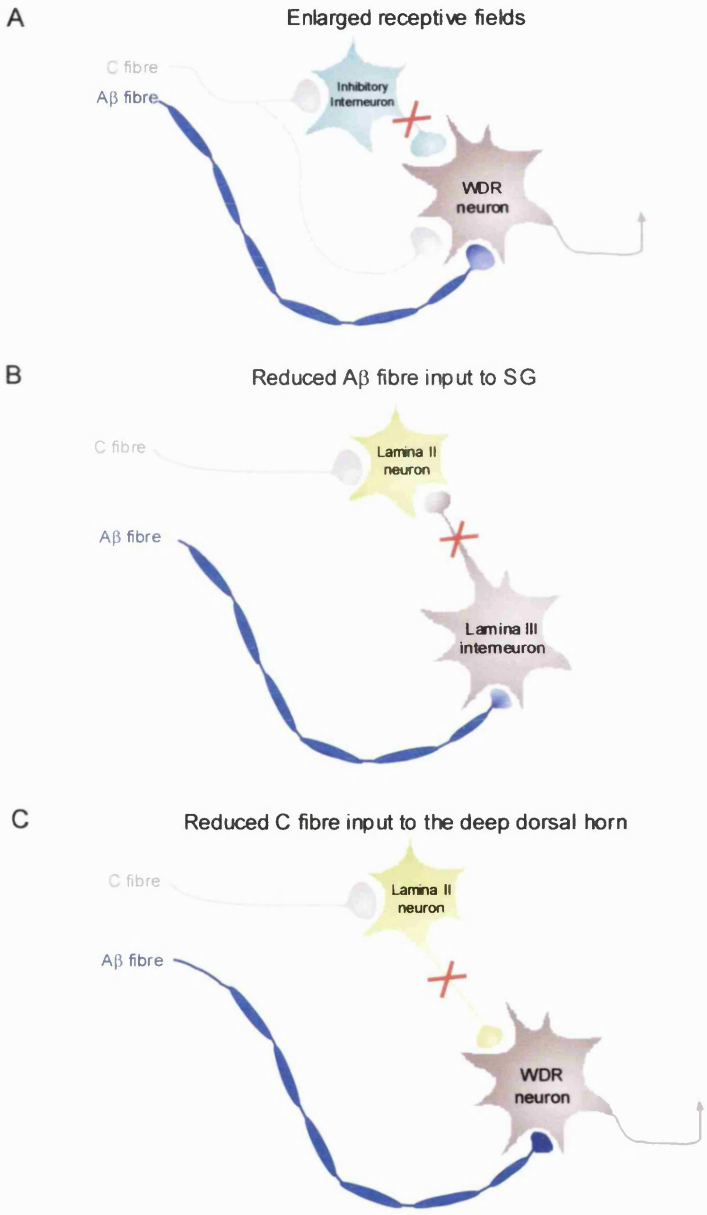

Figure 4.12
Diagrams demonstrating the possible disruptions to the interneuronal connectivity in the T286A mutant. The disruptions are illustrated by the red X. (A) Enlarged receptive sizes could be caused by reduced inhibitory control by lamina II interneurones. (B) Reduced Aβ fibre input caused by decreased axonal elaborations of deep dorsal horn interneurones. (C) Reduced C fibre input to the deep dorsal horn caused by decreased axonal elaborations of lamina II interneurones. Because of this disruption, the WDR neurone would now receive low-threshold input only.

Chapter 5

PAIN BEHAVIOUR IN THE T286A MUTANT

5.1 Introduction

In the last chapter we demonstrated that CaMKII autophosphorylation affects the development of dorsal horn circuitry in subtle ways, perhaps through interneuronal functional and structural alterations. The development of primary afferents and their central terminals, which is NMDA receptor-dependent, are unaffected by the T286A mutation, perhaps because young animals display parallel pathways of plasticity induction (Wikstrom *et al.*, 2003; see Chapter Three). The disruption to the spinal connectivity described might be predicted to produce behavioural alterations in the mutant, for example increasing the nociceptive thresholds as a result of the reduced C fibre-mediated spile activity, or perhaps lowering of the flexion withdrawal threshold due to the enlarged receptive fields (Figure 4.11). This reflex involves basic spinal circuitry, whereby activation of nociceptors produces activation of motorneurones via interneuronal depolarisation, and subsequent withdrawal of the limb (Woolf & Swett, 1984). We therefore explored the behavioural manifestations of the mutation with regard to mechanical and thermal noxious stimulation.

In the adult brain, CaMKII autophosphorylation appears to be essential for induction of many forms of plasticity (Giese *et al.*, 1998; Glazewski *et al.*, 2000; Hardingham *et al.*, 2003). We therefore decided to examine the role of CaMKII in the synaptic plasticity of the spinal cord. Plasticity in the dorsal horn often leads to pain- a phenomenon that is believed to display characteristics of memory- where the cord displays long-term changes in response to an external stimulus.

Perhaps the most studied form of plasticity in the spinal cord is central sensitisation, where increased nociceptive and C fibre input leads to long-lasting increases in the excitability of second-order dorsal horn neurones. This can be observed as an increased firing of cells to a fixed stimulus before and after C fibre stimulation or tissue injury and inflammation, and therefore has been associated with LTP. Central sensitisation is dependent on NMDA activation and subsequent calcium-mediated signalling within the neurone, and therefore may well involve autophosphorylation of CaMKII.

5.1.1 NMDA receptors and inflammatory pain

Tissue inflammation produces pain responses that can last from hours up to years. This is thought to be due to the fact that dorsal horn cells become sensitised in response to increased activity of primary afferents, which themselves are sensitised by

inflammatory agents in the periphery that are released in response to tissue damage (Scholz & Woolf, 2002; Hunt & Mantyh, 2001). The role of NMDA receptors in the mediation of dorsal horn excitability was first shown by Davies & Lodge (1987) and Dickenson & Sullivan (1987a). Here, *in vivo* electrophysiological recording revealed that, while afferent-mediated potentials were unaffected by application of NMDA receptor antagonists, facilitation in response to repeated firing (a phenomenon known as 'wind-up') was attenuated by NMDA receptor blockade. Further studies revealed that the increased long-term excitability induced by either repetitive stimulation or by mustard oil application (a substance that selectively activates a subpopulation of C fibres through binding to the TRP receptor, ANKTM1; (Jordt *et al.*, 2004) could be prevented by application of NMDA receptor antagonists (Woolf & Thompson, 1991). In addition, the facilitation returned to baseline by application of NMDA receptor antagonists to the cord after mustard oil treatment, implying a role for the receptors not just in induction of central sensitisation, but in its maintenance. Similar effects of NMDA receptor antagonists have been observed in hyperalgesia produced by formalin injection (Haley *et al.*, 1990; Coderre & Melzack, 1992). Finally, central hyperalgesia produced by injection of the inflammatory agent, carrageenan, has also been shown to be dependent on NMDA channel opening (Ren *et al.*, 1992a; Ren *et al.*, 1992b; Eisenberg *et al.*, 1994). In all these studies, NMDA receptor antagonists did not affect basal transmission or sensory processing, implying that the receptor enhances, rather than transmits, noxious information (Dickenson *et al.*, 1997).

The mechanisms by which the NMDA receptor mediates inflammatory pain are now becoming clear, in terms of both receptor pharmacology and downstream signalling. Injection of formalin, which elicits a primary acute phase of pain behaviour followed by a late centrally-mediated phase in rodents, causes an increase in NR2A mRNA expression accompanied by a decrease in NR2C mRNA expression (Gaunitz *et al.*, 2002). In addition, peripheral injection of complete Freund's adjuvant (CFA; a powerful inflammatory agent) produces a shift in the *I-V* relationship in a hyperpolarized direction and a weakened Mg^{2+} block, implying that the receptor could be activated at more negative membrane potentials (Guo & Huang, 2001). Inflammation also produces an increase in tyrosine phosphorylation of the NR2B, but not NR2A, subunit that is dependent on primary afferent drive and correlates with the development and maintenance of the hyperalgesia (Guo *et al.*, 2002). This is of

particular interest as the NR2B subunit has been associated with pain processing due to its abundance in the superficial dorsal horn (Momiyama, 2000; Yung, 1998). Indeed, blockade of NR2B-containing receptors produces powerful antinociceptive effects in a number of different pain states (Taniguchi *et al.*, 1997; Boyce *et al.*, 1999; Malmberg *et al.*, 2003). Inflammatory hyperalgesia, induced by the second phase of the formalin test, has also been shown to be attenuated in mice with a spatiotemporal deletion of postsynaptic NR1, implying a role for the receptor in mediating this form of central sensitisation (South *et al.*, 2003).

In addition to altering its pharmacology in response to tissue injury, and thus changing cell excitability, NMDA receptors also directly affect synaptic activity by producing synaptic potentiation and depression. The extent to which central sensitisation is similar to the classical hippocampal LTP is unclear, although synaptic potentiation has been observed in the dorsal horn, with characteristics that are similar to those seen in the brain (Randic *et al.*, 1993; Ikeda *et al.*, 2003). One such characteristic is the dependence on NMDA receptor activation as LTP, when elicited through high-frequency stimulation of primary afferents, cannot be induced in the presence of the NMDA receptor antagonist, APV. The level to which spinal cord LTP is specific to individual synapses, as is the case in the CA1 region of the hippocampus, or whether it is really whole cell sensitisation, is yet to be established.

5.1.1.1 Downstream mediators of inflammatory pain

Upon opening, NMDA channels allow entry of calcium ions into the dorsal horn cell. Calcium then acts to increase synaptic excitability through its interactions with downstream kinases such as PKC and CaMKII. PKC has been shown to play a role in increasing dorsal horn cell sensitivity by phosphorylating both the NR2B (Guo *et al.*, 2002) and NR1 (Brenner *et al.*, 2004) subunits of the NMDA receptor, leading to an increase in the probability of channel openings and a reduction in the voltage-dependent Mg^{2+} block (Chen & Huang, 1992). For example, CFA injection into the hindpaw of rats results in a 75-100% increase in PKCγ immunoreactivity in the ipsilateral superficial dorsal horn (Martin *et al.*, 1999). In addition, inhibition of the kinase inhibits behavioural hyperalgesia in response to capsaicin injection (Sluka & Willis, 1997), and the shift in *I-V* relationship of NMDA receptors after inflammation can be prevented by application of PKC inhibitors (Guo & Huang, 2001). These results imply

that calcium entry through NMDA receptors can activate PKC and subsequently increase excitability of dorsal horn neurones.

Less is known about the role of the other main calcium-dependent kinase, CaMKII, in mediating dorsal horn cell central sensitisation. Rather than acting to potentiate NMDA receptor-mediated responses, the acute action of CaMKII is on AMPA receptors, particularly the GluR1 subunit, which it phosphorylates and inserts into the membrane upon activation. In keeping with the idea that central sensitisation involves some form of synaptic potentiation, it could be hypothesised that CaMKII does indeed play a role in increasing dorsal horn cell excitability. For example, CaMKII mRNA is upregulated after injection of formalin, suggesting that the kinase plays a role in producing sensitisation after inflammation (Liang *et al.*, 2004b). In addition, a study by (Fang *et al.*, 2002) demonstrated that intradermal injection of capsaicin produced increases in total and phosphorylated CaMKII protein expression in the superficial dorsal horn. Additionally, the CaMKII inhibitor, KN-93, prevented the increase in dorsal horn cell excitability and the decrease in exploratory behaviour seen after capsaicin application. The upregulation of phospho-GluR1 was also prevented by prior administration of KN-93 (Fang *et al.*, 2002). These data imply that CaMKII plays a role in central sensitisation by phosphorylating the AMPA receptors subunit, GluR1 and suggest that the sensitisation will also be disrupted in the T286A mutant.

5.1.2 NMDA receptors and neuropathic pain

Neuropathic pain is a chronic condition that is caused by damage to the peripheral nerves themselves. This damage can include nerve lesion (Seltzer *et al.*, 1990; Decosterd & Woolf, 2000; Kim & Chung, 1992), demyelination (Wallace *et al.*, 2003) or diabetic neuralgia (Simmons & Feldman, 2002). Pain is manifested as hyperalgesia, allodynia and spontaneous pain, and is caused by both peripheral and central alterations. Although the measurable pain behaviour in rodents is similar in neuropathic pain and inflammatory pain, the two models are extremely different in their mechanisms of action. This is primarily because the pain results from nerve damage and subsequent deafferentation, which causes release of growth factors and neurotropins into the cord. Additionally, the damaged nerves produce high levels of spontaneous activity both within the DRG (Wallace *et al.*, 2003), where it is observed in both damaged and undamaged axons (Boucher *et al.*, 2000) and the dorsal horn (Chapman *et al.*, 1998).

The onset of neuropathic pain, therefore, is not observed until some days after the injury. Some similarities can be observed between the two models in terms of dorsal horn pharmacology. The NMDA receptor, for example, has been shown to play a role in both induction and maintenance of neuropathic pain, which can in some cases be treated with receptor antagonists such as ketamine (Hocking & Cousins, 2003). However it is important to note that, while inflammatory pain has been very well characterised in both rodents and humans, little is known about the mechanisms underlying the various forms of neuropathic pain.

Evidence of a role for NMDA receptors in induction of neuropathic pain has come from the finding that blockade of the receptors with intrathecally applied MK-801 or D-AP5 prior to sciatic nerve lesion produced a significant delay in the onset of neuropathic pain behaviour (Seltzer *et al.*, 1991). Thermal hyperalgesia could also be prevented by MK-801 treatment prior to chronic constriction injury (CCI) of the sciatic nerve (Davar *et al.*, 1991). Similarly, pain-related behaviour can be delayed by genetic knockdown of PSD-95/SAP90- a scaffolding protein known to bind the NMDA receptor to signalling molecules at the synapse- using intrathecal injection of antisense oligodeoxynuceotides (Tao *et al.*, 2001). In addition, potentiation of synaptic responses after nerve injury is blocked by prior application of D-AP5 (Sandkuhler & Liu, 1998). Neuropathic pain cannot be completely prevented by pre-emptive blockade of NMDA receptors, and this is probably due to the fact that peripheral nociceptors continue to display aberrant firing characteristics for long periods after the initial injury, and through alternative central mechanisms. This afferent barrage can therefore continue to drive plasticity in the dorsal horn through NMDA receptor-mediated processes.

Neuropathic pain can last for many years in humans, but treatment using the NMDA receptor antagonists ketamine and memantine can produce long-lasting relief, implying that NMDA receptors are involved in the maintenance of the pain. It has been shown that a variety of NMDA receptor antagonists can diminish the hyperexcitability of dorsal horn neurones in rats that have previously received spinal nerve ligation (SNL) treatment (Suzuki *et al.*, 2001). The antagonists were capable of producing the effects two weeks after surgery- a time when neuropathic pain behaviour is at its maximum- and is therefore unlikely to be caused by a prevention of further increasing excitability. The mechanism by which NMDA receptors contribute to neuropathic pain is not clear,

although recent evidence suggests that it could be caused by removal of inhibition from excitatory interneurones. Through this model slow, NMDA receptor-mediated currents are prevented from occurring by inhibitory interneurones that hyperpolarize the cell after fast, AMPA receptor-mediated depolarisations. After nerve injury, however, GABAergic interneurones may die due to excitotoxic events (Moore *et al.*, 2002), meaning that the NMDA receptor-mediated component of the EPSC is revealed, and repetitive action potentials can depolarise the projection neurones (Baba *et al.*, 2003).

5.1.2.1 Downstream mediators of neuropathic pain

As in inflammatory pain, the contribution of the NMDA receptor in neuropathic pain implies downstream mediation by calcium signalling. PKC has been strongly implicated in neuropathic pain as intrathecal injection of compounds that inhibit translocation of the kinase attenuated pain behaviour in rats that had received CCI (Mao *et al.*, 1993) or SNL (Hua *et al.*, 1999) surgery. It was later shown that the PKCγ isoform appeared to be essential for induction of pain, as mice lacking this gene displayed reduced hypersensitivity after partial sciatic nerve ligation (Malmberg *et al.*, 1997). As PKC is directly activated by calcium, the deficits in neuropathic behaviour after protein inhibition or gene deletion imply that calcium-mediated events play a major role in inducing and maintaining neuropathic pain states.

Evidence for a role for CaMKII in mediation of neuropathic pain comes from a study by Garry *et al.* (2003) that showing increases in both phosphorylated and total protein expression in the dorsal horn after CCI surgery. This upregulation was not seen in mice lacking the NMDA receptor anchoring protein, PSD-95, implying firstly that PSD-95 is required to assemble an in vivo connection between the receptor and the kinase, and secondly that CaMKII activation was dependent on calcium entry through the NMDA channel. Additionally, inhibition of the kinase produced attenuated hypersensitivity in behavioural tests.

5.1.3 Aims of this chapter

This chapter examines how the T286A mutant behaves in response to various painful stimuli. We first examined the animals' ability to respond to normal sensory stimulation (both mechanical and thermal) as the physiological profile described in Chapter Five implied that there might be alterations caused by altered synaptic circuitry

in the dorsal horn. We then went on to examine the responsiveness to different pain states. Because central sensitisation, inflammatory pain and neuropathic pain all appear to involve calcium signalling by way of the NMDA receptor, and this signalling appears to involve downstream calcium kinases, we hypothesised that the T286A mutant mouse may show diminished pain behaviour in response to various painful interventions.

5.2 Methods

5.2.1 Mechanical stimulation

All behavioural testing was performed blind to genotype. Mechanical sensitivity was assessed using the Von Frey hair test of the flexion withdrawal reflex (Chaplan *et al.*, 1994). Wild-type and T286A mutants were placed on a raised frame (constructed in-house) covered with a fine netting so that the paws could be mechanically stimulated from below. Glass histology pots were placed over each individual animal to limit movement. Animals were left on the frame for 1-2 hours on the two days prior to testing in order to acclimatise to their surroundings.

On the day of testing, the mice were placed on the netting and allowed to acclimatise for 1-2 hours. Mechanical sensitivity was examined by pressing Von Frey filaments onto the plantar aspect of the hindpaw. This was performed using a 'down-up' protocol, so very fine, sub-threshold filaments were used at first, and the thickness incrementally increased. The filaments were pressed onto the paw until they bent, and this bending-force could be measured by pressing each hair onto a set of electric scales. Responses were measured as a clear lifting of the paw in response to hair application (Chaplan *et al.*, 1994). Hairs were applied five times to each hindpaw in an alternating fashion. For baseline testing, data from each paw were pooled. The test was terminated when the animal displayed five out of five withdrawals to a hair application.

Because Von Frey hair force increases in a logarithmic fashion, the data were plotted in terms of log(g) against the percentage withdrawal. These data formed sigmoidal curves, and a line of best fit was applied to each curve using the Sigmoidal Dose-Response fit from GraphPad Prism 3.0, keeping the bottom (0) and top (100) values as constants. The effective force required to produce a response 50% of the time (the EF50) was then calculated (see Figure 5.2A for example). All experiments were performed blind to genotype.

5.2.2 Thermal stimulation

Sensitivity to heat stimulation was analysed using the Hargreaves radiant heat test (Hargreaves *et al.*, 1988). Mice were placed in perspex enclosures (10cm x 20cm) resting on a platform, beneath which was an infra red heat generator (Ugo Basile, Italy). They were left for 30 minutes in order to habituate to their surroundings, and then the

heat generator was placed under the paw and activated (stimulation intensity of 65). The generator produces a heat ramp that was automatically cut off when the animal withdrew its foot, and the latency of this withdrawal noted as the thermal threshold.

Each animal was stimulated three times on each paw, with at least a five minute interval between tests so as to avoid sensitisation. The withdrawal latency was measured as an average of the three tests.

5.2.3 Carrageenan inflammation

50µl of λ-carrageenan (2% in saline; Sigma) were injected into the plantar region of the left hind paw under brief halothane anaesthesia. The right hindpaw was left untreated. Animals were left to recover, and mechanical and heat sensitivity assessed four hours post-injection. Behavioural testing was performed as above, except that the contralateral side was plotted separately and used as a control. At the end of the test period, the animals were sacrificed with CO_2 and a piece of tail removed for verification of genotype.

5.2.4 Formalin test

Mice were placed on a platform made of fine netting and clear histology pots placed over them. They were acclimatised to the environment for 1 hour on the day before testing and another hour on the day of testing. 20µl of formalin (5% in saline, BDH) was injected into the left hindpaw of the awake animals and behavioural testing commenced immediately.

Pain behaviour was assessed as the amount of time spent licking or biting the injured paw during each 5 minute period after the injection. Testing was performed for 1 hour post-injection. At the end of each test, the animals were sacrificed with CO_2 and a piece of tail removed for verification of genotype.

5.2.5 Nerve injury

In order to induce neuropathic pain, we used the spared nerve injury model described by Decosterd and Woolf (2000). Mice were anaesthetised with halothane (2-3% in medical oxygen) and the skin and fascia of the left lower thigh incised, revealing the trifurcation of the sciatic nerve. The common perineal and tibeal nerves were then ligated with 5-0

Mersilk suture and then axotomised distal to the ligation. The sural nerve was left intact. Muscle and skin were then sutured with 5-0 Mersilk sutures.

