The co-localisation and distribution of glutamate decarboxylase isoforms in the rat spinal cord

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Summary

GABA is one of the two main inhibitory neurotransmitters in the central nervous system, CNS (along with glycine, which has a major role in the brainstem and spinal cord). GABA regulates a number of functions in the CNS, and in the spinal cord, it is responsible for presynaptic inhibition of primary afferents and postsynaptic inhibition of neurons. GABA is produced by decarboxylation of L-glutamate by glutamate decarboxylase (GAD). Two GAD isoforms have been identified, GAD65 and GAD67.

Antibodies raised against glutaraldehyde conjugates of GABA have been used to investigate the distribution of GABAergic cell bodies, whilst the distribution of GABAergic terminals has been examined with antibodies against GAD. Although GABAergic cell bodies are detected throughout the spinal grey matter, these are concentrated in laminae I-III of the spinal dorsal horn. GAD is present in axon terminals in all laminae of the rat spinal cord, but only a few immunoreactive cell bodies have been detected in the superficial dorsal horn. This differs from the situation in the brain, where many GAD-immunoreactive cell bodies can be found. Studies in the brain suggest that while most (if not all GABAergic neurons) synthesise both GAD isoforms, many have relatively high levels of one or other isoform. It is not known whether this is the case in the spinal cord. Until recently, most studies that have looked at the distribution of GAD have used antibodies that do not differentiate between the two isoforms, and such studies in the spinal cord have been qualitative and no attempt has been made to quantify GAD levels in individual laminae, or examine the co-localisation of GAD isoforms in individual boutons. The recent availability of antibodies that are directed against each isoform separately enables detailed studies to be performed that compare the distribution and co-localisation of the two isoforms. In this study, immunocytochemistry and confocal microscopy were used to examine the distribution and co-localisation of GAD65 and GAD67 in individual axonal boutons in each lamina of the rat spinal grey matter. The main finding of this part of the study was that although most GAD-

immunoreactive boutons were labelled with both GAD65 and GAD67 antibodies, some showed similar intensities of both types of immunoreactivity whilst others appeared to have relatively higher levels of one or other of the GAD isoforms. This suggests that GAD-immunoreactive neurons are a heterogeneous population. Also, GAD-immunoreactivity differed between each lamina of the spinal cord e.g. in the superficial dorsal horn, boutons that had relatively higher levels of either GAD65 or GAD67 were frequently found. In contrast, most boutons in the ventral horn displayed relatively high levels of GAD67, although discrete clusters of boutons that had high levels of GAD65 immunoreactivity were detected in lamina IX. Very few GAD-immunoreactive cell bodies were detected, and those that were found were generally GAD67-immunoreactive.

Populations of GABAergic neurons in the dorsal horn that differ in their neurochemistry have been identified. These have specific laminar distributions and are thought to be functionally different. GABAergic cells can be classified according to their enrichment with other substances. In many neurons, glycine co-localises with GABA, and at some synapses in the spinal cord, they may be released from the same vesicles. Therefore, GABA and glycine may act as co-transmitters at some inhibitory synapses in the CNS. The co-localisation of each GAD isoform with GLYT2 (a marker for glycinergic axons) was examined. In this study, no relationship was detected between GAD and GLYT2 expression in the dorsal horn, as some GLYT2-immunostained profiles showed strong GAD65-immunoreactivity whilst others displayed relatively higher levels of immunoreactivity for GAD67. In contrast, in the ventral horn, boutons that were immunoreactive for GLYT2 were more likely to have relatively high levels of GAD67-immunoreactivity whilst those that were GLYT2-negative were more likely to have relatively stronger GAD65-immunoreactivity.

The relationship between PV and NOS (2 markers of GABAergic populations) with GAD67 was investigated in cell bodies in laminae II and III. Although 83% of PV-

immunoreactive cell bodies were immunostained with the GAD67 antibody, none of the NOS-positive cells were GAD67-immunoreactive.

GABAergic axo-axonic synapses are the anatomical substrate of presynaptic inhibition of primary afferents and primary afferent depolarisation, and P boutons are responsible for this inhibition in group Ia primary muscle spindle afferents. A 'GAD65 intense' population in the ventral horn may be the P boutons as these form discrete clusters in lamina IX. This study examined their association with primary afferent terminals. This was done with immunocytochemistry, confocal microscopy and electron microscopy. Primary afferent terminals were identified by retrograde labelling with cholera toxin type b (CTb) and vesicular glutamate transporter type I (VGLUT1)-immunoreactivity. The main finding of this part of the study was that 88-89% of the 'GAD65 intense' boutons in lamina IX were in close contact with primary afferent terminals, and frequently formed clusters around them. Since these boutons lack GLYT2-immunoreactivity they are presumably not glycinergic. This is consistent with evidence that P boutons are not glycinergic. In conclusion, the 'GAD65 intense' boutons in lamina IX are the P boutons.

GABA, and glycine, may play very specific roles in the modulation of pain information in the spinal dorsal horn, as intrathecal administration of $GABA_A$ and glycine receptor antagonists results in a dose-dependent exaggerated response to light tactile stimulation (a symptom of neuropathic pain). In this study, investigations were carried out to establish whether there were any changes in GAD65- or GAD67-immunoreactivity in laminae I, II or III after peripheral nerve injury (with the chronic constriction injury (CCI) model) or complete nerve transection (with the sciatic nerve transection (SNT) model). Immunocytochemistry, confocal microscopy and image analysis were used to investigate these changes. This part of the study found that there was a significant reduction in GAD65- and GAD67-immunoreactivity in lamina II of SNT animals. A significant decrease in GAD67-immunoreactivity was also detected in lamina III of CCI animals

and laminae I and III of SNT animals. When these results are viewed in conjunction with the results of a study by Moore et al. (2002), it appears that this decrease in GAD65 and GAD67 in the spinal dorsal horn after nerve injury does not directly result in a reduction in GABA-mediated inhibitory transmission in neuropathic animals.

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Declaration

I declare that the work presented in this thesis is my own (except where otherwise stated) and has not been submitted previously for any higher degree.

List of Abbreviations

5HT	serotonin
CCI	chronic constriction injury
CNS	central nervous system
Confocal-EM	confocal electron microscopy
CTb	cholera toxin subunit b
Cy5	cyanine 5.18
DAB	diaminobenzidine
EPSPs	excitatory postsynaptic potentials
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
$GABA_A$	GABA type A
$GABA_B$	GABA type B
$GABA_C$	GABA type C
GAD	glutamate decarboxylase
GLYT2	glycine transporter type 2
HRP	horseradish peroxidase
IPSPs	inhibitory postsynaptic potential
Lamina IIi	lamina II inner
Lamina IIo	lamina II outer
NK-1	neurokinin-1
NOS	nitric oxide synthase
PAD	primary afferent depolarisation
PBS	phosphate buffered saline
PLP	pyridoxal 5'-phosphate

PV	parvalbumin
SNI	spared nerve injury
SNT	sciatic nerve transaction
SP	substance P
VGLUT1	vesicular glutamate transporter type 1
VGLUT2	vesicular glutamate transporter type 2

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Chapter 3 Distribution and co-localisation of glutamate decarboxylase isoforms in the rat spinal cord.

Chapter 4 GABA and the P boutons

Publications arising from this work

Papers:

Mackie M, Hughes DI, Maxwell DJ, Tillakaratne NJK and Todd AJ (2003) Distribution and co-localisation of glutamate decarboxylase isoforms in the rat spinal cord. Neuroscience 119: 461-472.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ (2003) The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. European Journal of Neuroscience 17: 13-27.

Hughes DI, Mackie M, Nagy GG, Riddell JS, Maxwell DJ, Szabó G, Erdélyi F, Veress G, Szűcs P, Antal M, Todd AJ (2005) P boutons in lamina IX of the rodent spinal cord express high levels of glutamic acid decarboxylase-65 and originate from cells in deep medial dorsal horn Proceedings of the National Academy of Sciences of the United States of America 102: 9038-9043.

Copies of these publications are included in appendix

Chapter 1: Introduction

1.1 GABA in the Central Nervous System.

γ-aminobutyric acid (GABA), and glycine, are the main inhibitory neurotransmitters in the mammalian central nervous system (CNS). GABA's role as a neurotransmitter was first established in the 1960s in the neuromuscular junction of the lobster. Investigation of this junction has allowed experimenters to demonstrate, using neurochemistry and electrophysiology, that GABA was released from inhibitory nerves in this species (Otsuka et al., 1966). It has since been shown that GABA has an important role as an inhibitory neurotransmitter in the mammalian CNS, where it regulates a number of functions including locomotion, learning, reproduction, development, pain and circadian rhythms. Areas such as the substantia nigra, globus pallidus and cerebellar cortex (all cell types except granular cells) have a strong GABAergic input (Storm-Mathisen et al., 1983). GABA is also transiently expressed in non-GABAergic neurons of the embryonic and adult CNS, suggesting that it has a role in the development and plasticity of the nervous system (Sloviter et al., 1996). In the spinal cord, GABA is responsible for producing both presynaptic inhibition of primary afferents (Eccles et al., 1963) and postsynaptic inhibition of spinal neurons (Curtis et al., 1968).

Outside the CNS, GABA, and its synthetic enzyme, glutamate decarboxylase (GAD), are found in peripheral tissues, including the testi (Persson, 1990), oviduct and ovary (Apud, 1984). GABA and GAD are also found in the islets of Langerhans of the pancreas, where insulin is produced and GAD65 (one of two isoforms of GAD) has been identified as an autoantigen in insulin dependent diabetes mellitus. This is an autoimmune condition where there is T-cell mediated destruction of pancreatic insulin-secreting β cells (Solimena, 1991). In the pancreas, GABA may act as an autocrine inhibitor of insulin release, as well as a paracrine inhibitor of glucagon and somatostatin release (Franklin and Wollheim, 2005).

In addition, GABA also functions as an intermediate in energy metabolism in GABAergic neurons. The synthesis and degradation of GABA (known as the GABA shunt) accounts for a small fraction of the tricarboxylic acid cycle in the brain. GAD, GABA-α-oxoglutarate transaminase and succinic semialdehyde dehydrogenase are the three main enzymes of this GABA pathway that provides a by-pass to avoid two steps of the tricarboxylic acid cycle (Martin and Rimvall, 1993).

1.2 GABA receptors and transporters

To date, three GABA receptor subtypes have been identified: GABA type A ($GABA_A$), GABA type B ($GABA_B$) and GABA type C ($GABA_C$). The $GABA_A$ receptor is a heteromeric ligand gated chloride channel that is based on a family of at least 15 subunits (e.g., α1, α2, α3, α5, β2,3, and γ2; Bohlhalter et al., 1996). GABA, acting on $GABA_A$ receptors, can depolarise primary afferent fibres that innervate muscle and skin via axo-axonic synapses in the dorsal horn, ventral horn, intermediate nucleus and Clarke's column. This results in inhibition of glutamate release from primary afferent terminals (Curtis et al., 1986), and is known as presynaptic inhibition. The actions of GABA at this receptor can be modulated by benzodiazepines, barbiturates, and neurosteroids (Malcangio and Bowery, 1996), and blocked by the receptor antagonists, bicuculline (Curtis et al., 1971b) and picrotoxin (Curtis et al., 1969).

The $GABA_B$ receptor is a GTP-binding protein coupled receptor that consists of two identified subunits, $GABA_{B1}$ and $GABA_{B2}$. $GABA_B$ is linked to membrane calcium and potassium channels (Bowery et al., 1993). GABA, acting on the $GABA_B$ receptor, can also reduce neurotransmitter release from primary afferent terminals, but with no associated depolarisation of the postsynaptic membrane (Curtis et al., 1981). The inhibitory effects of GABA mediated via this receptor can be selectively mimicked by β-chlorophenyl-GABA (baclofen; Bowery et al., 1993) and blocked by the antagonist CGP35348.

Both $GABA_A$ and $GABA_B$ receptor agonists selectively depress excitatory postsynaptic potentials (EPSPs) in motoneurons, and therefore have a role in spinal reflex activity (Malcangio and Bowery, 1996). Although $GABA_A$, and $GABA_B$ receptors are located at synapses, they are also found outside conventional synapses (Nusser et al., 1995), and this in conjunction with the high concentration of GABA found in synaptic clefts, indicates that GABA may also take part in non-synaptic signalling (volume transmission) at a distance away from the site of its release.

Much less is known about the $GABA_C$ receptor, which was pharmacologically identified based on its insensitivity to bicuculline ($GABA_A$ receptor antagonist) and baclofen ($GABA_B$ receptor agonist) (Drew et al., 1984). The $GABA_C$ receptor resembles the $GABA_A$ receptor, in that both are associated with fast chloride conductance (Johnston, 1994). $GABA_C$ receptors consist of a combination of three receptor subunits, named ρ1, ρ2 and ρ3 (Ogurusu et al., 1995; Ogurusu et al., 1997; Zhang et al., 1995). The ρ2 subunit is the most widely distributed of the subunits in the rat brain and retina. ρ1 has a restricted expression and ρ3 is expressed strongly in the embryonic brain, but has decreased expression towards adulthood (Ogurusu et al., 1997; Bouc-Grabot et al., 1998).

At present, four plasma membrane GABA transporters have been identified and cloned: GAT1, GAT2, GAT3 and GAT4. These are Na^+ dependent carrier-mediated transport systems and are presumed to take part in the inactivation and recycling of GABA found in the extracellular space (Kleinbergerdoron et al., 1994). In situ hybridisation histochemistry (Durkin et al., 1995; Jursky and Nelson, 1996; Yasumi et al., 1997) and immunocytochemistry (Ikegaki et al., 1994; Radian et al., 1990) have been used to investigate the distribution of the GABA transporters in the CNS. According to these studies, GAT1 is expressed throughout the CNS (Durkin et al., 1995) by neurons and glia (Jursky and Nelson, 1996) and is co-localised with GAD67 (one of two GAD isoforms) in most nuclei of the brain (Yasumi et al., 1997). GAT4 (which also transports beta alanine) is found at high concentrations in brain and is localised in

neurons (Jursky and Nelson, 1996). GAT3 is expressed in restricted regions of the brain (retina, olfactory bulb, subfornical organ, hypothalamus, midline thalamus and brainstem) and GAT2 is rarely found in the CNS (only over the pia and arachnoid meninges). The differential distribution of these four GABA transporters suggests that although each contributes to the uptake of GABA, they do so in distinct populations of neurons in the CNS.

1.3 Synthesis of GABA

In the adult brain, only neurons synthesise GABA. GABA is produced by decarboxylation of L-glutamate by GAD (Wingo and Awapara, 1950; Roberts and Frankel, 1951). GABA synthesis is complex, and different stages take place in separate subcellular compartments of the neuron. Firstly, the precursor of glutamate, glutamine, is synthesised in astrocytes, and then transported to neurons (Battaglioli et al., 1990). Glutamine then enters the mitochondria where it is hydrolysed to glutamate by phosphate-activated glutaminase (Kvamme et al., 1991). GAD is found in the cytosol (Fonnum, 1968), and as GABA synthesis is dependent on GAD, glutamate must move from the mitochondria to the cytosol for decarboxylation to occur.

Although GAD-independent synthesis of GABA does occur, this does not appear to contribute significantly to total brain GABA levels, at least during development, as the brains of GAD knockout mice (that lack GAD completely) contain only 0.02% of the GABA found in wild-type brains (Ji et al., 1999). GAD can exist as an active holoenzyme, holoGAD, and an inactive apoenzyme, apoGAD. All active GAD requires to be bound to the co-factor pyridoxal 5'-phosphate (PLP) (Roberts and Frankel, 1950) and short-term regulation of GABA synthesis is controlled by the interactions between GAD and PLP (Miller et al., 1977). This in turn may be regulated by substances, such as inorganic phosphate or adenosine triphosphate, which influence the conversion of apoGAD to holoGAD (Meeley and Martin, 1983).

After synaptic release, excess extracellular GABA is taken up and inactivated by neuronal and non-neuronal GABA transporters. Degradation of GABA occurs in the mitochondria, (Salganicoff et al., 1965) and depends upon GABA-transaminase, which converts GABA to succinic semialdehyde (Roberts and Bregoff, 1953).

1.4 GAD isoforms

GAD was initially extensively purified by Roberts and colleagues in mouse brain (Wu et al., 1979). In the CNS, two GAD isoforms have been identified, GAD65 and GAD67, which are named after their approximate molecular weights (65kDa and 67kDa). These isoforms are encoded by different genes (Bu et al., 1992). In humans, the gene for GAD67 is found on chromosome 2 at position 2q31, whilst the gene encoding GAD65 is found on chromosome 10 at position 10p11.23 (Bu et al., 1992; Edelhoff et al., 1993). In rats, each isoform has two distinct segments. These are residues 1-96 in GAD65 and 1-102 in GAD67; encoded by exons 1-3 and residues 97-585 in GAD65, and exons 4-16 and residues 103-594 in GAD67 (Erlander et al., 1991). This situation differs from that of other known neurotransmitters such as the catecholamines, acetylcholine and serotonin (5HT), as each of their synthetic enzymes are the product of a single gene (Soghomanian and Martin, 1998).

In addition, the two isoforms differ in their amino acid sequences, anatomical distribution, and regulatory control and in their responses to pathological conditions. GAD65 and GAD67 are highly conserved amongst vertebrates, with 95% amino acid sequence identity between the rat, cat, mouse and human forms of each protein (Erlander et al., 1991). Sequence analysis of the two GADs have shown that each is composed of two domains; a highly divergent N-terminal domain, which shows 23% identity between human GAD65 and GAD67, and a much larger C-terminal domain, which contains the catalytic centre and has 73% identity between isoforms in humans (Bu and Tobin, 1994).

Most, if not all, GABAergic neurons in the brain probably synthesise both GAD isoforms, most neurons preferentially express one or other of the isoforms (Soghomanian and Martin, 1998). It is not known whether this is also the case in the spinal cord. Esclapez et al. (1994) used immunohistochemistry and non-radioactive in situ hybridisation to examine the distribution of GAD65 and GAD67proteins and mRNAs within several subclasses of GABAergic neurons in the forebrain, midbrain, olfactory bulb, and cerebellum. They found that in most brain regions, both GADs were present in cell bodies and nerve terminals, although in some populations of neurons GAD67 immunoreactive cell bodies outnumbered those that were GAD65 immunoreactive (e.g., cerebellar Purkinje cells, non- pyramidal cells of the cerebral cortex and granule cells of the olfactory bulb). This was not always the case, as GAD65 immunoreactive cell bodies were present in high numbers in the reticular nucleus of thalamus and the olfactory bulb (periglomerular cells). In addition, the density of GAD65 immunoreactive axon terminals was higher than that of GAD67 immunoreactive terminals in most brain regions. A strong parallel was found between the patterns of cell body labelling found with immunohistochemistry and mRNA labelling detected using in situ hybridisation for each GAD. This supported the immunohistochemical findings that most brain regions contained fewer cell bodies that were predominantly GAD65 immunoreactive. The authors suggested that these differences in distribution within neurons might occur because GAD65 is transported to the axon terminal more readily than GAD67 from the neuronal cell body, or because GAD67 is degraded more rapidly at the terminal (Esclapez et al. 1994). Alternatively, it has been suggested that the two GAD isoforms may synthesise two separate pools of GABA in the brain that work by different mechanisms (Soghomanian and Martin, 1998). The high levels of both GAD67 protein and mRNA in cell bodies is consistent with a high rate of synthesis of GAD67, and therefore GAD67 may be more abundant in tonically firing cells that require a larger supply of GABA. This might provide a metabolic pool of GABA. In addition, some of the GABA produced by GAD67 may be

released through a non-vesicular mechanism, via the reversal of one of the plasma membrane bound GABA transporters, possibly GAT-1, whose distribution almost mirrors that of GAD67 in the brain (Yasumi et al., 1997). This GABA may take on the role of a paracrine signal (Gaspary et al., 1998), believed to be important in the inhibition of excessive neuronal firing in neurological disorders such as epilepsy. It has also been proposed that GAD65 may be more abundant in cells with phasic activity, where the supply of GAD within nerve terminals may be sufficient to meet the local needs for synaptically released GABA used in classical inhibitory transmission. (Feldblum et al., 1993; Esclapez et al., 1993; Esclapez et al., 1994; Hendrickson et al., 1994). It is not yet known whether there is a difference in the cellular and terminal concentrations of each isoform in the spinal cord.

The two GAD isoforms may also differ in their response to CNS injury. Alterations in GAD67 levels are detected more frequently than changes in GAD65 levels after certain types of injury to the nervous system (Soghomanian and Chesselet, 1992; Dumoulin et al., 1996; Feldblum et al., 1998; Tillakaratne et al., 2000) and it has been suggested that GAD67 may therefore have a protective role in neuronal plasticity of the CNS after injury. Studies by Soghomanian and Chesselet (1992), using in situ hybridisation histochemistry and immunohistochemistry have detected changes in GAD67 levels in sub-populations of basal ganglia neurons (in the globus pallidus) after unilateral injection of the neurotoxin, 6-hydroxydopamine into the substantia nigra of the rat. They found that in the ipsilateral globus pallidus, the number of cells labelled, and the intensity of individually labelled cells was increased after neurotoxin-induced lesions, whilst no change was detected on the contralateral side. No change in GAD65 levels were detected on either side. Studies by Dumoulin et al. (1996) and Feldblum et al. (1998) have investigated changes in GAD67 after partial deafferentation as a result of unilateral dorsal rhizotomy or neonatal capsaicin treatment respectively (Dumoulin et al., 1996; Feldblum et al., 1998). Dumoulin and colleagues found that three days after rhizotomy, there was a decrease in the

number of GAD67 mRNA expressing neurons in the superficial dorsal horn, with increased GABA immunostaining of axonal fibres in the same region. In contrast, seven days after lesion, there was an increase in GAD67 mRNA-expressing neurons in the deep dorsal and ventral horns in conjunction with a reduction of GABA-immunostained axons (Dumoulin et al., 1996). Feldblum and colleagues also found a transient increase in GAD67 mRNA levels following neonatal capsaicin treatment, whilst GAD65 mRNA levels remained low (Feldblum et al., 1998). In addition, in 2000, Tillakaratne and co-workers looked at changes in GABA, GAD65 and GAD67 levels after complete transection of the cat spinal cord between thoracic segments 12 and 13. Using in situ hybridisation, immunohistochemistry and Western blot analysis, they found an increase in GAD67 protein and mRNA, but not GAD65, in the dorsal horn of the lumbar spinal cord. It has been suggested that an increase in GAD67 after injury may lead to an increase in GABA production in the region of the injury, which would inhibit excessive neuronal firing during repair, and provide trophic support to the injured neurons (Soghomanian and Chesselet, 1992; Dumoulin et al., 1996; Feldblum et al., 1998; Tillakaratne et al., 2000).

The two GAD isoforms also differ in how they interact with their cofactor, PLP (Rimvall and Martin, 1992; Rimvall et al., 1993; Rimvall and Martin, 1994). Although both GADs require PLP as a cofactor, they differ in the rate at which holoGAD loses PLP to become apoGAD. In the brain, 80% of the apoGAD reservoir is GAD65, whilst it has been shown that GAD67 is saturated with PLP (Kaufman et al., 1991). In a study by Erlander et al. (1991) it was shown that GAD65 is more sensitive to the presence of PLP than is GAD67. Both GAD cDNAs were subcloned into vectors that allowed their expression in bacteria, and each bacterially expressed GAD was stimulated by exogenous PLP. Under these conditions, PLP stimulated the enzymic activity of GAD65 more than that of GAD67. The level of enzymatic activity achieved by stimulation of bacterially expressed GAD65 was similar to previous reports of stimulation of synaptosomal

preparations of rat substantia nigra with PLP, providing further evidence that PLP stimulated the enzymic activity of GAD65 more than that of GAD67 (Miller and Walters, 1979).

Knockout mice have been developed to assist in understanding the roles of the two isoforms (Asada et al., 1996; Kash et al., 1997; Asada et al., 1997; Condie et al., 1997; Ji et al., 1999; Choi et al., 2002). Mice lacking the GAD67 isoform died at birth from respiratory failure, although there were no detectable morphological brain abnormalities. The GAD and GABA content of the cerebral cortex of these mice were 20% and 7% respectively, of that found in wild-type mice (Asada et al., 1997). In mice lacking the GAD65 isoform, the knock out animals appeared to behave normally and no change in brain GABA content was detected. These mice had slightly enhanced susceptibility to seizures when compared to wild-type animals (Asada et al., 1996; Kash et al., 1997). Evidence from these studies suggests that GAD67 substantially controls the synthesis of GABA in the brain, more so than GAD65.

1.5 Spinal cord anatomy and physiology

The spinal cord, a cylindrical-shaped continuation of the medulla oblongata (the inferior part of the brainstem) extends down the vertebral canal to the second lumbar vertebra in adult humans. In transverse section, the cord is visualised as a column of grey matter, and is composed of neuronal cell bodies, dendrites, bundles of myelinated and unmyelinated axons, axon terminals and neuroglia. This is surrounded by a sheath of white matter (myelinated axons, supported by oligodendrocytes and astrocytes).