Animals were left to recover and then returned to their cages in the animal holding facility. Von Frey hair testing was performed prior to surgery, and then 2, 4, 8, 11 and 14 days after surgery. Because this model produces hypersensitivity in the lateral region of the paw (the sural nerve-innervated area; Figure 5.1A), this area was selectively stimulated with the Von Frey hairs. After the 14 day period, the animals were sacrificed by cervical dislocation and a piece of tail removed for verification of genotype.

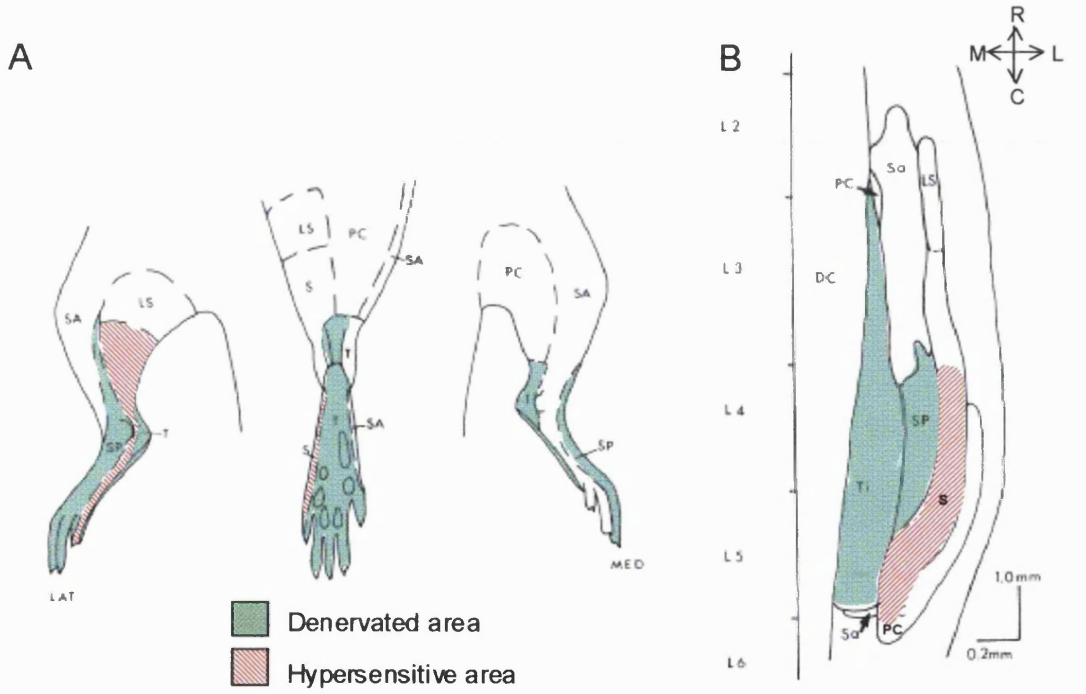

Figure 5.1
Diagram showing the dermatomes of the hindpaw and how they are affected by SNI surgery. (A) shows the area of the hindpaw that becomes hypersensitive after SNL, lying on the lateral aspect of the paw and leg. (B) shows the central innervation of the dermatomes in the spinal cord. The hypersensitive cells of the sural region are located on the lateral aspect of the dorsal horn, between L4 and L6. SA=Saphenous; LS=Lateral Sural; T=Tibeal; S=Sural; PC=Common Perineal; SP=Superficial Perineal. Adapted from (Swett & Woolf, 1985).

5.2.6 Physiological recording

In vivo electrophysiological recordings were performed in anaesthetised mice in the same manner as described in Chapter Five. However, because the experiments were performed on animals with spared nerve injury, only the cells that were innervated by the sural nerve were recorded (the others had lost primary afferent input as a result of the injury; Figure 5.1B). As a result the data were compared with cells from the naïve animal that had sural nerve receptive fields.

5.3 Results

5.3.1 Baseline behavioural responses

Acute sensory and pain behaviour was assessed in T286A mutant mice and their wild-type littermates in terms of mechanical (Von Frey hair test) and thermal (Hargreaves test) sensitivity. Plotting the number of withdrawals against force revealed clear sigmoidal curves for both mutant and wild type (Figure 5.2A). The mechanical EF50 (the force that caused withdrawal 50% of the time) was 1.66±0.3g for wild-type (n=7) and 1.70±0.2g for mutant (n=8; Figure 5.2B). The baseline heat withdrawal times were 2.34±0.4s for wild-type (n=6) and 2.29±0.3s for mutants (n=6; Figure 5.2C). Neither mechanical nor heat thresholds were significantly affected by the mutation ($p>0.05$, unpaired t tests).

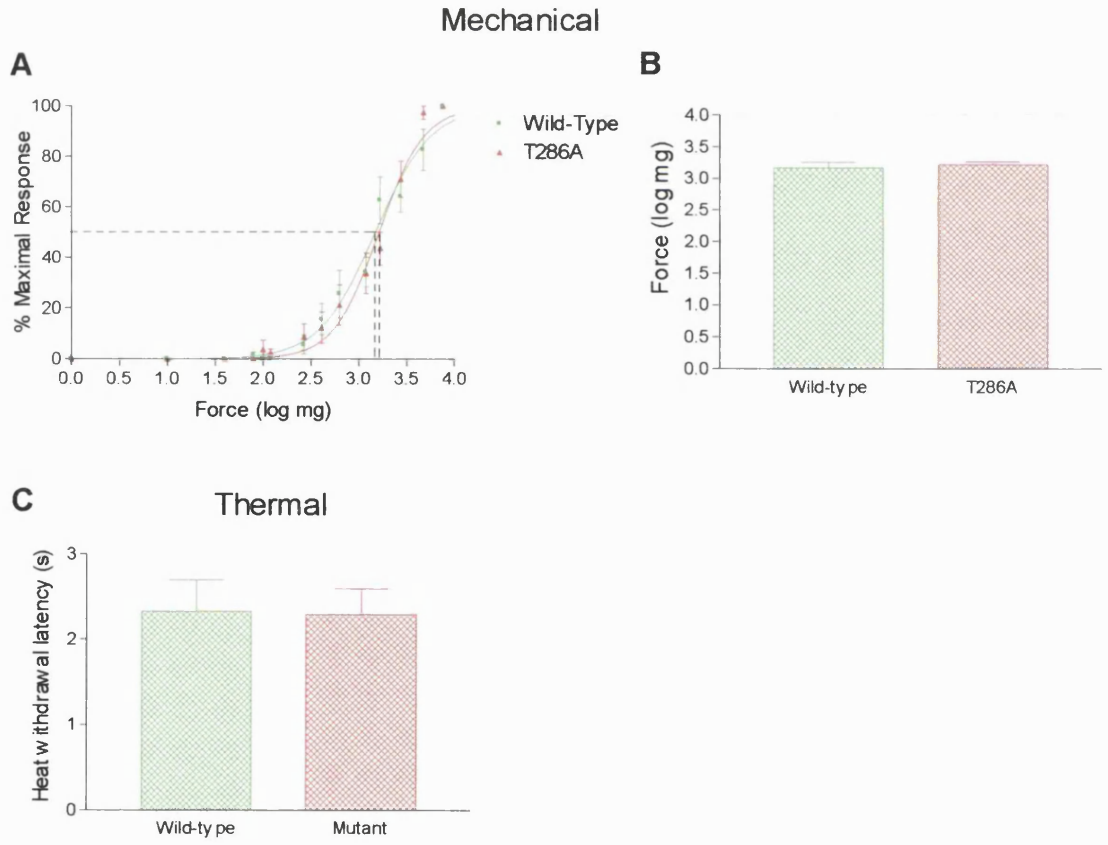

Figure 5.2
Baseline behavioural responses in the wild-type and T286A mutant. (A) shows the log force-response curve for the mechanical withdrawal test. Both wild-type and mutant responses display sigmoidal curves. The dotted line demonstrates the EF50 for each group. (B) shows the EF50s for mutant and wild-type. (C) shows the time taken for mice to withdraw their paws in response to radiant heat stimulation. No significant differences were seen between the genotypes in either behavioural test.

5.3.2 Responses to carrageenan inflammation

Inflammation was induced by injection of carrageenan into the plantar left hind-paw in a manner known to produce hyperalgesia (Hargreaves *et al.*, 1988; Meller *et al.*, 1994). Four hours after injection, both groups displayed a local inflammatory response characterised by oedema and reddening. Both groups developed thermal and mechanical hyperalgesia, with wild-types displaying a drop in EF50 to 0.272±0.08g (n=3) and mutants showing a drop to 0.228±0.06g (n=3; Figure 5.3A and 5.3B). This corresponds to a drop to 30.7±7% of baseline for wild-types and 18.8±6% for mutants. Neither the EF50s after inflammation, nor the percentage drop in threshold were significantly different between genotypes. Sensitivity to mechanical stimulation was not affected contralateral to the injury in either group (data not shown).

The mice also showed significant reductions in withdrawal latencies in response to radiant heat stimulation. The wild-types showed a drop in withdrawal latency to 0.72±0.1s (n=3), while mutants showed a drop to 0.70±0.3s (n=3; Figure 5.3C). This corresponds to a drop to 26.6±5% of baseline for wild-types and 20.9±11% for mutants. The reductions in latency were not significantly different between the genotypes. Heat sensitivity was not affected in the contralateral paw in either group (data not shown).

6.3.3 Responses to formalin injection

Injection of 5% formalin into the left hindpaw resulted in a clear biphasic response, as described previously. Pain behaviour was assessed as licking and biting of the treated paw, and was seen in both wild-types and mutants (n=4 for each group). The first phase started immediately after injection, and peaked during the first five minutes (time spent licking was 57.03 ±7.2s for wild-types and 70.02±12.3s for mutants during the first five minute period; Figure 5.4). This peak was not significantly different between the groups ($p>0.05$, two-way ANOVA with Bonferroni's posttest). The first phase was followed by a period of low activity in terms of attention paid to the injured paw, although the animals still displayed pain behaviour in the form of shivering, curling into a ball and piloerectus.

The second phase of licking/biting commenced at 15 minutes post-injection, and peaked at 35-40 minutes in the wild-type and 30 minutes in the mutant. Two-way ANOVA revealed a significantly reduced amount of licking/biting in the mutant compared to the wild-type. The peak time spent licking in the mutant was 55.67±21.7s, whereas in the

wild-type, it was 103.2±19s ($p<0.01$, two-way ANOVA; Figure 5.4). The results therefore show that, while the acute phase of the formalin test is intact in the T286A mutant, the second, chronic phase is significantly attenuated in these mice.

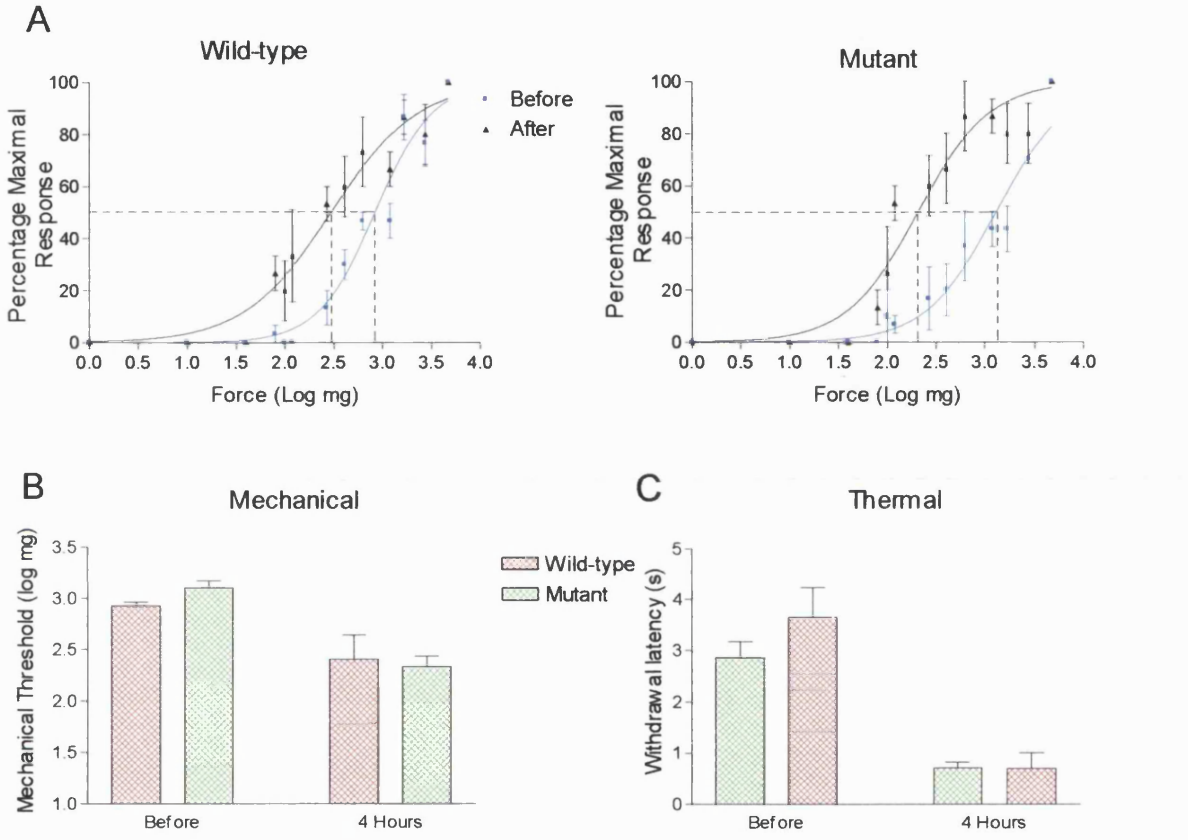

Figure 5.3
Effects of carrageenan inflammation on behavioural responses in the wild-type and T286A mutant. (A) shows pooled force-response curves for the wild-type (left panel) and mutant (right panel) before (blue lines) and after (black lines) inflammation. The dotted lines represent the EF50 for each condition. (B) shows the pooled EF50s for mutant and wild-type before and after inflammation (n=3 for each group). (C) shows withdrawal latencies to heat stimulation before and after inflammation (n=3 for each group).

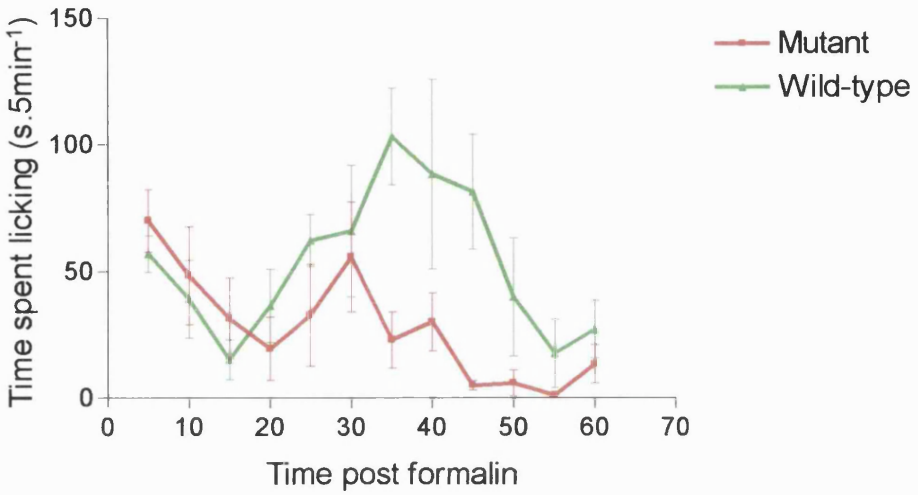

Figure 5.4
Pain behaviour in response to formalin injection in wild-type andT286A mutant mice. Time spent licking and biting the injured paw was timed in 5 minute blocks. The mutant response was significantly reduced compared to wild-type (n=4 for each group).

5.3.4 Responses to nerve injury

Cutting and ligating the common peroneal and tibeal nerves resulted in a loss in sensitivity of the denervated skin areas and allodynia in the intact skin (the area innervated by the sural nerve; Figure 5.1). This is in keeping with previous findings (Decosterd and Woolf, 2000). Significant drops in the EF50 were seen in wild-type at 4 days post-surgery (from 2.2±0.3g to 0.32±0.09g; n=4; $p<0.05$, repeated measures ANOVA with Tukey's Multiple Comparison Test; Figure 5.5A, left panel, and 5.5B) and lasted for the course of the experiment. In the mutant, significant drops in the EF50 were observed by 2 days post-surgery (from 1.8±0.3g to 0.23±0.1g in mutants; n=5; $p<0.01$, repeated measures ANOVA with Tukey's Multiple Comparison Test; Figure 5.5A, right panel, and 5.5B). By day 4, the mutant group had developed significantly enhanced allodynia compared with wild types (EF50 was 0.32±0.1g for wild-types and 0.03±0.008g for mutants; $p<0.001$, two-way ANOVA with Bonferroni's posttest; Figure 5.5B) and this was maintained for the duration of the experiment (up to day 14 post-surgery). Withdrawal latencies from radiant heat could not be measured in these animals as the hypersensitive area was seen on the lateral aspect of the paw and not on the plantar surface.

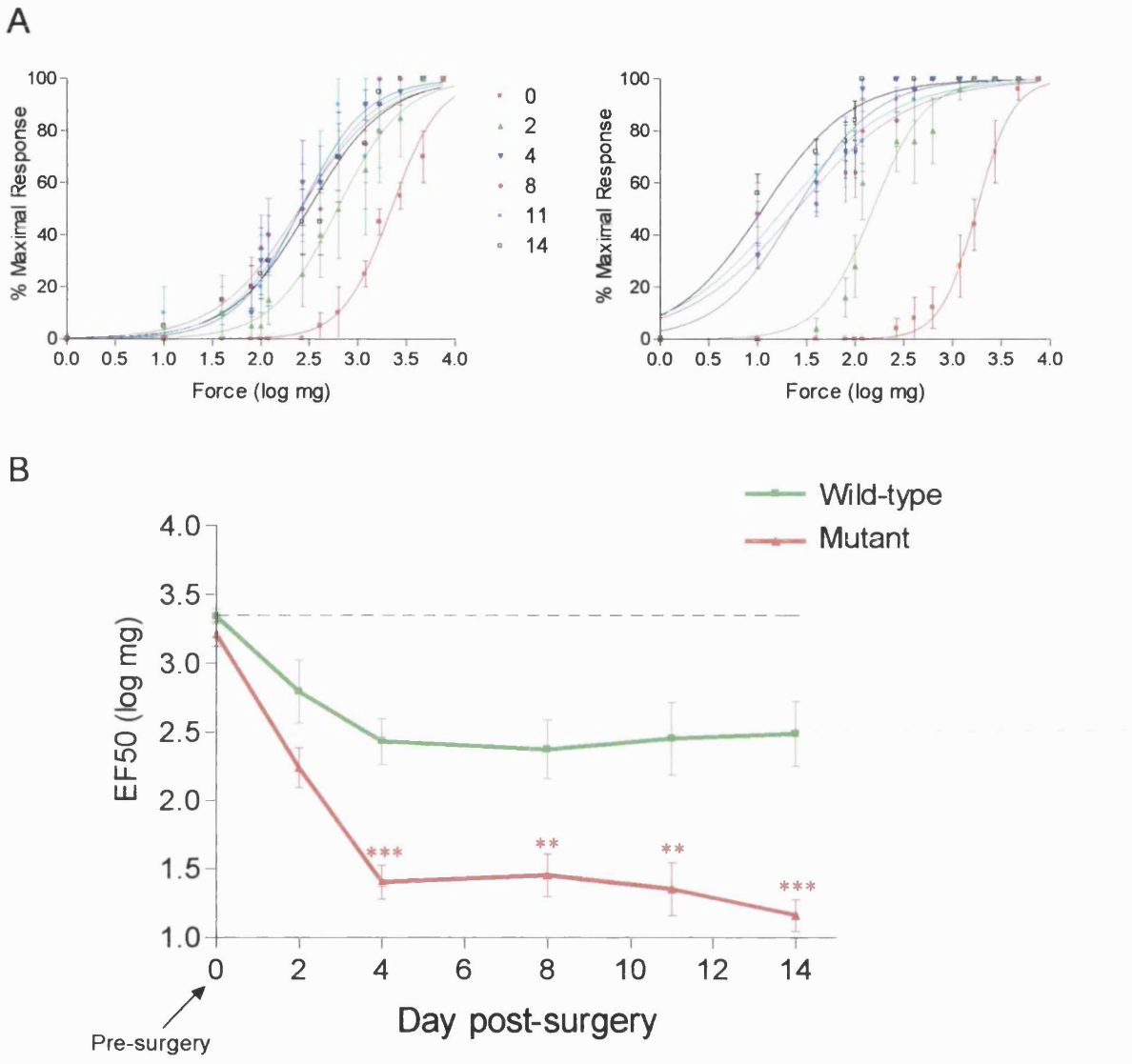

Figure 5.5
Mechanical responses after spared nerve injury in wild-type and T286A mutant mice. (A) shows the pooled force-response curves at each post-surgery day tested (day 0 is pre-surgery levels) in the wild-type (n=4; left panel) and mutant (n=5; right panel). The curve shifts to the right as the withdrawal threshold drops. (B) shows the pooled EF50s for mutant and wild-type in the two weeks post-surgery, showing a significantly larger drop in threshold in the mutant compared to wild-type.