The grey matter of the spinal cord divides the white matter into distinct anatomical regions know as the anterior, dorsal and lateral white columns, which are made up of fibre tracts that travel between the brain and the spinal cord, conveying sensory and motor information, in ascending and descending tracts respectively. The grey matter is subdivided into distinct areas called horns. The dorsal horn (the major receiving zone for sensory information) is in the area

closest to the posterior of the spinal cord, whilst the ventral horn (that is primarily responsible for motor control and locomotion) is closer to the anterior of the spinal cord.

The grey matter of the spinal cord is further subdivided into ten regions called laminae, as described by Rexed in 1952 and 1954 in the cat, based on differences in the size and packing density of neurons (cytoarchitectonics). This form of classification has since been extended to the rat spinal cord (Molander et al., 1984). Lamina I, known as the marginal layer, has neurons that vary extensively in size and shape. Lamina II corresponds to the substantia gelatinosa, and has a translucent appearance because of its lack of myelin. This lamina is further divided into lamina II inner (lamina IIi) and lamina II outer (lamina IIo), of which lamina IIo has a higher neuronal packing density. Laminae I and II are collectively known as the superficial dorsal horn. Laminae III-VI are also part of the dorsal horn, but whilst lamina III has a high density of small neurons, laminae IV to VI, and VIII have a more heterogeneous population, as neurons here vary in size, and the packing density is less that that of laminae I- III. Lamina VII is named the intermediate zone, and contains groups of nuclei, including those of Clarke's column and the intermedio-lateral nucleus (in the appropriate spinal segments). Cells in this lamina are generally triangular or star-shaped. Lamina IX consists of groups of motor nuclei and smaller short-axoned inhibitory interneurons, Renshaw cells (Matsushita, 1969). The somata of motor neurons are amongst the largest in the CNS, and these neurons have extensive dendritic trees. Lamina X is the grey matter surrounding the central canal (Rexed, 1952; 1954).

The anatomical substrate for communication between the spinal cord and the periphery is the spinal nerves. Each spinal nerve is connected to the cord by two spinal roots, the dorsal (sensory) root, and the ventral (motor) root. The dorsal root contains sensory nerve fibres only, and conducts nerve impulses from the periphery to the dorsal horn of the spinal cord. In contrast, the ventral root contains nerve fibres that carry commands regarding motor control, and conducts impulses from the ventral horn to the periphery. Each dorsal root has a swelling that contains the

cell bodies of primary afferent fibres that convey sensory information from the periphery. This swelling is known as the dorsal root ganglion.

Primary afferent fibres that pass sensory information from the periphery to the spinal cord enter through the dorsal roots and terminate in specific laminae according to their axonal diameter and receptive field modality (Todd and Koerber, 2005). All primary afferents are thought to use glutamate as their main transmitter (Broman et al., 1993), and these form excitatory synapses on neurons when entering the spinal cord. Primary afferents can be classified according to their conduction velocity and whether or not they are myelinated. Sensory afferents are named Aα-, Aβ-, Aδ- and C-fibres. Although each group has a wide range of functional types (e.g., a combination of fibres that carry noxious and innocuous stimuli), the majority of axons within each group share a similar sensory modality. Aα- and Aβ-fibres have the fastest conduction velocities and mainly respond to innocuous mechanical stimuli (Todd and Koerber, 2005). The majority of these afferents are low-threshold mechanoreceptors. After entering the spinal cord, afferents from the skin (cutaneous) bifurcate into ascending and descending branches that run in the dorsal columns. Collaterals arise from these and turn ventrally to terminate in laminae IIi-V, depending on their sensory modality. Those innervating hair follicle afferents terminate more superficially than those that innervate slowly adapting receptors (Light and Perl, 1979; Brown, 1981). Examples of low-threshold mechanoreceptive afferents include those that innervate Meisner's corpuscles (rapidly adapting, sensitive to skin deformation), those that innervate Merkel cells (slowly adapting type I afferents, sensitive to prolonged tactile stimuli) and Ruffini corpuscles (slowly adapting type II afferents, responsive to stretching of the skin). In addition, myelinated afferents innervating specific receptors of muscle (e.g., muscle spindle afferents, Golgi tendon organs) project further ventrally to laminae IV-VII and the ventral horn.

Aδ fibres, first described by Burgess and Perl in 1967 have an intermediate conduction velocity (when compared to A- and C-fibres) and are thinly myelinated. Aδ- fibres may contain a

number of neuropeptides as well as glutamate, including substance P (SP) and calcitonin gene related peptide (Lawson et al., 1997). Light and Perl (1979) showed that in the cat, the main branches of Aδ-nociceptor afferents travelled in the lateral dorsal column and in Lissauer's tract. After entering the spinal cord, these sent collaterals that terminated mainly in laminae I and IIo, with some terminal arbors in lamina V. Although the majority of thinly myelinated primary afferents respond to noxious stimulation and changes in temperature, and are Aδ-fibres, some respond to innocuous stimulation (Burgess and Perl, 1967).

The slowest conducting group of sensory afferents are the C-fibres, and these have been studied in detail by Sugiura and colleagues (1986) in the guinea pig. Cutaneous C-fibres enter the spinal cord and run rostrally and/or caudally in the region of the tract of Lissauer. Several collaterals branch ventrally into laminae I and II. Fibres innervating muscle project to parts of the deep dorsal horn (Ling et al., 2003). The majority of C-fibres respond to noxious stimuli and temperature, and the smallest of C-fibres are responsive to histamine and cause the perception of itch (Bessou et al., 1969). C-fibre afferents can be divided into two groups based on their neurochemical phenotype. C-fibres of the first group are sensitive to the neurotrophin, nerve growth factor and may contain a combination of neuropeptides, calcitonin gene related peptide, SP and galanin (Averill et al., 1995). These are known as peptidergic C-fibres. C-fibres of the second group are responsive to the neurotrophin, glial cell line-derived nerve growth factor and bind the lectin Bandeiraea simplicifolia isolectin, IB4 (Bennett et al., 1998). Most of these do not contain neuropeptides (Averill et al., 1995) and are therefore generally known as non-peptidergic C-fibres. It is still not clear whether these two groups correspond to two functional types, although a segregation of their central projections has been noted, with non-peptidergic fibres projecting mainly to lamina IIi, whilst peptidergic fibres terminate in laminae I and IIo with scattered projections in laminae II-V (Averill et al., 1995).

Motor output is continuously modified by input from muscle spindle afferents and other sensory neurons. Spindles in cat hind limb contain two types of sensory endings: primary (innervated by low-threshold group I diameter axons, named Ia afferents) and secondary (innervated by group II afferents), as classified by Lloyd in 1943. Another population of Group I axons, known as Ib afferents, innervate Golgi tendon organs (Hunt and Kuffler, 1951). These are slowly adapting mechanoreceptors that respond to muscle tension. These are excited by muscle contraction. On entering the spinal cord, these arborize widely into laminae V-VIII. Group Ia and group II afferents differ in their conduction velocity: over 80m s^{-1} for group Ia fibres, in cats (Fyffe, 1979), and under 80m s^{-1} for group II in the same species (Matthews, 1963). These also have different properties, including the location of their axonal arborizations. On entering the dorsal horn, through its dorsal or medial border, Ia collaterals descend through laminae I-V. Intra-axonal injection with horseradish peroxidase (HRP) has shown that Ia fibres send collaterals to interneurons in lamina VI, lamina VII, where the Ia inhibitory interneurons originate (Jankowska and Lindström, 1972), and lamina IX. Group II afferents descend to lamina IV before they branch and project to the dorsal horn, intermediate zone and ventral horn (Fu and Schomburg, 1974). They also have terminations on neurons of Clarke's column and the spinocerebellar tracts. Both groups of fibres make monosynaptic excitatory connections with α-motoneurons, and have important roles in the monosynaptic reflex, studied by Renshaw (1940) and Eccles et al. (1962). Mendell and Henneman (1971) provided evidence that a single Ia fibre innervating the medial gastrocnemius muscle was capable of exciting approximately 300 motoneurons that innervated the same muscle. Although the main mode of action of group II afferents is to excite flexor muscles and inhibit extensors concurrently (Lloyd, 1946), the opposite is also true (Eccles and Lundberg, 1959).

Within the spinal dorsal horn some types of primary afferent are under intense presynaptic control from surrounding axons and vesicle-containing dendrites (at axo-axonic and dendro-

axonic synapses) in what are known as synaptic glomeruli. A glomerulus is described as a complex synaptic structure with several peripheral profiles contacting a central axon. Ralston (1965) first described these in the cat. Ribeiro-da-Silva and Coimbra (1982) have shown that the central axon in each synaptic glomerulus is of primary afferent origin, and that two types of glomeruli can be identified (type I and type II). These two types have different localisations and ultrastructural features. Type I glomeruli are found in the middle and ventral parts of lamina II whilst type II glomeruli are found in the ventral part of lamina II and the dorsal part of lamina III. The central axon of type I glomeruli contains densely packed synaptic vesicles that vary in diameter. In contrast, central axons of type II glomeruli have an electron-lucent cytoplasm, and vesicles are of a more uniform size. Also, many peripheral axons in type II glomeruli form axo-dendritic synapses in synaptic triads (Todd, 1996), where the presynaptic axon terminal, and the central primary afferent terminal both synapse on the same postsynaptic dendrite (Barber et al., 1978). In a study by Ribeiro-da-Silva et al. (1986), fluoride-resistant acid phosphatase (a marker for non-peptidergic primary afferents) was found in 80% of the central axons of type I glomeruli, but was not found in those of type II glomeruli, providing evidence that the primary afferents in type I glomeruli are non-peptidergic. Ribeiro-da-Silva et al. (1986) subsequently showed that neuropeptide containing primary afferents receive few axo-axonic/dendro-axonic synapses and seldom form glomeruli. It is also possible that at least some of the central axons in type I glomeruli are nociceptive afferents (Todd, 1996) as the majority of small neurons in the saphenous nerve are fluoride-resistant acid phosphatase positive (O'Brien et al., 1989), and at least 70% of the fibres in this nerve are nociceptors (Lynn and Carpenter, 1982). Type II central axons originate from Aδ down-hair afferents, which have the same laminar distribution and ultrastructural appearance in the monkey (Réthelyi et al., 1982).

The two main types of neurons in the spinal cord are projection neurons and interneurons. The highest number of projection neurons is found in lamina I in the spinal grey matter, although

they are also scattered throughout laminae III-VI and the ventral horn. Some projection neurons have axons that cross the midline and extend upwards to various supraspinal areas including the thalamus, the periaqueductal grey matter of the midbrain, the lateral parabrachial area of the pons, and the reticular formation of the medulla (Craig, 1995). Approximately 80% of lamina I projection neurons express the NK-1 receptor (Todd et al., 2000), although large NK-1 expressing projection neurons are also present in laminae III and IV. The latter group also send dorsal dendrites to lamina I. Both groups of projection neurons are heavily innervated by substance P-containing primary afferents and therefore may assist in the transmission of nociceptive information from the spinal cord to the brain. Less is known about lamina I projection neurons that do not express the NK-1 receptor, although some may also be activated by noxious input (Naim et al., 1997; Todd et al., 2002). Interestingly, some lamina I projection neurons are also involved in local spinal cord circuitry, and have axon collaterals in the vicinity of their cell bodies (Hylden et al., 1986).

The majority of neurons in the spinal dorsal horn are interneurons, and the packing density of these is highest in laminae I-III. Studies involving the Golgi method (where neurons are visualised by impregnating them with heavy metal salts, generally silver or gold) or intracellular injection methods (where cells are injected with tracer substances such as HRP) have shown that the majority of axons derived from interneurons in these laminae are short and arborize close to their cell body (Beal and Cooper, 1978; Gobel, 1978; Light et al., 1979). In addition, a few send their axons one or two segments rostrally or caudally through the dorsolateral fasiculus (Cervero and Iggo, 1980). It is also quite common for interneurons to give rise to axons that enter different laminae within the same segment. Some lamina II interneurons send axons to lamina I or laminae III-V (Gobel, 1978; Grudt and Perl, 2002), whilst other interneurons in laminae III-V have axons that arborize ventral to their cell body (Schneider, 1992).

Dorsal horn interneurons can be divided into two categories based on their function: inhibitory neurons that use GABA and/or glycine as their main neurotransmitters, and excitatory neurons, which are glutamatergic (Todd and Spike, 1993). It is highly probable that spinal projection neurons use glutamate as their main transmitter, and therefore it is presumed that all GABAergic and glycine enriched neurons in the dorsal horn are interneurons (Todd and Koerber, 2005).

1.6 Distribution of GABA and GAD in the spinal cord.

In early immunohistochemical studies, the distribution of GABA-containing synaptic terminals and cell bodies in the rat spinal cord was examined using antibodies against GAD (McLaughlin et al., 1975; Barber et al., 1978; Hunt et al., 1981). These early antibodies did not distinguish between the two GAD isoforms, and were generally prepared from mouse brain synaptosomal fraction. Light microscopy showed that GAD was present in axon terminals in all laminae of the rat spinal cord, although the highest concentration of GAD-immunoreactive terminals were found in laminae I-III, with moderate GAD-immunoreactivity in laminae IV-VI, lamina VII and lamina X. GAD staining in laminae VIII and IX was less dense. In general, a much higher concentration of GAD-immunoreactive terminals was found in the dorsal horn, when compared to that found in the ventral horn.

Further studies involving electron microscopy confirmed these findings and showed that higher numbers of GAD-labelled terminals were present in the superficial dorsal horn than in laminae IV-VI and more ventral laminae. It was established that GAD was localised in synaptic terminals, and that the majority of GAD-positive terminals contained pleomorphic (flattened) vesicles, and made symmetrical synaptic contacts. In the dorsal horn, GAD-containing terminals were found presynaptic to dendrites (termed axo-dendritic synapses), cell bodies (axo-somatic synapses), and other axons (axo-axonic synapses). Axo-axonic synapses were more numerous in

laminae II and III than in other laminae. In the ventral horn, the same synaptic arrangements were evident. Some GAD positive terminals occurring presynaptic to dendrites and other axons, were in turn presynaptic to motoneuron cell bodies (McLaughlin et al., 1975).

Many studies of GABA distribution in the spinal cord now use antibodies raised against each of the GAD isoforms separately (e.g., Feldblum et al., 1995; Tillakaratne et al., 2000). These studies carried out in rat and cat respectively, found that both GAD65 and GAD67 were present throughout the spinal grey matter, with especially high levels of both isoforms in the superficial dorsal horn and in lamina X, whilst GAD67 was the predominant form found in the ventral horn (Feldblum et al., 1995; Tillakaratne et al., 2000). In the study by Feldblum et al. (1995), GAD immunostaining in the rat spinal cord was mainly found in axon terminals, although a few immunoreactive cell bodies were detected with each GAD antibody in the superficial dorsal horn without the use of colchicine (Feldblum et al., 1995). This differed significantly from the situation in the brain, where many GAD-immunoreactivity cell bodies can be found, particularly with the GAD67 antibody (Esclapez et al., 1994).

Although many GABAergic axons in the dorsal horn of the spinal cord are thought to originate from local GABAergic interneurons (Hunt et al., 1981), there are also GABAergic projections from the brain that terminate in the spinal dorsal horn (Holstege, 1991; Antal et al., 1996; Maxwell et al., 1996). Many of these descending GABAergic fibres contain 5HT, and originate from the raphe nuclei and the nucleus paragigantocellularis in the brainstem (Bowker et al., 1982; Antal et al., 1996). Other descending GABAergic fibres lack 5HT and also project from the rostral ventromedial medulla. Those projections where GABA and 5HT co-localised were found to terminate selectively in lamina I and II of the dorsal horn (Maxwell et al., 1996), whilst the non-serotonergic GABAergic fibres terminated in laminae I/IIo and IV-V (Antal et al., 1996). Some of the descending GABAergic fibres (with or without 5HT) that terminated in the dorsal horn may be involved in nociception (Antal et al., 1996; Maxwell et al., 1996). Furthermore, a

study by Holstege (1991) showed that GABAergic projections from the ventromedial reticular formation of the lower brainstem terminate on motoneuronal cell groups in the lumbar spinal cord, where they are responsible for general inhibition of these spinal motoneurons.

In the study by Barber and colleagues (1978), unilateral dorsal rhizotomies were used in the lumbar spinal cord of adult rats to study degenerating primary afferent terminals in the dorsal horn ipsilateral to the injury. Electron microscopy studies of these degenerating afferents showed that they were commonly found at the centre of rosette-like structures, and formed synaptic contacts with axon terminals and the surrounding dendrites. GAD-immunoreactive terminals were often found presynaptic to these degenerating primary afferent terminals, and sometimes a triadic arrangement was noted, in which the presynaptic GAD positive terminal and the primary afferent contacted by that GAD positive terminal both synapsed on the same dendrite. The authors concluded that this was direct evidence that GABAergic axon terminals are involved in presynaptic inhibition.

Levels of GAD in neuronal cell bodies are very low in the spinal cord, so in some early studies colchicine was administered to block the axoplasmic flow of GAD so that GAD-immunoreactivity could be visualised in cell bodies. The reliability of results obtained when using colchicine has been disputed, as the use of this substance may cause abnormal synthesis of certain peptides in some areas of the brain (Kiyama and Emson, 1991; Cortes et al., 1990), and therefore may also alter the synthesis of other substances, including GABA. Following colchicine treatment, GAD-immunoreactive cell bodies have been observed in laminae I-III (Hunt et al., 1981; Barber et al., 1982) and in all other laminae of the spinal grey matter (except lamina IX), the ependymal layer and the dorsolateral funiculus. Small GAD-positive cell bodies were found in laminae I-III, although the size of immunoreactive somata increased for cells located in more ventral laminae, with the largest cell bodies detected in lamina VII (Barber et al., 1982).

It is now possible to detect GABAergic cell bodies directly using antibodies against glutaraldehyde conjugates of GABA (Storm-Mathisen et al., 1983). Before these antibodies were produced, direct visualisation of amino acid neurotransmitters with immunocytochemistry was not possible. Initially, the specificity of these antibodies was tested in the hippocampus, as this region of the brain is believed to have a well defined network of excitatory and inhibitory neurons that use glutamate and GABA, respectively, as transmitters. Storm-Mathisen et al. (1983) showed that the tissue localisation of GABA-like immunoreactivity matched previously defined uptake sites for GABA (substantia nigra, globus pallidus and some cerebellar nuclei). The staining also matched that found using antibodies against GAD in previous studies (McLaughlin et al., 1975; Barber et al., 1978). Also, they found no immunoreactivity for GABA in structures previously identified as glutamatergic (Storm-Mathisen et al., 1983). This type of GABA antibody was tested further by Hodgson et al. (1985) using the unlabelled antibody enzyme method on tissue sections. Hodgson et al. (1985) found that the anti-GABA sera were highly specific and did not cross-react with several of other amino acids (including glutamate and glycine). The GABA immunostaining achieved was completely abolished by absorption of the antibody with GABA conjugated to polyacrylamide beads by glutaraldehyde. Somogyi et al. (1985) compared the immunostaining obtained with antibodies against GAD and glutaraldehyde conjugates of GABA in the cerebellum, and found comparable staining with both antibodies, in that the types, distribution and proportion of neurons and axon terminals stained with each antibody was the same. The conclusion reached by both studies was that the GABA antisera are a reliable marker for GABAergic neurons within the CNS (Hodgson et al., 1985; Somogyi et al., 1985).

Information gathered using these types of antibodies and non-stereological counts of GABAergic cell bodies were carried out by Magoul et al. (1987) and Todd and McKenzie (1989) in the rat. Both studies showed that GABAergic cell bodies are predominantly found in laminae I-III of the spinal dorsal horn. Furthermore, GABA immunoreactivity has a similar distribution in

the cervical, thoracic, lumbar and sacral segments of the spinal cord (Magoul et al., 1987). In addition, it was shown that approximately 24% of neurons in lamina I, 29% of those in lamina II, and 33% of neurons in lamina III are GABA-immunoreactive (Todd and McKenzie, 1989), and that the majority of these had cell bodies that were round or fusiform in shape (Magoul et al., 1987). GABA-immunoreactive cell bodies were found in smaller numbers throughout the rest of the dorsal horn and ventral horn. In lamina IX, the occasional GABA-immunoreactive cell body was detected, but motoneurons were never GABA-immunoreactive. The distribution of GABA-immunoreactive cell bodies was similar to that of GABA-immunoreactive axon terminals in the superficial dorsal horn, suggesting that both belong to local interneurons, and a local decrease in GABA has been shown after ischaemic destruction of interneurons in the dorsal horn, supporting this concept. Both sets of results (Magoul et al., 1987; Todd and McKenzie, 1989) confirm data obtained from earlier immunocytochemical studies with antibodies against GAD (McLaughlin et al., 1975; Barber et al., 1978). Recently Polgar et al. (2003) have used a stereological method to investigate the proportion of GABA-immunoreactive cell bodies in laminae I-III of the rat spinal cord. The results of this quantitative analysis were similar to those of Todd and McKenzie (1989), as 25% of cells in lamina I, 31% of those in lamina II and 40% of cells in lamina III were found to be GABA-immunoreactive.

1.7 GABA receptors in the spinal cord

$GABA_A$ and $GABA_B$ receptors are both found in the rat spinal cord. $GABA_A$ receptors are evenly distributed throughout the spinal grey matter, including on dorsal horn interneurons and ventral horn motoneurons (Price et al., 1984). Bohlhalter et al. (1996) analysed the expression of many of the $GABA_A$ receptor subunits in the spinal cord and found widespread expression of the subunits $\alpha 3$, $\beta 2,3$ and $\gamma 2$. In contrast, the $\alpha 1$ and $\alpha 5$ subunits are mostly found in the intermediate zone, whilst the $\alpha 2$ subunit predominates in the superficial dorsal horn. $GABA_A$ receptors are also

found in the dorsal columns of the white matter (where GABA acts to assist in the modulation of axonal conduction in the tracts of myelinated fibres; Sakatini et al., 1991). By acting on $GABA_A$ receptors, GABA can depolarise afferent fibre terminals at axo-axonic synapses in the dorsal and ventral horn of the spinal cord (Curtis et al., 1986), resulting in presynaptic inhibition of primary afferents.

In contrast, $GABA_B$ receptors are concentrated in laminae I-III of the dorsal horn (Price et al., 1984). $GABA_B$ receptor subunits $GABA_{B1}$ and $GABA_{B2}$ are found in the spinal cord and dorsal root ganglion (Towers et al., 2000). GABA can also reduce neurotransmitter release from primary afferent terminals through $GABA_B$ activation, although this action is not associated with any depolarisation (Curtis et al., 1981). Instead, activation of this receptor probably reduces Ca^{2+} influx into the terminals, and subsequently reduces the release of excitatory neurotransmitters (Dolphin et al., 1990). Both receptor types can be presynaptic to primary afferent terminals, whilst $GABA_B$ receptors are also found presynaptic to GABAergic interneurons that in turn synapse on primary afferent fibres (Malcangio and Bowery, 1996). Activation of $GABA_A$ or $GABA_B$ receptors can result in muscle relaxation. Although baclofen, a $GABA_B$ receptor agonist, is used therapeutically, agonists of $GABA_A$ are not used clinically as the resultant increase in chloride conductance has a significant effect on the excitability of motoneurons in the ventral horn. Instead, modulators of the receptor's allosteric site (e.g., benzodiazepines, such as diazepam) are used, which increases $GABA_A$ receptor-mediated presynaptic inhibition (Pole et al., 1974).

Electrophysiological studies in adult (Johnston, 1996) and neonatal (Rozzo et al., 2002) rats; in conjunction with the identification of the ρ1 and ρ2 subunits transcripts of the $GABA_C$ receptor in the adult spinal cord (Enz et al., 1995) have provided evidence that this receptor is present in the spinal cord. Rozzo et al. (2002) have investigated the distribution of ρ1 and ρ2 in the neonatal (postnatal days 1 and 7) and adult lumbar spinal cord of rats, using a combination of

immunocytochemistry and in situ hybridisation. At postnatal day 1, mainly lamina IX cells (presumably motoneurons) were stained with the $GABA_C$ receptor antibody (although in situ hybridisation identified a few positive cells scattered throughout the rest of the grey matter). At postnatal day 7, motoneurons and dorsal horn interneurons appeared to be stained using immunocytochemistry, although the staining intensity in lamina IX neurons had decreased. The number of dorsal horn cells stained for the ρ1 and ρ2 mRNAs had increased. In the adult spinal cord, labelled neurons were found in all laminae, although the staining pattern had changed, and intensely immunoreactive boutons predominated over weakly stained cell bodies. In addition, electrophysiological experiments were carried out which showed that the $GABA_C$ receptors expressed on motoneurons at postnatal day 1 were functional.

1.8 Functions of GABA in the spinal cord

The CNS is constantly exposed to a barrage of incoming information from the periphery. The amount of information received probably exceeds its processing abilities and therefore 'surplus' irrelevant input has to be abolished. The most direct way in which sensory afferent input is modulated in the spinal cord is through presynaptic control of transmitter release from the central terminals of primary afferent neurons, as is thought to occur in GABA-mediated presynaptic inhibition. In presynaptic inhibition, primary afferent depolarisation (PAD) is induced by a GABA-mediated increase in chloride conductance at the central terminal of the afferent axon. This results in the initiation of an intense inhibitory process and an increase in afferent excitability. The voltage-dependent currents of the invading action potential are shunted, which subsequently decreases excitatory transmitter release from the central terminal.