5.3.4.1 Electrophysiology of dorsal horn cells in SNI mice

It order to test whether the nerve injuery-induced pain behaviour was produced by alterations in sensory rather than motor circuits, we performed *in vivo* extracellular recordings of dorsal horn neurones in anaesthetised wild-type and mutant mice 8-14 days after SNI surgery.

Because SNI surgery results in a large proportion of the leg, except that area innervated by the sural nerve, becoming denervated (Figure 5.1), the baseline data were restricted to those cells that responded to stimulation of the sural region, to allow comparison between the groups of cells. Spontanteous activity of dorsal horn neurones was signicantly increased in animals that had received SNI surgery. This could be seen as both the number of cells that displayed spontaneous activity (from 18% and 12.5% to 84% and 96% in wild-types and mutants respectively; Table 5.1), and in the frequency of this activity (from 0.65±0.3Hz and 0.31±0.2Hz to 4.2±1.1Hz and 6.8±1.4Hz in wild-type and mutant respectively; Figure 6.6). No significant difference, however, was observed between mutant and wild-type (p=0.06, unpaired t test).

	Wild-type	Mutant
Naïve	4/23 (18%)	2/16 (12.5%)
SNI	20/24 (83%)	21/22 (96%)

Table 5.1
Proportions of cells that displayed spontaneous activity

The cells that displayed spontaneous activity could be divided into those that had a cutaneous input and receptive fields (ie those that received input from the intact sural region), and those that did not display a receptive field (those that received input from the denervated from region of the paw). Cells that had been denervated showed significantly higher levels of spontaneous activity than those that had intact receptive fields. In the wild-type, the denervated cells showed activity at a mean frequency of 8.98±1.9Hz, while the mutant showed mean frequencies of 12.67±2.8Hz (Figure 5.7A). There was no significant difference between genotypes ($p>0.05$, unpaired t test). Of the cells that had intact connections, significantly higher levels of activity were observed in the mutant (2.77±0.6Hz) compared to wild-type (1.33±0.3Hz;$p>0.05$, unpaired t test; Figure 5.7B).

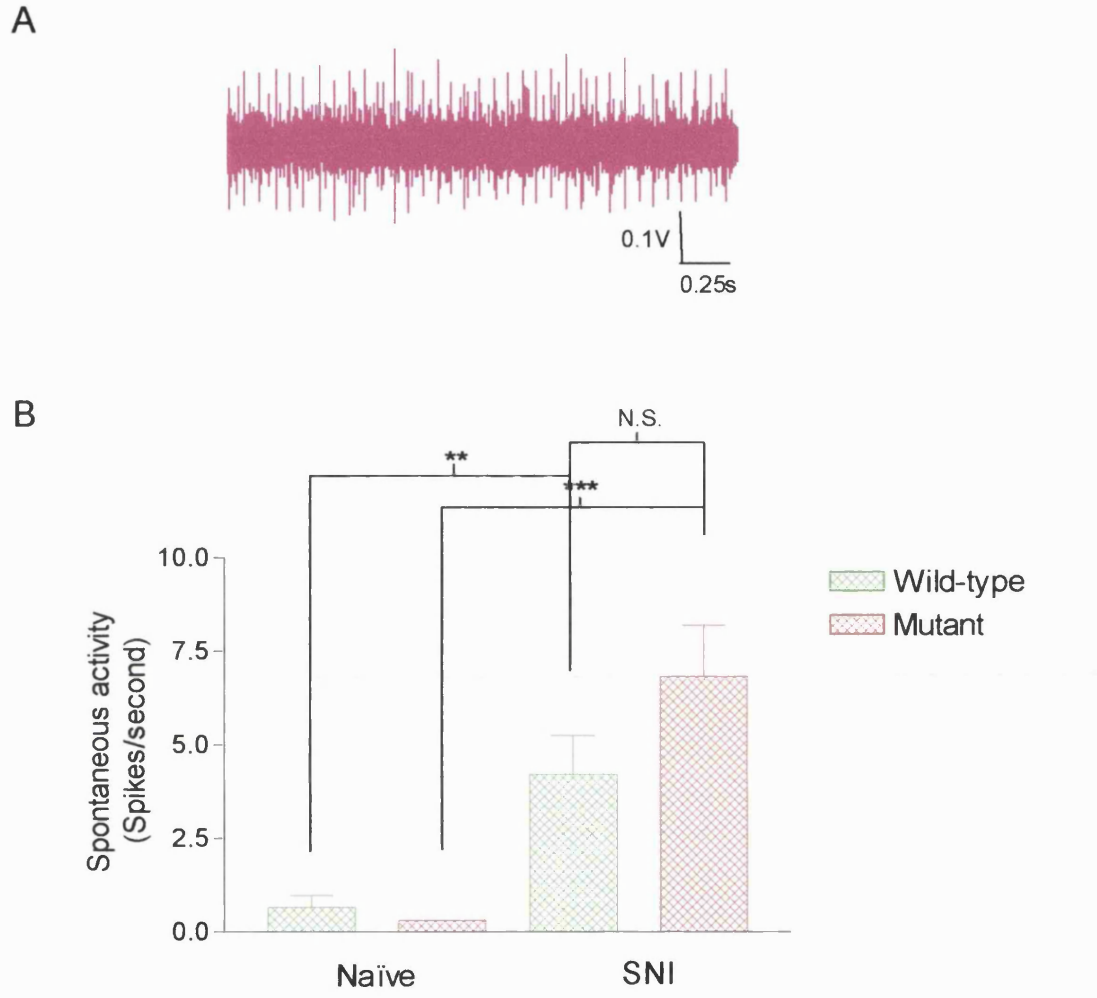

Figure 5.6
Total spontaneous activity in dorsal horn cells from naïve and SNI-treated wild-type and mutant mice. (A) shows an example of spontaneous activity from a denervated cell from a mutant mouse. (B) shows a comparison of spontaneous activity between states and genotype. While the SNI-treated group displayed significantly higher levels of activity compared with naïve, there was no difference between the genotypes.

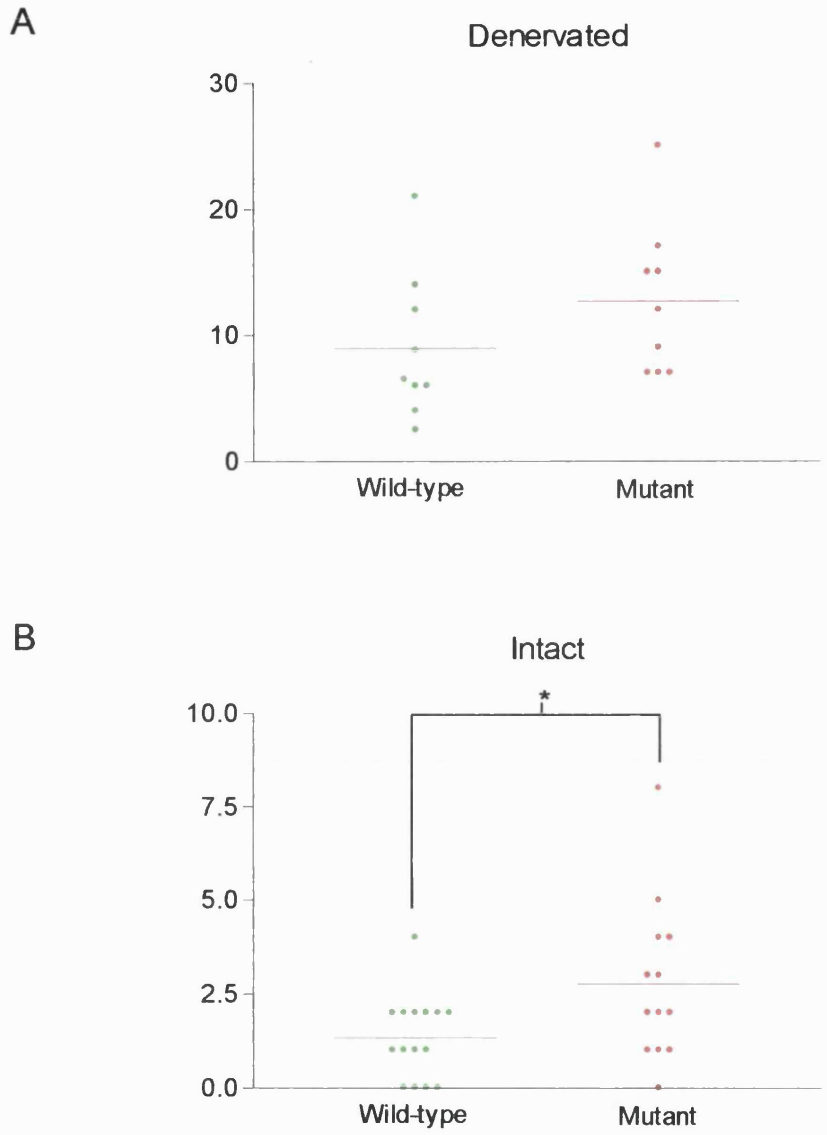

Figure 5.7
Spontaneous activity in cells from the dorsal horn of wild-type and mutant mice, as defined by their receptive field properties. (A) shows the activity from cells that had lost their primary afferent input as a result of the SNI surgery. No significant difference was seen between genotypes. (B) shows the activity of cells that had an intact afferent input. The activity was reduced compared to those that had been deinervated (A), but was significantly higher in the mutant compared to the wild-type.

Sural nerve receptive fields sizes were significantly decreased in SNI-treated animals. In the wild-type, the naïve receptive field size was 2173±245 units, and this was significantly reduced to 880±133 units after injury (Figure 5.8; $p<0.01$; one-way ANOVA with Tukey's multiple comparison test). In the mutant, the naïve receptive field size was 3342±484 units, and 1072±230 units in SNI-treated animals (Figure 5.8;

$p<0.001$; one-way ANOVA with Tukey's multiple comparison test). The receptive field sizes of SNI-treated mutants were not significantly different from the SNI-treated wild-types.

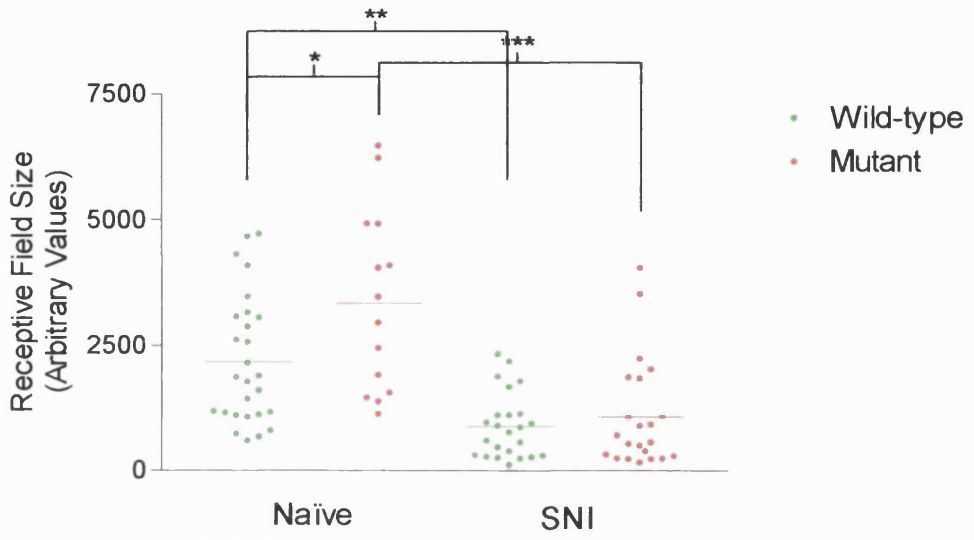

Figure 5.8
Receptive field sizes of naïve and SNI-treated wild-type and mutant mice. Both genotypes showed significantly smaller receptive fields after SNI compared with naïve. No significant difference, however, was observed between the genotypes after SNI.

The Von Frey thresholds were also reduced in both wild-type and mutant mice. In the naïve wild-type, the mechanical threshold to elicit spike activity was 0.85±0.36g (n=21), and this was reduced to 0.24±0.08g (n=15) after SNI surgery, although this reduction did not reach significance (Figure 5.9; $p>0.05$, one-way ANOVA with Tukey's multiple comparison test). In the mutant, the threshold was 0.65±0.16g in the naïve (n=11) and 0.13±0.02g after SNI (n=15; Figure 5.9; $p<0.01$, one-way ANOVA with Tukey's multiple comparison test). No significant differences were observed between the genotypes after SNI surgery.

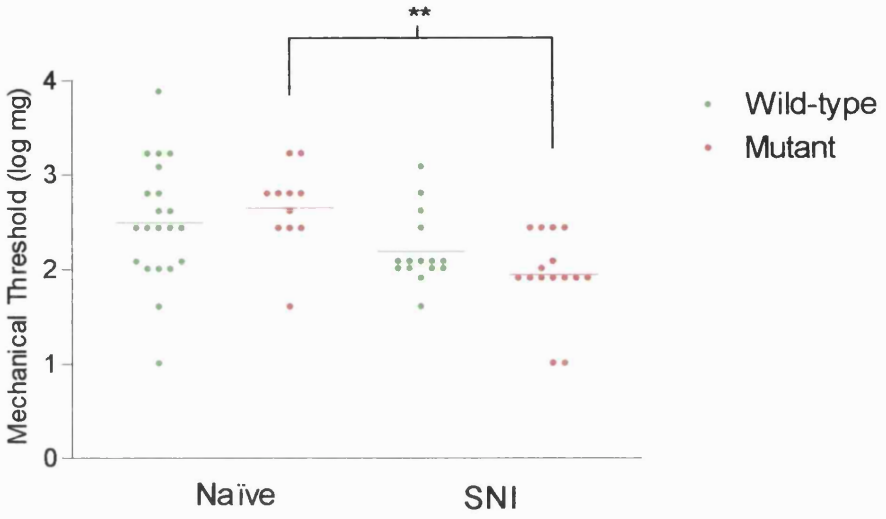

Figure 5.9
Von Frey hair thresholds in naïve and SNI-treated wild-type and mutant mice. Both genotypes displayed a significant reduction in mechanical threshold after SNI surgery, although no significant differences were seen between the SNI-treated groups.

Finally, we examined the number of spikes elicited by Aβ intensity electrical and mechanical stimulation. Because stimulation of naïve animals could produce firing of nerves that were cut in the SNI-treated group, a fair comparison between the states could not be made. Therefore, only data from the SNI-treated groups were analysed. Electrical stimulation of the skin produced significantly more spike activity in mutant compared to the wild-type animals. In the wild-type, electrical stimulation at twice the threshold required to produce Aβ firing elicited 2.67±0.3 spikes (n=15), and in the mutant, this stimulation elicited 5.18±0.9 spikes (n=11; $p<0.01$, unpaired t test; Figure 5.10A). Mechanical skin stimulation at two Von Frey hairs higher than threshold also elicited significantly more spikes in the mutant (6.07±0.7 spikes; n=15) compared to the wild-type (4.0±0.3 spikes; n=14; $p<0.05$, unpaired t test; Figure 5.10B). Because naïve data were not available in these experiments, the SNI results were analysed with unpaired t tests, as opposed to one-way ANOVAs, and is therefore less accurate than the earlier experiments, where variances could be analysed across a number of situations.

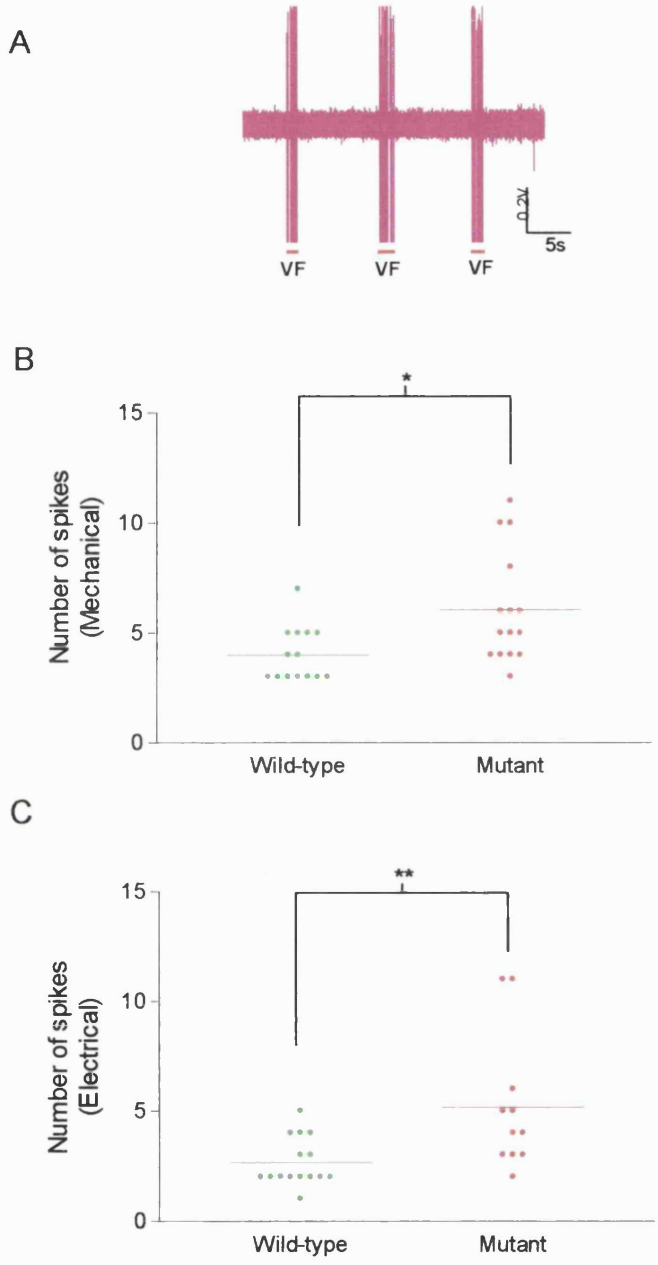

Figure 5.10
Number of spikes evoked by A fibre stimulation in wild-type and mutant SNI-treated mice. (A) shows an example of the spikes elicited by stimulation at two Von Frey hairs above threshold. (B) shows the pooled number of spikes elicited by this mechanical stimulation. The mutant displayed a significantly higher number of spikes elicited compared to wild-type ($p<0.05$). (C) shows the number of spikes produced by electrical stimulation of the skin at twice A fibre intensity. The mutant displayed a significantly higher amplitude than the wild-type ($p<0.01$).

5.4 Discussion

We have shown that the T286A mutant displays altered behavioural and electrophysiological profiles to various forms of injury. In summary, we have demonstrated that,

- Acute pain processing is not affected by the T286A mutation.
- Carrageenan-induced inflammatory pain is not affected by the T286A mutation.
- The second phase of the formalin response is significantly attenuated in the T286A mutant compared to wild-type.
- Neuropathic pain as a result of spared nerve injury is enhanced in the T286A mutant compared to wild-type, and is accompanied by increased excitability of the dorsal horn neurones.

These findings suggest a complex role for the kinase in mediating different forms of noxious processing, as will be discussed below.

5.4.1 Acute pain processing in the T286A mutant

Despite displaying larger receptive field sizes and altered connectivity in the dorsal horn, the T286A mutant did not show any gross deficits in its responsiveness to mechanical or thermal stimulation. While this implies that the altered circuitry does not affect spinal sensory processing, it is worth noting that the behavioural testing is by no means an indicator of tactile sensitivity. Von Frey hair and thermal testing is used to study the flexion withdrawal reflex in animals and neonatal humans, and this reflex involves simple spinal circuitry. In the mouse, stimulation with Von Frey hairs causes lifting of the paw when the force is great enough to activate Aδ and C nociceptors, which in turn activate interneurones that result in firing of motorneurones and subsequent withdrawal of the limb (Woolf & Swett, 1984). Therefore, while the signalling to noxious mechanical stimulation appears to be intact, the behavioural test gives little idea as to the more subtle aspects of tactility. For example, large receptive fields are associated with a reduction in spatial discrimination, as stimulation produces activation of a greater number of dorsal horn neurones, thus making for a less specific firing profile. Such alterations in these subtle characteristics of tactile sensitivity would be detectable by the relatively crude Von Frey test.

The flexion withdrawal reflex in response to heat stimulation shares similar mechanisms to the mechanical withdrawal reflex, so that C fibres depolarise the superficial dorsal horn neurone, which in turn produces firing in the motorneurone (Woolf & Swett, 1984). It is somewhat surprising that this reflex was not affected by the mutation, considering the reduced C fibre input to the dorsal horn in these animals. However previous studies that have ablated C fibres by neonatal application of capsaicin, have also shown residual heat and mechanical processing (Nagy & van der Kooy, 1983), suggesting alternative mechanisms for transmission. As the C fibre input to lamina II appeared normal in the mutant animal (see Chapter Four), it is possible that the signal may not require transmission to the deep dorsal horn, but rather transmits to the projection neurones of lamina I.

5.4.2 Inflammatory pain in the T286A mutant

We have demonstrated that two models of inflammation in the mouse produce very different effects in the T286A mutant. Intradermal injection of carrageenan produced pain-related behaviour of a magnitude similar to that seen in the wild-type. Both groups displayed local reddening and oedema, and responded to lower mechanical forces and heat ramps ipsilateral to the injury. Therefore, it would appear that CaMKII autophosphorylation is not required for this type of noxious signalling. Pain behaviour in response to formalin injection, however, was significantly attenuated in the mutant. While the first phase, which is associated with hyperactivity of primary afferents, was unaffected, the second phase, which is often characterised as an inflammatory response, was suppressed in the T286A mutant. Such a discrepancy between models has not been shown previously, and suggests that disparate mechanisms occur in the different models.

5.4.2.1 Carrageenan inflammation

Carrageenan inflammation produces a cascade of biochemical processes that act to remove injured tissue and to promote healing. The cascade includes the release of cytokines, prostaglandins, bradykinin and histamine which sensitise the primary afferents, thus making them more excitable to external stimulation (Chuang *et al.*, 2001; Julius & Basbaum, 2001; Gold *et al.*, 1998). The increased activity of the afferents can then produce central sensitisation in the dorsal horn. Injection of carrageenan does not produce high-frequency bursting of C fibres, therefore, but rather produces a slow-onset

sensitisation through peripheral effects producing central alterations (Julius & Basbaum, 2001). While the evidence for a role of NMDA receptors in carrageenan-induced inflammatory pain are ample (see Introduction), the majority of studies have demonstrated this role as mediation of the sensitisation, as opposed to an involvement in induction. Rather than the NMDA receptor acting as the gateway for high levels of calcium entry and subsequent alterations in AMPA receptor funtion, therefore, it instead becomes more capable of contributing to the postsynaptic depolarisation at basal transmission levels, leading to a potentiation of the synapse.

NMDA receptors are phosphorylated by tyrosine kinases, specifically by Src acting at the NR2B subunit (Suzuki & Okumura-Noji, 1995; Moon *et al.*, 1994). Tyrosine kinase phosphorylation acts to increase the NMDA receptor-activated conductance, as intracellular application of activated tyrosine kinases causes potentiation of NMDA currents (Wang & Salter, 1994). Therefore, increases in phosphorylated NR2B allows increased excitatory drive through the NMDA receptor, and subsequent increases in synaptic efficacy. Recent evidence has demonstrated that NMDA receptor function is increased after carrageenan inflammation, as application of exogenous NMDA produces a significantly greater facilitation of C fibre-evoked currents in carrageenan-treated rats compared to controls (Rygh *et al.*, 2001). This increased function is probably caused by tyrosine phosphorylation as intrathecal application of the selective inhibitor, lavendustin A, reduces carrageenan/kaolin-induced mechanical hyperalgesia (Sato *et al.*, 2003). Furthermore, CFA-induced hyperalgesia is accompanied by tyrosine phosphorylation of NR2B (Guo *et al.*, 2002). This phosphorylation was found to follow a timecourse similar to the persistence of peripheral noxious stimulation, early development of hyperalgesia and stimulus-matched plasma extravasation. Additionally, the phosphorylation, along with the pain behaviour, was blocked by application of lidocaine, but returned after the anaesthetic had worn off, implying a dependence on ongoing primary afferent drive. The authors proposed that the tyrosine phosphorylation of NR2B was mediated by group I metabotropic glutamate receptors (mGluRs) and NK1 receptors, whereby activation of these G protein-coupled receptors activated phospholipase C and caused calcium release from intracellular stores. Consistent with this hypothesis, blockade of mGluRs and NK1 receptors prevented the phosphorylation of NR2B (Guo *et al.*, 2002). Behaviourally, mechanical hyperalgesia can be blocked by intrathecal application of group I mGluR (Young *et al.*, 1997) or NK1 (Gao *et al.*, 2003)

antagonists. These effects were only seen if the antagonists were applied after the induction of inflammation, in keeping with the requirement for continued afferent drive.

The evidence described here strongly suggests that central sensitisation, as a response to carrageenan inflammation, is manifest as increased current flow through the NMDA receptor as a result of tyrosine phosphorylation (Figure 5.11A). This hypothesis would explain why NMDA receptor antagonists are effective in treating inflammatory pain (Eisenach & Gebhart, 1995; Laird *et al.*, 1996), and suggests that this form of sensitisation is different from LTP, where NMDA receptors are only involved in induction and have a limited role in maintentance (Bliss & Collingridge, 1993). In support of this, there is little evidence to suggest that inflammatory pain is mediated by increases in AMPA receptor function. One group have reported that intrathecally-applied AMPA receptor antagonists have a larger effect in carrageenan-treated animals compared to controls, suggesting an increase in receptor function (Stanfa & Dickenson, 1999), although it is worth noting that a similar results was obtained with application of kainate receptor antagonists. Because kainate receptors are only found presynaptically in the adult (Stegenga & Kalb, 2001; Hwang *et al.*, 2001), it is possible that the AMPA receptor antagonists were acting on a presynaptic locus.

5.4.2.2 Response to formalin

In contrast to the carrageenan test, the T286A mutant displayed a reduction in pain behaviour after injection of formalin. Although this test is also used as a measure for inflammatory pain, our data suggest that the mechanisms mediating the response are very different for those mediating carrageenan-induced nociception.

The two phases of the formalin test were originally believed to reflect an initial acute phase of C fibre activity, which produced an immediate pain response as characterised by shaking and licking of the injected paw, followed by a second, longer-lasting phase mediated by central mechanisms (Coderre & Melzack, 1992; Coderre *et al.*, 1993). This second phase was believed to be a result of the bursting activity of C fibres during the first phase, which produced central sensitisation in the dorsal horn in a manner similar to LTP induction in the hippocampus (Coderre *et al.*, 1993; Le Bars *et al.*, 2001; Ji *et al.*, 2003). However, recent evidence suggests a more complex mechanism by which formalin elicits pain behaviour in rats. For example, it was shown that the C and Aδ fibres themselves responded to formalin in a biphasic manner with a timecourse that

matched the pain-related behaviour (Puig & Sorkin, 1996; McCall *et al.*, 1996), suggesting that both phases of the response were dependent on primary afferent activity. Secondly, it was found that blockade of the first phase with local anaesthetics did not block pain behaviour in the second phase, again suggesting ongoing afferent hyperexcitability (Taylor *et al.*, 1997; Dallel *et al.*, 1995). Other groups, however, have reported that blockade of the first phase with opioid analgesia (followed by naloxone treatment to restore sensitivity) did attenuate pain behaviour in the second phase (Abram & Yaksh, 1993; Dickenson & Sullivan, 1987b). Finally, the second phase of the response elicits an inflammatory reaction (Taylor *et al.*, 2000) that can be attenuated by application of non-steroidal anti-inflammatory drugs (Malmberg & Yaksh, 1992; Jourdan *et al.*, 1997). Therefore it appears that a large component of the second phase of pain behaviour is caused by peripheral hypersensitivity.

The attenuation of the second phase of the formalin test in T286A mutants could be explained by the fact that central sensitisation is not occurring in response to the burst activity of the C fibres during the first phase. By this mechanism, the high-frequency C fibre activity would depolarise the postsynaptic dorsal horn neurone to an extent that allows for calcium entry through NMDA receptors. This calcium entry would be of a level that activates CaMKII, allowing it to enter its autophosphorylated state. Once phosphorylated, the kinase would translocate to the membrane and phosphorylate/insert AMPA receptors, producing an increase in synaptic strength. This would lead to increased activity of dorsal horn neurones in response to the tonic firing of primary afferents caused by the inflammatory component of the formalin response. Preventing the autophosphorylation of CaMKII would prevent the AMPA receptor mediated, but not the inflammation-induced NMDA receptor-mediated increase in synaptic strength or the tonic firing, which would contribute to the residual pain behaviour seen in the mutants (Figure 5.11B). In support of this theory, a recent study that performed a spatiotemporal knockout of NR1 in the adult dorsal horn found that the second phase of the formalin test was ameliorated to a level similar to that seen in the present study (South *et al.*, 2003). Secondly, induction of high-frequency firing of C fibres by capsaicin produces central sensitisation in the dorsal horn and accompanying pain behaviour in a manner that can be blocked by CaMKII inhibitors (Fang *et al.*, 2002).

Some controversy surrounds the precise mechanisms of induction of the second phase of the formalin test. As stated previously, a large body of evidence shows that blockade of the first phase of the formalin test with lidocaine or remifentanil does not decrease the pain behaviour in the second phase, implying that the second phase is only mediated by afferent drive (Taylor *et al.*, 1997; Dallel *et al.*, 1995). A possible explanation for such a disparity could be that a mouse that has not experienced the first phase of the response may behave more fervently than one that has experienced it. Indeed, the interphase of the response is believed to be caused by activation of descending inhibitory pathways (Matthies & Franklin, 1992; Henry *et al.*, 1999), and so it is possible that animals that do not feel the acute phase would not receive any analgesia from higher centres, and would thus feel the inflammatory phase more acutely.

The contribution made by enhanced AMPA receptor function to the second phase of the formalin test could be further tested with biochemical assays of phospho-GluR2 at different stages of the formalin test. Our hypothesis would predict that there would be an increase in phosphorylated AMPA receptors in the dorsal horn soon after injection of formalin, and that this increase would last for the course of the experiment. Whole-cell electrophysiological profiles of dorsal horn cells could also lead to a better understanding of the contribution of various types of receptor, although it would be difficult to find the neurones that had been potentiated in response to the stimulus.

In conclusion, we hypothesise that the two models of chemical-induced pain described here require slightly different mechanisms of action. Carrageenan inflammation induces central sensitisation of a different to form to that classically described, whereby long-term primary afferent activity causes phosphorylation of NMDA receptors, and subsequent increases in synaptic strength through Na^+ gating, through mGlu and NK1 receptor-mediated processes (Figure 5.11A). The calcium entry through the NMDA receptors during this process is of an insufficient magnitude to activated CaMKII and cause AMPA receptor phosphorylation/insertion. The formalin test shares this mechanism during the second phase, and this contributes to the spinal hyperexcitability, but an additional form of plasticity is induced by the burst firing during phase one. This firing releases large amounts of glutamate and allows the postsynaptic NMDA receptors to shed their Mg^{2+} blocks and for calcium to enter the cell. This calcium entry is sufficient to activate CaMKII, which subsequently phosphorylates and inserts AMPA

receptors in an LTP-like manner (Figure 5.11B). This form of central sensitisation is blocked in the T286A mutant, but the inflammation-induced sensitisation is not, resulting in an attenuated, but not abolished, second phase of pain behaviour.

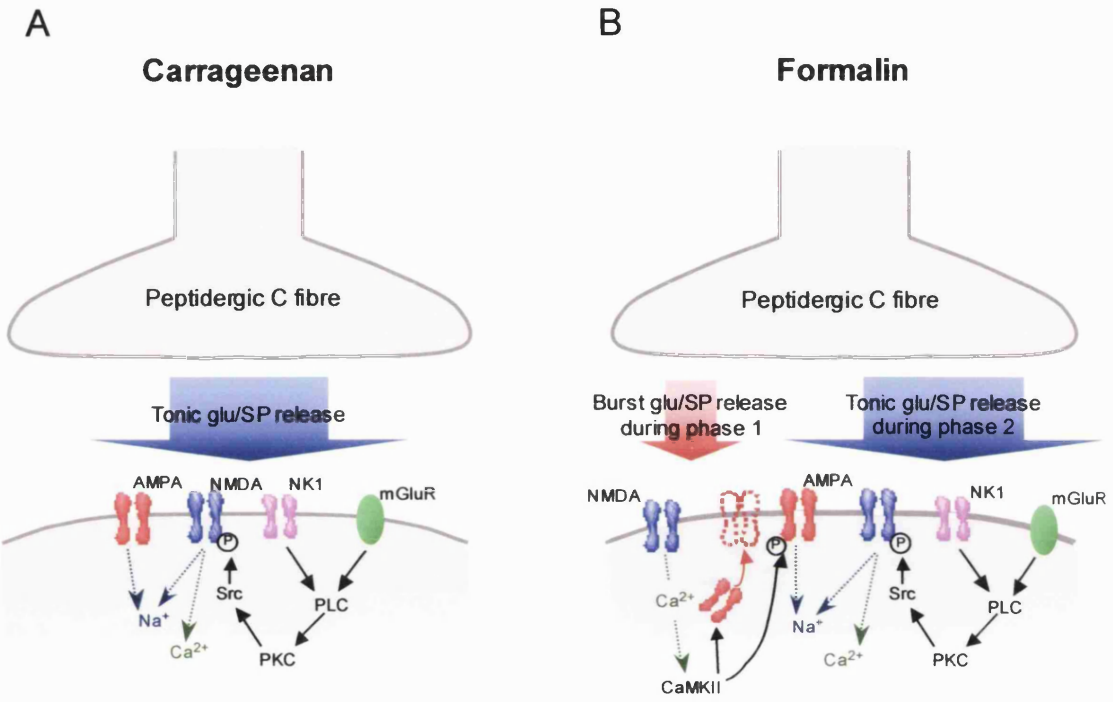

Figure 5.11
Types of central sensitisation induced by different inflammatory agents. (A) Intraplantar carrageenan injection causes a local inflammatory response that sensitises the C fibres and produces tonic firing. The release of glutamate and substance P activates mGlurRs and NK1 receptors, which signal a release from intracellular calcium stores, and subsequent tyrosine phosphorylation of NMDA receptors. This phosphorylation produces increased conductance and a lower affinity to Mg^{2+}, thus allowing the NMDA receptor to contribute to baseline transmission. While Ca^{2+} can also enter through the NMDA receptors, it is of an insufficient magnitude to produce CaMKII autophosphorylation. (B) Intraplantar formalin injection causes burst firing of C fibres that causes a large release of glutamate and subsequent opening of NMDA channels. This allows high levels of Ca^{2+} into the cell, which activates CaMKII and leads to AMPA receptor phosphorylation and insertion. The second phase also causes an inflammatory response that produces tonic glutamate/substance P release and subsequent phosphorylation of NMDA receptors.

5.4.3 Neuropathic pain in the T286A mutant

We have shown here that neuropathic pain behaviour is significantly enhanced in the T286A mutant compared to wild-type littermates. This is supported by the physiological data that demonstrate increased spontaneous activity and amplitudes of responses to low-threshold electrical and mechanical stimulation. The finding is the converse of our hypothesis, which suggested a decrease in pain behaviour after neuropathic injury because of the known attenuation of some forms of neuropathic pain after NMDA blockade and PKC deletion. The results are also difficult to interpret in light of our finding that the formalin response was attenuated in the mutant, and suggests that very different mechanisms mediate neuropathic pain behaviour.

With regard to the physiological data, an important finding was that the spontaneous activity was significantly increased in the dorsal horn neurones. The increase was seen in both cells that had intact input and those that had been denervated. Increased spontaneous activity has been previously observed in both the dorsal horn (Chapman *et al.*, 1998) and in the DRGs of both damaged and undamaged axons (Boucher *et al.*, 2000). Because of this latter finding, we could hypothesise that the increased postsynaptic activity is caused by ectopic activity in the primary afferents, perhaps as a result of upregulation of sodium channels (Gold *et al.*, 2003). It is worth noting that it is the neurones with the intact input that have increased spontaneous activity in the mutant compared with the wild-type. This is in accordance with the behavioural data, as it would be this population of cells that are activated by Von Frey hair stimulation.

With regard to the decrease in receptive field size as a result of the nerve injury, the result is almost certainly artefactual. As can be seen in Figure 5.1, the sural nerve innervates only a tiny portion of the lateral hindpaw. Therefore removal of all other innervation would mean that the receptive fields could only be contained within this region. Despite the fact that we attempted to only record from cells that had sural innervation, they would probably have also received input from neighbouring nerves. A more useful control, therefore, would have been to acutely cut the nerves during the experiment, thus removing innervation but not allowing for the long-term pain effects. Nerve damage, such as after partial sciatic nerve ligation (Takaishi *et al.*, 1996) and spinal nerve ligation (Suzuki *et al.*, 2000) has often been described to produce *increases* in receptive field size. Even if the SNI model resulted in an attempted expansion of the

receptive fields, the effects of removing all surrounding input would still result in an overall decrease in receptive field size- they would have nowhere to expand to. It is worth noting, however, that SNI surgery results in different alterations in dorsal horn physiology than do other models (Kohno *et al.*, 2003), and so it remains possible that the reduction in size is somehow a result of these changes.

Neuropathic pain is well known to be a complex phenomenon, involving peripheral sensitisation (Gold *et al.*, 2003), microglial activation (Tsuda *et al.*, 2003), excitotoxic cell death (Moore *et al.*, 2002), and structural reorganisation (Woolf *et al.*, 1992). The role of CaMKII in such a process is therefore difficult to elucidate simply from behavioural and electrophysiological studies. It is possible, however, to hypothesise the possible locations of involvement of the enzyme.

There are two explanations for the increases in dorsal horn excitability after nerve injury in the mutant: 1) increases in excitatory transmission or 2) decreases in inhibitory transmission.

5.4.3.1 Increases in excitatory transmission

It is possible that the T286A mutation of CaMKII produces hyperphosphorylation of AMPA receptors through autophosphorylation-independent mechanisms. For example, it is known that the kinase can be rendered permanently active by its interaction with the NR2B subunit, which allows for translocation to the postsynaptic density and subsequent phosphorylation/insertion of AMPA receptors (Bayer *et al.*, 2001). This form of activation cannot be reversed by phosphatases such as PP1, and may therefore allow the protein to remain active for very long periods of time, as would fit a profile of neuropathic pain. Such a hypothesis seems unlikely as in no other part of the T86A mutant brain has a hyperphosphorylation of AMPA been described, but only a loss in phosphorylation in response to various stimulation protocols (Hardingham *et al.*, 2003; Giese *et al.*, 1998). Secondly, such a theory would predict similar effects in response to the formalin test, so that the central sensitisation produced by the first phase would produce even larger pain responses in the second phase compared with wild-type littermates. Instead, we saw a clear attenuation of the second phase.

An alternative model for the facilitatory effect of the T286A mutation could involve presynaptic alterations. For example, a recent study has shown that mice with a specific

deletion of the kinase at presynaptic terminals displayed enhanced activity-dependent increases in the probability of neurotransmitter release at CA3-CA1 synapses of the hippocampus (Hinds *et al.*, 2003). The authors found that, while baseline transmission was unaffected in these mutants, the EPSCs were potentiated after high-frequency stimulation to a greater extent in the mutant compared to the wild-type. This potentiation was associated with increases in Pr, and implies that CaMKII places an inhibitory constraint on presynaptic release in response to high-frequency activity. It is known that primary afferents to the spinal cord fire spontaneous high-frequency bursts after neuropathic injury. As the kinase is present in the DRG, it is possible that the mutation is preventing the inhibitory constraint of the presynaptic release during this bursting, resulting in increased postsynaptic activity. However, this hypothesis is again unlikely to produce the results described here, as dorsal rhizotomy produces no noticeable reduction in expression in the dorsal horn, implying a primarily postsynaptic role for the kinase in dorsal horn processing (see Chapter 3), and the presynaptic functions of CaMKII are believed to be independent of autophosphorylation (Y. Elgersma, personal communication). Additionally, the study described above used a spatiotemporal deletion of the kinase, which is very different from the point mutation that was used in this study. It therefore appears that the enhanced sensitivity of the dorsal horn in response to nerve injury is produced by a disinhibition of the dorsal horn neurones.

5.4.3.2 Decreases in inhibitory transmission

The theory that nerve injury produces disinhibition in the spinal cord was first suggested by Devor & Wall in (Wall & Devor, 1981). In support of this, it has been shown that nerve damage by CCI produces reductions in GABA and GAD immunoreactivity (Eaton *et al.*, 1998; Ibuki *et al.*, 1997), although this finding has been disputed by another group (Polgar *et al.*, 2003). This is accompanied by reductions in the proportion of neurones that display primary afferent-induced IPSCs, and in the size and duration of these IPSCs (Moore *et al.*, 2002). Analysis of spontaneous inhibitory currents revealed a reduction on the frequency, but not amplitude, of GABA-mediated IPSCs, consistent with a loss of GABA cells, but not receptors, in the superficial dorsal horn. The authors also demonstrated that the loss was caused by selective excitotoxic cell death of GABAergic neurones (Moore *et al.*, 2002). It therefore appears that the hyperexcitability seen in the dorsal horn is produced, at least in part, by a loss in

GABAergic synaptic transmission. This hypothesis would explain the increased spontaneous activity that we observed in the SNI-treated mice.

Because our results demonstrated *increased* behavioural pain sensitivity and dorsal horn excitability in the T286A mutant, it is possible that the mutation produces increased excitotoxic cell death of GABAergic cells after spared nerve injury. Indeed, CaMKII has been implicated in neuronal death in the visual system (Laabich *et al.*, 2000) and in cortical neurones (Hajimohammadreza *et al.*, 1995), although generally as a promoter of apoptosis rather than as a neuroprotector (Wright *et al.*, 1997), as would be expected if mutation of the kinase were to increase cell death in the dorsal horn. In addition, the lack of colocalisation between CaMKII and GABA described in Chapter Three strongly suggests that the kinase does not play a role in excitotoxic GABAergic cell death in response to nerve injury.