The concept of what is now known as presynaptic inhibition emerged when Frank and Fuortes (1957) described a depression of mono-synaptic EPSPs occurring without any change in postsynaptic potential or in motoneuronal excitability, and named this "remote inhibition".

Research into this phenomenon was extended by Eccles and his colleagues (1961), who showed that during "remote inhibition", no change in the time course of the depressed primary afferent-induced EPSP was detected. In 1962, a study by Gray found axo-axonic contacts between axonal profiles and profiles of primary afferent origin in the cat spinal cord and concluded that the anatomical substrate of presynaptic inhibition is the axo-axonic synapse (Gray, 1962). Subsequently, Conradi showed in the ventral horn that primary afferent terminals were synaptically linked to motoneuron membranes, and that these were themselves postsynaptic to other terminals (Conradi, 1969a). Eccles et al. (1963) originally hypothesised that GABA-induced presynaptic depolarisation was responsible for EPSP depression. Evidence of this was provided by Barber and colleagues in an electron microscopy study in the rat spinal dorsal horn that showed GAD positive presynaptic terminals in contact with primary afferent terminals. The authors concluded that presynaptic inhibition is mediated by axo-axonic synapses formed between GABAergic neurons and primary sensory neurons (Barber et al., 1978). Pharmacological evidence supported this finding, as PAD can be mimicked by topical application of GABA to the spinal cord, and antagonists of $GABA_A$ receptors, bicuculline and picrotoxin, reduce PAD (Eccles et al., 1963; Curtis et al., 1971b) suggesting that presynaptic inhibition is mediated through the $GABA_A$ receptor. In addition, diazepam, a $GABA_A$ agonist increases presynaptic inhibition (Polc et al., 1974). The $GABA_B$ receptor seems to have a minor role or no role in presynaptic inhibition (Stuart and Redman, 1992). More recently, GABA or GAD-immunoreactivity boutons have been found that are presynaptic to the central terminals of a number of sensory afferent neurons including Aβ hair follicle afferents (Maxwell and Noble, 1987; Sutherland et al., 2002), high threshold Aδ mechanoreceptors (Alvarez et al., 1992), group I and group II muscle afferents (Maxwell et al., 1990), group Ia muscle afferents (Pierce and Mendell, 1993; Destombes, 1996; Watson and Bazzaz, 2001) and type I and type II glomeruli (Ribeiro-da-Silva and Coimbra, 1982; Todd, 1996). Sutherland et al. (2002) characterised the transmitter content of presynaptic

structures apposed to cutaneous afferent terminals in the spinal dorsal horn and found that approximately 80-100% of boutons presynaptic to these primary afferents were immunoreactive for GABA. This provided evidence that GABA is probably responsible for presynaptic inhibition of large cutaneous afferents.

Pierce and Mendell (1993) and Watson and Bazzaz (2001) have investigated axo-axonic contacts on Ia primary afferents, and found that the majority of these received at least one axo-axonic contact. These contacts were from GABAergic P boutons (as described by Conradi, 1969a). Pierce and Mendell (1993) also found that the number of presynaptic contacts on a primary afferent bouton was directly proportionate to its size, i.e. the larger boutons received more contacts (Pierce and Mendell, 1993). In addition, Watson and Bazzaz (2001) showed that in the ventral horn, 91% of P boutons were GABA-immunoreactive, whilst the remaining 9% of boutons were GABA and glycine-immunoreactive. In the deep dorsal horn, 58% of boutons presynaptic to Ia terminals were immunoreactive for GABA only, 31% were GABA and glycine-immunoreactive, and 11% were only immunoreactive for glycine. They concluded that different groups of Ia afferent boutons are modulated by neurochemically distinct populations of presynaptic neurons.

Demonstration that group 1b fibres receive substantial numbers of contacts from inhibitory presynaptic boutons came from work by Lamotte d' Incamps et al. (1998) on identified 1b fibres in the anaesthetized cat. They found at least 69 contacts from GABAergic interneurons on the two examined 1b collaterals from a single fibre, and concluded that 1b fibres are subject to GABA-mediated presynaptic inhibition. No electron microscopy was done in this study and therefore it is not known if these contacts were axo-axonic.

Maxwell and Riddell (1999) examined the terminations of group II afferents in laminae IV-VII of the cat, and found that these were all postsynaptic to GABAergic axon terminals, and that frequently there was more than one presynaptic axon per group II terminal. Sometimes the

presynaptic axon was also enriched with glycine. Triadic arrangements were also observed in 25% of cases. The authors concluded that it was very possible that group II primary afferents are controlled by GABA-induced presynaptic inhibition.

In 1996, Todd examined the complex interconnections between primary afferent fibres and dorsal horn interneurons using a quantitative post-embedding immunogold technique. This method has many advantages when used for certain types of quantitative EM studies, when compared to pre-embedding immunocytochemical techniques. For example, the ultrastructure of labelled terminals is often concealed by the reaction product in pre-embedding techniques, and this problem is not common when using the post-embedding immunogold method. Todd examined the co-localisation of GABA and glycine in presynaptic axons and vesicle containing dendrites in type I and type II glomeruli, to determine if there are selective connections between different inhibitory interneurons with the two types of glomeruli. He showed that, in type I glomeruli, the majority of peripheral axons and vesicle-containing dendrites were immunoreactive for GABA, whilst in type II glomeruli, these structures were generally both GABA- and glycine-immunoreactive. Central axons of both types of glomeruli were presumably under GABA-mediated presynaptic control. Although glycine was found in the peripheral axons/dendrites of type II glomeruli, it is not thought that it has a role in presynaptic inhibition, as strychnine has no effect on this (Levy 1977).

GABA-induced increases in chloride permeability in the spinal cord may result in membrane depolarisation, as occurs in presynaptic inhibition of myelinated and unmyelinated primary afferents in the dorsal horn via axo-axonic synapses, as discussed previously. Changes in chloride permeability may also result in membrane hyperpolarisation (generating an inhibitory postsynaptic potential, IPSP), as occurs in postsynaptic inhibition of spinal motoneurons and interneurons via axo-dendritic and axo-somatic synapses. Which type of membrane potential change occurs depends on the intracellular chloride conductance. It has been shown that IPSPs

can be reversed by injecting chloride ions intracellularly (Curtis et al., 1968a). Postsynaptic inhibition is described as a temporary decrease in the excitability of spinal neurons. There are various known postsynaptic inhibitory pathways, including those that contribute to the inhibition of spinal motoneurons and interneurons, including Renshaw cells (by excitatory afferents of muscle and cutaneous origin). Also, recurrent inhibition, after excitation of Renshaw cells, and descending inhibition, from supraspinal areas directed at motoneurons and interneurons (Curtis, 1969) are examples of these pathways.

Initially, glycine was considered to be solely responsible for postsynaptic inhibition (Eccles et al., 1963). Evidence for the role of GABA in this process was first provided by Kellerth and Szumski (1966), who used intracellular recordings in cat popliteal, common perineal and hamstring motoneurons. They investigated the hyperpolarisation of the postsynaptic membrane and changes in the size of monosynaptic EPSPs and the firing rate of neurons after stretching of the triceps surae, tibalis anterior or semitendinosus muscles. They described a type of stretch-activated postsynaptic inhibition in motoneurons that was resistant to strychnine but sensitive to picrotoxin. Previous to this study, strychnine-resistant postsynaptic inhibition had been described in many supraspinal areas, including the hippocampus (Andersen et al., 1963), and cerebellar Purkinje cells (Crawford et al., 1963). Subsequently, Curtis and colleagues (1968b) showed that GABA inhibited motoneurons just as effectively as glycine, and was also as effective in producing the depression of Renshaw cell firing in the anaesthetised cat (Curtis et al., 1968b). Although glycine has been shown to be more effective in producing the hyperpolarisation of dorsal horn neurons than GABA (Curtis et al., 1968b), bicuculline- and picrotoxin- sensitive IPSPs have also been recorded from these neurons (Curtis et al., 1969). Further analysis of the postsynaptic actions of GABA and glycine suggested that both amino acids produced the same alteration in membrane permeability of spinal motoneurons, as they both produced an increase in the permeability of the neuronal membrane towards potassium and chloride ions (Curtis et al.,

1968b). In addition, in motoneurons, some of the postsynaptic inhibition recorded by Curtis et al. (1971b) was also sensitive to bicuculline. This provides evidence that GABA acts as an inhibitory postsynaptic transmitter in the dorsal horn and in the ventral horn.

Cullheim and Kellerth (1981) have also studied the effects of strychnine and bicuculline/ picrotoxin on the recurrent inhibition of α-motoneurons in the cat lumbosacral spinal cord. They described a type of postsynaptic inhibition that had strychnine-sensitive and bicuculline-/ picrotoxin-sensitive components, suggesting that both glycine and GABA are used as neurotransmitters by Renshaw cells. More recently, Yoshimura and Nishi (1995) have examined the inhibitory role of GABA and glycine in the spinal dorsal horn using intracellular recordings from substantia gelatinosa neurons in the adult rat spinal cord. They found that after stimulation of Aδ fibres and an initial EPSP, there was a short and/or long IPSP. Further investigation showed that the short IPSP was reversibly blocked by strychnine (and resulted from activation of the glycine receptor) whilst the long IPSP was reversibly blocked by bicuculline (and resulted from activation of the $GABA_A$ receptor). In conclusion, the authors showed that both glycine and GABA are responsible for postsynaptic inhibition of dorsal horn neurons. There is therefore, substantial evidence that glycine and GABA both contribute to postsynaptic inhibition of spinal motoneurons and dorsal horn interneurons.

1.9 GABA and glycine in the spinal cord

Glycine is a major neurotransmitter in the spinal cord. It is commonly believed that all cells contain glycine, which is involved in protein synthesis and some metabolic reactions (Shank, 1970). Immunocytochemistry has shown that many cell bodies and axons in the spinal cord (in both the grey and white matter), brainstem, cerebellum (granule and molecular layers), hypothalamus and retina are enriched with glycine (Ottersen and Storm-Mathisen, 1987; van den Pol and Gores, 1988) and use it as a neurotransmitter. Glycine is released by neurons (Mulder,

1974) and is removed from the extracellular space by active uptake mechanisms (Neal and Pickles, 1969), two of the major criteria that show it is a neurotransmitter. Electrophysiological investigations have also shown inhibitory responses to glycine that can be blocked by the application of strychnine (Curtis et al., 1968a), a glycine receptor antagonist (Curtis et al., 197Ia).

Three glycine transporters have been identified to date, glycine transporter type Ia, glycine transporter type 1b (both derived from the same gene) and glycine transporter type 2 (GLYT2; encoded by a separate gene). GLYT2 is of particular interest as it is found at high concentrations in the spinal cord and brainstem. This transporter has an extended N-terminus with multiple phosphorylation sites, and has a predominantly neuronal distribution (Jursky et al., 1994).

The glycine receptor is a ligand-gated chloride channel, and consists of α and β subunits and gephyrin, a membrane-associated protein that anchors glycine receptor at synapses. The main form of the α-subunit is α1, and this is restricted to glycinergic synapses, whilst the β-subunit is found in many regions where glycinergic transmission is not thought to occur. In some parts of the CNS, gephyrin is found without the α1 subunit (Kirsch and Betz, 1993). However, in the spinal dorsal horn, gephyrin is co-localised with this subunit (Alvarez et al., 1997), making it a reliable marker for glycine receptors here. Since antibodies directed against the glycine receptor α1 subunit give sub-optimal staining when used on fixed tissue, antibodies against gephyrin are often used instead in immunocytochemistry of spinal cord tissue.

Antibodies against glycine conjugated to protein carriers have been developed using the same technique as that of Storm-Mathisen and colleagues (Ottersen et al., 1987; van den Pol and Gores, 1988). These antibodies proved to be highly specific for glycine, and the antibody raised by Ottersen et al. showed no detectable cross-reactivity with other amino acids including GABA (Ottersen et al., 1987), the antibody described by van den Pol and Gores showed only 1% cross-reactivity with GABA. Although glycine is part of a number of proteins, these antibodies did not recognise glycine when it was incorporated into peptides in ELISA assays (van den Pol and

Gores, 1988), and it is therefore thought to be unlikely that non-specific staining would occur with these antibodies. Van den Pol and Gores (1988) showed a wide distribution of glycine-immunoreactivity throughout all laminae of the spinal cord in the cervical, thoracic, lumbar and sacral segments. Profiles that were immunoreactive for glycine in the spinal cord showed a much higher intensity of staining than those that were immunoreactive in the brain. Glycine-immunoreactive boutons and cell bodies were observed in both the dorsal and ventral grey matter of the rat and primate spinal cord. In the dorsal horn, immunoreactive cell bodies and axons were predominantly found in the deeper laminae, although immunoreactivity was still apparent in laminae I and II. In the ventral horn, intensely immunoreactive terminals were observed in close contact with large motoneuron cell bodies and dendrites. In addition, glycine-immunoreactivity was also detected in axons in the white matter of the spinal cord, with the highest number of immunoreactive axons found adjacent to the grey matter in the lateral and ventral white matter. Further immunocytochemistry using an antibody raised against the glycine receptor, showed that staining achieved with this was similar to the staining found using the glycine antibody. This provided evidence that the immunoreactive axons and cell bodies detected with the antibodies raised against glutaraldehyde conjugates of glycine were using glycine as a neurotransmitter, rather than for general metabolic purposes. The widespread distribution of both glycine- and glycine receptor-immunoreactivity in the dorsal and ventral horns has led to the assumption that glycine has an important role in both sensory and motor circuits in the spinal cord. Interestingly glycine is enriched in approximately 30% of GABAergic neurons in lamina I, 45% of those in lamina II, and 65% of those in lamina III. Furthermore, all glycine-immunoreactive cell bodies within laminae I-III of the spinal dorsal horn are also immunoreactive for GABA, whilst cells in deeper laminae are often glycine-immunoreactive, but do not use GABA as a transmitter (Todd and Sullivan, 1990).

Evidence exists that GABA and glycine may act as co-transmitters at some inhibitory synapses in the spinal cord (Taal and Holstege, 1994; Örnung et al., 1996; Todd et al., 1996; Jonas et al., 1998). GABA and glycine are enriched in some axons that are presynaptic to the central terminals of type Ia muscle afferents (Watson and Bazzaz, 2001), group II muscle afferents (Maxwell and Riddell, 1999) and the peripheral axons and vesicle-containing dendrites of type II glomeruli (Todd, 1996). In addition, Renshaw cells, have immunoreactivity for GABA and glycine, and exert an intense inhibitory effect on motoneurons (Cullheim and Kellerth, 1981; Schneider and Fyffe, 1992; Örnung et al., 1996). Some types of postsynaptic inhibition are blocked by both strychnine and bicuculline (Game and Lodge, 1975; Yoshimura and Nishi, 1995), which would suggest that the inhibition is mediated via $GABA_A$ and glycine receptors. Todd et al. (1996) were the first to show that, at some synapses in the spinal cord, co-localisation of GABA and glycine receptors occurred. By comparing the distribution of the $GABA_A\beta_3$ receptors and gephyrin, combined with post-embedding detection of GABA and glycine, the authors illustrated that many synapses in the dorsal and ventral horn showed both $GABA_A\beta_3$ and gephyrin immunoreactivity, and that GABA and glycine were enriched in the same presynaptic terminal at these synapses. This was not always the case, as frequently synapses were observed that had only $GABA_A\beta_3$ or gephyrin immunoreactivity, indicating that GABA and glycine may act separately or together at synapses in the spinal cord.

Co-localisation of GABA and glycine has been quantitatively assessed in axon terminals of lamina IX of the cat. In a study by Taal and Holstege (1994), it was concluded that within this area of the ventral horn, approximately one third of the terminals were immunoreactive for GABA and glycine, one third were immunoreactive for GABA only, and one third were only immunoreactive for glycine. Whilst Örnung et al. (1996) also showed co-localisation of GABA and glycine in approximately one third of the terminals, they noted that slightly less than two thirds of terminals were immunoreactive for glycine only (a higher proportion than that stated by

Taal and Holstege, 1994) and that very few (only 2%) were immunoreactive for GABA alone. Many of the terminals examined in these studies are likely to be involved in postsynaptic inhibition of motoneurons, although some may represent P boutons. Although the results of these studies differ, both support the concept that GABA and glycine may act as co- transmitters at some synapses on motoneurons.

More recently, the co-transmission theory was examined in rat spinal cord slices using dual whole-cell patch-clamp recordings from synaptically coupled pairs of interneurons and large cells in lamina IX, that were presumed motoneurons (Jonas et al., 1998). Strychnine, bicuculline and baclofen were used to examine any actions on the glycine receptors and $GABA_A$ receptors, and the contribution that these made to unitary IPSCs and spontaneous miniature IPSCs detected in the pairs of interneurons. Results from this study showed, that at some synapses in the spinal cord, unitary IPSCs and some miniature IPSCs have glycine receptor- and $GABA_A$ receptor-mediated components, supporting the hypothesis that co-transmission of glycine and GABA occurs at some synapses, and that they may be released from the same vesicles. This cotransmission may be important in motor control, where regulation of presynaptic GABA and glycine release may influence the timecourse of postsynaptic conductance (Jonas et al., 1998) and minimise the risk of irrelevant activity in motoneurons. Fluctuations in the inhibitory control of motoneurons (caused by alterations in GABA and glycine levels) may be important in maintaining the tonic versus phasic properties of these cells (Örnung et al., 1996). Co-transmission may also allow feedback control of transmitter release by $GABA_B$ receptors, probably not possible at purely glycinergic synapse. It may also compensate in genetic glycine receptor subunit defects (Brune et al., 1996).

1.10 Sub-populations of GABAergic neurons

Because of its complexity, relatively little is known about spinal dorsal horn circuitry, e.g. the connections between dorsal horn neurons with each other, and with neurons in other laminae. It is therefore helpful to categorise these cells into groups that share common features, and examine their anatomical and physiological properties further. Cells can be categorised by morphology, physiological response to stimuli, and their neurochemistry.

Studies in the superficial dorsal horn that have addressed the morphological characteristics of neurons have used Golgi staining or intracellular labelling in conjunction with electrophysiological recordings from cells. These studies have led to the categorisation of lamina I neurons as fusiform, pyramidal, flattened and multipolar, based on the shape of their somata and the origin of its dendrites (Gobel, 1978; Beal et al., 1989, Lima et al., 1986). There is disagreement between studies on the number of types of neurons in lamina II. In 1978, Gobel described four main categories in lamina II of the trigeminal nucleus caudalis of the cat. These were 'islet' cells (with abundant sagitally orientated dendrites), 'stalked cells' (with ventrally and sagitally orientated dendrites), 'arboreal' (stellate-like) and 'border cells'. In contrast, a study by Price et al. (1979) found only islet and stalked cells in lamina II of the monkey spinal cord. Analysis of the substantia gelatinosa in the primate by Beal and Cooper (1978) concluded that classification of the cells in this lamina was not possible as they were so heterogeneous, and that instead a gradient of cell types existed, based on cell morphology and the orientation of the primary dendrites. In the rat, a study by Todd and Lewis (1986) concluded that lamina II neurons consisted of two main groups, that resembled Gobel's stalked and islet cells, whilst the remainder had some characteristics of Gobel's minority groups. They also stated that a number of neurons possessed characteristics of more than one group.

In 2002, a study by Grudt and Perl, used electrophysiology to categorise neurons in the superficial dorsal horn. Using tight-seal, whole-cell recordings in hamsters, they measured the

spontaneous synaptic potentials, evoked postsynaptic currents, and the pattern of discharge resulting from depolarising pulses. They also examined the current-voltage relationships of cells whilst noting the morphological characteristics of each cell (its location and the size of its somata, and the pattern of arborisation of its dendrites and axons). They found that there was a correlation between the neuronal morphology of cells and their electrophysiological features. Using the described criteria, lamina II neurons were categorised into one of five categories (islet, central, medial-lateral, radial or vertical).

Neuronal populations can also be categorised on a neurochemical basis, and studies have shown that the majority of large islet cells are GABAergic (Todd and McKenzie, 1989; Todd and Spike, 1993) and that some also use glycine as their co-transmitter (Spike and Todd, 1992). Smaller islet cells (that correspond to the central cells described by Grudt and Perl, 2002) have been observed that are not immunoreactive for either inhibitory neurotransmitter. Studies of stalked cells have shown that they contain neither GABA nor glycine, and it has been suggested that these are excitatory interneurons (Todd and Spike, 1993).

Many immunocytochemical studies have identified neurochemically different populations of GABAergic neurons in the dorsal horn that have a specific laminar distribution. This suggests that these populations are also functionally different. GABAergic cells have been classified according to their enrichment with other substances, including glycine, acetylcholine, nitric oxide synthase (NOS), parvalbumin (PV; a calcium binding protein), galanin, thyrotrophin releasing hormone and neuropeptide Y. Co-localisation with glycine is particularly important in the classification of GABAergic neurons as this neurotransmitter is found in specific populations of GABAergic neurons, and may be used as a cotransmitter in these.

Two distinct populations of GABAergic neurons that are enriched with glycine and are found in the superficial dorsal horn are those that display immunoreactivity for NOS and those that are immunoreactive for PV (Laing et al., 1994). PV is found at high concentrations in

laminae I-III of the spinal dorsal horn. Antal et al. (1991) investigated the distribution of PV and calbindin (another calcium binding protein) in the spinal cord and found that although 70% of PV-containing neurons in the superficial dorsal horn were GABAergic, calbindin was largely restricted to non-GABAergic neurons. There are several forms of NOS that synthesise the cell messenger, nitric oxide. These include calcium-dependent nNOS and endothelial NOS, and the calcium independent isoform (Diaz-Ruiz et al., 2005). Neuronal NOS, is used in some neurochemical studies as a convenient marker for one of the two identified populations of GABAergic neurons in the superficial dorsal horn that uses glycine as a cotransmitter. NOS-immunoreactive cell bodies are numerous in laminae II and III, and in lamina II all NOS immunoreactive boutons are immunoreactive for GABA (Valtschanoff et al., 1992). Some NOS immunoreactive cell bodies in deeper laminae (including lamina III) are GABA-immunoreactive but are not enriched with glycine (Todd, 1991). Although both of these GABAergic populations have a similar anatomical distribution within the spinal dorsal horn, with cell bodies predominantly found in laminae II and III, PV and NOS are not co-localised in this region of the spinal cord, and make up two distinct non-overlapping GABAergic populations (Laing et al., 1994).

The neurotransmitter, acetylcholine is synthesised by choline acetyl transferase. Cholinergic neurons are found in the deep dorsal horn (laminae III-VI), and are detected using antibodies directed against choline acetyl transferase. Evidence that GABA and acetylcholine may act as co-transmitters was produced by examining the sparse collection of cholinergic cell bodies in lamina III of the rat spinal cord. It became apparent that these cholinergic cell bodies were GABA-immunoreactive, but were never glycine-immunoreactive (Todd 1991), and so they represent a population of GABAergic neurons that do not use glycine as a transmitter. In addition, it has been shown that all cholinergic cells in lamina III contain NOS (Spike et al., 1993).

Many neuropeptides are present within GABAergic axons and cell bodies in the spinal cord, particularly in laminae I-II, where peptide-containing neurons give rise to plexuses of axons (Hunt et al., 1981; Harkness and Brownfield, 1986; Rowan et al, 1993; Tuscherer and Seybold, 1989). Axons containing substance P, somatostatin or galanin may also be of primary afferent origin (Barber et al., 1979; Averill et al., 1995).

Cell bodies and axons belonging to NPY, galanin- and enkephalin-containing neurons are found predominantly in laminae I and II, with some cell bodies in lamina III. When glutaraldehyde is included in the primary fixative, a dense plexus of TRH-containing axons are observed in laminae II and III. The cell bodies of origin of TRH are found in laminae II-IV (Fleming and Todd, 1994). Immunocytochemical studies that have examined peptidergic cell bodies in laminae I and II, have shown, that all of the cells that were immunoreactive for NPY or galanin, 87% of those that contained TRH and 69% of the enkephalin-immunoreactive cells were also GABA-immunoreactive (Todd et al., 1992; Rowan et al., 1993; Fleming and Todd, 1994; Simmons et al., 1995). In addition none of these GABAergic populations appear to use glycine as a cotransmitter (Laing et al., 1994).

In contrast, two other neuropeptides, somatostatin (found in laminae I and II) and neurotensin (laminae I-III), are not found in neurons that are GABA-immunoreactive or glycine-immunoreactive, and neurons that contain these are thought to be excitatory and use glutamate as their main transmitter (Proudlock et al., 1993; Todd et al., 2003).