A more likely role for CaMKII in the inhibitory processes that occur after injury is as a phosphorylator of $GABA_A$ receptors. While this is a relatively new concept, there is a growing body of evidence that CaMKII can indeed phosphorylate $GABA_A$ receptors in different parts of the brain. An interaction between the two proteins was first shown McDonald & Moss (McDonald & Moss, 1994), who demonstrated that the kinase phosphorylated the β subunit of the $GABA_A$ receptor at sites S384 and S409 in *Escherichia coli*. It was later shown that injection of an activated form of the kinase into cultured dorsal horn neurones produced increases in GABA-mediated IPSCs (Wang *et al.*, 1995). A similar increase has since been observed in cerebellar granule cells (Houston *et al.*, 2003) and cortex (Churn *et al.*, 2002; Aguayo *et al.*, 1998). Additionally, it has been shown that long-term potentiation of GABA-induced currents in cerebellar cells (a phenomenon known as 'rebound potentiation') is mediated by CaMKII, as intracellular injection of inhibitors of the enzyme block the potentiation (Kano *et al.*, 1996; Kawaguchi & Hirano, 2002). In these neurones, the potentiation of GABA currents is produced by activation of excitatory synaptic inputs, which causes calcium influx through VGCCs and subsequent CaMKII activation. Additional evidence in the cortex suggests that the calcium influx could also occur through NMDA receptors (Aguayo *et al.*, 1998). This model therefore suggests that increases in excitation can lead to increased inhibitory transmission, thus allowing for increased inhibitory tone in response to an overexcited system.

The mechanism by which phosphorylation produces increases in GABAergic currents is unclear. It has been suggested that the phosphorylation produces increase in single-channel conductance (Moss & Smart, 1996), although some evidence suggests that the action of CaMKII on $GABA_A$ receptors involves increasing the binding coefficient (B_{max}) of allosteric modulators such as muscimol (Churn & Delorenzo, 1998). The authors found that the increase in B_{max} was not accompanied by changes in K_D, implying that the phosphorylation caused an increase in the number of functional GABA receptors at the membrane.

The idea that neuropathic injury leads to increased $GABA_A$ receptor-mediated currents seems to contradict the evidence suggesting decreases in inhibition after SNI (Moore *et al.*, 2002). However, this study focused solely on the presynaptic effects of GABA transmission, and so it is possible that there is an increase in GABA receptor activity in response to the decreased levels of GABA in the cord. Indeed, it has been shown that intrathecal application of the $GABA_A$ receptor antagonist, bicuculline, at doses that did not affect basal transmission, significantly increased C fibre-mediated activity in spinal nerve ligated animals (Kontinen *et al.*, 2001). This suggests that nerve injury causes increases in inhibitory tone in the spinal cord.

Figure 5.12 shows a possible role for CaMKII in the mediation of neuronal excitablilty after nerve injury. Neurones with an intact afferent input would be more depolarised due to the loss of GABAergic inhibitory control, meaning that high levels of calcium could enter the glutamatergic synapse in response to the increased activity in the primary afferents. The calcium could then activate CaMKII leading to phosphorylation of $GABA_A$ receptors at neighbouring synapses in order to counter the hyperexcitability. In addition, there might be a CaMKII-mediated insertion of $GABA_A$ receptors in response to the reduced presynaptic inhibitory drive. Blockade of CaMKII autophosphorylation would prevent this mechanism, meaning that the dorsal horn cell would be even more excitable than in wild-type animals, resulting in a reduction in mechanical withdrawal thresholds.

Why would the kinase play a selective role in GABAergic inhibition after nerve injury, but a completely different role in response to formalin injection? The dichotomy between the results does indeed appear puzzling, but in fact the two pain states have very little in common with one another. Firstly, the timecourses of the pain behaviour

are drastically different between groups. While formalin elicits an immediate behavioural response, pain behaviour is not seen after nerve injury until day four. Secondly, the neurochemical properties of both peripheral and central neurones are drastically different after nerve injury compared with inflammation. Substance P, for example, is known to upregulated in the dorsal horn and DRG after injection of formalin (McCarson & Goldstein, 1990), whereas it is significantly downregulated after nerve injury (Sanderson Nydahl *et al.*, 2004). The neurochemical changes are accompanied by drastic increases in spontaneous activity, as described above. Such increases are not seen after inflammation, and would result in increases in general excitability within the dorsal horn, meaning that the cells would fire more easily in response to afferent stimulation.

In humans, neuropathic pain is often characterised by acute mechanical allodynia combined with thermal *hypo*algesia (Baron, 2000), which could be caused by C fibre degeneration and subsequent Aβ fibre sprouting. Combined, these data demonstrate very different mechanisms with which neuropathic and inflammatory damage lead to pain symptoms, and the discrepancies between the pain behaviour seen in the T286A mutant are not unexpected.

In conclusion, we have shown that the spared nerve injury model of neuropathic pain results in enhanced excitability in the T286A mutant compared to controls, and this is accompanied by decreased receptive field sizes and increased A fibre-mediated activity in dorsal horn neurones. I hypothesise that nerve injury induces phosphorylation of GABA receptors in response to the reduction in GABA transmission in the cord, and that this is mediated by CaMKII.

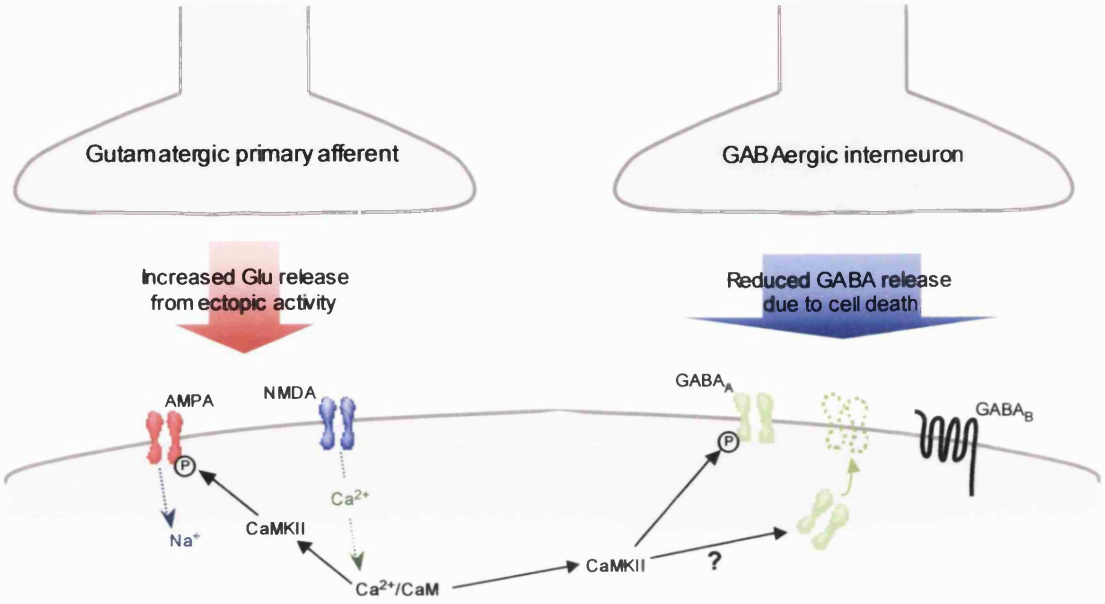

Figure 5.12
A possible role for CaMKII in nerve injury-induced pain. Calcium could enter the cell as a result of membrane depolarisation caused by decreases in inhibitory tone, combined with increased glutamate release from the intact primary afferent. This could lead to CaMKII activation and subsequent phosphorylation of AMPA receptors, but also of $GABA_A$ receptors, thereby maximising the inhibitory control in response to decreased GABA release. There may also be a CaMKII-mediated insertion of GABA receptors in response to the reduced GABAergic drive.

Chapter 6

CONCLUSIONS

6.1 Introduction

The work described here provides a comprehensive analysis of the sensory phenotype of the T286A mutant, and, as such, begins to elucidate the role of CaMKII autophosphorylation in the development and plasticity of the dorsal horn. It demonstrates firstly that mutant animals rarely display the phenotype expected from pharmacological studies, but do often reveal previously unexpected characteristics that provide insights into the subtlety of the nervous system machinery. More generally, we have discovered that CaMKII does indeed play a role, albeit a subtle one, in the development of the dorsal horn, and that this role allows for fine tuning of the neural connectivity in postnatal life.

The results presented in this thesis have been extensively discussed within each chapter, and so the aim of this chapter is to summarise our findings, and to provide a broader overview of their significance.

6.2 Summary of findings

We have demonstrated that CaMKII is present in the spinal cord from birth, and that its expression is upregulated during the first two weeks of life, to peak at P14. This expression profile matches those seen in the brain (Herms *et al.*, 1993), as well as the expression patterns of many iGluRs, consistent with the idea that synaptic activity drives expression of downstream machinery. It would be interesting to see whether blockade of NMDA receptors during this period results in lower expressions of CaMKII. As protein levels can be upregulated by interventions to the sensory system, such as in nerve injury, one might expect similar changes in response to deficient developmental plasticity.

We have examined the developmental profile of Aβ fibre terminations in the spinal cord. While such experiments have been performed before, we employed a novel technique of selectively labelling the fibres with a lipophilic tracer, thus allowing for large populations of collaterals to be measured, but also for accurate analysis of individual fibre terminals. Such a technique confirmed previous reports that Aβ fibres project into the superficial dorsal horn during early postnatal life, but then withdraw during development (Fitzgerald *et al.*, 1994). This finding offers a cellular mechanism

to explain the early hyperexcitability of the neonatal nervous systems of both rats and humans.

Contrary to our hypothesis, we found that CaMKII autophosphorylation does not play a role in the withdrawal, as the mutant displayed similar Aβ fibre termination patterns to the wild-type. Additionally, no difference was observed in the termination of C fibres, as characterised with IB4 labelling. Therefore, despite structural alterations occurring in animals that had been chronically exposed to NMDA receptor antagonists during development (Beggs *et al.*, 2002), disruption of a major downstream signalling cascade appeared not to affect the termination profile. The normal developmental profile could reflect the parallel pathways that exist in the postsynaptic density, so that abolition of one would allow the other to mediate plasticity in the newborn spinal cord.

Although no gross anatomical alterations to the primary afferent input to the spinal cord of the mutant were observed, closer physiological analysis did reveal some subtle changes in the neuronal circuitry of the dorsal horn. Our primary observations were that Aβ fibre-mediated input to the superficial dorsal horn via polysynaptic circuitry was reduced in the mutant compared to the wild-type, and that the receptive fields of dorsal horn neurones of the mutant were enlarged compared to wild-type. Both these findings suggest disruptions of the interneuronal circuitry within the dorsal horn, with respect to a reduced inhibitory modulation of dorsal horn cells resulting in larger receptive field sizes, and reduced axodendritic ranges of interneurones transmitting Aβ-mediated information to the superficial dorsal horn. Such hypotheses could be confirmed with anatomical labelling of the interneurones, and would support the findings that cultured neurones with mutations of the CaMKII protein display more aberrant patterns of axodendritic morphology compared to wild-type (Zou & Cline, 1999).

Despite the alterations to the synaptic circuitry within the dorsal horn, the T286A failed to show any alterations in the flexion withdrawal reflex or responses to the radiant heat test, implying that basic noxious processing is fully functional. However, these behavioural tests are only rudimentary, and may not distinguish the finer discriminatory aspects of tactile and thermal sensation. The larger receptive field sizes may well produce deficits in the animal's ability to spatially discriminate a tactile or noxious

stimulation, and the reduced Aβ fibre input to the superficial dorsal horn might alter some of the fine control of C fibre input to the synapse-rich glomeruli.

The finding that the mutant responds in completely different ways to different pain-inducing stimuli sheds light onto the variety of mechanisms that exist in the perception of pain. The reduction in pain behaviour in the mutant in response to the second phase of the formalin test strongly argues a case for the first phase of response mediating behaviour in the second phase. While this area clearly requires more investigation, for example by analysing the levels of phosphorylated GluR2 and NR2B, our results are the first to demonstrate a clear dichotomy between carrageenan- and formalin-induced pain, and suggest that the formalin test is not simply a model for inflammatory pain. Finally, the mutants' enhanced response to nerve injury suggests a role for CaMKII in applying a 'brake' to peripheral nerve injury-induced pain. It is possible that it does so through phosphorylation of $GABA_A$ receptors, although such a theory requires further research.

The ubiquitous nature of CaMKII makes it unlikely that CaMKII inhibitors/activators could ever be used as therapies for persistent pain states. However, this research does help to elucidate the role of this fascinating kinase in sensory processing and development, and could lead to better understanding of how the nervous system adapts to alterations in sensory input both during development and in the adult.

PUBLICATIONS

Pattinson DT, Dhayatker V, Giese KP & Fitzgerald M (2003) Calcium/calmodulin-Dependent Kinase IIα Plays a Role in Inhibition after Neuropathic Injury. *Soc. Neurosci. Abst.*

Pattinson DT & Fitzgerald M (2003) The Neurobiology of Infant Pain: Development of Excitatory and Inhibitory Neurotransmission in the Spinal Dorsal Horn. *Regional Anesthesia and Pain Medicine* **29**: 36-44.

Parsley SL, Pilgram SM, Pattinson DT, Fitzgerald M, Edwards F, Soto F (2002) Distribution Of Alpha Calcium/Calmodulin-Dependent Protein Kinase II (CaMKII) In Mouse Brain And Spinal Cord Through Postnatal Development. *Soc. Neurosci. Abst.*

Pattinson DT & Fitzgerald M (2002) Developmental Profile of Large Myelinated Fibre Terminals in the Dorsal Horn of the Rat Spinal Cord. *IASP Abst.*
DT & Fitzgerald M (2001) CaMKIIα is Expressed from Birth in the Developing Mouse Spinal Cord. *FENS Abst.*

REFERENCES

Abbott, L. F., Varela, J. A., Sen, K., & Nelson, S. B. (1997). Synaptic depression and cortical gain control. *Science* **275**, 220-224.

Abram, S. E. & Yaksh, T. L. (1993). Morphine, but not inhalation anesthesia, blocks post-injury facilitation. The role of preemptive suppression of afferent transmission. *Anesthesiology* **78**, 713-721.

Agmon, A., Yang, L. T., Jones, E. G., & O'Dowd, D. K. (1995). Topological precision in the thalamic projection to neonatal mouse barrel cortex. *J.Neurosci.* **15**, 549-561.

Agrawal, S. G. & Evans, R. H. (1986). The primary afferent depolarizing action of kainate in the rat. *Br.J.Pharmacol.* **87**, 345-355.

Aguayo, L. G., Espinoza, F., Kunos, G., & Satin, L. S. (1998). Effects of intracellular calcium on GABAA receptors in mouse cortical neurons. *Pflugers Arch.* **435**, 382-387.

Albuquerque, C., Lee, C. J., Jackson, A. C., & MacDermott, A. B. (1999). Subpopulations of GABAergic and non-GABAergic rat dorsal horn neurons express Ca2+-permeable AMPA receptors. *Eur.J.Neurosci.* **11**, 2758-2766.

Allcorn, S., Catsicas, M., & Mobbs, P. (1996). Developmental expression and self-regulation of Ca2+ entry via AMPA/KA receptors in the embryonic chick retina. *Eur.J.Neurosci.* **8**, 2499-2510.

Altman, J. & Bayer, S. A. (1984). The development of the rat spinal cord. *Adv.Anat.Embryol.Cell Biol.* **85**, 1-164.

Andrews, K. & Fitzgerald, M. (1994). The cutaneous withdrawal reflex in human neonates: sensitization, receptive fields, and the effects of contralateral stimulation. *Pain* **56**, 95-101.

Averill, S., McMahon, S. B., Clary, D. O., Reichardt, L. F., & Priestley, J. V. (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur.J.Neurosci.* **7**, 1484-1494.

Baba H, Doubell TP, Moore KA, & Woolf CJ (2000). Silent NMDA receptor-mediated synapses are developmentally regulated in the dorsal horn of the rat spinal cord. *J.Neurophysiology* **83**, 955-962.

Baba, H., Doubell TP, Moore KA, & Woolf CJ (2000). Silent NMDA receptor-mediated synapses are developmentally regulated in the dorsal horn of the rat spinal cord. *J.Neurophysiology* **83**, 955-962.

Baba, H., Doubell, T. P., & Woolf, C. J. (1999). Peripheral inflammation facilitates Abeta fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord. *J.Neurosci.* **19**, 859-867.

Baba, H., Ji, R. R., Kohno, T., Moore, K. A., Ataka, T., Wakai, A., Okamoto, M., & Woolf, C. J. (2003). Removal of GABAergic inhibition facilitates polysynaptic A fiber-mediated excitatory transmission to the superficial spinal dorsal horn. *Mol.Cell Neurosci.* **24**, 818-830.

Baccei, M. L., Bardoni, R., & Fitzgerald, M. (2003). Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. *J.Physiol* **549**, 231-242.

Baccei, M. L. & Fitzgerald, M. (2003). Development of GABAergic and glycinergic transmission in the neonatal rat dorsal horn. *Soc.Neurosci.Abs.*

Baccei, M. L. & Fitzgerald, M. Development of GABAergic and glycinergic transmission in the neonatal rat dorsal horn. J.Neurosci. 2004.
Ref Type: In Press

Bahn, S., Volk, B., & Wisden, W. (1994). Kainate receptor gene expression in the developing rat brain. *J.Neurosci.* **14**, 5525-5547.

Bao, L., Wang, H. F., Cai, H. J., Tong, Y. G., Jin, S. X., Lu, Y. J., Grant, G., Hokfelt, T., & Zhang, X. (2002). Peripheral axotomy induces only very limited sprouting of coarse myelinated afferents into inner lamina II of rat spinal cord. *Eur.J.Neurosci.* **16**, 175-185.

Bardoni R, Magherini PC, & MacDermott AB (1998). NMDA EPSCs at glutamatergic synapses in the spinal cord dorsal horn of the postnatal rat. *J.Neuroscience* **18**, 6558-6567.

Bardoni R, Magherini PC, & MacDermott AB (2000). Activation of NMDA receptors drives action potentials in superficial dorsal horn from neonatal rats. *NeuroReport* **11**, 1721-1727.

Baron, R. (2000). Peripheral neuropathic pain: from mechanisms to symptoms. *Clin.J.Pain* **16**, S12-S20.

Barria, A., Muller, D., Derkach, V., Griffith, L. C., & Soderling, T. R. (1997). Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science* **276**, 2042-2045.

Bayer, K. U., De Koninck, P., Leonard, A. S., Hell, J. W., & Schulman, H. (2001). Interaction with the NMDA receptor locks CaMKII in an active conformation. *Nature* **411**, 801-805.

Bayer, K. U., Lohler J, Schulman H, & Harbers K (1999). Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. *Mol.Brain Res.* **70**, 147-154.

Beal, J. A. & Bice, T. N. (1994). Neurogenesis of spinothalamic and spinocerebellar tract neurons in the lumbar spinal cord of the rat. *Brain Res.Dev.Brain Res.* **78**, 49-56.

Beggs, S. Plasticity of primary sensory neurons and their central connections: normal development and the effects of neonatal axotomy. 2000. University College London. 2000.

Beggs, S., Torsney, C., Drew, L. J., & Fitzgerald, M. (2002). The postnatal reorganization of primary afferent input and dorsal horn cell receptive fields in the rat spinal cord is an activity-dependent process. *Eur.J.Neurosci.* **16**, 1249-1258.

Ben Ari, Y. (2002b). Excitatory actions of gaba during development: the nature of the nurture. *Nat.Rev.Neurosci.* **3**, 728-739.

Ben Ari, Y. (2002a). Excitatory actions of gaba during development: the nature of the nurture. *Nat.Rev.Neurosci.* **3**, 728-739.

Ben Ari, Y. (2002c). Excitatory actions of gaba during development: the nature of the nurture. *Nat.Rev.Neurosci.* **3**, 728-739.

Ben Ari, Y., Cherubini, E., Corradetti, R., & Gaiarsa, J. L. (1989). Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J.Physiol* **416**, 303-325.

Benke, T. A., Luthi, A., Isaac, J. T., & Collingridge, G. L. (1998). Modulation of AMPA receptor unitary conductance by synaptic activity. *Nature* **393**, 793-797.

Bennett, D. L., Michael, G. J., Ramachandran, N., Munson, J. B., Averill, S., Yan, Q., McMahon, S. B., & Priestley, J. V. (1998). A distinct subgroup of small DRG cells

express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J.Neurosci.* **18**, 3059-3072.

Bice, T. N. & Beal, J. A. (1997a). Quantitative and neurogenic analysis of neurons with supraspinal projections in the superficial dorsal horn of the rat lumbar spinal cord. *J.Comp Neurol.* **388**, 565-574.

Bice, T. N. & Beal, J. A. (1997b). Quantitative and neurogenic analysis of the total population and subpopulations of neurons defined by axon projection in the superficial dorsal horn of the rat lumbar spinal cord. *J.Comp Neurol.* **388**, 550-564.

Bicknell, H. R. & Beal, J. A. (1984). Axonal and dendritic development of substantia gelatinosa neurons in the lumbosacral spinal cord of the rat. *J.Comp Neurol.* **226**, 508-522.

Birgbauer, E., Sechrist, J., Bronner-Fraser, M., & Fraser, S. (1995). Rhombomeric origin and rostrocaudal reassortment of neural crest cells revealed by intravital microscopy. *Development* **121**, 935-945.