Interestingly, there is evidence that some populations of GABAergic neurons have specific postsynaptic targets. A study by Polgar et al. (1999) showed that GABAergic axons that contain NPY selectively target projection neurons in laminae III and IV that express the neurokinin-1 (NK-1) receptor. The relationship between NPY and NK-1 expressing neurons was shown to be specific as postsynaptic dorsal column neurons (which are located in the same laminae as these NK-1 expressing neurons, but do not possess the NK-1 receptor) received very

few contacts from NPY-containing axons. Laminae III and IV NK-1-expressing neurons received few contacts from NOS immunoreactive axons (which belong to another population of GABAergic neurons in the dorsal horn). This provided further evidence that the contacts made between the NPY-immunoreactive axons and the NK-1 expressing neurons were highly specific.

Another example of the association between individual GABAergic populations and specific postsynaptic targets is illustrated in a study by Puskar et al. (2001), which identified a population of very large lamina I projection neurons that had high numbers of gephyrin puncta associated with them, and lacked NK-1 receptors. These were found to be selectively innervated by NOS-containing axons.

1.11 Neuropathic pain and GABA

Pain is normally elicited in response to noxious or damaging stimulation, and has a protective role, as it warns of potential or actual tissue damage and results in the initiation of withdrawal responses to avoid or minimise damage. In contrast, neuropathic pain is a pathological pain state that may occur spontaneously, and is the result of previous damage to the peripheral or central nervous systems. Central neuropathic pain can occur after injury to the brainstem, thalamus, or cerebral cortex. This type of neuropathic pain is less common than peripheral neuropathic pain (that results from damage to a peripheral nerve, dorsal root, or the dorsal root ganglion) and may have different underlying mechanisms (Woolf and Mannion, 1999).

This group of pain states (peripheral neuropathic pain) affects approximately 1% of the United Kingdom population, approximately 500,000 people (Karlsten and Gordh, 1997). This may occur in conjunction with diabetic neuropathy, postherpatic neuralgia, cancer, spinal cord injury, multiple sclerosis or human immunodeficiency virus. In addition, lower back pain and phantom pain may have elements of neuropathic pain associated with them (Bennett, 1997). It can be difficult to predict which individuals will be affected by neuropathic pain, as it is not linked to

any specific type of nerve injury, and symptoms and underlying mechanisms often differ between individuals (Woolf and Mannion, 1999). It has been proposed that a genetic component may determine who is predisposed to neuropathic pain. In experiments by Devor and Raber (1990), male and female rats underwent ligation of the sciatic and saphenous nerves. Many of the animals developed autotomy, where self-mutilation of the denervated area occurred. This is presumed to represent an index of pain or dysesthesia. Animals with low levels of autotomy were interbred, as were those with high levels. After six generations of breeding, animals had either consistently high levels or consistently low levels of autotomy, and the ratios obtained of each type suggested that autotomy is inherited as a single-gene autosomal recessive trait.

Symptoms of neuropathic pain are classed as negative symptoms (sensory deficits) and positive symptoms. The latter include spontaneous pain (where there is no apparent noxious stimulus), allodynia (where a normally innocuous stimulus is perceived as painful) and hyperalgesia (where there is an exaggerated response to a painful sensation). Neuropathic pain is difficult to treat using mainstream analgesia as non-steroidal anti-inflammatory drugs and opiates are relatively ineffective in easing the pain experienced (Woolf and Mannion, 1999).

There are many theories surrounding the mechanisms of peripheral neuropathic pain. It has been suggested that it is the result of spontaneous activity in C-fibre nociceptors and large myelinated A-fibres (Ochoa et al., 1982), that it occurs after central sensitisation of dorsal horn neurons (Woolf, 1983), or that there is abnormal sprouting of A-fibres into lamina II (Woolf et al., 1992). There may also be reduced inhibitory control of dorsal horn neurons (disinhibition) (Woolf and Mannion, 1999) after nerve injury. Profound changes in sensory function will occur after traumatic injury to a nerve and may result in alterations in sensitivity, excitability and transmission in the injured axons. It is difficult to investigate which types of afferent are preferentially affected after partial nerve injury, as after axotomy afferent fibres are disconnected from their sensory receptor endings. Tal et al. (1999) compared the prevalence of spontaneous

and ectopic firing in nerve-end neuromas originating from nerves serving muscle (medial gastrocnemius nerve) and skin (saphenous and sural nerves) in the rat. They found that although spontaneous firing in the medial gastrocnemius had a higher incidence, ectopic mechanosensitivity was found more often in neuromas of cutaneous nerves. The authors concluded that the development of spontaneous or ectopic firing after nerve injury depended on the type of myelinated afferent fibres involved.

After peripheral nerve injury, ongoing ectopic firing from sensory fibres in the injured nerve may increase the synaptic efficacy of somatosensory neurons in the spinal dorsal horn. This is known as central sensitisation (Ji et al., 2003). This commences immediately after intense peripheral noxious stimuli or nerve injury, and is not restricted to the area of the injury (Woolf, 1983). It may result in a decreased pain threshold, amplification of future pain responses, and the spread of pain sensitivity to neighbouring non-injured axons (Gracely, 1992). Central sensitisation may be both triggered and maintained by ectopic discharges from axotomised A-fibres (Liu et al., 2000). After its onset, low threshold sensory fibres, which are normally activated by light touch, appear to innervate high-threshold nociceptive neurons due to the increased excitability of CNS neurons. This results in decreased pain threshold and the onset of allodynia.

Woolf et al. (1992) hypothesised that there is A-fibre sprouting into lamina IIo after peripheral nerve injury. After injection into the sciatic nerve, cholera toxin subunit b (CTb) binds to the GM1 ganglioside, which is selectively found on the surface of intact myelinated somatic primary afferents (Robertson and Grant, 1989) and transported to their cell bodies and central terminals. Under normal circumstances, these terminals are present in all laminae of the spinal dorsal horn, except lamina IIo that receives input from nociceptive C-fibres. After peripheral nerve injury, Woolf and his colleagues found that the CTb labelling expanded into lamina IIo, and interpreted this as sprouting of A-fibres into this lamina. The authors concluded that if lamina IIo,

which normally receives nociceptive information, began to receive non-noxious information, this might be misinterpreted as noxious input, and result in the onset of allodynia (Woolf et al., 1992; Woolf et al., 1995). Since this study, many studies have shown that A-fibres do not sprout into lamina IIo after peripheral nerve injury, but instead, there is a phenotypic switch and CTb is taken up and transported by axotomised C-fibres that terminate in lamina IIo (Tong et al., 1999; Bao et al., 2002; Hughes et al., 2003; Shehab et al., 2004).

Dorsal horn neurons, which process and transfer noxious information, are subject to many excitatory and inhibitory inputs from local and supraspinal neurons. An increase in inhibitory input to these neurons is believed to decrease activity in the dorsal horn neurons that relay the noxious information and thus act as a spinal 'gate' that can reduce the firing of these neurons and therefore diminish the sensation of pain (Melzack and Wall, 1965). Synaptic connections are frequently made between GABAergic terminals and GABA-containing cell bodies, providing a feedback system, which may have a role in inhibition (Roberts et al., 1978). Disinhibition occurs when the inhibitory control of dorsal horn neurons decreases. This could result from decreased levels of GABA (possibly from apoptosis of inhibitory interneurons through excitotoxicity, due to increased levels of glutamate) (Sugimoto et al., 1990) or down-regulation of GABA receptors.

Most people who have a partial peripheral nerve injury experience neuropathic pain (Decosterd and Woolf, 2000), and therefore a feature of animal models of neuropathic pain is partial denervation, where there is a mixture of intact and injured fibres (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000). These models are commonly used in studies into the underlying mechanisms of neuropathic pain, and are useful when comparing dorsal horn levels of GABA and GAD before and after peripheral nerve injury, to determine whether there are alterations in the levels of these substances that might result in disinhibition of dorsal horn neurons and the onset of neuropathic pain.

In the chronic constriction injury (CCI) model (Bennett and Xie, 1988), four ligatures are tied loosely around the whole sciatic nerve, spaced approximately 1mm apart proximal to the trifurcation of the nerve. This results in nerve constriction, swelling and partial strangulation of the nerve, and damage of only some of the sciatic axons. With this model, behavioural signs corresponding to hyperalgesia, allodynia and possibly spontaneous pain (which is more difficult to detect accurately in rats) continue for over two months. The process involved in CCI is similar to that of entrapment neuropathies (e.g., carpal tunnel syndrome) although the onset and progression of symptoms is much faster under experimental conditions. Another model is the partial sciatic nerve ligation model, which involves tight ligation of approximately half of the sciatic nerve. Symptoms of neuropathic pain (allodynia, mechanical hyperalgesia, and possibly spontaneous pain) develop within a few hours of the procedure and continue for several months afterwards (Seltzer et al., 1990). Both models allow investigators to analyse any changes is thermal and mechanical sensitivity after nerve injury, but can be difficult to reproduce. The spinal nerve ligation model involves tight ligation of the entire fifth, and sometimes sixth, lumbar spinal nerves close to the dorsal root ganglion, leaving the fourth lumbar (L4) nerve and third lumbar (L3) root intact. After this procedure, long-lasting thermal hyperalgesia (for at least 5 weeks), mechanical allodynia (lasting approximately 10 weeks), and signs of spontaneous pain are apparent (Kim and Chung, 1992). More recently, Decosterd and Woolf (2000) have developed the spared nerve injury (SNI) model, in which two of the terminal branches of the sciatic nerve are tightly ligated and sectioned distal to the ligation (tibial and common peroneal), sparing the sural nerve. This model allows behavioural testing of the neighbouring nerve territories surrounding the denervated area. The onset of symptoms (increased mechanical and thermal responses in the ipsilateral sural and, to some extent, saphenous territories) occurs within 24 hours and lasts for over six months.

Studies have also investigated changes in animals with complete sciatic nerve transection (SNT) although controversy surrounds whether this is a model of neuropathic pain or not. For SNT, the sciatic nerve is ligated (usually at the mid-thigh level) and completely transected by removing a few millimetres of the nerve. This generally results in autotomy.

GABA, and glycine, may play very specific roles in the modulation of pain information in the spinal dorsal horn. Yaksh (1989) showed that intrathecal administration of $GABA_A$ and glycine receptor antagonists resulted in a dose-dependent exaggerated response to light tactile stimulation. Subsequently, Hwang and Yaksh (1997) demonstrated that intrathecal administration of $GABA_A$ and $GABA_B$ receptor agonists (muscimol and baclofen respectively) resulted in a dose-dependent antagonism of the allodynia experienced by Chung model rats. Furthermore, injection of the $GABA_A$ antagonist, bicuculline, or the $GABA_B$ antagonist, CGP 35348, prior to the respective receptor agonist had little effect on normal pain thresholds, but effectively reversed the anti-allodynic state that had been produced by injection of muscimol or baclofen respectively. Wilson and Yaksh (1978) showed that baclofen had a dose-dependent anti-nociceptive effect on unoperated rats when administered into the lumbar spinal subarachnoid space via an intrathecal catheter. It is therefore believed that spinal $GABA_A$ and $GABA_B$ receptors may be responsible for the modulation of spinal systems that mediate the allodynia resulting from peripheral nerve injuries.

Further studies examining the role of GABA in neuropathic pain, investigated whether there was any change in GABA or GAD levels in the spinal dorsal horn after peripheral nerve injury, and whether any loss of GABA-immunoreactivity was detected in conjunction with behavioural signs of the condition (Kontinen et al., 2001; Castro-Lopes et al., 1993; Satoh and Omote, 1996; Ibuki et al., 1997; Eaton et al., 1998; Moore et al., 2002; Somers and Clemente, 2002; Polgar et al., 2003). The results of these studies were conflicting, as some showed a decrease in GABA or GAD levels in the dorsal horn (Castro-Lopes et al., 1993; Ibuki et al., 1997;

Eaton et al., 1998; Moore et al., 2002) whilst some studies showed an increase in GABA or GAD after peripheral nerve injury (Kontinen et al., 1992; Satoh and Omote, 1996). Furthermore, other studies showed that there was no change in GABA or GAD levels after nerve injury (Somers and Clemente, 2002; Polgar et al., 2003). Both Ibuki et al. (1997) and Eaton et al. (1998) found a dramatic bilateral decrease in GABAergic cell numbers after CCI, whilst Castro-Lopez et al. (1993) found a modest unilateral decrease after SNT. Although the reduction in immunoreactivity found in the studies by Ibuki and Eaton and their colleagues paralleled heightened sensitivity to innocuous stimuli, behavioural responses were restricted to the ipsilateral side of the nerve injury. More recently, a study by Moore et al. (2002) used the CCI, SNI and SNT models to investigate changes in GABAergic inhibition in the superficial dorsal horn after nerve injury. They found a decrease in dorsal horn levels of GAD65 ipsilateral to both partial nerve injuries, but only a slight reduction in GAD67 levels in the CCI model.

Although, the majority of studies suggest that GABA and GAD levels decrease after nerve injury, other studies contradict this. In 2001, Kontinen and colleagues detected an increase in endogenous GABAergic inhibitory tone in rats after spinal nerve ligation. Also, an ipsilateral increase in the concentration of GABA in rat dorsal horn homogenates has been described after CCI by Satoh and Omote (1996). Interestingly, in another study using the same experimental methods as Satoh and Omote (1996) it was reported that GABA levels were unaltered (Somers and Clemente, 2002). In addition, no significant difference in the packing density of GABAergic cell bodies was found after CCI, when compared to that calculated pre-injury using the optical dissector method (Polgar et al., 2003). It is therefore important that further studies are conducted to clarify the role of GABA in neuropathic pain.

A novel explanation for the underlying mechanisms of disinhibition, involves the possibility that there are alterations in anion homeostasis after nerve injury that leads to the onset of the symptoms of neuropathic pain. In the nervous system, intracellular cation-chloride

cotransporters set the reverse potential for $GABA_A$- and glycine- receptors. In neurons, this controls intracellular Cl^- anion gradients. Two examples of such transporters are the potassium chloride transporters (KCCs) and the sodium potassium chloride transporters (NKCCs). Under normal circumstances, KCCs decrease intracellular levels of K^+ and Cl^- ions, whilst NKCCs increase intracellular levels of Na^+, K^+ and Cl^- ions. In the brain in early development, GABA acts mainly as an excitatory neurotransmitter. This is thought to occur due to high NKCC1 expression and low KCC2 expression during development. This results in high intracellular Cl^- levels that cause membrane depolarisation after activation of the $GABA_A$ receptor, and a resultant net outward flow of anions (Price et al., 2005). Coull et al (2003) hypothesised that down-regulation of the KCC2 transporter may occur after peripheral nerve injury, resulting in disruption of anion homeostasis in lamina I nociceptive neurons. The resultant shift in transmembrane anion gradient could cause normally hyperpolarising synaptic currents to become depolarising, causing disinhibition of dorsal horn projection neurons. The authors used immunoblotting to compare KCC2 protein levels before and after nerve injury. Peripheral neuropathy was used in this study, and involved surgically implanting a polyethylene cuff around the sciatic nerve. This resulted in nerve constriction, similar to that produced in the CCI model. After injury, KCC2 levels in the lumbar spinal dorsal horn were reduced on the ipsilateral side to approximately half of that found on the contralateral side. Electrophysiological patch-clamp recordings were used to examine the excitability of dorsal horn neurons after blockade or knockdown of the KCC2 transporter in intact rats. Evidence from behavioural studies suggested that the nociceptive threshold was decreased in animals following reduced efficacy of this transporter in intact rats (Coull et al., 2003).

1.12 Aims and Objectives

Investigation 1:

Studies in the brain have shown that although most, if not all GABAergic neurons, probably synthesise both GAD isoforms, most preferentially express one or other of these. It is not known if this is the case in the spinal cord. It would be of interest to examine if there is co-localisation of the two isoforms in individual axons in each lamina of the spinal cord. GAD-immunoreactive cell bodies are found in lower numbers in the spinal cord than in the brain. This study sought to investigate these issues further with immunocytochemistry and confocal microscopy.

Investigation 2:

GABA and glycine are likely to be co-transmitters. In addition, GABA co-exists with many other neurotransmitters and neuropeptides in sub-populations of GABAergic neurons. It would be interesting to examine whether either GAD isoform predominates in different populations of GABAergic neurons. The co-localisation of each of the GAD isoforms with GLYT2 (a marker for glycinergic axons), NOS and PV was examined. This was done using immunocytochemistry, and confocal microscopy.

Investigation 3:

GABA has an important role in the presynaptic inhibition of primary afferent terminals. This occurs at axo-axonic synapses made by GABAergic P boutons on Ia afferent terminals. This part of the study sought to confirm that a GAD65-intense population in lamina IX (identified in investigation 1) are the P boutons. This was done by examining their association with primary afferent terminals using immunocytochemistry, confocal microscopy and electron microscopy. Primary afferent terminals were identified by retrograde labelling with CTb and vesicular glutamate transporter type I (VGLUT1)-immunoreactivity.

Investigation 4:

GABA modulates incoming sensory information in the spinal cord, and changes in GABAergic transmission may contribute to neuropathic pain. We therefore examined any laminar changes in either GAD isoform in the spinal dorsal horn after nerve injury. Immunocytochemistry, confocal microscopy and image analysis were used to investigate these changes.

Chapter 2: General Materials and Methods

The following protocols were used in all of the experiments described in the following chapters, unless stated otherwise.

2.1 Animals

For all immunocytochemical studies, adult male Wistar or Sprague Dawley rats (Harlan UK Ltd, Bicester, UK) were deeply anaesthetised with pentobarbitone (60mg/kg intraperitoneal). After pre-rinsing with mammalian Ringer solution for 5 seconds, animals were perfused through the left cardiac ventricle with a fixative containing 4% freshly depolymerised formaldehyde (in 0.1M PB). Experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and performed in accordance with the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimise the number of animals used and their suffering.

2.2 Immunocytochemistry

Mid-lumbar spinal cord segments (L3-L5) were removed and post-fixed overnight in a fixative containing 4% formaldehyde (as before). Transverse sections (60- 70 μm thick) were cut with a Vibratome and immersed in 50% ethanol for 30 minutes to enhance antibody penetration. This technique was first used by Llewellyn-Smith and Minson (1992) on sections obtained from various regions of the CNS, including the spinal cord. They found that the use of ethanol in this way enhanced the penetration of immunoreagents through tissue sections, without any significant adverse effects on tissue ultrastructure. For immunoperoxidase staining, free-floating sections were incubated for 48-72 hours at 4°C in primary antibodies. The sections were then rinsed and incubated for 24 hours in species-specific biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA; diluted 1:500). After rinsing, the sections were agitated in avidin-peroxidase conjugate (Sigma, Poole, Dorset, U.K., diluted 1:1000) for 24 hours, before

further rinsing. All antibody solutions and avidin peroxidase were made up in phosphate buffer with 0.3 M Saline (PBS) containing 0.3M NaCl and 0.3% Triton-X100. Also PBS containing 0.3M NaCl was used for all rinsing. This solution was used, as high salt concentrations decrease the amount of non-specific staining experienced in immunocytochemistry due to the high ionic strength. Peroxidase activity was revealed with 0.125% of 3,3'-diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide for 5-10 minutes. Sections were then rinsed with PB, dehydrated in graded concentrations of ethanol (5 minutes in each of the following: distilled water, 70% ethanol, 90% ethanol, then 100% ethanol (3 times)), cleared in Histoclear (2 times) (National Diagnostics, Raymond Lamb, London) and mounted on gel coated slides using Histomount (Agar Scientific, Stansted, UK). In some cases the DAB reaction product was intensified by including 3.5% nickel chloride in the DAB incubation (4-5 minutes).

For immunofluorescence staining, free-floating sections were incubated for 48-72 hours at 4°C in a cocktail of primary antibodies. The sections were then rinsed and incubated overnight in species specific anti-IgG secondary antibodies (Jackson immunoresearch; all raised in donkey; diluted 1:100), conjugated to Rhodamine Red-X, fluorescein isothiocyanate (FITC) and if a third primary antibody was used, cyanine 5.18 (Cy5). In some cases secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) was used instead of those conjugated to FITC. The sections were then mounted in anti-fade medium (Vectashield, Vector Laboratories, Peterborough, U.K.) and stored at -20°C. All antibodies were made up in PBS containing 0.3M NaCl and 0.3% Triton-X100, and all rinsing was done in PBS containing 0.3M NaCl.

2.3 Antibodies

The GAD6 monoclonal antibody is highly selective for GAD65 and does not appear to cross-react with GAD67 (Chang and Gottlieb, 1988). This antibody was produced after the immunization of mice with the 59kDa GAD protein taken from rat brains. The specificity of the

GAD6 antibody has been shown by reacting spinal cord sections from GAD65 knock-out mice (Kash et al., 1997) with GAD6, with no resultant staining (Hughes et al., 2005). Mouse and rat GAD65 are highly homologous, and the GAD6 antibody can be used to recognise GAD65 specifically, in both mouse and rat tissue (manufacturer's specifications). The polyclonal GAD65 antibody Sigma (G4913) was developed in rabbit against a synthetic peptide corresponding to the C-terminal region of human GAD65, using a sequence not found in GAD67. Use of this antibody in immunocytochemistry with spinal cord sections, showed a similar staining pattern as when the GAD6 monoclonal antibody was used and the same structures were stained with both antibodies (unpublished observation). No staining was detected on tissue from GAD65 knock-out mice when incubated with this antibody (Hughes et al., 2005).

The GAD67 polyclonal antibody, K2 (produced by Dr Allan Tobin, UCLA) was generated in rabbits against the N-terminal portion of GAD67, a sequence not shared by GAD65. K2 therefore recognizes GAD67 specifically (Kaufman et al., 1991). The antigen was produced by transfecting bacteria with nucleotides 122-2265 of GAD67 cDNA. Slight cross-reactivity with GAD65 has been noted (Esclapez et al.; 1994) (see chapter 4). The GAD67 monoclonal antibody, Chemicon, shows no detectable cross-reactivity with GAD65 when used in western blotting of rat brain lysates (manufacturer's specification). When used in immunocytochemistry on rat spinal cord sections, this antibody gives the same general pattern of staining as the GAD67 rabbit antibody (K2) although these antibodies do not always label the same structures. For further discussion of this, see chapter 4.

The guinea pig GLYT2 antibody, Chemicon, was raised against a peptide corresponding to amino acids 780-799 of rat GLYT2. Pre-absorption with this peptide completely abolishes GLYT2-immunostaining (manufacturer's specification). Double immunofluorescence staining using this antibody and a well-characterised rabbit anti-GLYT2 antibody (Zafra et al., 1995) resulted in staining of identical structures by both antibodies (unpublished observations).

Immunocytochemistry using the guinea-pig anti-VGLUT1 antibody gives a staining pattern that matches that described using other characterised VGLUT1 antibodies (Tong et al., 1999; Varoqui et al., 2002; Todd et al., 2003). The staining pattern obtained using the guinea-pig anti-vesicular glutamate transporter type 2 (VGLUT2) similarly matched that described using other characterised VGLUT2 antibodies (Tong et al., 2001; Varoqui et al., 2002; Todd et al., 2003). Preabsorption of either antibody with its immunogen peptide abolishes all staining (manufacturer's specifications).

The polyclonal NOS antibody (sheep; Herbison et al., 1996) was raised by immunising sheep with the complete rat neuronal NOS (nNOS) protein, which was cloned in an insect expression system. This antibody is highly specific for rat nNOS.

The monoclonal PV antibody (mouse IgG1 isotype) was produced by the fusion of mouse myeloma cells and splenocytes from mice immunised with purified frog muscle PV. According to the manufacturer, this antibody does not recognise other similar substances, such as calmodulin or intestinal calcium binding proteins.

The goat anti-choleragenoid antibody (List Biological Laboratories) recognises CTb with the same specificity as other anti-choleragenoid antibodies (manufacturer's specification).

2.4 Confocal microscopy and analysis

Sections reacted for immunofluorescence were examined with a Bio-Rad MRC 1024 confocal microscope equipped with a Krypton-Argon laser (Bio-Rad, Hemel Hempstead, U.K.). In most instances, sections were initially scanned with dry lenses (4×, 10×, and 20×) using a transmitted light detector and a dark field condenser. These scans were used in the identification of laminar boundaries (Todd et al., 1998). Since lamina II lacks myelin, this appears as a darker region of the dorsal horn, and its dorsal and ventral borders can be identified because of this. Other laminar boundaries were adapted from an atlas of the rat CNS (Paxinos and Watson, 1986). Sections were

also scanned sequentially with the 488 568 and 647 nm lines of the laser (to reveal rhodamine, fluorescein and Cy5, respectively) through a 60× oil-immersion lens.

Fig. 2.1 Primary antibodies used in immunocytochemistry.

Antibody	Species	Dilution (peroxidase)	Dilution (fluorescence)	Source
GAD65 (GAD6)	mouse	1/500- 1/1000	1/100- 1/200	Developmental Studies Hybridoma Bank, University of Iowa
GAD65 (G4913)	rabbit	N/A	1/5000	Sigma, Poole, Dorset, UK
GAD67 (K2)	rabbit	1/40,000	1/5000	Chemicon International, Harrow, UK
GAD67	mouse	N/A	1/10,000	Chemicon
GLYT2	guinea pig	N/A	1/10,000	Chemicon
VGLUT1	guinea pig	N/A	1/20,000	Chemicon
VGLUT2	guinea pig	N/A	1/5000	Chemicon
NOS	sheep	N/A	1/1000	Dr P. Emson
PV	mouse	N/A	1/1000	Sigma
CTb	goat	N/A	1/5000	List Biological Laboratories, Campbell, CA

Subsequent analysis was performed using Neurolucida for Confocal (Microbrightfield, Colchester, VT, USA) software and Metamorph (Molecular Devices Corporation, Downington, PA, USA). Initially, drawings of the grey matter outline were constructed using Neurolucida. The low magnification images were used to identify the boundaries of laminae I and II.

Chapter 3: Distribution and co-localisation of glutamate decarboxylase isoforms in the rat spinal cord.

3.1 Background

Some boutons in the spinal dorsal horn probably have significantly higher levels of GAD65 than GAD67, and GABA production may be particularly reduced in these axons after partial nerve injury. It is therefore important to determine the distribution of the two GAD isoforms and the extent to which they are co-localised within individual axon terminals in each lamina of the spinal cord grey matter. This information would assist in assessing whether either GAD would be able to compensate in GABA production if there were a reduction in the other GAD isoform after nerve injury.

Until recently, studies that have looked at the distribution of GAD have used antibodies that have not differentiated between the two isoforms. The recent availability of antibodies that are directed against each isoform separately (e.g., rabbit anti-GAD67 polyclonal 'K2' and anti-GAD65 monoclonal 'GAD6') now enables detailed studies to be performed that compare the distribution and co-localisation of the two isoforms. To date, such studies in the spinal cord have been qualitative (Feldblum et al., 1995) and no attempt has been made to quantify GAD levels in individual laminae. In this part of the study, immunocytochemistry and confocal microscopy were used to examine the distribution and co-localisation of GAD65 and GAD67 in individual axonal boutons in each lamina of the rat spinal grey matter in an attempt to understand more about the GABAergic system in the spinal cord.

The inhibitory neurotransmitter, glycine is co-localised with GABA in many axon terminals and cell bodies in the spinal cord, and it has been shown that GABA and glycine may act as co-transmitters in some neurons (Taal and Holstege, 1994; Örnung et al., 1996; Todd et al., 1996; Jonas et al., 1998). GABA is also thought to co-exist with other substances in the spinal dorsal horn. These include NOS, acetylcholine, PV, galanin, enkephalin and thyrotrophin

releasing hormone. Different sub-populations of GABAergic neurons can be classified on a neurochemical basis (see chapter 1 for more detail) depending on which substances they co-express. These populations are predominantly non-overlapping although all cholinergic neurons in lamina III (which are GABAergic) are thought to contain NOS (Spike et al., 1993). Investigations into the co-localisation of each GAD isoform with glycine, NOS and PV formed another part of this study. Analysis was carried out to determine whether either GAD is preferentially expressed within the cell bodies of NOS and PV neurons or axonal boutons of glycinergic neurons.

The principal excitatory neurotransmitter in the CNS is glutamate and this is believed to be the main neurotransmitter used by primary afferents (Broman et al., 1993), excitatory interneurons and possibly all projection neurons in the spinal cord (Broman and Adahl, 1994). Until recently suitable markers for glutamatergic neurons were not available. The production of antibodies against VGLUT1 and VGLUT2 now allows further investigations into the excitatory circuitry of the CNS, as these selectively label largely non-overlapping populations of glutamatergic axons. Another aim of this part of the study was to examine whether VGLUT1 or VGLUT2 immunoreactivity was ever found in GAD-immunoreactive axon terminals, or whether these markers for excitatory (VGLUT1 and VGLUT2) and inhibitory (GAD65 and GAD67) neurons label strictly separate populations of axons.

3.2 Materials and Methods

The protocol described in chapter 2 for the preparation of spinal cord sections for immunoperoxidase and immunofluorescence staining was used for all experiments.

For the investigations into the distribution and co-localisation of the GAD isoforms and experiments that examined the co-localisation of GAD and GLYT2, six adult male Wistar rats (230-300g) were perfused with fixative. For immunoperoxidase and immunofluorescence

reactions, spinal cord sections and sections from the neocortex were incubated with antibodies against GAD65 (GAD6, mouse) and/or GAD67 (K2, rabbit). In some cases of immunofluorescence with the spinal cord sections, guinea pig anti-GLYT2 was included in the cocktail of primary antibodies as a marker for glycinergic axons (Spike et al., 1997). The secondary antibodies used in all of these reactions were anti-mouse and anti-rabbit IgGs, and for immunofluorescence, these were conjugated to Rhodamine Red-X and FITC respectively. When anti-GLYT2 was included in the primary incubation, anti-guinea pig IgG conjugated to Cy5 was added to the secondary antibodies (see chapter 2 for antibody information and experimental protocols). NB. Some of the neocortex sections were not exposed to Triton-X100, as primary and secondary antibodies were diluted with PBS without Triton-X100, and all rinsing was also done in this solution.

For investigations into the GAD content of different neurochemical populations of GABAergic neurons, three adult male Wistar rats (260g) were perfused and sections of spinal cord were incubated in a cocktail of either a) rabbit anti-GAD65, sheep anti-NOS and mouse anti-PV antibodies or b) rabbit anti-GAD67, sheep anti-NOS and mouse anti-PV antibodies. Secondary antibodies used in these experiments were anti-rabbit, anti-mouse and anti-goat/sheep IgGs conjugated to Rhodamine Red-X, FITC and Cy5 respectively.

In experiments that examined VGLUT1- and VGLUT2-immunoreactivity in GAD-positive terminals, three adult male Wistar rats (190g) were perfused and spinal cord sections were reacted with a guinea-pig antibody raised against VGLUT1 or VGLUT2, with mouse anti-GAD65 and rabbit anti-GAD67. The secondary antibodies used were anti-guinea-pig, anti-mouse and anti-rabbit IgGs conjugated to Rhodamine Red-X, Cy5 and FITC respectively.

3.3 Confocal microscopy and analysis

For quantitative analysis of the co-existence of GAD65 and GAD67 in axon terminals of the spinal cord, sections were examined using the Bio-Rad MRC 1024 confocal laser scanning microscope. One section was chosen from each rat and the grey matter of one side was scanned with the 488 and 568 nm lines of the laser (to reveal rhodamine and fluorescein, respectively) through a 60× oil-immersion lens. Each Z-series consisted of 11 optical sections and covered a field of 103 × 103 μm. These were averaged over 4 scans and Z-steps were separated by 0.5 μm. By scanning approximately 20 overlapping fields, a vertical strip encompassing lamina I to lamina VIII was obtained. Individual areas from laminae IX and X were also scanned as described above. It was important not to saturate the signal for either of the two GAD antibodies, and so the gain of each of the photomultipliers was carefully set to ensure that the maximum pixel luminance value found anywhere in each section was less than the maximum possible value of 255. Each section was also scanned with dry lenses (4 ×, 10 × and 20 ×). Neurolucida for confocal software was used in subsequent analysis.

Initially, laminar boundaries were identified, as described in chapter 2, and plotted onto an outline of the relevant spinal cord section. Each pair of confocal z- series captured with the 60× lens for GAD65 and GAD67 was initially merged to give a greyscale image so that GAD-containing boutons could be identified, but it was impossible to distinguish between the staining for either GAD. This avoided a bias toward sampling boutons with a particular pattern of GAD staining. One hundred GAD-immunoreactive boutons from each lamina in each animal were then chosen randomly from these files and their locations were drawn onto the outline of that section. The initial confocal images were then overlaid on the drawing of the same section. The luminance value of the brightest pixel in each channel was recorded for each of the 1000 selected boutons per animal (100 each from laminae I-X) and expressed as a percentage of the value of the brightest pixel found in any of these boutons in that section, for the relevant channel. These

calculated values for GAD65 and GAD67 were added for each of the sampled boutons, and the percentage of this end value corresponding to GAD65-immunostaining was determined. Boutons were termed predominantly GAD65-immunoreactive if the GAD65 component represented 50% of the calculated end value, and the remainder were described as predominantly GAD67 immunoreactive. One way ANOVA was used to establish whether the proportion of boutons that were classified as predominantly GAD65-immunoreactive in each lamina differed significantly from that found in other laminae ($p<0.05$). Tukey's pairwise test was then used post hoc to determine whether these differences were significant ($p<0.05$). The mean of these end values corresponding to predominantly GAD65-immunoreactive and predominantly GAD67-immunoreactive was calculated for the 100 selected boutons in each lamina from each animal.

For investigations into the co-localisation of GAD with GLYT2, PV or NOS, two sections were scanned from each of the three rats. Each z-series consisted of 11 sections and had 0.5µm z-spacing. These were obtained by scanning sequentially with the 488-, 568- and 647 nm lines of the Bio-Rad MRC 1024 confocal microscope (as before) through a 60× oil- immersion lens. For sections that had been reacted with the GLYT2, GAD65 and GAD67 antibodies, one z-series was scanned from each lamina, and these were examined to determine whether boutons that were labelled with both GAD and GLYT2 antibodies were mainly GAD65- or GAD67- positive. For sections that were reacted with the PV, NOS and GAD65 or GAD67 antibodies, an area covering lamina IIi and dorsal lamina III was scanned from the selected sections. From the sections that had the GAD67 antibody in the primary incubation, a total of 97 PV-immunoreactive cells and 89 NOS-immunoreactive cells were examined for GAD-immunoreactivity. For PV, 38 were analysed from animal 1, 29 from animal 2 and 30 from animal 3. For NOS, 34 were analysed from animal 1, 20 from animal 2 and 35 from animal 3. From sections that were reacted against GAD65, PV and NOS, quantitative analysis was not carried out as careful inspection showed that in all sections PV- and NOS-immunoreactive cells were never GAD65-positive. Quantitative

analysis of boutons that were immunmoreactive for PV and GAD65/GAD67 was carried out to establish if these boutons showed a specific pattern of GAD-immunoreactivity.

For the part of the study that examined the expression of VGLUT1 and VGLUT2 in GAD-immunoreactive terminals, one section from each rat was scanned with the 60× oil-immersion lens. The pairs of confocal images for GAD65 and GAD67 were initially merged to give a greyscale image thus avoiding bias toward sampling boutons that had a particular pattern of GAD-immunoreactivity. One hundred GAD-immunoreactive boutons from each of seven regions from each rat were then selected. These regions were laminae I/II, III/IV, V/VI, VII, VIII, IX and X. Initially confocal images were studied to determine whether any GAD-immunoreactive boutons were labelled with either of the VGLUT antibodies. Careful inspection did not reveal any GAD/VGLUT1 double-labelled boutons and so subsequent analysis was only carried out on sections incubated with the anti-VGLUT2 antibody to determine the proportion of GAD-immunoreactive boutons that were VGLUT2 positive.

3.4 Results

3.4.1 Immunoperoxidase staining for GADs

Staining with both GAD antibodies was observed throughout laminae I-X of the L4 and L5 segments of the rat spinal cord, although the distribution of GAD65- and GAD67-immunoreactivity differed between laminae (fig. 3.1, fig. 3.2). At high magnification (60×), punctate immunostaining was observed with both antibodies and this presumably corresponded to axonal boutons.

Fig. 3.1 Immunoperoxidase staining of GAD67 in a section taken from the mid-lumbar region of the rat spinal cord. (a) Immunostaining is found throughout the grey matter, although it is particularly dense in the superficial part of the dorsal horn. Moderate levels of immunoreactivity are detected in the deep dorsal horn and lamina X, while immunostaining is less dense in lamina VIII. (b-d) Higher-magnification images of the superficial dorsal horn, the medial aspect of the deep dorsal horn, and the ventral horn (lamina IX) respectively. In each lamina, the staining detected is punctate, and few immunolabelled cell bodies are found. (b) in the superficial dorsal horn, a dense band of immunostaining is visible. This corresponds to in inner part of lamina II (lamina IIi). (c) Punctate staining in the deep dorsal horn is shown. (d) Numerous immunostained puncta are visible in lamina IX, and some of these surround large unlabelled cell bodies. These presumably belong to motoneurons, and one is marked with an arrow. Scale bars (a) = 200μm, (b-d) = 50μm.

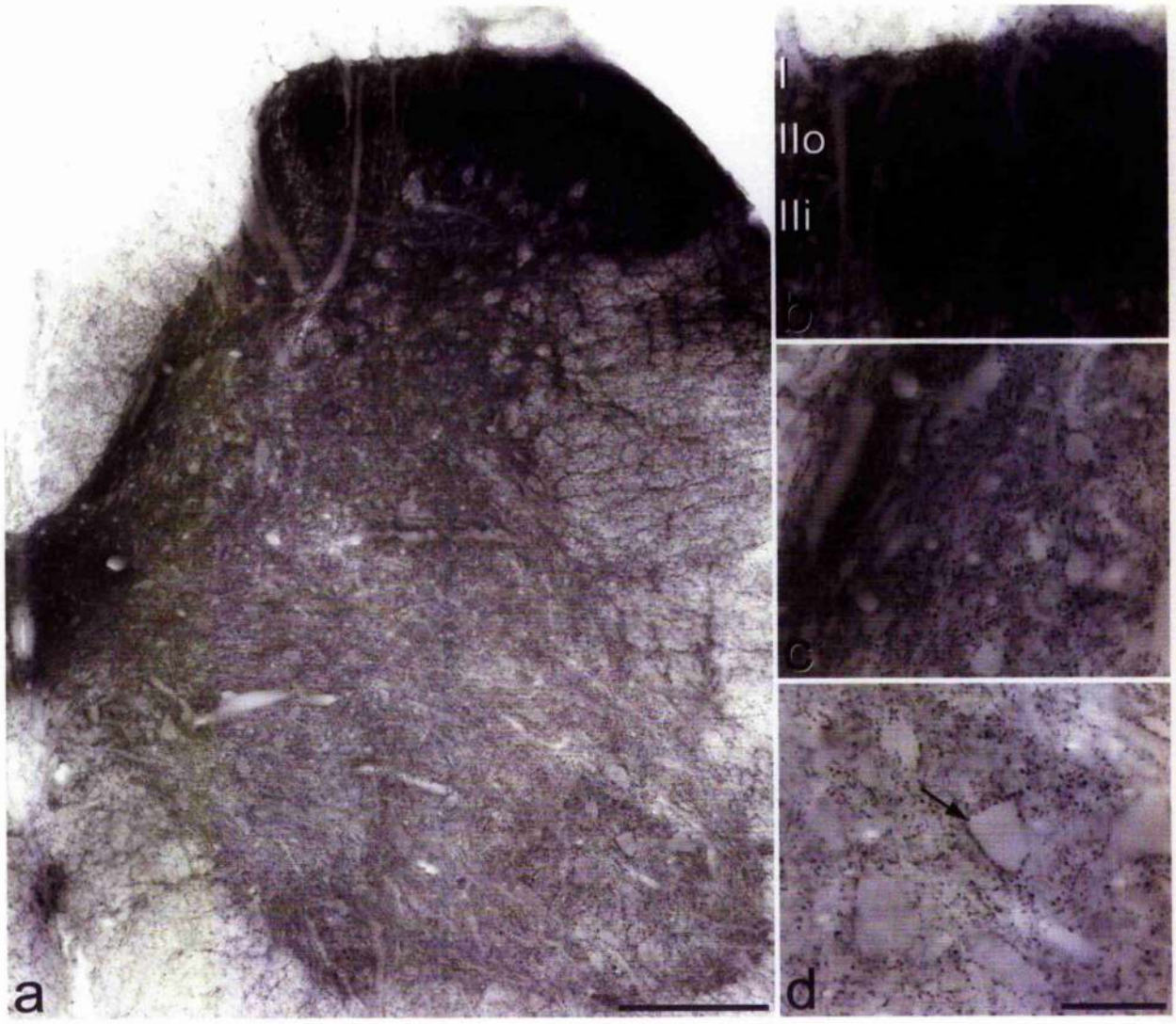
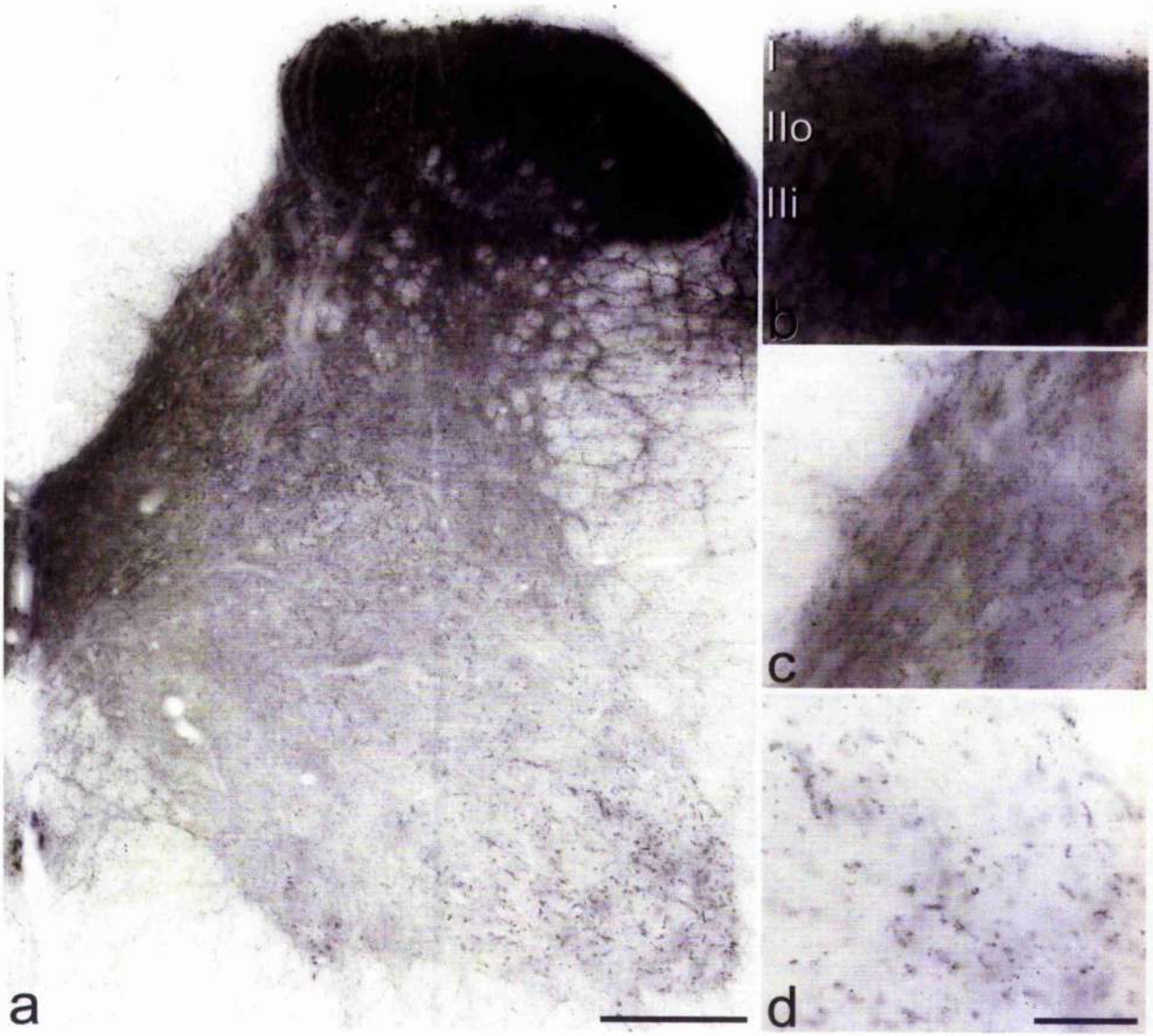

a
I
IIo
IIi
b
c
d

Fig. 3.2 Immunoperoxidase staining for GAD65 in the mid-lumbar region of the rat spinal cord. (a) Generally, the staining is similar to that detected for GAD67. However, much lower levels of GAD65-immunoreactivity are detected in laminae VII-IX (the intermediate area and the ventral horn). Clusters of immunostained puncta are visible in the area corresponding to the motor nucleus in lamina IX. As was the case with the GAD67-antibody, the staining detected was punctate, but in this case no immunoreactive cell bodies were detected. (b) In the superficial dorsal horn, a high density of immunolabelled puncta were detected in laminae I and IIi, with fewer found in the outer part of lamina II (lamina IIo). (c) immunostained puncta in the medial aspect of the deep dorsal horn. (d) small clusters of immunoreactive puncta are found in the motor nucleus. Unlike the pattern seen with the GAD67 antibody, unlabelled cell bodies are not outlined by these puncta.

Scale bars (a) = 200μm (b-d) = 50μm.


a
b
I
IIo
IIi
c
d

In contrast, only GAD67-immunoreactive somata were found in laminae I and II, and these were only detected occasionally. Although GAD67-immunoreactive puncta were found in all laminae of the spinal grey matter, strong GAD67-immunoreactivity was concentrated in laminae I-III. In lamina II, immunostaining was stronger in the inner (ventral) part (lamina IIi), when compared to the outer (dorsal) part (lamina IIo). Moderate numbers of strongly immunoreactive GAD67 puncta were also found in the deep dorsal horn and to a lesser extent, around the central canal. In the ventral horn, GAD67-immunoreactive puncta often surrounded large unstained cell bodies in lamina IX (probably belonging to motoneurons).

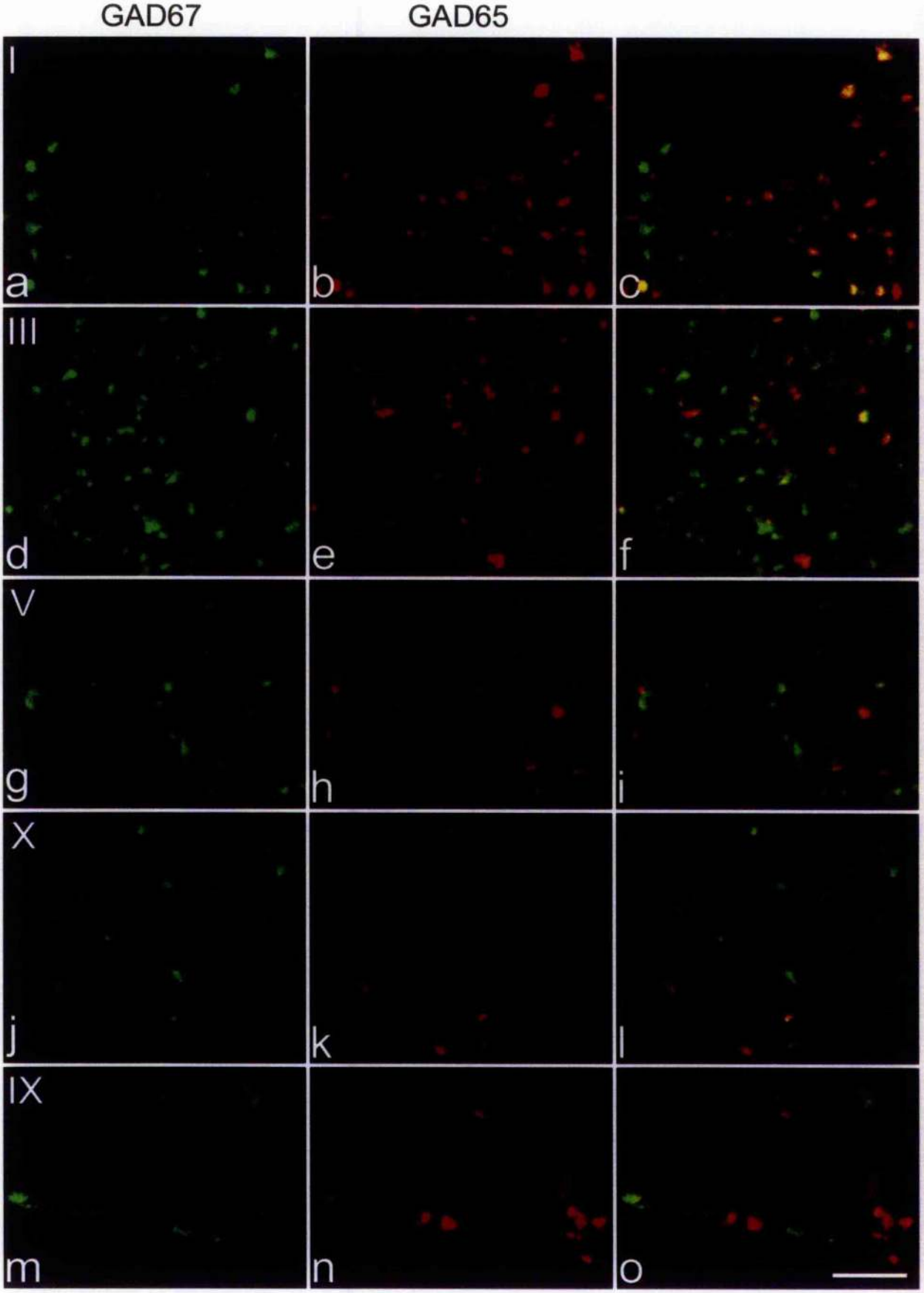
The distribution of GAD65-immunoreactive puncta differed between laminae (fig. 3.2). The highest concentration of strongly immunoreactive puncta was found in laminae I-III where two distinct bands of intense GAD65-immunoreactivity were detected: one in lamina I and another in lamina IIi. Moderate numbers of immunoreactive puncta were observed in laminae IV-VI (this was particularly strong in the medial part) and lamina X. Although puncta with strong GAD65-immunoreactivity were less frequent in laminae VII or IX, discrete clusters of profiles with strong GAD65-immunoreactivity were found in lamina VII and IX. These puncta were often found close to large unstained cell bodies, presumably motoneurons. Many boutons that were weakly immunoreactive for GAD65 were also observed throughout the grey matter, and these were predominantly found in the more ventral laminae.