Bleakman, D., Brorson, J. R., & Miller, R. J. (1990). The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dorsal root ganglion cells. *Br.J.Pharmacol.* **101**, 423-431.

Bliss, T. V. & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31-39.

Blue, M. E. & Parnavelas, J. G. (1983). The formation and maturation of synapses in the visual cortex of the rat. II. Quantitative analysis. *J.Neurocytol.* **12**, 697-712.

Boucher, T. J., Okuse, K., Bennett, D. L., Munson, J. B., Wood, J. N., & McMahon, S. B. (2000). Potent analgesic effects of GDNF in neuropathic pain states. *Science* **290**, 124-127.

Boyce, S., Wyatt, A., Webb, J. K., O'Donnell, R., Mason, G., Rigby, M., Sirinathsinghji, D., Hill, R. G., & Rupniak, N. M. (1999). Selective NMDA NR2B antagonists induce antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. *Neuropharmacology* **38**, 611-623.

Braun, A. P. & Schulman, H. (1995). The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu.Rev.Physiol* **57**, 417-445.

Bregman, B. S. (1987). Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. *Brain Res.* **431**, 245-263.

Brenner, G. J., Ji, R. R., Shaffer, S., & Woolf, C. J. (2004). Peripheral noxious stimulation induces phosphorylation of the NMDA receptor NR1 subunit at the PKC-dependent site, serine-896, in spinal cord dorsal horn neurons. *Eur.J.Neurosci.* **20**, 375-384.

Brocke, L., Chiang, L. W., Wagner, P. D., & Schulman, H. (1999). Functional implications of the subunit composition of neuronal CaM kinase II. *J.Biol.Chem.* **274**, 22713-22722.

Brown, A. G. (1981). *Organisation in the Spinal Cord: The Anatomy and Physiology of Identified Neurones* Springer-Verlag, Berlin.

Brown, K. M., Wrathall, J. R., Yasuda, R. P., & Wolfe, B. B. (2002). Quantitative measurement of glutamate receptor subunit protein expression in the postnatal rat spinal cord. *Brain Res.Dev.Brain Res.* **137**, 127-133.

Bruggemann, I., Schulz, S., Wiborny, D., & Hollt, V. (2000). Colocalization of the mu-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Brain Res.Mol.Brain Res.* **85**, 239-250.

Burgin, K. E., Waxham, M. N., Rickling, S., Westgate, S. A., Mobley, W. C., & Kelly, P. T. (1990). In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. *J.Neurosci.* **10**, 1788-1798.

Cain, D. M., Khasabov, S. G., & Simone, D. A. (2001). Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J.Neurophysiol.* **85**, 1561-1574.

Carlton, S. M. (2002). Localization of CaMKIIalpha in rat primary sensory neurons: increase in inflammation. *Brain Res.* **947**, 252-259.

Carlton, S. M. & Hargett, G. L. (2002). Stereological analysis of Ca(2+)/calmodulin-dependent protein kinase II alpha -containing dorsal root ganglion neurons in the rat: colocalization with isolectin Griffonia simplicifolia, calcitonin gene-related peptide, or vanilloid receptor 1. *J.Comp Neurol.* **448**, 102-110.

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., & Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288**, 306-313.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816-824.

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., & Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *J.Neurosci.Methods* **53**, 55-63.

Chapman, V., Ng, J., & Dickenson, A. H. (1998). A novel spinal action of mexiletine in spinal somatosensory transmission of nerve injured rats. *Pain* **77**, 289-296.

Chen, G., Trombley, P. Q., & van den Pol, A. N. (1996). Excitatory actions of GABA in developing rat hypothalamic neurones. *J.Physiol* **494 (Pt 2)**, 451-464.

Chen, L. & Huang, L. Y. (1992). Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. *Nature* **356**, 521-523.

Cheunsuang, O., Maxwell, D., & Morris, R. (2002). Spinal lamina I neurones that express neurokinin 1 receptors: II. Electrophysiological characteristics, responses to primary afferent stimulation and effects of a selective mu-opioid receptor agonist. *Neuroscience* **111**, 423-434.

Cheunsuang, O. & Morris, R. (2000). Spinal lamina I neurons that express neurokinin 1 receptors: morphological analysis. *Neuroscience* **97**, 335-345.

Chuang, H. H., Prescott, E. D., Kong, H., Shields, S., Jordt, S. E., Basbaum, A. I., Chao, M. V., & Julius, D. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* **411**, 957-962.

Chung, K., McNeill, D. L., Hulsebosch, C. E., & Coggeshall, R. E. (1989). Changes in dorsal horn synaptic disc numbers following unilateral dorsal rhizotomy. *J.Comp Neurol.* **283**, 568-577.

Churn, S. B. & Delorenzo, R. J. (1998). Modulation of GABAergic receptor binding by activation of calcium and calmodulin-dependent kinase II membrane phosphorylation. *Brain Res.* **809**, 68-76.

Churn, S. B., Rana, A., Lee, K., Parsons, J. T., De Blas, A., & Delorenzo, R. J. (2002). Calcium/calmodulin-dependent kinase II phosphorylation of the GABAA receptor alpha1 subunit modulates benzodiazepine binding. *J.Neurochem.* **82**, 1065-1076.

Cinquanta, M., Frittoli, E., Mennini, T., & Gobbi, M. (1997). Further evidence of Ca(2+)-dependent, exocytotic-like serotonin release induced by D-fenfluramine. *Pharmacol.Res.* **35**, 439-442.

Coderre, T. J., Fundytus, M. E., McKenna, J. E., Dalal, S., & Melzack, R. (1993). The formalin test: a validation of the weighted-scores method of behavioural pain rating. *Pain* **54**, 43-50.

Coderre, T. J. & Melzack, R. (1992). The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. *J.Neurosci.* **12**, 3665-3670.

Coggeshall, R. E., Jennings, E. A., & Fitzgerald, M. (1996). Evidence that large myelinated primary afferent fibers make synaptic contacts in lamina II of neonatal rats. *Brain Res.Dev.Brain Res.* **92**, 81-90.

Coimbra, A., Ribeiro-da-Silva, A., & Pignatelli, D. (1986). Rexed's laminae and the acid phosphatase (FRAP)-band in the superficial dorsal horn of the neonatal rat spinal cord. *Neurosci.Lett.* **71**, 131-136.

Colman, H., Nabekura, J., & Lichtman, J. W. (1997). Alterations in synaptic strength preceding axon withdrawal. *Science* **275**, 356-361.

Constantine-Paton, M. & Cline, H. T. (1998). LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. *Curr.Opin.Neurobiol.* **8**, 139-148.

Constantine-Paton, M., Cline, H. T., & Debski, E. (1990). Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. *Annu.Rev.Neurosci.* **13**, 129-154.

Crair, M. C. & Malenka, R. C. (1995). A critical period for long-term potentiation at thalamocortical synapses. *Nature* **375**, 325-328.

Cramer, K. S. & Sur, M. (1995). Activity-dependent remodeling of connections in the mammalian visual system. *Curr.Opin.Neurobiol.* **5**, 106-111.

Dallel, R., Raboisson, P., Clavelou, P., Saade, M., & Woda, A. (1995). Evidence for a peripheral origin of the tonic nociceptive response to subcutaneous formalin. *Pain* **61**, 11-16.

Davar, G., Hama, A., Deykin, A., Vos, B., & Maciewicz, R. (1991). MK-801 blocks the development of thermal hyperalgesia in a rat model of experimental painful neuropathy. *Brain Res.* **553**, 327-330.

Davies, J., Evans, R. H., Francis, A. A., & Watkins, J. C. (1979). Excitatory amino acid receptors and synaptic excitation in the mammalian central nervous system. *J.Physiol (Paris)* **75**, 641-654.

Davies, S. N. & Lodge, D. (1987). Evidence for involvement of N-methylaspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat. *Brain Res.* **424**, 402-406.

De Lima, J. C. Developmental aspects of NMDA antagonist sensitivity in rats- an electromyographic study using the withdrawal reflex. 2001. University College London.

Decosterd, I. & Woolf, C. J. (2000). Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* **87**, 149-158.

Derkach, V., Barria, A., & Soderling, T. R. (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc.Natl.Acad.Sci.U.S.A* **96**, 3269-3274.

Dickenson, A. H., Chapman, V., & Green, G. M. (1997). The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. *Gen.Pharmacol.* **28**, 633-638.

Dickenson, A. H. & Sullivan, A. F. (1987a). Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. *Neuropharmacology* **26**, 1235-1238.

Dickenson, A. H. & Sullivan, A. F. (1987b). Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. *Pain* **30**, 349-360.

Ding, Y. Q., Takada, M., Shigemoto, R., & Mizumo, N. (1995). Spinoparabrachial tract neurons showing substance P receptor-like immunoreactivity in the lumbar spinal cord of the rat. *Brain Res.* **674**, 336-340.

Dingledine, R., Borges, K., Bowie, D., & Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacol.Rev.* **51**, 7-61.

Durand, G. M., Kovalchuk, Y., & Konnerth, A. (1996). Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* **381**, 71-75.

Eaton, M. J., Plunkett, J. A., Karmally, S., Martinez, M. A., & Montanez, K. (1998). Changes in GAD- and GABA- immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. *J.Chem.Neuroanat.* **16**, 57-72.

Eisenach, J. C. & Gebhart, G. F. (1995). Intrathecal amitriptyline acts as an N-methyl-D-aspartate receptor antagonist in the presence of inflammatory hyperalgesia in rats. *Anesthesiology* **83**, 1046-1054.

Eisenberg, E., LaCross, S., & Strassman, A. M. (1994). The effects of the clinically tested NMDA receptor antagonist memantine on carrageenan-induced thermal hyperalgesia in rats. *Eur.J.Pharmacol.* **255**, 123-129.

Engelman, H. S., Allen, T. B., & MacDermott, A. B. (1999). The distribution of neurons expressing calcium-permeable AMPA receptors in the superficial laminae of the spinal cord dorsal horn. *J.Neurosci.* **19**, 2081-2089.

Erondu, N. E. & Kennedy, M. B. (1985). Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *J.Neurosci.* **5**, 3270-3277.

Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., & Luscher, B. (1998). Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat.Neurosci.* **1**, 563-571.

Evans, R. H., Evans, S. J., Pook, P. C., & Sunter, D. C. (1987). A comparison of excitatory amino acid antagonists acting at primary afferent C fibres and motoneurones of the isolated spinal cord of the rat. *Br.J.Pharmacol.* **91**, 531-537.

Fang, L., Wu, J., Lin, Q., & Willis, W. D. (2002). Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J.Neurosci.* **22**, 4196-4204.

Feldman, D. E. & Knudsen, E. I. (1998). Experience-dependent plasticity and the maturation of glutamatergic synapses. *Neuron* **20**, 1067-1071.

Fields, H. L., Basbaum, A. I., Clanton, C. H., & Anderson, S. D. (1977). Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. *Brain Res.* **126**, 441-453.

Fink, C. C., Bayer, K. U., Myers, J. W., Ferrell, J. E., Jr., Schulman, H., & Meyer, T. (2003). Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron* **39**, 283-297.

Fitzgerald, M. (1985). The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. *J.Physiol* **364**, 1-18.

Fitzgerald, M. (1987). Cutaneous primary afferent properties in the hind limb of the neonatal rat. *J.Physiol* **383**, 79-92.

Fitzgerald, M. (1988). The development of activity evoked by fine diameter cutaneous fibres in the spinal cord of the newborn rat. *Neurosci.Lett.* **86**, 161-166.

Fitzgerald, M. & Beggs, S. (2001). The neurobiology of pain: developmental aspects. *Neuroscientist.* **7**, 246-257.

Fitzgerald, M., Butcher, T., & Shortland, P. (1994). Developmental changes in the laminar termination of A fibre cutaneous sensory afferents in the rat spinal cord dorsal horn. *J.Comp Neurol.* **348**, 225-233.

Fitzgerald, M. & Fulton, B. P. (1992). The physiological properties of developing sensory neurons. In *Sensory Neurons*, ed. Scott, S. A., pp. 287-306. Oxford University Press, Oxford.

Fitzgerald, M. & Jennings, E. (1999). The postnatal development of spinal sensory processing. *Proc.Natl.Acad.Sci.U.S.A* **96**, 7719-7722.

Fitzgerald, M., King, A. E., Thompson, S. W., & Woolf, C. J. (1987). The postnatal development of the ventral root reflex in the rat; a comparative in vivo and in vitro study. *Neurosci.Lett.* **78**, 41-45.

Fitzgerald, M. & Koltzenburg, M. (1986). The functional development of descending inhibitory pathways in the dorsolateral funiculus of the newborn rat spinal cord. *Brain Res.* **389**, 261-270.

Fitzgerald, M., Millard, C., & MacIntosh, N. (1988a). Hyperalgesia in premature infants. *Lancet* **1**, 292.

Fitzgerald, M., Millard, C., & McIntosh, N. (1989). Cutaneous hypersensitivity following peripheral tissue damage in newborn infants and its reversal with topical anaesthesia. *Pain* **39**, 31-36.

Fitzgerald, M., Reynolds, M. L., & Benowitz, L. I. (1991). GAP-43 expression in the developing rat lumbar spinal cord. *Neuroscience* **41**, 187-199.

Fitzgerald, M., Shaw, A., & MacIntosh, N. (1988b). Postnatal development of the cutaneous flexor reflex: comparative study of preterm infants and newborn rat pups. *Dev.Med.Child Neurol.* **30**, 520-526.

Fitzgerald, M. & Swett, J. (1983). The termination pattern of sciatic nerve afferents in the substantia gelatinosa of neonatal rats. *Neurosci.Lett.* **43**, 149-154.

Fox, K. (1995). The critical period for long-term potentiation in primary sensory cortex. *Neuron* **15**, 485-488.

Fox, K. (2002). Anatomical pathways and molecular mechanisms for plasticity in the barrel cortex. *Neuroscience* **111**, 799-814.

Fox, K., Schlaggar, B. L., Glazewski, S., & O'Leary, D. D. (1996). Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. *Proc.Natl.Acad.Sci.U.S.A* **93**, 5584-5589.

Ganguly, K., Schinder, A. F., Wong, S. T., & Poo, M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* **105**, 521-532.

Gao, Y. J., Zhang, Y. Q., & Zhao, Z. Q. (2003). Involvement of spinal neurokinin-1 receptors in the maintenance but not induction of carrageenan-induced thermal hyperalgesia in the rat. *Brain Res.Bull.* **61**, 587-593.

Gardoni, F., Caputi, A., Cimino, M., Pastorino, L., Cattabeni, F., & Di Luca, M. (1998). Calcium/calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities. *J.Neurochem.* **71**, 1733-1741.

Garry, E. M., Moss, A., Delaney, A., O'Neill, F., Blakemore, J., Bowen, J., Husi, H., Mitchell, R., Grant, S. G., & Fleetwood-Walker, S. M. (2003). Neuropathic Sensitization of Behavioral Reflexes and Spinal NMDA Receptor/CaM Kinase II Interactions Are Disrupted in PSD-95 Mutant Mice. *Curr.Biol.* **13**, 321-328.

Gaudilliere, B., Konishi, Y., de, l., I, Yao, G., & Bonni, A. (2004). A CaMKII-NeuroD Signaling Pathway Specifies Dendritic Morphogenesis. *Neuron* **41**, 229-241.

Gaunitz, C., Schuttler, A., Gillen, C., & Allgaier, C. (2002). Formalin-induced changes of NMDA receptor subunit expression in the spinal cord of the rat. *Amino.Acids* **23**, 177-182.

Giese, K. P., Fedorov, N. B., Filipkowski, R. K., & Silva, A. J. (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279**, 870-873.

Glazewski, S., Bejar, R., Mayford, M., & Fox, K. (2001). The effect of autonomous alpha-CaMKII expression on sensory responses and experience-dependent plasticity in mouse barrel cortex. *Neuropharmacology* **41**, 771-778.

Glazewski, S., Chen, C. M., Silva, A., & Fox, K. (1996). Requirement for alpha-CaMKII in experience-dependent plasticity of the barrel cortex. *Science* **272**, 421-423.

Glazewski, S., Giese, K. P., Silva, A., & Fox, K. (2000). The role of alpha-CaMKII autophosphorylation in neocortical experience- dependent plasticity. *Nat.Neurosci.* **3**, 911-918.

Gobel, S. (1975). Golgi studies in the substantia gelatinosa neurons in the spinal trigeminal nucleus. *J.Comp Neurol.* **162**, 397-415.

Gobel, S. (1978). Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). *J.Comp Neurol.* **180**, 395-413.

Godement, P., Vanselow, J., Thanos, S., & Bonhoeffer, F. (1987). A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. *Development* **101**, 697-713.

Gold, M. S., Levine, J. D., & Correa, A. M. (1998). Modulation of TTX-R INa by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. *J.Neurosci.* **18**, 10345-10355.

Gold, M. S., Weinreich, D., Kim, C. S., Wang, R., Treanor, J., Porreca, F., & Lai, J. (2003). Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain. *J.Neurosci.* **23**, 158-166.

Gordon, J. A., Cioffi, D., Silva, A. J., & Stryker, M. P. (1996). Deficient plasticity in the primary visual cortex of alpha- calcium/calmodulin-dependent protein kinase II mutant mice. *Neuron* **17**, 491-499.

Gordon, J. A. & Stryker, M. P. (1996). Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. *J.Neurosci.* **16**, 3274-3286.

Green, G. M. & Gibb, A. J. (2001). Characterization of the single-channel properties of NMDA receptors in laminae I and II of the dorsal horn of neonatal rat spinal cord. *Eur.J.Neurosci.* **14**, 1590-1602.

Grudt, T. J. & Perl, E. R. (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J.Physiol* **540**, 189-207.

Gu, J. G., Albuquerque, C., Lee, C. J., & MacDermott, A. B. (1996). Synaptic strengthening through activation of Ca2+-permeable AMPA receptors. *Nature* **381**, 793-796.

Guo, A., Vulchanova, L., Wang, J., Li, X., & Elde, R. (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur.J.Neurosci.* **11**, 946-958.

Guo, H. & Huang, L. Y. (2001). Alteration in the voltage dependence of NMDA receptor channels in rat dorsal horn neurones following peripheral inflammation. *J.Physiol* **537**, 115-123.

Guo, W., Zou, S., Guan, Y., Ikeda, T., Tal, M., Dubner, R., & Ren, K. (2002). Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *J.Neurosci.* **22**, 6208-6217.

Gwyn, D. G. & Waldron, H. A. (1968). A nucleus in the dorsolateral funiculus of the spinal cord of the rat. *Brain Res.* **10**, 342-351.

Haghighi, A. P., McCabe, B. D., Fetter, R. D., Palmer, J. E., Hom, S., & Goodman, C. S. (2003). Retrograde control of synaptic transmission by postsynaptic CaMKII at the Drosophila neuromuscular junction. *Neuron* **39**, 255-267.

Hajimohammadreza, I., Probert, A. W., Coughenour, L. L., Borosky, S. A., Marcoux, F. W., Boxer, P. A., & Wang, K. K. (1995). A specific inhibitor of calcium/calmodulin-dependent protein kinase-II provides neuroprotection against. *J.Neurosci.* **15**, 4093-4101.

Haley, J. E., Sullivan, A. F., & Dickenson, A. H. (1990). Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res.* **518**, 218-226.

Hardingham, N., Glazewski, S., Pakhotin, P., Mizuno, K., Chapman, P. F., Giese, K. P., & Fox, K. (2003). Neocortical long-term potentiation and experience-dependent synaptic plasticity require alpha-calcium/calmodulin-dependent protein kinase II autophosphorylation. *J.Neurosci.* **23**, 4428-4436.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., & Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**, 77-88.

Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., & Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287**, 2262-2267.

Hebb, D. O. (1949). *The Organisation of Behaviour* John Wiley & Sons, New York.

Henry, J. L., Yashpal, K., Pitcher, G. M., & Coderre, T. J. (1999). Physiological evidence that the 'interphase' in the formalin test is due to active inhibition. *Pain* **82**, 57-63.

Herms, J., Zurmohle, U., Brysch, W., & Schlingensiepen, K. H. (1993). Ca2+/calmodulin protein kinase and protein kinase C expression during development of rat hippocampus. *Dev.Neurosci.* **15**, 410-416.

Hinds, H. L., Goussakov, I., Nakazawa, K., Tonegawa, S., & Bolshakov, V. Y. (2003). Essential function of alpha-calcium/calmodulin-dependent protein kinase II in neurotransmitter release at a glutamatergic central synapse. *Proc.Natl.Acad.Sci.U.S.A* **100**, 4275-4280.

Hocking, G. & Cousins, M. J. (2003). Ketamine in chronic pain management: an evidence-based review. *Anesth.Analg.* **97**, 1730-1739.

Hollmann, M., Hartley, M., & Heinemann, S. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science 252[5007], 851-853. 1991.

Hori, Y. & Kanda, K. (1994). Developmental alterations in NMDA receptor-mediated [Ca2+]i elevation in substantia gelatinosa neurons of neonatal rat spinal cord. *Brain Res.Dev.Brain Res.* **80**, 141-148.