3.4.2 Immunofluorescence staining for GADs

The pattern of GAD67- and GAD65-immunoreactivity observed with immunofluorescence was equivalent to that found with immunoperoxidase staining (fig. 3.3). Although most immunoreactive profiles were stained for both GAD isoforms, some boutons showed similar levels of immunofluorescence for both, whilst others had a much higher level of immunostaining for either GAD65 or GAD67. Measuring pixel luminance values showed that

Fig. 3.3 Immunofluorescence staining for GAD65 and GAD67

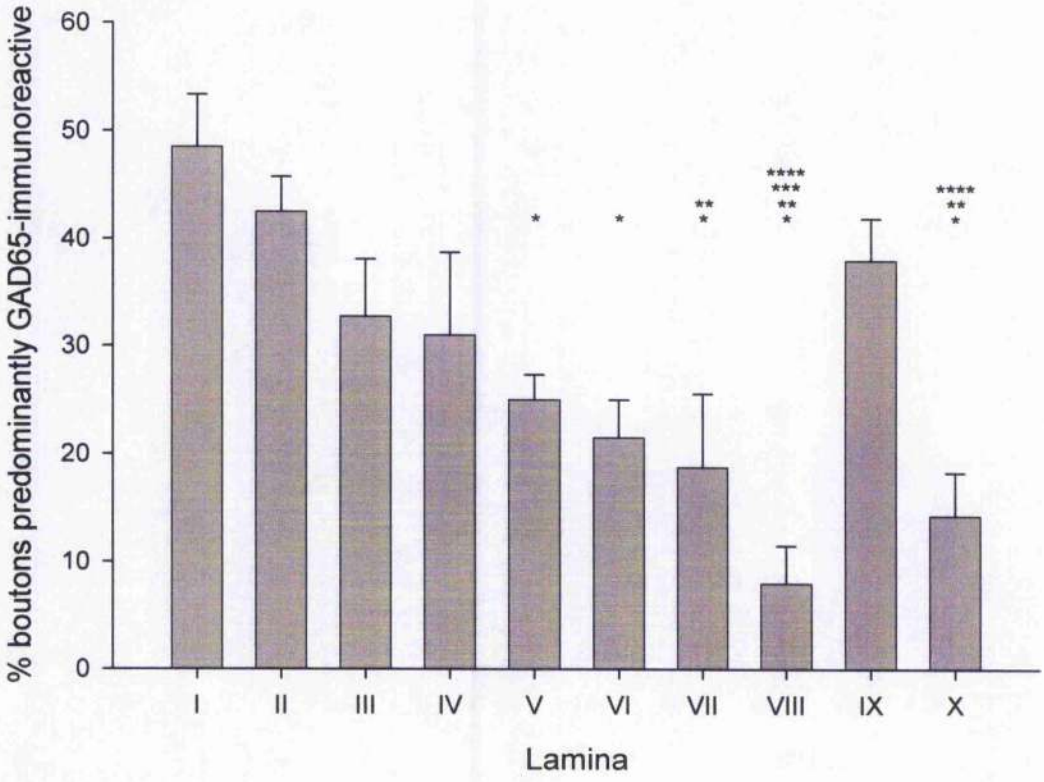
Confocal microscopy images show the co-localisation of GAD65-immunoreactivity and GAD67-immunoreactivity in individual boutons in different laminae of the rat spinal cord. These illustrate the relative intensity of immunolabelling with each GAD antibody in several parts of the grey matter. (a-c: lamina I; d-f: lamina III; g-i: lamina V; j-l: lamina X; m-o: lamina lX). All images are constructed from the projections of 3 optical sections, at z-spacing of o.5μm. Note the high density of labelled puncta in lamina I-III, and also the wide range of intensities displayed by each GAD isoform in each lamina. Scale bar = 5 μm


GAD67
GAD65
I
a
b
c
III
d
e
f
V
g
h
i
X
j
k
l
IX
m
n
o

although some boutons appeared to be unstained with one of the antibodies, even these had a low level of immunoreactivity that was so weak that it was difficult to see on the confocal images. The highest concentration of puncta that showed strong GAD65-immunoreactivity were found in laminae I and II and these became progressively less common in laminae III-VIII and X. In contrast, a large proportion of profiles in all laminae contained relatively high levels of GAD67-immunoreactivity. Although it appeared that there was a difference in GAD67-immunoreactivity between laminae IIi and IIo, closer examination of confocal images demonstrated that the relative staining intensity for the two GADs did not differ between these two areas, and that the difference in appearance was observed because GAD-immunoreactive profiles were less densely packed in lamina IIo than in lamina IIi. Interestingly, in lamina IX, the majority of GAD-immunoreactive profiles had relatively high levels of GAD67-immunoreactivity, but clusters of puncta with strong GAD65 immunostaining were also observed, and these had relatively low levels of GAD67-immunostaining.

Statistical analysis with one-way ANOVA showed that there was a highly significant difference ($p<0.001$) in the proportions of boutons that were defined as predominantly GAD65- or GAD67-immunoreactive between different laminae. Tukey's post-hoc pairwise test gave further information on the differences between some of the individual laminae ($p<0.05$). Lamina I contained more profiles that were predominantly GAD65-immunoreactive than did laminae V, VI, VII, VIII and X whilst lamina II had significantly more of these profiles than laminae VII, VIII and X. In addition, lamina IX contained considerably more profiles that were predominantly GAD65-immunoreactive than did laminae VIII and X. Finally, lamina VIII contained the lowest proportion of these profiles, and analysis showed that this was significantly different from laminae I, II, III and IX (fig. 3.4).

Fig. 3.4 Histogram of the proportion of boutons in each lamina that were defined as predominantly GAD65-immunoreactive (see materials and methods). The mean (and S.E.M.) percentages are shown for each lamina. Significant differences ($P<0.05$) are indicated by symbols. *= different from lamina I, **= different from lamina II, ***= different from lamina III, **** = different from lamina IX (n=4).


% boutons predominantly GAD65-immunoreactive
60
50
40
30
20
10
0
I
II
III
IV
V
VI
VII
VIII
IX
X
Lamina
*
*
**
*
****
***
**
*
****
**
*

3.4.3 Co-localisation of GAD with GLYT2

The pattern of immunostaining obtained with the GLYT2 antiserum was similar to that found previously (Zafra et al., 1995, Spike et al., 1997). Relatively low levels of immunofluorescence were observed in laminae I and II compared to the staining found in all the other spinal laminae. At high magnification, GLYT2-immunoreactivity encircled many profiles that resembled axonal boutons. Although many of these boutons were GAD-immunoreactive, many were not. Also there did not appear to be any relationship between GLYT2-immunoreactivity and the pattern of GAD expression in individual boutons in the dorsal horn, intermediate area and lamina X, as examples of profiles that were immunoreactive for GLYT2 and GAD included some that were predominantly immunoreactive for GAD65 and others that were predominantly immunoreactive for GAD67 (fig. 3.5).

Although fewer boutons in the ventral horn were double labelled with GAD and GLYT2 antibodies, those that were, were more likely to express predominantly GAD67 with relatively low levels of GAD65. Many of these boutons surrounded presumed motoneuron cell bodies. The clusters of boutons with intense GAD65-immunoreactivity in lamina IX were never GLYT2-immunoreactive (fig. 3.6).

3.4.4 Co-localisation of GAD with PV and NOS

The immunostaining observed using the PV and NOS antibodies was similar to that found in previous studies with these antibodies (Antal et al., 1990; Antal et al., 1991; Dun et al., 1993; Spike et al., 1993). With both antibodies, immunoreactive cell bodies were mainly found in laminae II and III. Occasionally cell bodies in lamina I were PV-immunoreactive, while many in deeper laminae were NOS immunoreactive. In addition, a dense band of punctate staining was observed in lamina IIi with both antibodies. Cell bodies that were NOS- immunoreactive exhibited cytoplasmic staining but had an unstained nucleus, as described in previous studies

(Spike et al., 1993) whilst those that were PV-immunoreactive had both cytoplasmic and nuclear staining.

In this study, no examples of GAD65/ PV- or GAD65/NOS- double-labelled somata were found. This was expected as GAD65-immunoreactive cell bodies were rarely seen in the spinal cord. Quantitative analysis of these populations of cells (NOS or PV) showed that none of the NOS- immunoreactive cells (34 cells from animal 1, 20 from animal 2 and 35 from animal 3) were GAD67-positive whilst 81% of PV-immunoreactive cells were GAD67-positive (fig. 3.7) (28 of 34 from animal 1, 23 of 29 from animal 2 and 25 of 30 from animal 3). Also, it appeared that all of the GAD67-immunoreactive cells in laminae II and III were PV-immunoreactive. Interestingly, PV-immunoreactive cells that were GAD67-immunonegative were more likely to be found in lamina III than in lamina II. In addition, qualitative analysis of the PV/GAD double-labelled axonal boutons showed relatively higher levels of GAD65-immunoreactivity when compared to the GAD67-immunoreactivity detected in these boutons. NOS-/GAD-double-labelled boutons were difficult to identify as the NOS antibody used gave extensive dendritic labelling.

3.4.5 Co-localisation of GAD with VGLUT1 and VGLUT2

Immunostaining obtained using the VGLUT antibodies was similar to that described in a previous study by Varoqui et al. in 2002. Both VGLUT1 and VGLUT2 immunostaining was detected on spinal cord sections but the laminar distribution of the staining differed for each transporter. Whilst VGLUT2 was detected throughout the grey matter, VGLUT1 labelling was sparse in laminae I and II, and more dense in laminae III-IV, the intermediate grey matter and the ventral horn (especially the medial aspect). After careful inspection of all regions of the grey matter in the sections reacted with theVGLUT1 antibody, it was concluded that there were no axonal boutons that were immunoreactive for both GAD and VGLUT1. Quantitative analysis of

Fig. 3.5 GLYT2 and GAD immunostaining in laminae III, V and X

Confocal images show that there did not appear to be any relationship between GLYT2-immunoreactivity (green) and the pattern of GAD expression in individual boutons in lamina III (a-c), V (d-f) and X (g-i), as examples of profiles that were immunoreactive for GLYT2 and GAD included some that were predominantly immunoreactive for GAD65 (red) and others that were predominantly immunoreactive for GAD67 (blue). Images in a-c are constructed from 2 optical sections (0.5 μm z-steps), while all other images are the product of a single optical section. Scale bar = 5 μm.

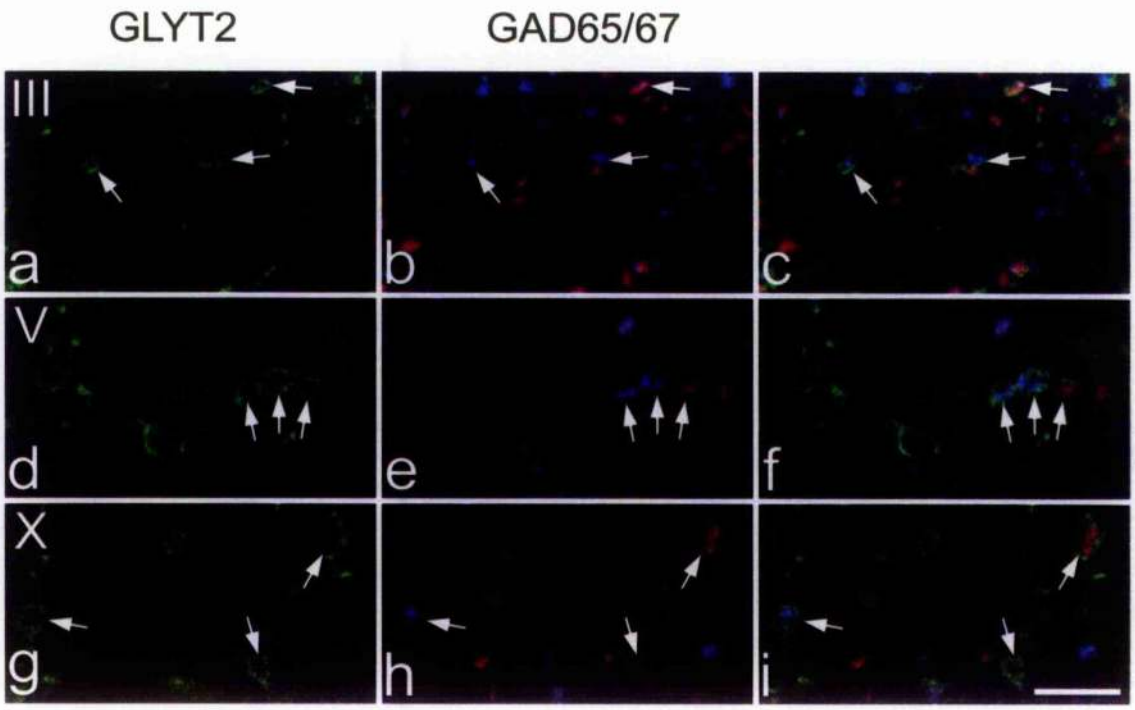

GLYT2
GAD65/67
III
a
b
c
V
d
e
f
X
g
h
i

Fig. 3.6 GLYT2 and GAD immunostaining in lamina IX

(a) numerous GLYT2-immunoreactive boutons are visible, many of which surround a large unstained cell body (presumably a motoneuron). (b) GAD65- (red) and GAD67- (blue) immunoreactive puncta. The majority of GAD-immunostained terminals in this region have relatively higher levels of GAD67- than of GAD65-immunoreactivity, although two discrete clusters of GAD65-intense boutons are shown (large arrows). (c) This merged image of GLYT2- and GAD65/67-immunoreactivity shows that many of the profiles that are predominantly labelled with the GAD67 antibody are also GLYT2- positive (small arrows). In contrast, the discrete clusters of boutons with relatively high levels of GAD65-immunoreactivity are never GLYT2 positive. Scale bar = 10 μm

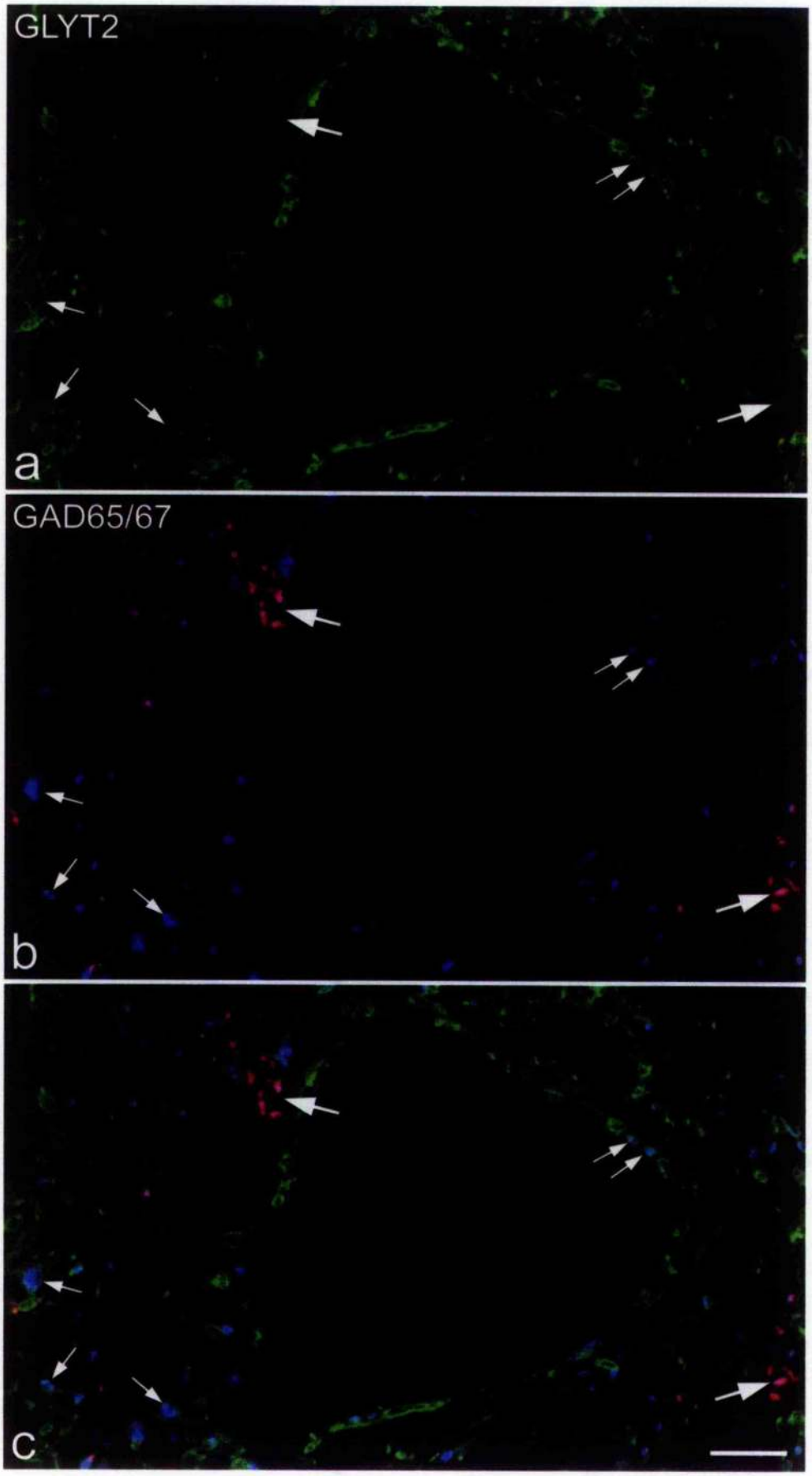

GLYT2
a
GAD65/67
b
c

Fig. 3.7 GAD and PV immunostaining in laminae II/III

Confocal images showing GAD67 (green) and parvalbumin, PV (red). The use of the GAD67 antibody resulted in cytoplasmic staining whilst the PV antibody gave nuclear and cytoplasmic staining. Although the majority of PV-immunoreactive cells were GAD67-immunoreactive (two of these are shown), some were GAD67-negative (one example is shown). All images are constructed from a single optical section. Scale bar = 10 μm

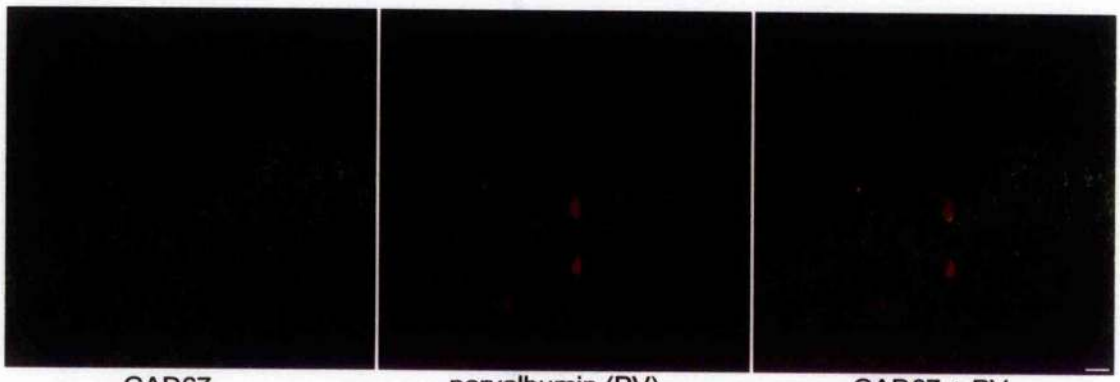

GAD67
parvalbumin (PV)
GAD67 + PV

Fig. 3.8 NOS and GAD immunostaining in laminae II/III

Confocal images showing GAD67 (green) and NOS (blue). The use of the GAD67 and NOS antibodies resulted in cytoplasmic staining. No GAD67-/NOS-double-labelled cell bodies were detected in this study. All images are constructed from a single optical section. Scale bar = 10 μm


GAD67
NOS
GAD67+NOS

sections reacted with GAD65, GAD67 and VGLUT2 showed that although the vast majority of GAD-immunoreactive boutons were VGLUT2-negative, one GAD/VGLUT2 double-labelled bouton was found. This VGLUT2/GAD double-labelled bouton was detected in lamina IX and was the only one found in the 2100 GAD immunoreactive-profiles examined from the 3 animals in this part of the study.

3.5 Discussion

The distribution of GAD65- and GAD67-immunoreactivity that was observed here was similar to that reported by Feldblum et al. (1995), who also used the GAD6 and K2 antibodies. The combined distribution of both GADs also resembled that found previously using antibodies that recognised both isoforms (McLaughlin et al., 1975; Barber et al., 1978). The main finding of this part of the study was that although most GAD-immunoreactive boutons were labelled with both GAD65 and GAD67 antibodies, some showed similar intensities of both types of immunoreactivity whilst others appeared to have higher levels for one or other of the GAD isoforms. This differed between laminae of the spinal cord. For example, in the superficial dorsal horn, boutons that had relatively higher levels of either GAD65 or GAD67 were frequently found. In contrast, most boutons in the ventral horn displayed relatively higher levels of GAD67, although discrete clusters of boutons that had high levels of GAD65 immunoreactivity were detected in lamina IX. It was subsequently found that these are the P boutons (see chapter 4 and Hughes et al. 2005). With regards to the investigation into whether GLYT2-immunoreactive puncta display a particular pattern of GAD expression, no relationship was detected in the dorsal horn, as some GLYT2-immunostained profiles showed strong GAD65-immunoreactivity whilst others displayed relatively higher immunoreactivity for GAD67. In contrast, in the ventral horn there was a relationship between GLYT2-immunoreactive boutons and the pattern of GAD-immunoreactivity found, as whilst the GAD65-intense boutons in the ventral horn were never

GLYT2 positive, boutons with relatively high levels of GAD67-immunoreactivity were often GLYT2-immunoreactive.

It was not possible to examine the relationship between GAD65 and NOS or PV in cell bodies since GAD65-immunoreactivity was rarely detected in somata. In contrast, in laminae II/III, all NOS-immunoreactive-cell bodies were GAD67-negative whilst 83% of PV-immunoreactive- cell bodies were immunostained with the GAD67 antibody. In contrast, profiles that resembled axonal boutons that were PV-immunoreactive, had relatively high levels of GAD65. This difference in distribution of the two GADs within the cell bodies and axon terminals of PV-containing-neurons is similar to the pattern of GAD staining found in some populations of GABAergic neurons in the brain (Esclapez et al., 1994). Although it is not known why this pattern of GAD-immunoreactivity occurs in this population of PV-immunoreactive neurons, it may be because GAD65 is transported to the axon terminal more readily than GAD67 from the neuronal cell body, or because GAD67 is degraded more rapidly at the terminal within this sub-type of GABAergic neuron. Alternatively, GAD65 and GAD67 may synthesise two separate pools of GABA in these neurons, similar to the arrangement that is thought to occur in the brain (Esclapez et al., 1994).

Although qualitative analysis of confocal images from tissue stained with VGLUT1, GAD65 and GAD67 showed that there were no structures double-labelled for VGLUT1 and GAD, and that the majority of GAD-immunoreactive profiles were also VGLUT2 negative, one example of a GAD/VGLUT2 double-labelled bouton was found in lamina IX. This finding suggests that although GABA and glutamate are normally found in different populations of axon terminals, very occasionally these substances may be co-expressed in the same neuron.

3.5.1 Technical considerations

The antibody used in this study to detect GAD67-immunoreactivity (K2) shows very weak cross-reactivity with GAD65 (Esclapez et al., 1994), and it is therefore possible that boutons with strong GAD65- and very weak GAD67-immunoreactivity may have contained only GAD65. This possibility was explored further. In the four sections used to examine the distribution of the two GAD isoforms, the number of boutons in which the GAD67-immunoreactivity was less than 10% of the total GAD-immunostaining in that bouton was never more than 1% of the total boutons sampled in each section. It is therefore probable that only a few boutons, if any, contained only GAD67. After this study was completed, a monoclonal GAD67 antibody became available commercially, which has been reported to show no cross-reactivity with GAD65.

As the relationship between antigen concentration and the strength of immunostaining is not known in immunocytochemical studies, it was not possible to estimate the absolute concentration of either GAD. Instead, the ratio between pixel luminance values obtained for GAD65- and GAD67- immunoreactivity in individual boutons was used to determine if they were predominantly GAD65- or GAD67-immunoreactive. We can therefore only assume that boutons with a higher level of immunoreactivity for one of the GADs, predominantly expresses that isoform. Also, as the intensity of immunofluorescence can vary between sections, depending on the exact experimental conditions used, comparisons were not made between sections, and semi-quantitative analysis only allowed comparisons to be made between boutons in different laminae. Finally, since it was difficult to resolve the outline of each immunoreactive bouton, the luminance value of the brightest pixel in each bouton was noted as a measure of immunoreactivity instead of the mean pixel luminance value for each bouton.

In the spinal cord, GLYT2-immunoreactive boutons are glycine-enriched (Spike et al., 1997) and therefore presumably use glycine as their neurotransmitter. GLYT2 antibodies are therefore considered to be a reliable marker for glycinergic axons in this area of the CNS (Puskár

et al., 2001; Geiman et al., 2002). In the sections used for confocal microscopy, GLYT2 was present in the plasma membrane, and surrounded the GAD labelling in individual boutons, as expected (Fig. 3.6).

With regard to the PV/NOS study, it is important to note that although GAD65-immunoreactivity was not detected in either population of cell bodies, GAD65 may be rapidly transported to the axon terminals after synthesis in these neurons. Antibodies against GAD65 may therefore not be sensitive enough to detected the remaining GAD65 in these populations of cells.

In a previous study in the brain, Esclapez et al. (1994) showed that higher numbers of GAD labelled cell bodies were detected when detergent was not added to the solution of antibodies. Although Triton-X100 was used in this study to enhance the penetration of the antibodies, immunostaining of spinal cord sections without Triton-X100 was also performed, and even then few cell bodies were labelled with either GAD antibody. As sections of neocortex were also immunostained with these antibodies (with and without Triton-X100) and numerous immunoreactive cell bodies were detected with both GAD antibodies (Mackie and Todd, unpublished observations), it is not likely that the low number of GAD-immunoreactive cell bodies detected in the spinal cord is attributable to the use of detergent. Tillakaratne et al. (2000) reported that significant numbers of neuronal cell bodies in the cat spinal cord were stained with the GAD67 antibody that was used in this study, and so there may be a species difference regarding the levels of GAD67 in cell bodies within the spinal cord.

3.5.2 Comparisons between the present study and previous studies

3.5.2.1 GAD65 and GAD67

In 1994, Esclapez et al. examined the distribution of GAD65 and GAD67 within several subclasses of GABAergic neurons in the forebrain, midbrain, olfactory bulb, and cerebellum. They found that although both GADs were present in cell bodies and nerve terminals in most

brain regions, in some populations of neurons GAD67 immunoreactive cell bodies outnumbered those that were GAD65 immunoreactive. In addition, the density of GAD65 immunoreactive axon terminals was higher than the GAD67 immunoreactive terminals in most of the brain regions examined. The authors therefore concluded that the difference in distribution of the two isoforms within many neurons suggested that the two GAD isoforms may synthesise two separate pools of GABA in the brain. (Soghomanian and Martin, 1998). Until now, it was not known whether this was also the case in the spinal cord. In this part of the study, both GADs were detected mainly in axonal boutons. In addition, in the ventral horn, GAD67 seemed to be the predominant form found in GABAergic boutons. These results are in agreement with a study conducted by Feldblum et al. (1995), which also only detected few GAD-immunoreactive cell bodies. Both sets of results suggest that a different arrangement is present in the rat spinal cord, from that described in the brain by Esclapez et al., 1994, with significant numbers of GAD67-immunoreactive boutons and undetectable levels of each GAD in most GABAergic cell bodies.

The results of this study indicate that GABAergic axons are particularly concentrated in laminae I-III of the rat spinal cord. Moderate numbers are also found in the medial part of laminae IV-VI, in lamina X and in the motor nuclei. Relatively fewer GABAergic axons are found in other regions such as laminae VII and VIII. This distribution of GABAergic axonal boutons is similar to that of GABA-immunoreactive cell bodies (Todd and McKenzie, 1989; Todd and Sullivan, 1990). It is therefore likely that many GABAergic axons are derived from local interneurons. Intracellular and whole-cell studies have looked at interneurons in laminae I-II and showed that these have local axons (e.g., Light et al., 1979; Schneider, 1992) Some of the GAD-immunoreactive terminals in this study probably originate from the descending GABAergic input from the medulla to both the ventral and dorsal horns of the spinal cord (Holstege, 1991; Antal et al., 1996; Maxwell et al., 1996).

Moore et al. (2002) have reported that there was a significant depletion of GAD65, but not GAD67, in the ipsilateral dorsal horns of rats after CCI and SNI. The results of this investigation into the distribution and co-localisation of the two GAD isoforms in each lamina of the rat spinal cord showed that some GABAergic boutons in the superficial dorsal horn appear to have relatively high levels of GAD65 and low levels of GAD67. These boutons may therefore be principally affected after nerve injury, as the low level of GAD67 found in them is unlikely to compensate for GAD65 depletion. This may therefore lead to a reduced GABA synthesis in these boutons, and this could result in a reduction in GABAergic inhibition of lamina II neurons after nerve injury.

3.5.2.2 Glycine

In the ventral horn, GAD67 was the predominant isoform found in most GAD-immunoreactive boutons, and many of these were also immunoreactive for GLYT2. This suggests that these boutons originate from neurons that contain both GABA and glycine, such as Renshaw cells, that were found to have immunoreactivity for GABA and glycine (Cullheim and Kellerth, 1981; Schneider and Fyffe, 1992; Örnung et al., 1996). Some of these boutons were in close contact with large unstained cell bodies that were presumably motoneurons, although many of these boutons that contacted motoneurons were GLYT2- but not GAD-immunoreactive. This agrees with the results of an electron microscopy immunocytochemical study in the cat spinal cord by Örnung et al. (1996), who stated that 43% of the boutons in contact with motoneuron somata were only immunoreactive for glycine, while 25% were both GABA- and glycine-immunoreactive, and 2% were only GABA-immunoreactive.

The boutons that showed strong immunoreactivity for GAD65, and frequently occurred in clusters in lamina IX, have not been described in previous studies. As these lacked GLYT2, this

suggests that they belong to neurons that are GABAergic, but not glycinergic. This is explored further in chapter 4.

3.5.2.3 NOS/PV

The main finding of this part of the study was that 83% of the PV labelled cell bodies were immunoreactive for GAD67, whilst none of the NOS-immunoreactive cell bodies were stained with the GAD67 antibody. In addition, all GAD67-immunoreactive cells in laminae II and III that were examined in this study were PV-immunoreactive. Although there is no published data on the expression of each GAD isoform in NOS- or PV-immunoreactive neurons, results from previous studies on the GABAergic content of PV-immunoreactive cells are in general agreement with the results in this study. Antal et al. (1991) showed that approximately 75% of PV-immunoreactive cells in laminae II and III were GABAergic. In contrast, Laing et al. (1994) stated that in lamina II, most cells that were positive for reduced nicotinemide adenine dinucleotide phosphate (NADPH) diaphorase, that is thought to be a reliable marker for NOS (Spike et al., 1993), were also GABA-immunoreactive. The data from this study suggests that GAD67 is not expressed in NOS- immunoreactive cell bodies in this area of the dorsal horn. As most NOS-immunoreactive cells in this area of the spinal cord are GABAergic, these cells must express one or both of the GAD isoforms. It is obvious that the level of GAD present in these cells is below the threshold level at which they are recognised by the currently available GAD antibodies.

3.5.2.4 VGLUT1/ VGLUT2

VGLUT1 and VGLUT2 are found in axons belonging to largely non-overlapping populations of glutamatergic neurons. It is thought that these neurons are always excitatory, and it is not expected that axons of this origin would contain the inhibitory neurotransmitter, GABA or its

synthesising enzyme, GAD. Interestingly, in this study 1 bouton of the 2100 analysed was immunostained with the VGLUT2 antiserum and both GAD antibodies, and this was in lamina IX. It is therefore possible that occasionally excitatory and inhibitory substances may be co-localised within the same neuron. This is in good agreement with a previous study that found co-expression of VGLUT1 and VGLUT2 genes with genes for GAD in neurons in the rat brain (Danik et al., 2005). In addition, Somogyi (2006) found co-localisation of GABA and glutamate in mossy fibre terminals, and some nerve terminals of the retina, brainstem and spinal cord. It is not yet clear if these are released from the same terminals or packaged in the same vesicles.

Chapter 4: GABA and the P boutons

4.1 Background

Studies by Feldblum et al. (1995) and Mackie et al. (2003) have shown that although both GAD isoforms are present in the ventral horn of the rat, GAD67 is the predominant form in this region and the majority of GABAergic boutons show moderate-strong GAD67 immunoreactivity, but only weak immunoreactivity for GAD65. However, some boutons in lamina VII and IX show very high levels of GAD65, and many of these form clusters. These boutons will be referred to as 'GAD65 intense'.

In 1969, an ultrastructural study in the lumbosacral spinal cord of the cat classified boutons that were in contact with motoneurons into six groups. This was done on the basis of bouton size, vesicular shape, synaptic cleft width, the type of postsynaptic thickening and any association with specialized postsynaptic structures (table 4.1; Conradi, 1969a). Boutons with spherical synaptic vesicles were named S type, M type, T type or C type depending on these criteria. S type boutons normally had some contrast-rich material attached to the presynaptic membrane and the size of their synaptic clefts varied significantly. Studies into the distribution of S type boutons on motoneurons have provided conflicting evidence, as Conradi (1969a) and Kellerth et al. (1979) both reported that these were found predominantly on distal dendrites, whilst Conradi et al. (1983) and Fyffe and Light (1984) stated that they were located much closer to the cell body. Other studies have documented a much wider distribution of these boutons, with a small number in contact with the motoneuron cell body (Burke et al., 1979; Pierce and Mendell, 1993). Large boutons containing scattered spherical synaptic vesicles that had an extensive synaptic complex of an irregular shape were named M boutons. These had a thick postsynaptic membrane. Originally, it was believed that M boutons were Ia afferent terminals (Conradi, 1969b). These are still believed to be of primary afferent origin, and may be group II afferents.

Later studies, in which intra-axonal HRP labelling was used, identified Ia afferent terminals as Conradi's S type boutons

Table 4.1

Bouton type	Bouton size	Vesicle shape	Synaptic cleft width	Associated structures
S	varies	spherical	varies	contrast rich material on presynaptic membrane
M	large	spherical	varies	thick postsynaptic membrane
T	varies	spherical	large	postsynaptic dense bodies
C	large	spherical	very large and narrow	no postsynaptic density
F	small	flattened	narrow	thin layer of postsynaptic dense rich material
P	small	flattened	narrow	no pronounced synaptic specialization

Table 4.1 Lamina IX boutons

This table shows the main defining features of the six classes of boutons in lamina IX, as described by Conradi (1969a).

(Conradi et al., 1983; Fyffe and Light, 1984). M boutons were almost always located on the proximal dendrites of motoneurons (Conradi et al., 1969a). Fyffe and Light (1984) found that M boutons and large S boutons formed only approximately 1% of the synaptic coverage of the motoneuron's surface. In Conradi's study, C type boutons were the largest bouton type described, and were frequently grouped together on the motoneuron surface. These were never observed adjacent to smaller neurons in the neuropil. In addition, they were associated with an extensive cistern underlying the postsynaptic membrane, and the synaptic cleft was very large and narrow. The spherical vesicles in C type boutons were tightly packed and intermingled with some dense core vesicles. In addition, they lacked a postsynaptic density. Boutons classified as T type were similar to the S type boutons, although the synaptic cleft/complexes were larger. These boutons exhibited postsynaptic dense bodies situated below a thick layer of postsynaptic material. F type boutons contained flattened synaptic vesicles, and usually established several synaptic complexes. The synaptic cleft was much narrower than that of the S type boutons, and a thin layer of postsynaptic contrast-rich material was normally apparent. Interestingly, the distribution of each bouton type differed along the motoneuron surface, as T and F type boutons were located on all regions, whilst C type boutons were restricted to the cell body and its proximal dendrites.

In contrast, P boutons were small with flattened vesicles of an irregular shape and had a clear cytoplasm. S boutons are the major postsynaptic targets of P boutons (Conradi et al., 1969b; Fyffe and Light, 1984), although P boutons also occasionally synapse on M boutons (Destombes et al., 1996). Also, the size of Ia afferents appears to determine the number of contacts received from P boutons. In 1983, Conradi showed that Ia boutons that received no contacts from P boutons were relatively small, whilst larger terminals received the greatest number of contacts (Conradi et al., 1983). In cases where a single Ia afferent received more than one contact from P boutons, these originated from the same parent fibre (Conradi, 1969c).

It is now assumed that boutons with flattened vesicles contain inhibitory substances (i.e., F boutons and P boutons) whilst those with spherical vesicles are excitatory (S, T, M and C type boutons) (Uchizono, 1965). In addition, in 1962, Gray showed that the thickness of pre- and postsynaptic densities related to the type of the event taking place at each synapse, i.e. if the synaptic density on the postsynaptic side of the synapse was thick with no synaptic density on the presynaptic side, this was an asymmetrical synapse (type I), and was typical of an excitatory synapse. If the synaptic densities were equally thick on both sides of the synapse, this was a symmetrical synapse (type II), and was a feature of inhibitory synapses. Although these concepts devised by Uchizono et al. and Gray are still used to determine whether synapses are excitatory or inhibitory, it must be noted that the type of fixative used and the length of the fixation procedure can alter the fine ultrastucture of synapses and their associated vesicles. Now, immunocytochemistry with specific markers for excitatory, e.g., VGLUT1 and VGLUT2, and inhibitory, e.g., GABA and GLYT2, substances is used to classify synapses. Many studies have shown that P boutons are GABA- or GAD-immunoreactive (Destombes et al., 1996; Örnung et al., 1996; Watson and Bazzaz, 2001), but are not enriched with glycine (Watson and Bazzaz, 2001). In contrast, F boutons can be immunoreactive for both GABA and glycine, although these are predominantly glycinergic (Örnung et al., 1996; Watson and Bazzaz, 2001). Örnung et al. (1996), used post-embedding immunogold histochemistry to examine the distribution of glycine- and GABA-like immunoreactivity in nerve terminals on α- motoneurons in the lumbar spinal cord. They found that, of the 405 terminals examined, 69% were immunoreactive for GABA or glycine, and that, 43% were immunoreactive for glycine only, 25% were immunoreactive for both GABA and glycine, and 1% were immunoreactive for GABA only. Terminals that were glycine-immunoreactive (with or without GABA) contained flat or oval synaptic vesicles, and were therefore classified as F type. No examples of F boutons that were immunonegative for GABA and glycine were detected in the study. Of the six synaptic boutons that were purely GABA-

immunoreactive, one was of the C type, while the remaining five were described as P boutons as they made synaptic contact with large S or M type boutons, but made no visible synapses with motoneurons. Destombes et al. (1996) found that on the membrane of α-motoneurons, 20% of F type boutons and all P boutons, but no M boutons contained GABA. Also, the study by Watson and Bazzaz (2001) examined GABA and glycine-immunoreactivity at axo-axonic synapses on Ia muscle afferent terminals. They found that in the ventral horn, 91% of these P boutons were immunoreactive to GABA only, and only 9% were also enriched with glycine. This differed from results found in the deep dorsal horn, where 58% of boutons presynaptic to Ia muscle afferent terminals were immunoreactive for GABA alone, 31% were GABA and glycine-immunoreactive and 11% showed only glycine-immunoreactivity. This suggests that Ia afferent boutons are modulated by different populations of presynaptic neurons, depending on where they are located within the spinal cord (Watson and Bazzaz, 2001) and that P boutons in the ventral horn are derived from cells that use GABA, but not glycine, as their neurotransmitter. With regard to the other types of boutons, that were classified by Conradi (1969), C boutons are primarily cholinergic (Lewis et al., 1970) whilst S type and M type boutons are glutamatergic (Fyffe and Light 1984).

Motor output is continuously modified by input from muscle spindle afferents and other sensory neurons, as well as from descending supraspinal pathways. Small, but appropriate alterations in motor output enable patterns of locomotion to be contextually appropriate. This is achieved in part via presynaptic control of Ia muscle spindle afferents. For example, PAD in these afferents may assist in damping motor responses to a low level of sensory stimulation, therefore reducing the risk of irrelevant motoneuron activity. There is evidence that individual axon branches of the same afferent may be under different levels of presynaptic control, as a study by Eguibar et al. (1994) showed that the size of effect achieved by inducing PAD via cortical stimulation, differed between collaterals of the same neuron. It is now generally accepted that

GABAergic axo-axonic synapses are the anatomical substrate of presynaptic inhibition of primary afferents and PAD, (see chapter 1) and that P boutons are responsible for this inhibition in group Ia primary muscle spindle afferents in the motor nuclei (Rudomin and Schmidt, 1999). To date, the cells that mediate presynaptic inhibition of these afferents, i.e., the cells of origin of P boutons, have not been identified, although stimulation of neurons in laminae V and VI leads to PAD in Ia afferent terminals (Jankowska et al., 1981), and this suggests that these cells may be found in this region of the spinal cord.

Several studies have observed P boutons forming part of a triadic arrangement, in which the P bouton is presynaptic to both the Ia bouton and the same dendrite that is postsynaptic to the primary afferent terminal (Fyffe and Light, 1984; Pierce and Mendell, 1993). The extent to which P boutons are thought to take part in triads differs between studies, with Pierce and Mendell stating that 66% of these formed a triadic arrangement, whilst other studies have found many fewer examples of this (Fyffe and Light, 1984; Watson and Bazzaz, 2001). The involvement of P boutons in synaptic triads indicates that they are not solely involved in presynaptic inhibition (Pierce and Mendell, 1993) and that they may also be capable of producing postsynaptic inhibition of motoneurons. This inhibition is picrotoxin (but not strychnine) sensitive (Rudomin et al., 1990), consistent with findings that P boutons are not enriched with glycine (Watson and Bazzaz, 2001).

Group II afferents, from muscle spindle secondary endings, also terminate in the ventral horn (Fyffe, 1979) and synapse on α-motoneurons (Kirkwood and Sears, 1974), although intracellular studies have shown that projections to lamina IX are sparse, and that these afferents primarily project to lamina IV and the intermediate grey matter. Occasionally, boutons that are presynaptic to group II afferent terminals are part of synaptic triads (Maxwell and Riddell, 1999). Immunocytochemistry has shown that all terminals presynaptic to group II afferents contain GABA, and that co-localisation with glycine occurs in the majority of these terminals (Maxwell

and Riddell, 1999). Although GABA is also frequently co-localised with glycine in boutons that are presynaptic to Ia afferents in the deep dorsal horn, it has been shown that approximately 11% of these do not contain GABA. Furthermore in the ventral horn, all P boutons are GABAergic, and only 9% of these are enriched with glycine (Watson and Bazzaz, 2001). Group II afferents are also subject to PAD and presynaptic inhibition by GABAergic spinal interneurons via axo-axonic synapses. In addition, they are also controlled by supraspinal monoaminergic neurons, that release 5-HT, dopamine and norepinephrine (Jankowska and Riddell, 1995). Little is known about the stimuli that induce PAD in these muscle afferents, although it has been proposed that activity in group I and group II flexor afferents or cutaneous afferents may have a role (Harrison and Jankowska, 1989). The neurotransmitter content of boutons that are presynaptic to Ia and group II afferents in the deep dorsal horn, and P boutons in the ventral horn differ. This would suggest that different populations of presynaptic interneurons are involved in PAD in these afferents (Rudomin, 1990).

P boutons form a different functional population from the majority of GABAergic boutons in the ventral horn, which form axo-somatic and axo-dendritic synapses, and include some of the F type boutons. Mackie et al. (2003) suggested that the 'GAD65 intense' population in the ventral horn might be the P boutons due to the extent that these form discrete clusters, and their distinct lack of glycine. In this part of the study, the GAD65-intense population in lamina IX have been investigated by using retrograde tracing, immunocytochemistry, confocal microscopy and electron microscopy, to confirm that these are the P boutons.

4.2 Materials and methods

4.2.1 Analysis of GAD65-intense boutons in lamina IX

Immunocytochemistry was performed to examine the GAD65-intense boutons in lamina IX. Firstly, three adult male Sprague-Dawley rats (300-350g) were anaesthetised with halothane

(1-2%) and in each case, CTb was injected into the left sciatic nerve. This procedure was carried out by Professor D.J. Maxwell. CTb is commonly used to selectively label myelinated afferents in normal animals (for more details, see chapter 1). Using a sterile technique, the left sciatic nerve was uncovered by incising the skin of the lateral thigh and retracting the hamstring muscle. The nerve was then injected with 4μl of 1% CTb (Sigma, Poole, Dorset, UK) in distilled water, using a glass micropipette placed approximately 15-18mm distal to the piriformis tendon. The skin incision was sutured using 3/0 Mersilk Sutures (AW-online). Analgesia was then administered.

Three days later, the rats were deeply anaesthetised with pentobarbitone and perfused with a fixative containing 4% formaldehyde followed by post-fixation in the same fixative. The spinal cords were removed and transverse 60μm Vibratome sections were cut from the L3-L5 segments and immunocytochemistry was performed to detect CTb, GAD65 (using GAD6) and VGLUT1 (see Chapter 2 for antibody information). VGLUT1 antibodies are known to label the central terminals of myelinated proprioceptive primary afferents (Todd et al., 2003). However, not all VGLUT1 immunoreactive terminals in lamina IX are from primary afferents and some originate from axons belonging to the corticospinal tract (Hughes and Enriquez-Denton, unpublished observations). Therefore CTb labelling was used to identify primary afferents in some cases.

Selected areas from the lateral motoneuronal cell groups of the L4 segment were scanned for each rat (n=3) using the Bio-Rad MRC1024 confocal microscope through a 60× oil-immersion lens. Quantitative analysis was carried out using Neurolucida for Confocal software. The first part of this study involved determining the proportion of 'GAD65 intense' boutons that were in close contact with individual Ia afferent terminals in lamina IX. Primary afferent terminals were identified by the presence of both CTb and VGLUT1, and sixty of these were randomly selected from each animal, without viewing the GAD65- immunoreactivity for each section. The number of 'GAD65 intense' boutons in close contact with each primary afferent

terminal was determined by examining every optical section that each afferent terminal appeared in. Afferent terminals that appeared in the first and last optical sections of the z-series were not included in the sample, as it was not possible to determine how many contacts these received from 'GAD65 intense' boutons.

A separate analysis was performed to determine the proportion of 'GAD65 intense' boutons that were associated with primary afferent terminals, and could therefore be P boutons. Sections from the animals in the first part of this study were used for this analysis. Boutons (in the lateral motoneuronal cell groups) that showed strong GAD65- immunoreactivity were selected (between 253 and 397 from each animal), and the proportion that was in contact with a VGLUT1-immunoreactive terminal was determined. It is possible that not all primary afferents were labelled with CTb, and therefore contacts with VGLUT1-immunoreactive axons were included in the analysis. To avoid a bias in sampling, e.g. a preference towards those GAD65-intense boutons that formed clusters, Metamorph software was used to set an arbitrary threshold value for pixel luminance for GAD65-immunofluorescence in each confocal stack. All boutons with at least one pixel brighter than this value were included in the analysis.

4.2.2 GAD67 expression in P boutons

In order to determine whether the weak GAD67-immunoreactivity observed in GAD65-intense boutons by Mackie et al., 2003 was genuine, a further two male Wistar rats (240-250g) were fixed with a solution containing 4% formaldehyde followed by 4 hours of post fixation in the same fixative. Spinal cord sections were incubated in a cocktail of GAD67 antibodies (K2 polyclonal rabbit and mouse monoclonal, see chapter 2) followed by rabbit rhodamine and mouse Alexa 488 secondary antibodies.

Sections were scanned with the Bio-Rad MRC1024 confocal microscope using the 488- and 568-nm lines of the laser (to reveal Alexa 488 and rhodamine respectively). For each animal,

a single image was captured through a 10× lens to compare the staining pattern achieved with both antibodies, and two additional image stacks were scanned with the 60× oil-immersion lens for detailed examination. These image stacks each consisted of 11 z-steps with 0.5μm spacing. All of these confocal images were carefully examined to determine whether both GAD67 antibodies label the same structures in spinal cord sections. P boutons were identified as clusters of boutons with very weak GAD67-immunoreactivity when using the K2 antibody.

4.2.3 Combined confocal-electron microscopy studies of GAD65-intense contacts in lamina IX

For combined confocal and electron microscopy (confocal EM; Todd, 1997), an additional six adult male Sprague Dawley rats (250g) received CTb injections into the left sciatic nerve (as above) and after three days were anaesthetised with pentobarbitone and perfused. The first two rats were perfused with a solution containing 4% formaldehyde. At a later time, two rats were perfused with a solution containing 0.2% glutaraldehyde and 4% formaldehyde. Subsequently, another two animals were perfused with a solution containing 0.1% glutaraldehyde, 4% formaldehyde and 15% v/v of saturated picric acid. In all cases, lumbar cord segments were post-fixed for 4 hours in the same solution as the animals were perfused with followed by overnight immersion in 4% formaldehyde. Transverse Vibratome sections (70μm) were cut from the mid-lumbar spinal cord and immediately bathed in 50% ethanol for 30 minutes. After rinsing, these were treated with 1% sodium borohydride for 30 minutes (to reduce non-specific staining by blocking free aldehyde sites) before being thoroughly rinsed for 90 minutes. Sections were subsequently incubated in antibodies raised against GAD65 (rabbit), CTb (goat) and VGLUT1 (guinea pig). After rinsing, sections were incubated in fluorescent secondary antibodies (LRSC-rabbit, FITC-goat and Cy5- guinea pig) and biotinylated rabbit IgG for 24 hours at 4°C (for antibody details, see Chapter 2). The sections were then rinsed and placed in avidin peroxidase

conjugate for 24 hours at 4°C. No Triton X-100 was included in any of the solutions used on sections being prepared for confocal EM.