Hori, Y. & Watanabe, S. (1987). Morphine-sensitive late components of the flexion reflex in the neonatal rat. *Neurosci.Lett.* **78**, 91-96.

Houston, C. J., Jovanovic, J., & Smart, T. G. Calcium/calmodulin dependent protein kinase II (CaMKII) dependent modulation of the -aminobutyric acid type A receptor (GABA-A). Program No. 472.13. *2003 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2003. Online. 2003.

Hua, X. Y., Chen, P., & Yaksh, T. L. (1999). Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. *Neurosci.Lett.* **276**, 99-102.

Huang, L. & Pallas, S. L. (2001). NMDA antagonists in the superior colliculus prevent developmental plasticity but not visual transmission or map compression. *J.Neurophysiol.* **86**, 1179-1194.

Hubel, D. H. & Wiesel, T. N. (1969). Anatomical demonstration of columns in the monkey striate cortex. *Nature* **221**, 747-750.

Hubner, C. A., Stein, V., Hermans-Borgmeyer, I., Meyer, T., Ballanyi, K., & Jentsch, T. J. (2001). Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron* **30**, 515-524.

Hudmon, A. & Schulman, H. (2002). Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu.Rev.Biochem.* **71**, 473-510.

Huettner, J. E. (1990). Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. *Neuron* **5**, 255-266.

Hunt, S. P. & Mantyh, P. W. (2001). The molecular dynamics of pain control. *Nat.Rev.Neurosci.* **2**, 83-91.

Hwang, S. J., Pagliardini, S., Rustioni, A., & Valtschanoff, J. G. (2001). Presynaptic kainate receptors in primary afferents to the superficial laminae of the rat spinal cord. *J.Comp Neurol.* **436**, 275-289.

Ibuki, T., Hama, A. T., Wang, X. T., Pappas, G. D., & Sagen, J. (1997). Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. *Neuroscience* **76**, 845-858.

Ikeda, H., Heinke, B., Ruscheweyh, R., & Sandkuhler, J. (2003). Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. *Science* **299**, 1237-1240.

Isaac, J. T., Crair, M. C., Nicoll, R. A., & Malenka, R. C. (1997). Silent synapses during development of thalamocortical inputs. *Neuron* **18**, 269-280.

Isaac, J. T., Nicoll, R. A., & Malenka, R. C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15**, 427-434.

Jackman, A. & Fitzgerald, M. (2000). Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. *J.Comp Neurol.* **418**, 281-298.

Jakowec, M. W., Fox, A. J., Martin, L. J., & Kalb, R. G. (1995a). Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. *Neuroscience* **67**, 893-907.

Jakowec, M. W., Yen, L., & Kalb, R. G. (1995b). In situ hybridization analysis of AMPA receptor subunit gene expression in the developing rat spinal cord. *Neuroscience* **67**, 909-920.

Jancso, G., Santha, P., & Gecse, K. (2002). Peripheral nerve lesion-induced uptake and transport of choleragenoid by capsaicin-sensitive c-fibre spinal ganglion neurons. *Acta Biol.Hung.* **53**, 77-84.

Jansen, A. S. & Loewy, A. D. (1997). Neurons lying in the white matter of the upper cervical spinal cord project to the intermediolateral cell column. *Neuroscience* **77**, 889-898.

Jennings, E. & Fitzgerald, M. (1998). Postnatal changes in responses of rat dorsal horn cells to afferent stimulation: a fibre-induced sensitization. *J.Physiol* **509 (Pt 3)**, 859-868.

Ji, R. R., Kohno, T., Moore, K. A., & Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci.* **26**, 696-705.

Jia, W. G., Beaulieu, C., Liu, Y. L., & Cynader, M. (1992). Calcium calmodulin dependent kinase II in cat visual cortex and its development. *Dev.Neurosci.* **14**, 238-246.

Jiang, M. C., Liu, L., & Gebhart, G. F. (1999). Cellular properties of lateral spinal nucleus neurons in the rat L6-S1 spinal cord. *J.Neurophysiol.* **81**, 3078-3086.

Jonas, P., Bischofberger, J., & Sandkuhler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. *Science* **281**, 419-424.

Jordt, S. E., Bautista, D. M., Chuang, H. H., McKemy, D. D., Zygmunt, P. M., Hogestatt, E. D., Meng, I. D., & Julius, D. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427**, 260-265.

Jourdan, D., Ardid, D., Bardin, L., Bardin, M., Neuzeret, D., Lanphouthacoul, L., & Eschalier, A. (1997). A new automated method of pain scoring in the formalin test in rats. *Pain* **71**, 265-270.

Julius, D. & Basbaum, A. I. (2001). Molecular mechanisms of nociception. *Nature* **413**, 203-210.

Kano, M., Kano, M., Fukunaga, K., & Konnerth, A. (1996). Ca(2+)-induced rebound potentiation of gamma-aminobutyric acid-mediated currents requires activation of Ca2+/calmodulin-dependent kinase II. *Proc.Natl.Acad.Sci.U.S.A* **93**, 13351-13356.

Katz, L. C. & Crowley, J. C. (2002). Development of cortical circuits: lessons from ocular dominance columns. *Nat.Rev.Neurosci.* **3**, 34-42.

Katz, L. C. & Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. *Science* **274**, 1133-1138.

Kawaguchi, S. Y. & Hirano, T. (2002). Signaling cascade regulating long-term potentiation of GABA(A) receptor responsiveness in cerebellar Purkinje neurons. *J.Neurosci.* **22**, 3969-3976.

Keller, A. F., Coull, J. A., Chery, N., Poisbeau, P., & De Koninck, Y. (2001). Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. *J.Neurosci.* **21**, 7871-7880.

Kerchner, G. A., Wilding, T. J., Li, P., Zhuo, M., & Huettner, J. E. (2001). Presynaptic kainate receptors regulate spinal sensory transmission. *J.Neurosci.* **21**, 59-66.

Kerr, R. C., Maxwell, D. J., & Todd, A. J. (1998). GluR1 and GluR2/3 subunits of the AMPA-type glutamate receptor are associated with particular types of neurone in laminae I-III of the spinal dorsal horn of the rat. *Eur.J.Neurosci.* **10**, 324-333.

Kidd, F. L., Coumis, U., Collingridge, G. L., Crabtree, J. W., & Isaac, J. T. (2002). A presynaptic kainate receptor is involved in regulating the dynamic properties of thalamocortical synapses during development. *Neuron* **34**, 635-646.

Kim, S. H. & Chung, J. M. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**, 355-363.

Kirkwood, A., Lee, H. K., & Bear, M. F. (1995). Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience. *Nature* **375**, 328-331.

Kitao, Y., Robertson, B., Kudo, M., & Grant, G. (1996). Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurons including neurons projecting to the dorsal column nuclei. *J.Comp Neurol.* **371**, 249-257.

Klausner, R. D. & Wolf, D. E. (1980). Selectivity of fluorescent lipid analogues for lipid domains. *Biochemistry* **19**, 6199-6203.

Kohno, T., Moore, K. A., Baba, H., & Woolf, C. J. (2003). Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn. *J.Physiol* **548**, 131-138.

Kolaj, M., Cerne, R., Cheng, G., Brickey, D. A., & Randic, M. (1994). Alpha subunit of calcium/calmodulin-dependent protein kinase enhances excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. *J.Neurophysiol.* **72**, 2525-2531.

Kolodziej, S. J., Hudmon, A., Waxham, M. N., & Stoops, J. K. (2000). Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIalpha and truncated CaM kinase IIalpha reveal a unique organization for its structural core and functional domains. *J.Biol.Chem.* **275**, 14354-14359.

Koltzenburg, M. & Lewin, G. R. (1997). Receptive properties of embryonic chick sensory neurons innervating skin. *J.Neurophysiol.* **78**, 2560-2568.

Koltzenburg, M., Stucky, C. L., & Lewin, G. R. (1997). Receptive properties of mouse sensory neurons innervating hairy skin. *J.Neurophysiol.* **78**, 1841-1850.

Kontinen, V. K., Stanfa, L. C., Basu, A., & Dickenson, A. H. (2001). Electrophysiologic evidence for increased endogenous gabaergic but not glycinergic inhibitory tone in the rat spinal nerve ligation model of neuropathy. *Anesthesiology* **94**, 333-339.

Kumar, S. S., Bacci, A., Kharazia, V., & Huguenard, J. R. (2002). A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J.Neurosci.* **22**, 3005-3015.

Laabich, A., Li, G., & Cooper, N. G. (2000). Calcium/calmodulin-dependent protein kinase II containing a nuclear localizing signal is altered in retinal neurons exposed to N-methyl-D-aspartate. *Brain Res.Mol.Brain Res.* **76**, 253-265.

Laird, J. M., Mason, G. S., Webb, J., Hill, R. G., & Hargreaves, R. J. (1996). Effects of a partial agonist and a full antagonist acting at the glycine site of the NMDA receptor on inflammation-induced mechanical hyperalgesia in rats. *Br.J.Pharmacol.* **117**, 1487-1492.

Lanuza, M. A., Garcia, N., Santafe, M., Gonzalez, C. M., Alonso, I., Nelson, P. G., & Tomas, J. (2002). Pre- and postsynaptic maturation of the neuromuscular junction during neonatal synapse elimination depends on protein kinase C. *J.Neurosci.Res.* **67**, 607-617.

Larsson, M. D. & Broman, J. (2003). Ultrastructural localization of autophosphorylated CaMKII in the superficial dorsal horn. *Soc.Neurosci.New Orleans*.

Lawson, S. N., Caddy, K. W., & Biscoe, T. J. (1974). Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. *Cell Tissue Res.* **153**, 399-413.

Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacol.Rev.* **53**, 597-652.

Lee, C. J., Kong, H., Manzini, M. C., Albuquerque, C., Chao, M. V., & MacDermott, A. B. (2001). Kainate receptors expressed by a subpopulation of developing nociceptors rapidly switch from high to low Ca2+ permeability. *J.Neurosci.* **21**, 4572-4581.

Lee, H. K., Barbarosie, M., Kameyama, K., Bear, M. F., & Huganir, R. L. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405**, 955-959.

Lewin, G. R., Mckintosh, E., & McMahon, S. B. (1994). NMDA receptors and activity-dependent tuning of the receptive fields of spinal cord neurons. *Nature* **369**, 482-485.

Li, G., Laabich, A., Liu, L. O., Xue, J., & Cooper, N. G. (2001a). Molecular cloning and sequence analyses of calcium/calmodulin-dependent protein kinase II from fetal and adult human brain. Sequence analyses of human brain calciuum/calmodulin-dependent protein kinase II. *Mol.Biol.Rep.* **28**, 35-41.

Li, M. X., Jia, M., Jiang, H., Dunlap, V., & Nelson, P. G. (2001b). Opposing actions of protein kinase A and C mediate Hebbian synaptic plasticity. *Nat.Neurosci.* **4**, 871-872.

Li, P., Wilding, T. J., Kim, S. J., Calejesan, A. A., Huettner, J. E., & Zhuo, M. (1999). Kainate-receptor-mediated sensory synaptic transmission in mammalian spinal cord. *Nature* **397**, 161-164.

Li, P. & Zhuo, M. (1998). Silent glutamatergic synapses and nociception in mammalian spinal cord. *Nature* **393**, 695-698.

Li, Y. & Burke, R. E. (2001). Short-term synaptic depression in the neonatal mouse spinal cord: effects of calcium and temperature. *J.Neurophysiol.* **85**, 2047-2062.

Li, Y. & Burke, R. E. (2002). Developmental changes in short-term synaptic depression in the neonatal mouse spinal cord. *J.Neurophysiol.* **88**, 3218-3231.

Li, Y., Erzurumlu, R. S., Chen, C., Jhaveri, S., & Tonegawa, S. (1994). Whisker-related neuronal patterns fail to develop in the trigeminal brainstem nuclei of NMDAR1 knockout mice. *Cell* **76**, 427-437.

Liang, D., Li, X., & Clark, J. D. (2004a). Increased expression of Ca2+/calmodulin-dependent protein kinase II alpha during chronic morphine exposure. *Neuroscience* **123**, 769-775.

Liang, D. Y., Li, X., & Clark, J. D. (2004b). Formalin-induced spinal cord calcium/calmodulin-dependent protein kinase IIalpha expression is modulated by heme oxygenase in mice. *Neurosci.Lett.* **360**, 61-64.

Lichtman, J. W. & Colman, H. (2000). Synapse elimination and indelible memory. *Neuron* **25**, 269-278.

Light, A. R. & Perl, E. R. (1979). Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. *J.Comp Neurol.* **186**, 133-150.

Lima, D. & Coimbra, A. (1988). The spinothalamic system of the rat: structural types of retrogradely labelled neurons in the marginal zone (lamina I). *Neuroscience* **27**, 215-230.

Lisman, J., Schulman, H., & Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat.Rev.Neurosci.* **3**, 175-190.

Lisman, J. E. & Zhabotinsky, A. M. (2001). A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. *Neuron* **31**, 191-201.

Liu, X. B. & Jones, E. G. (1996). Localization of alpha type II calcium calmodulin-dependent protein kinase at glutamatergic but not gamma-aminobutyric acid

(GABAergic) synapses in thalamus and cerebral cortex. *Proc.Natl.Acad.Sci.U.S.A* **93**, 7332-7336.

Loftis, J. M. & Janowsky, A. (2003). The N-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. *Pharmacol.Ther.* **97**, 55-85.

Lund, L. M. & McQuarrie, I. G. (1997). Calcium/calmodulin-dependent protein kinase II expression in motor neurons: effect of axotomy. *J.Neurobiol.* **33**, 796-810.

Lynn, B. & Carpenter, S. E. (1982). Primary afferent units from the hairy skin of the rat hind limb. *Brain Res.* **238**, 29-43.

Ma, W., Behar, T., Chang, L., & Barker, J. L. (1994). Transient increase in expression of GAD65 and GAD67 mRNAs during postnatal development of rat spinal cord. *J.Comp Neurol.* **346**, 151-160.

Ma, W., Saunders, P. A., Somogyi, R., Poulter, M. O., & Barker, J. L. (1993). Ontogeny of GABAA receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. *J.Comp Neurol.* **338**, 337-359.

Malenka, R. C. & Nicoll, R. A. (1997). Silent synapses speak up. *Neuron* **19**, 473-476.

Malmberg, A. B., Chen, C., Tonegawa, S., & Basbaum, A. I. (1997). Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. *Science* **278**, 279-283.

Malmberg, A. B., Gilbert, H., McCabe, R. T., & Basbaum, A. I. (2003). Powerful antinociceptive effects of the cone snail venom-derived subtype-selective NMDA receptor antagonists conantokins G and T. *Pain* **101**, 109-116.

Malmberg, A. B. & Yaksh, T. L. (1992). Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. *J.Pharmacol.Exp.Ther.* **263**, 136-146.

Malosio, M. L., Marqueze-Pouey, B., Kuhse, J., & Betz, H. (1991). Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J.* **10**, 2401-2409.

Mao, J., Mayer, D. J., Hayes, R. L., & Price, D. D. (1993). Spatial patterns of increased spinal cord membrane-bound protein kinase C and their relation to increases in 14C-2-deoxyglucose metabolic activity in rats with painful peripheral mononeuropathy. *J.Neurophysiol.* **70**, 470-481.

Martin, W. J., Liu, H., Wang, H., Malmberg, A. B., & Basbaum, A. I. (1999). Inflammation-induced up-regulation of protein kinase Cgamma immunoreactivity in rat spinal cord correlates with enhanced nociceptive processing. *Neuroscience* **88**, 1267-1274.

Masson, R. L., Sparkes, M. L., & Ritz, L. A. (1991). Descending projections to the rat sacrocaudal spinal cord. *J.Comp Neurol.* **307**, 120-130.

Matthies, B. K. & Franklin, K. B. (1992). Formalin pain is expressed in decerebrate rats but not attenuated by morphine. *Pain* **51**, 199-206.

McCall, W. D., Tanner, K. D., & Levine, J. D. (1996). Formalin induces biphasic activity in C-fibers in the rat. *Neurosci.Lett.* **208**, 45-48.

McCarson, K. E. & Goldstein, B. D. (1990). Time course of the alteration in dorsal horn substance P levels following formalin: blockade by naloxone. *Pain* **41**, 95-100.

McDonald, A. J., Muller, J. F., & Mascagni, F. (2002). GABAergic innervation of alpha type II calcium/calmodulin-dependent protein kinase immunoreactive pyramidal neurons in the rat basolateral amygdala. *J.Comp Neurol.* **446**, 199-218.

McDonald, B. J. & Moss, S. J. (1994). Differential phosphorylation of intracellular domains of gamma-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. *J.Biol.Chem.* **269**, 18111-18117.

McDonald, B. J. & Moss, S. J. (1997). Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase protein kinase C and Ca2+/calmodulin type II-dependent protein kinase. *Neuropharmacology* **36**, 1377-1385.

McKemy, D. D., Neuhausser, W. M., & Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52-58.

McLeod, A. L., Krause, J. E., Cuello, A. C., & Ribeiro-da-Silva, A. (1998). Preferential synaptic relationships between substance P-immunoreactive boutons and neurokinin 1 receptor sites in the rat spinal cord. *Proc.Natl.Acad.Sci.U.S.A* **95**, 15775-15780.

Mehta, A. K. & Ticku, M. K. (1999). An update on GABAA receptors. *Brain Res.Brain Res.Rev.* **29**, 196-217.

Meller, S. T., Cummings, C. P., Traub, R. J., & Gebhart, G. F. (1994). The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplantar injection of carrageenan in the rat. *Neuroscience* **60**, 367-374.

Meyer, T., Hanson, P. I., Stryer, L., & Schulman, H. (1992). Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* **256**, 1199-1202.

Michaelis, E. K. (1998). Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Prog.Neurobiol.* **54**, 369-415.

Miller, B. A. & Woolf, C. J. (1996). Glutamate-mediated slow synaptic currents in neonatal rat deep dorsal horn neurons in vitro. *J.Neurophysiol.* **76**, 1465-1476.

Mirnics, K. & Koerber, H. R. (1995). Prenatal development of rat primary afferent fibers: II. Central projections. *J.Comp Neurol.* **355**, 601-614.

Mirnics, K. & Koerber, H. R. (1997). Properties of individual embryonic primary afferents and their spinal projections in the rat. *J.Neurophysiol.* **78**, 1590-1600.

Momiyama, A. (2000). Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *J.Physiol* **523 Pt 3**, 621-628.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., & Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**, 529-540.

Moon, I. S., Apperson, M. L., & Kennedy, M. B. (1994). The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc.Natl.Acad.Sci.U.S.A* **91**, 3954-3958.

Moore, K. A., Kohno, T., Karchewski, L. A., Scholz, J., Baba, H., & Woolf, C. J. (2002). Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J.Neurosci.* **22**, 6724-6731.

Moss, S. J. & Smart, T. G. (1996). Modulation of amino acid-gated ion channels by protein phosphorylation. *Int.Rev.Neurobiol.* **39**, 1-52.

Nagy, J. I. & Hunt, S. P. (1982). Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience* **7**, 89-97.

Nagy, J. I. & van der Kooy, D. (1983). Effects of neonatal capsaicin treatment on nociceptive thresholds in the rat. *J.Neurosci.* **3**, 1145-1150.

Naim, M., Spike, R. C., Watt, C., Shehab, S. A., & Todd, A. J. (1997). Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents. *J.Neurosci.* **17**, 5536-5548.

Naim, M. M., Shehab, S. A., & Todd, A. J. (1998). Cells in laminae III and IV of the rat spinal cord which possess the neurokinin-1 receptor receive monosynaptic input from myelinated primary afferents. *Eur.J.Neurosci.* **10**, 3012-3019.

Nakatsuka, T., Ataka, T., Kumamoto, E., Tamaki, T., & Yoshimura, M. (2000). Alteration in synaptic inputs through C-afferent fibers to substantia gelatinosa neurons of the rat spinal dorsal horn during postnatal development. *Neuroscience* **99**, 549-556.

Nayak, A. S., Moore, C. I., & Browning, M. D. (1996). Ca2+/calmodulin-dependent protein kinase II phosphorylation of the presynaptic protein synapsin I is persistently increased during long-term potentiation. *Proc.Natl.Acad.Sci.U.S.A* **93**, 15451-15456.

Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C., & Greengard, P. (1990). Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. *Nature* **343**, 647-651.

Ninan, I. & Arancio, O. (2004). Presynaptic CaMKII Is Necessary for Synaptic Plasticity in Cultured Hippocampal Neurons. *Neuron* **42**, 129-141.

O'Leary, D. D., Ruff, N. L., & Dyck, R. H. (1994). Development, critical period plasticity, and adult reorganizations of mammalian somatosensory systems. *Curr.Opin.Neurobiol.* **4**, 535-544.

Obata, K., Oide, M., & Tanaka, H. (1978). Excitatory and inhibitory actions of GABA and glycine on embryonic chick spinal neurons in culture. *Brain Res.* **144**, 179-184.

Ogata, N. & Tatebayashi, H. (1992). Ontogenic development of the TTX-sensitive and TTX-insensitive Na+ channels in neurons of the rat dorsal root ganglia. *Brain Res.Dev.Brain Res.* **65**, 93-100.

Ottersen, O. P., Zhang, N., & Walberg, F. (1992). Metabolic compartmentation of glutamate and glutamine: morphological evidence obtained by quantitative immunocytochemistry in rat cerebellum. *Neuroscience* **46**, 519-534.

Owens, D. F., Boyce, L. H., Davis, M. B., & Kriegstein, A. R. (1996). Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J.Neurosci.* **16**, 6414-6423.

Park, J. S., Nakatsuka, T., Nagata, K., Higashi, H., & Yoshimura, M. (1999). Reorganization of the primary afferent termination in the rat spinal dorsal horn during post-natal development. *Brain Res.Dev.Brain Res.* **113**, 29-36.

Pattinson, D. & Fitzgerald, M. (2004). The neurobiology of infant pain: development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. *Reg Anesth.Pain Med.* **29**, 36-44.

Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Andersson, D. A., Story, G. M., Earley, T. J., Dragoni, I., McIntyre, P., Bevan, S., & Patapoutian, A. (2002). A TRP channel that senses cold stimuli and menthol. *Cell* **108**, 705-715.

Penn, A. A., Riquelme, P. A., Feller, M. B., & Shatz, C. J. (1998). Competition in retinogeniculate patterning driven by spontaneous activity. *Science* **279**, 2108-2112.

Petersen, J. D., Chen, X., Vinade, L., Dosemeci, A., Lisman, J. E., & Reese, T. S. (2003). Distribution of postsynaptic density (PSD)-95 and Ca2+/calmodulin-dependent protein kinase II at the PSD. *J.Neurosci.* **23**, 11270-11278.

Polgar, E., Hughes, D. I., Riddell, J. S., Maxwell, D. J., Puskar, Z., & Todd, A. J. (2003). Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. *Pain* **104**, 229-239.

Polley, D. B., Kvasnak, E., & Frostig, R. D. (2004). Naturalistic experience transforms sensory maps in the adult cortex of caged animals. *Nature* **429**, 67-71.

Poncer, J. C., Esteban, J. A., & Malinow, R. (2002). Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/calmodulin-dependent protein kinase II. *J.Neurosci.* **22**, 4406-4411.

Porter, F. L., Grunau, R. E., & Anand, K. J. (1999). Long-term effects of pain in infants. *J.Dev.Behav.Pediatr.* **20**, 253-261.

Pratt, K. G., Watt, A. J., Griffith, L. C., Nelson, S. B., & Turrigiano, G. G. (2003). Activity-dependent remodeling of presynaptic inputs by postsynaptic expression of activated CaMKII. *Neuron* **39**, 269-281.

Puig, S. & Sorkin, L. S. (1996). Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. *Pain* **64**, 345-355.

Rajendra, S., Lynch, J. W., & Schofield, P. R. (1997). The glycine receptor. *Pharmacol.Ther.* **73**, 121-146.

Ramón y Cajal (1909). *Histologie du Systeme Nerveux de L'Homme et des Vertebres*, pp. 908-911. Inst. Cajal, Madrid.

Randic, M., Jiang, M. C., & Cerne, R. (1993). Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. *J.Neurosci.* **13**, 5228-5241.

Reichling, D. B., Kyrozis, A., Wang, J., & MacDermott, A. B. (1994). Mechanisms of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. *J.Physiol* **476**, 411-421.

Ren, K., Hylden, J. L., Williams, G. M., Ruda, M. A., & Dubner, R. (1992a). The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal horn neuronal activity in rats with unilateral inflammation. *Pain* **50**, 331-344.

Ren, K., Williams, G. M., Hylden, J. L., Ruda, M. A., & Dubner, R. (1992b). The intrathecal administration of excitatory amino acid receptor antagonists selectively attenuated carrageenan-induced behavioral hyperalgesia in rats. *Eur.J.Pharmacol.* **219**, 235-243.

Reynolds, M. L. & Fitzgerald, M. (1992). Neonatal sciatic nerve section results in thiamine monophosphate but not substance P or calcitonin gene-related peptide depletion from the terminal field in the dorsal horn of the rat: the role of collateral sprouting. *Neuroscience* **51**, 191-202.

Rich, R. C. & Schulman, H. (1998). Substrate-directed function of calmodulin in autophosphorylation of Ca2+/calmodulin-dependent protein kinase II. *J.Biol.Chem.* **273**, 28424-28429.

Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., & Kaila, K. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* **397**, 251-255.

Rumpel, S., Kattenstroth, G., & Gottmann, K. (2004). Silent synapses in the immature visual cortex: layer-specific developmental regulation. *J.Neurophysiol.* **91**, 1097-1101.

Rygh, L. J., Svendsen, F., Hole, K., & Tjolsen, A. (2001). Increased spinal N-methyl-D-aspartate receptor function after 20 h of carrageenan-induced inflammation. *Pain* **93**, 15-21.

Sanderson Nydahl, K., Skinner, K., Julius, D., & Basbaum, A. I. (2004). Co-localization of endomorphin-2 and substance P in primary afferent nociceptors and effects of injury: a light and electron microscopic study in the rat. *Eur.J.Neurosci.* **19**, 1789-1799.

Sandkuhler, J. & Liu, X. (1998). Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. *Eur.J.Neurosci.* **10**, 2476-2480.

Santha, P. & Jancso, G. (2003). Transganglionic transport of choleragenoid by capsaicin-sensitive C-fibre afferents to the substantia gelatinosa of the spinal dorsal horn after peripheral nerve section. *Neuroscience* **116**, 621-627.

Sato, E., Takano, Y., Kuno, Y., Takano, M., & Sato, I. (2003). Involvement of spinal tyrosine kinase in inflammatory and N-methyl-D-aspartate-induced hyperalgesia in rats. *Eur.J.Pharmacol.* **468**, 191-198.

Schaffner, A. E., Behar, T., Nadi, S., Smallwood, V., & Barker, J. L. (1993). Quantitative analysis of transient GABA expression in embryonic and early postnatal rat spinal cord neurons. *Brain Res.Dev.Brain Res.* **72**, 265-276.

Scheibel, M. E. & Scheibel, A. B. (1968). Terminal axonal patterns in cat spinal cord. II. The dorsal horn. *Brain Res.* **9**, 32-58.

Scholz, J. & Woolf, C. J. (2002). Can we conquer pain? *Nat.Neurosci.* **5 Suppl**, 1062-1067.

Seltzer, Z., Cohn, S., Ginzburg, R., & Beilin, B. (1991). Modulation of neuropathic pain behavior in rats by spinal disinhibition and NMDA receptor blockade of injury discharge. *Pain* **45**, 69-75.

Seltzer, Z., Dubner, R., & Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **43**, 205-218.

Shehab, S. A., Spike, R. C., & Todd, A. J. (2003). Evidence against cholera toxin B subunit as a reliable tracer for sprouting of primary afferents following peripheral nerve injury. *Brain Res.* **964**, 218-227.

Shen, K. & Meyer, T. (1999). Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284**, 162-166.

Shin, J. B., Martinez-Salgado, C., Heppenstall, P. A., & Lewin, G. R. (2003). A T-type calcium channel required for normal function of a mammalian mechanoreceptor. *Nat.Neurosci.* **6**, 724-730.

Shortland, P., Woolf, C. J., & Fitzgerald, M. (1989). Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. *J.Comp Neurol.* **289**, 416-433.

Silva, A. J., Paylor, R., Wehner, J. M., & Tonegawa, S. (1992a). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**, 206-211.

Silva, A. J., Stevens, C. F., Tonegawa, S., & Wang, Y. (1992b). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**, 201-206.

Simmons, Z. & Feldman, E. L. (2002). Update on diabetic neuropathy. *Curr.Opin.Neurol.* **15**, 595-603.

Sluka, K. A. & Willis, W. D. (1997). The effects of G-protein and protein kinase inhibitors on the behavioral responses of rats to intradermal injection of capsaicin. *Pain* **71**, 165-178.

Snider, W. D. & Palavali, V. (1990). Early axon and dendritic outgrowth of spinal accessory motor neurons studied with DiI in fixed tissues. *J.Comp Neurol.* **297**, 227-238.

South, S. M., Kohno, T., Kaspar, B. K., Hegarty, D., Vissel, B., Drake, C. T., Ohata, M., Jenab, S., Sailer, A. W., Malkmus, S., Masuyama, T., Horner, P., Bogulavsky, J., Gage, F. H., Yaksh, T. L., Woolf, C. J., Heinemann, S. F., & Inturrisi, C. E. (2003). A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. *J.Neurosci.* **23**, 5031-5040.

Spike, R. C., Puskar, Z., Andrew, D., & Todd, A. J. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur.J.Neurosci.* **18**, 2433-2448.

Stanfa, L. C. & Dickenson, A. H. (1999). The role of non-N-methyl-D-aspartate ionotropic glutamate receptors in the spinal transmission of nociception in normal animals and animals with carrageenan inflammation. *Neuroscience* **93**, 1391-1398.

Stegenga, S. L. & Kalb, R. G. (2001). Developmental regulation of N-methyl-D-aspartate- and kainate-type glutamate receptor expression in the rat spinal cord. *Neuroscience* **105**, 499-507.

Strack, S. & Colbran, R. J. (1998). Autophosphorylation-dependent targeting of calcium/ calmodulin- dependent protein kinase II by the NR2B subunit of the N-me. *J.Biol.Chem.* **273**, 20689-20692.

Suzuki, R., Kontinen, V. K., Matthews, E., Williams, E., & Dickenson, A. H. (2000). Enlargement of the receptive field size to low intensity mechanical stimulation in the rat spinal nerve ligation model of neuropathy. *Exp.Neurol.* **163**, 408-413.

Suzuki, R., Matthews, E. A., & Dickenson, A. H. (2001). Comparison of the effects of MK-801, ketamine and memantine on responses of spinal dorsal horn neurones in a rat model of mononeuropathy. *Pain* **91**, 101-109.

Suzuki, T. & Okumura-Noji, K. (1995). NMDA receptor subunits epsilon 1 (NR2A) and epsilon 2 (NR2B) are substrates for Fyn in the postsynaptic density fraction isolated from the rat brain. *Biochem.Biophys.Res.Commun.* **216**, 582-588.

Swett, J. E. & Woolf, C. J. (1985). The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *J.Comp Neurol.* **231**, 66-77.

Szentagothiai, J. (1964). Neuronal and synaptic arrangement in the substantia gelatinosa Rolandi. *J.Comp Neurol.* **122**, 219-239.

Taddio, A., Goldbach, M., Ipp, M., Stevens, B., & Koren, G. (1995). Effect of neonatal circumcision on pain responses during vaccination in boys. *Lancet* **345**, 291-292.

Taha, S., Hanover, J. L., Silva, A. J., & Stryker, M. P. (2002). Autophosphorylation of alphaCaMKII is required for ocular dominance plasticity. *Neuron* **36**, 483-491.

Takahashi, T., Momiyama, A., Hirai, K., Hishinuma, F., & Akagi, H. (1992). Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. *Neuron* **9**, 1155-1161.

Takaishi, K., Eisele, J. H., Jr., & Carstens, E. (1996). Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats. *Pain* **66**, 297-306.

Taniguchi, K., Shinjo, K., Mizutani, M., Shimada, K., Ishikawa, T., Menniti, F. S., & Nagahisa, A. (1997). Antinociceptive activity of CP-101,606, an NMDA receptor NR2B subunit antagonist. *Br.J.Pharmacol.* **122**, 809-812.

Taylor, B. K., Peterson, M. A., & Basbaum, A. I. (1997). Early nociceptive events influence the temporal profile, but not the magnitude, of the tonic response to subcutaneous formalin: effects with remifentanil. *J.Pharmacol.Exp.Ther.* **280**, 876-883.

Taylor, B. K., Peterson, M. A., Roderick, R. E., Tate, J., Green, P. G., Levine, J. O., & Basbaum, A. I. (2000). Opioid inhibition of formalin-induced changes in plasma extravasation and local blood flow in rats. *Pain* **84**, 263-270.

Terashima, T., Ochiishi, T., & Yamauchi, T. (1994). Immunohistochemical detection of calcium/calmodulin-dependent protein kinase II in the spinal cord of the rat and monkey with special reference to the corticospinal tract. *J.Comp Neurol.* **340**, 469-479.

Terashima, T., Ochiishi, T., & Yamauchi, T. (1995). Alpha calcium/calmodulin-dependent protein kinase II immunoreactivity in corticospinal neurons: combination of axonal transport method and immunofluorescence. *Anat.Embryol.(Berl)* **192**, 123-136.

Todd, A. J. (1989). Cells in laminae III and IV of rat spinal dorsal horn receive monosynaptic primary afferent input in lamina II. *J.Comp Neurol.* **289**, 676-686.

Todd, A. J. (2002). Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Exp.Physiol* **87**, 245-249.

Todd, A. J., McGill, M. M., & Shehab, S. A. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *Eur.J.Neurosci.* **12**, 689-700.

Todd, A. J. & Sullivan, A. C. (1990). Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J.Comp Neurol.* **296**, 496-505.

Torsney, C. & Fitzgerald, M. (2002). Age-dependent effects of peripheral inflammation on the electrophysiological properties of neonatal rat dorsal horn neurons. *J.Neurophysiol.* **87**, 1311-1317.

Torsney, C. & Fitzgerald, M. (2003). Spinal dorsal horn cell receptive field size is increased in adult rats following neonatal hindpaw skin injury. *J.Physiol* **550**, 255-261.

Torsney, C., Meredith-Middleton, J., & Fitzgerald, M. (2000). Neonatal capsaicin treatment prevents the normal postnatal withdrawal of A fibres from lamina II without affecting fos responses to innocuous peripheral stimulation. *Brain Res.Dev.Brain Res.* **121**, 55-65.

Tran, T. S., Alijani, A., & Phelps, P. E. (2003). Unique developmental patterns of GABAergic neurons in rat spinal cord. *J.Comp Neurol.* **456**, 112-126.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W., & Inoue, K. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* **424**, 778-783.

Vaillant, A. R., Zanassi, P., Walsh, G. S., Aumont, A., Alonso, A., & Miller, F. D. (2002). Signaling mechanisms underlying reversible, activity-dependent dendrite formation. *Neuron* **34**, 985-998.

Waldenstrom, A., Thelin, J., Thimansson, E., Levinsson, A., & Schouenborg, J. (2003). Developmental learning in a pain-related system: evidence for a cross-modality mechanism. *J.Neurosci.* **23**, 7719-7725.

Wall, P. D. (1967). The laminar organization of dorsal horn and effects of descending impulses. *J.Physiol* **188**, 403-423.

Wall, P. D. (1977). The presence of ineffective synapses and the circumstances which unmask them. *Philos.Trans.R.Soc.Lond B Biol.Sci.* **278**, 361-372.

Wall, P. D. & Devor, M. (1981). The effect of peripheral nerve injury on dorsal root potentials and on transmission of afferent signals into the spinal cord. *Brain Res.* **209**, 95-111.

Wallace, V. C., Cottrell, D. F., Brophy, P. J., & Fleetwood-Walker, S. M. (2003). Focal lysolecithin-induced demyelination of peripheral afferents results in neuropathic pain behavior that is attenuated by cannabinoids. *J.Neurosci.* **23**, 3221-3233.

Wang, G. D. & Zhuo, M. (2002). Synergistic enhancement of glutamate-mediated responses by serotonin and forskolin in adult mouse spinal dorsal horn neurons. *J.Neurophysiol.* **87**, 732-739.

Wang, J., Reichling, D. B., Kyrozis, A., & MacDermott, A. B. (1994). Developmental loss of GABA- and Glycine-induced depolarization and Ca2+ transients in embryonic rat dorsal horn neurons in culture. *Eur.J.Neurosci.* **6**, 1275-1280.

Wang, R. A., Cheng, G., Kolaj, M., & Randic, M. (1995). Alpha-subunit of calcium/calmodulin-dependent protein kinase II enhances gamma-aminobutyric acid and inhibitory synaptic responses of rat neurons in vitro. *J.Neurophysiol.* **73**, 2099-2106.

Wang, R. A. & Randic, M. (1996). alpha-subunit of CaM-KII increases glycine currents in acutely isolated rat spinal neurons. *J.Neurophysiol.* **75**, 2651-2653.

Wang, Y. T. & Salter, M. W. (1994). Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369**, 233-235.

Watanabe, E. & Akagi, H. (1995). Distribution patterns of mRNAs encoding glycine receptor channels in the developing rat spinal cord. *Neurosci.Res.* **23**, 377-382.

Watanabe, M., Mishina, M., & Inoue, Y. (1994). Distinct spatiotemporal distributions of the N-methyl-D-aspartate receptor channel subunit mRNAs in the mouse cervical cord. *J.Comp Neurol.* **345**, 314-319.

Wikstrom, M. A., Matthews, P., Roberts, D., Collingridge, G. L., & Bortolotto, Z. A. (2003). Parallel kinase cascades are involved in the induction of LTP at hippocampal CA1 synapses. *Neuropharmacology* **45**, 828-836.

Willis, W. D. & Coggeshall, R. E. (1991). *Sensory mechanisms of the spinal cord* Plenum Press, New York.

Willis, W. D., Leonard, R. B., & Kenshalo, D. R., Jr. (1978). Spinothalamic tract neurons in the substantia gelatinosa. *Science* **202**, 986-988.

Woodbury, C. J. & Koerber, H. R. (2003). Widespread projections from myelinated nociceptors throughout the substantia gelatinosa provide novel insights into neonatal hypersensitivity. *J.Neurosci.* **23**, 601-610.

Woodbury, C. J., Ritter, A. M., & Koerber, H. R. (2000). On the problem of lamination in the superficial dorsal horn of mammals: a reappraisal of the substantia gelatinosa in postnatal life. *J.Comp Neurol.* **417**, 88-102.

Woodbury, C. J., Ritter, A. M., & Koerber, H. R. (2001). Central anatomy of individual rapidly adapting low-threshold mechanoreceptors innervating the "hairy" skin of newborn mice: early maturation of hair follicle afferents. *J.Comp Neurol.* **436**, 304-323.

Woolf, C. J. (1987). Central terminations of cutaneous mechanoreceptive afferents in the rat lumbar spinal cord. *J.Comp Neurol.* **261**, 105-119.

Woolf, C. J. & Fitzgerald, M. (1983). The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord. *J.Comp Neurol.* **221**, 313-328.

Woolf, C. J. & King, A. E. (1987). Physiology and morphology of multireceptive neurons with C-afferent fiber inputs in the deep dorsal horn of the rat lumbar spinal cord. *J.Neurophysiol.* **58**, 460-479.

Woolf, C. J. & King, A. E. (1990). Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. *J.Neurosci.* **10**, 2717-2726.

Woolf, C. J. & Salter, M. W. (2000). Neuronal plasticity: increasing the gain in pain. *Science* **288**, 1765-1769.

Woolf, C. J., Shortland, P., & Coggeshall, R. E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* **355**, 75-78.

Woolf, C. J. & Swett, J. E. (1984). The cutaneous contribution to the hamstring flexor reflex in the rat: an electrophysiological and anatomical study. *Brain Res.* **303**, 299-312.

Woolf, C. J. & Thompson, S. W. (1991). The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. *Pain* **44**, 293-299.

Wright, S. C., Schellenberger, U., Ji, L., Wang, H., & Larrick, J. W. (1997). Calmodulin-dependent protein kinase II mediates signal transduction in apoptosis. *FASEB J.* **11**, 843-849.

Wu, G. Y. & Cline, H. T. (1998). Stabilization of dendritic arbor structure in vivo by CaMKII. *Science* **279**, 222-226.

Xu, G. Y. & Huang, L. Y. (2002). Peripheral Inflammation Sensitizes P2X Receptor-Mediated Responses in Rat Dorsal Root Ganglion Neurons. *J.Neurosci.* **22**, 93-102.

Yasuda, H., Barth, A. L., Stellwagen, D., & Malenka, R. C. (2003). A developmental switch in the signaling cascades for LTP induction. *Nat.Neurosci.* **6**, 15-16.

Yokoyama, C. T., Sheng, Z. H., & Catterall, W. A. (1997). Phosphorylation of the synaptic protein interaction site on N-type calcium channels inhibits interactions with SNARE proteins. *J.Neurosci.* **17**, 6929-6938.

Yoshimura, M. & Jessell, T. M. (1989). Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons in vitro. *J.Neurophysiol.* **62**, 96-108.

Yoshimura, M. & Nishi, S. (1993). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. *Neuroscience* **53**, 519-526.

Young, M. R., Fleetwood-Walker, S. M., Dickinson, T., Blackburn-Munro, G., Sparrow, H., Birch, P. J., & Bountra, C. (1997). Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord. *Brain Res.* **777**, 161-169.

Yung, K. K. (1998). Localization of glutamate receptors in dorsal horn of rat spinal cord. *NeuroReport* **9**, 1639-1644.

Zou, D. J. & Cline, H. T. (1996). Expression of constitutively active CaMKII in target tissue modifies presynaptic axon arbor growth. *Neuron* **16**, 529-539.

Zou, D. J. & Cline, H. T. (1999). Postsynaptic calcium/calmodulin-dependent protein kinase II is required to limit elaboration of presynaptic and postsynaptic neuronal arbors. *J.Neurosci.* **19**, 8909-8918.

Zou, D. J., Greer, C. A., & Firestein, S. (2002). Expression pattern of alpha CaMKII in the mouse main olfactory bulb. *J.Comp Neurol.* **443**, 226-236.