Spinal cord sections from each of the rats were examined with a confocal microscope. Areas with strong CTb labelling in lamina IX were identified and scanned using the 60× lens and terminals that were double-labelled for CTb and VGLUT1 were identified in Photoshop. Sections were removed from the slides and reacted with a DAB solution, in the presence of hydrogen peroxide (15µl/50ml water) for 5-10 minutes and rinsed in PB. Afterwards sections were post-fixed for EM in 1% osmium tetroxide in PB for 20 minutes, rinsed in water, and dehydrated using a gradient of concentrations of acetone (70% for 30 minutes, 90% for 10 minutes and 100% for 3x10 minutes). Sections were block- stained with uranyl acetate, placed in a 1:1 mixture of acetone and Durcupan for one hour and then in pure Durcupan overnight, before being flat embedded and cured at 60°C for 48 hours. The areas of lamina IX that contained the GAD65-intense boutons identified earlier in confocal images were photographed through a light microscope and drawings were made of one section from each animal, noting any landmark feature such as blood vessels and their location relative to previously identified clusters of 'GAD65 intense' boutons, so that these could be easily identified on EM images. A series of ultrathin sections was cut from each block and these were stained with lead citrate to contrast the tissue. The selected boutons were followed through serial ultrathin sections with a Philips CM100 electron microscope to examine whether they formed axo-axonic synapses with large unlabelled structures, that had been identified as primary afferents (because of their VGLUT1/CTb-immunoreactvity) on the confocal images.

4.3 Results

The pattern of GAD65 and GAD67 immunoreactivity detected on sections from CTb injected animals was the same as that which has been previously reported in the ventral horn of

unoperated rats (Mackie et al., 2003). Examination of confocal images revealed that in most cases GAD-immunoreactive boutons showed relatively high level of immunostaining with the GAD67 antibody, and relatively lower levels with the antibody against GAD65. Many boutons in lamina IX were also found that showed very strong GAD65 immunoreactivity. These are referred to as 'GAD65 intense'. These often formed clusters. These could easily be distinguished from the terminals that displayed high levels of GAD67, and only low levels of GAD65.

The VGLUT1-immunostaining observed was also consistent with that found in previous studies (Varoqui et al., 2002; Todd et al., 2003). Immunoreactive terminals were sparse in laminae I and II, but more dense in laminae III-VI, particularly in the medial aspect. A moderate density of terminal staining was observed in lamina X, lamina VII and lamina IX. Also, a few immunoreactive profiles were observed in the spinal white matter, although this was observed mainly in the deepest part of the dorsal columns.

The distribution of CTb labelling was similar to that found previously in normal rats after sciatic nerve injections (LaMotte et al., 1991; Woolf et al., 1995). In L5, labelling was seen in laminae I and IIi to V, with virtually no labelling in lamina IIo. Labelling was restricted to terminals in the medial two-thirds to three-quarters of the dorsal horn, and labelled cell bodies of motoneuronal size were detected in lamina IX alongside large CTb-immunoreactive terminals (presumed Ia afferents).

4.3.1 Analysis of GAD intense boutons in lamina IX

Further examination showed that many 'GAD65 intense' boutons clustered around individual VGLUT1 immunoreactive axon terminals, many of which contained transganglionically transported CTb (Fig. 4.1). The majority of 'GAD65 intense' boutons that were not in clusters were also adjacent to VGLUT1/ CTb immunoreactive terminals. Quantitative analysis showed that VGLUT1/CTb double-labelled terminals in lamina IX received between 0

and 10 contacts from 'GAD65 intense' boutons, with the mean number per terminal equalling 2.5 (animal 1), 2.98 (animal 2) and 3.02 (animal 3), with an average of 2.83. Between 82-93% (mean 88%) of VGLUT1/CTb double-labelled terminals were in contact with at least one 'GAD65 intense' bouton (49/60 for animal 1, 53/60 for animal 2 and 56/60 for animal 3).

In a separate analysis, it was found that on average 89% (mean of n=3) of 'GAD65 intense' boutons contacted a VGLUT1 immunoreactive terminal. That is 76/87 for animal 1, 81/92 for animal 2 and 112/121for animal 3. Although the quantitative data was obtained from the lateral motoneuronal cell groups in L4, clusters of GAD65-intense boutons were observed in all motor nuclei in the L3-L5 segments.

4.3.2 GAD67 expression in P boutons

Spinal cord sections that were reacted with both GAD67 antibodies showed the same general pattern of staining for each antibody on low magnification images (×10). Closer inspection using high magnification images (×60) showed that some boutons were not double-labelled (fig. 4.2) and that fewer profiles with GAD67-immunoreactivity were detected in laminae I and IIo when using the monoclonal antibody, as compared to that obtained when K2 was used. A band of intense GAD67 staining in lamina IIi was detected with both antibodies. In lamina IX, P boutons were identified as clusters of boutons that were slightly GAD67-immunoreactive with the K2 antibody. These were not immunostained with the monoclonal GAD67 antibody.

Fig. 4.1 'GAD65 intense boutons' cluster around VGLUT1-labelled terminals

This confocal image shows GAD65 (red) and VGLUT1 (blue) in lamina IX of the rat spinal cord. This image is constructed from 11 optical z-sections that were merged. These had 0.5 μm spaces between them. Scale bar = 10 μm

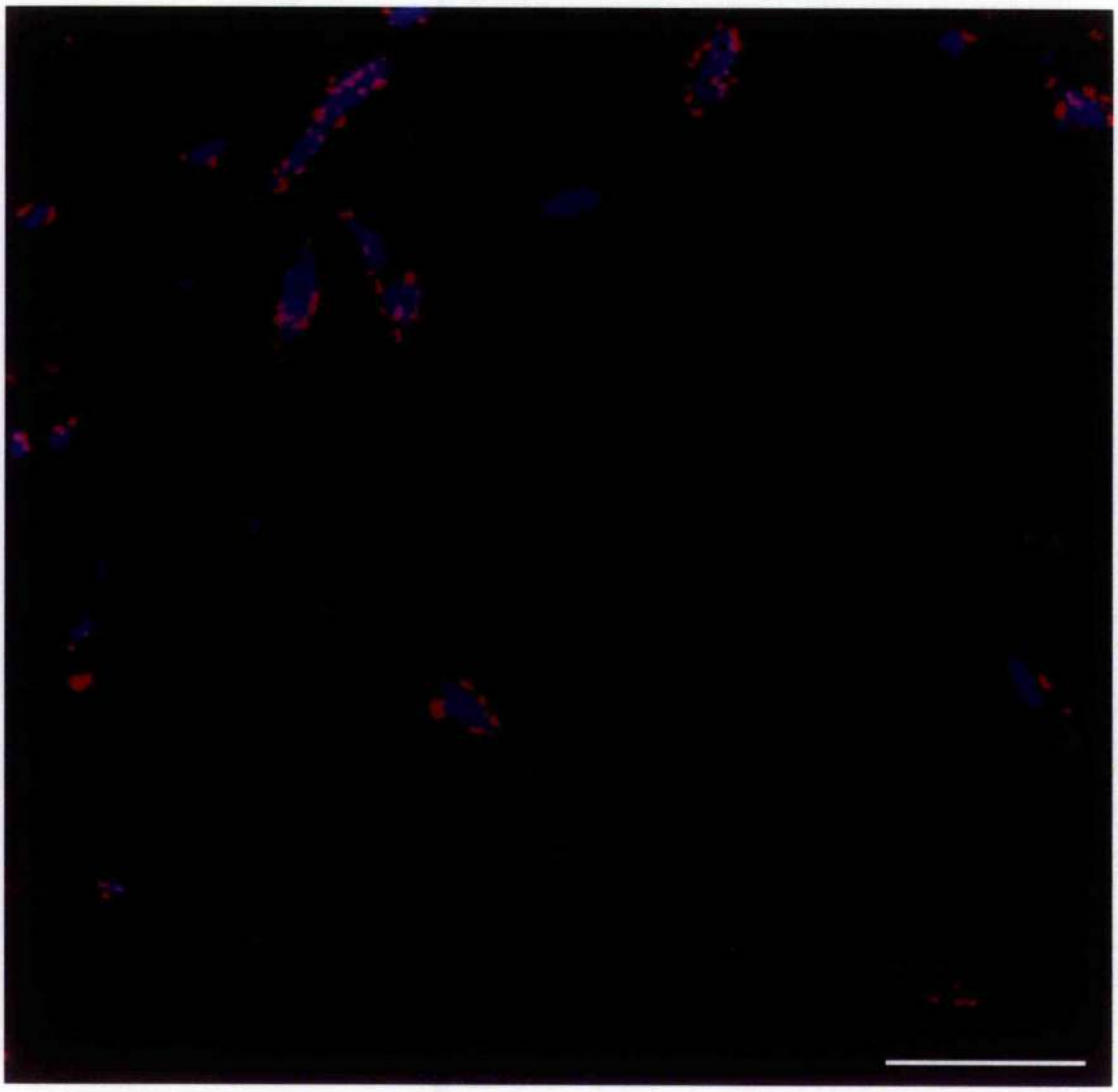
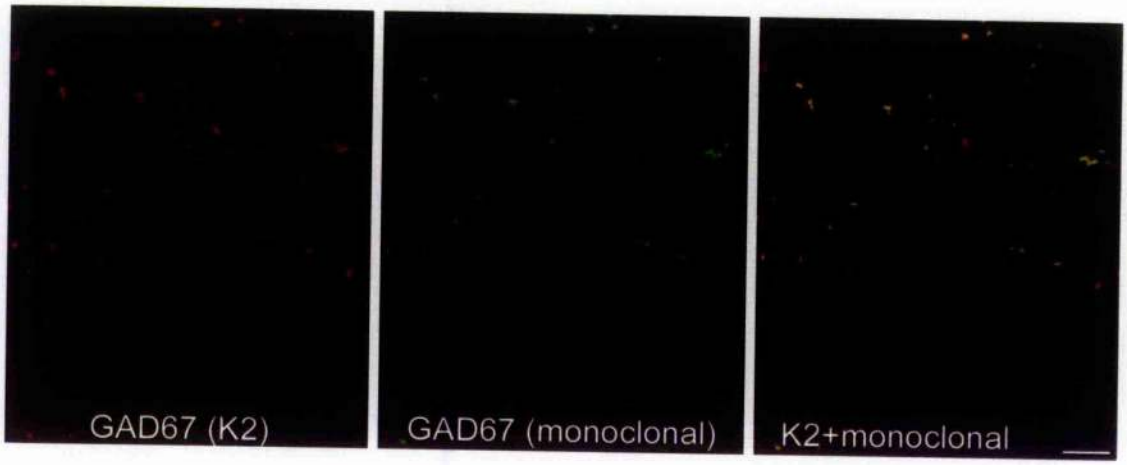


Fig. 4.2 Confocal images of immunostaining resulting from the use of GAD67 (K2) and GAD67 monoclonal antibodies.

Confocal images show that although the K2 antibody (red) and the monoclonal antibody (green) give the same general pattern of staining (shown in yellow in the third image) when used in immunocytochemistry, some boutons are not double-labelled with both antibodies. In lamina IX, boutons were identified that were GAD67 immunoreactive with the K2 antibody, that were not stained with the mouse antibody (red). Images were constructed from a single optical section. Scale bar = 10 μm


GAD67 (K2)
GAD67 (monoclonal)
K2+monoclonal

4.3.3 Combined confocal EM studies of GAD65-intense contacts in lamina IX

When spinal cord sections from animals perfused with a solution containing 4% formaldehyde were examine with the electron microscope, the ultrastructure of the tissue was poor. When spinal cord sections from animals perfused with a solution containing 0.2% glutaraldehyde and 4% formaldehyde were examine with the confocal microscope, the CTb-immunostaining was sub-optimal, since the glutaraldehyde in the fixative had affected this.

In the spinal cord sections from animals perfused with a solution containing 0.1% glutaraldehyde, 4% formaldehyde and 15% v/v of saturated picric acid, the CTb-immunostaining was good. In addition, the ultrastructure of the tissue was satisfactory when examining this with the electron microscope, and DAB-labelling of the proposed P boutons was of good quality. It was possible to recognise individual 'GAD65 intense' boutons that had been previously identified on confocal images (fig. 4.3, fig. 4.4), and 22 putative P boutons were identified. DAB-labelled 'GAD65 intense' profiles were observed in close apposition to large unlabelled terminals that were the neighbouring VGLUT1/CTb immunoreactive terminals observed on the confocal images. On at least one occasion the synapse between the DAB-labelled 'GAD65 intense' profile and the presumed primary afferent was visible.

Fig. 4.3 Confocal image of 'GAD intense' boutons in close opposition to VGLUT1/CTb labelled terminals in material prepared for confocal-EM.

Immunostaining for CTb (green), VGLUT1 (blue) and GAD65 (red) in lamina IX of the rat. CTb was injected into the ipsilateral sciatic nerve and has been retrogradely transported to several proprioceptive afferent terminals. VGLUT1 also labels these terminals. 'GAD65 intense' boutons are shown surrounding a VGLUT1-/CTb-labelled terminal (presumed primary afferent). A, B and C indicate the terminals seen by electron microscopy in Fig 4.4. This image is taken from a single optical section. Scale bar= 10μm

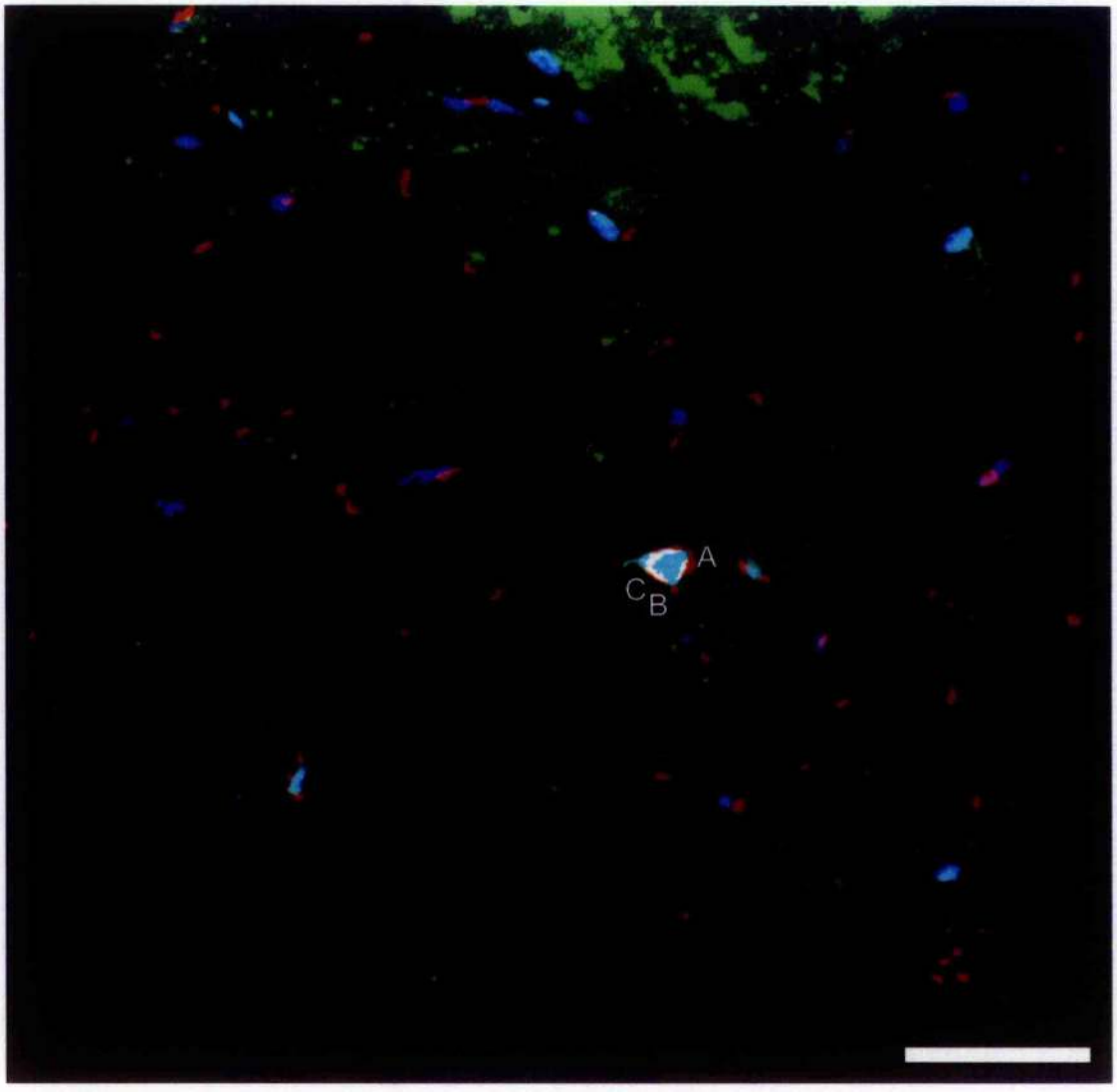
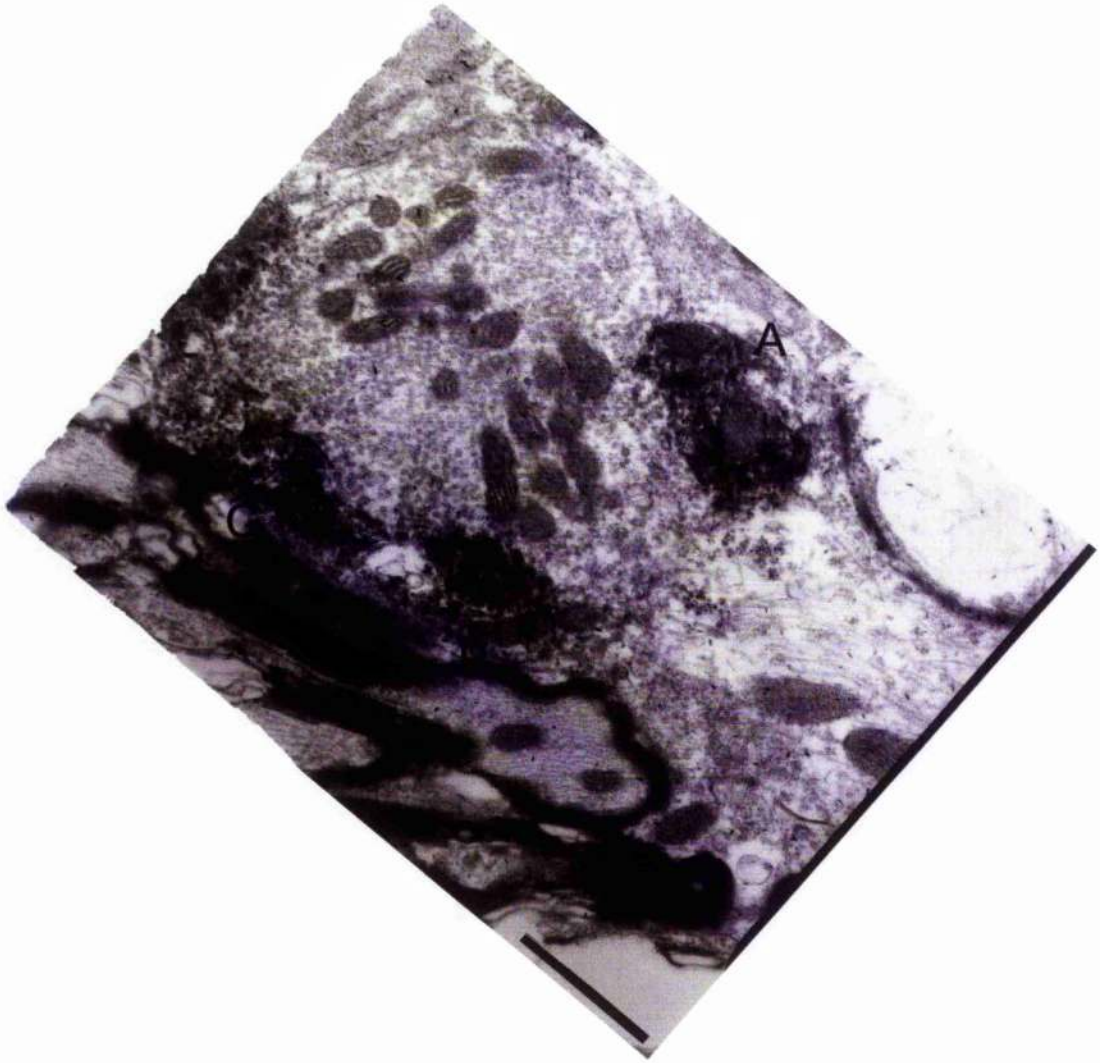

A
C
B

Fig. 4.4 Electron microscopic image of same VGLUT1-/CTb-labelled terminal as in fig. 4.3 and associated GAD65 terminals

This image shows DAB-labelling of GAD-immunostained axonal boutons in lamina IX. These surround a presumed primary afferent that was identified in confocal images of the same area of the same section. P boutons (GAD65 intense) from the confocal image (Fig. 4.3) are labelled with A, B and C. Scale bar = 10μm


A
C

4.4 Discussion

The main finding of this part of the study was that the 'GAD65 intense' boutons in lamina IX are the P boutons, as 88-89% of them are in close contact with primary afferent terminals, and they frequently form clusters around them. Hughes et al. (2005) have subsequently shown that 'GAD65 intense' boutons associated with Ia terminals, identified by VGLUT1-/CTb-immunoreactivity, make axo-axonic synapses on primary afferent terminals in lamina IX. As all P boutons are strongly GABAergic (Destombes et al., 1996; Örnung et al., 1996; Watson and Bazzaz, 2001), they must contain at least one GAD isoform, and although the majority of GAD-containing boutons in the ventral horn are predominantly GAD67 immunoreactive, these are seldom adjacent to VGLUT1-labelled terminals, which include all proprioceptive afferents (Todd et al., 2003). This study has shown that the great majority of 'GAD65 intense' boutons in lamina IX are in close contact with VGLUT1 terminals. This supports the hypothesis of Mackie et al. (2003), who stated that the 'GAD65 intense' population were likely to be the P boutons, since they frequently formed discrete clusters and were unlikely to be glycinergic as they lacked GLYT2. The present study also demonstrated that the P boutons are easily distinguished from other GABAergic terminals in lamina IX by their high level of GAD65, and that this provides a way of identifying P boutons that can be used in subsequent studies. For example, it would be of interest to compare the number of P boutons on Ia terminals before and after nerve injury, to investigate whether P boutons reduce in number after nerve injury. If these were to reduce in number after nerve injury, this would suggest that this contributes to the disinhibition that occurs in the spinal dorsal horn after nerve injury. Also, the easy identification of P boutons will allow studies to compare the number of P boutons on Ia afferents from different muscle groups. As 'GAD65 intense' boutons also frequently cluster around Ia afferents in the mouse, and show relatively high levels of GAD65- immunoreactivity when compared to the other GAD-containing boutons in the ventral horn, P boutons can also be easily identified in this species (Hughes et al.,

2005) which is of importance since GAD knock-out and other genetically modified animals are generally mice.

4.4.1 GAD67 in P boutons

In this part of the study, 'GAD65 intense' boutons displayed low levels of immunoreactivity for GAD67 when the K2 antibody was used. However, it is thought that this antibody cross-reacts weakly with GAD65 (Esclapez et al., 1994). In spinal cord sections reacted with the monoclonal antibody against GAD67, the majority of 'GAD65 intense' boutons showed no detectable GAD67, whilst a few showed very weak labelling (Hughes et al., 2005). Although failure to detect GAD67 with this antibody may be the result of a lack of sensitivity of the mouse antibody, it is more likely that the apparent GAD67-labelling detected in the 'GAD65 intense' boutons in the present study was due to cross-reactivity of the K2 antibody with GAD65, as this has previously been reported (Esclapez et al., 1994). In addition, the GAD67 monoclonal antibody shows no detectable cross-reactivity with GAD65 on western blots of rat brain lysate (manufacturer's specification). The GAD65 antibody used in this study (GAD6) has been tested for specificity (Hughes et al., 2005) on tissue from GAD65 knock-out mice (Kash et al., 1997) and no staining was detected after incubation, which confirms that this antibody specifically detects GAD65, and does not cross-react with GAD67. These two lines of evidence, when combined, suggest that most P boutons in the rat do not contain detectable levels of GAD67. In contrast, it appears that P boutons in the mouse do contain GAD67, as these are GAD67-immunoreactive with the monoclonal GAD67 antibody (Hughes et al., 2005).

4.4.2 Other studies of P boutons

Many studies have examined the organization of synapses on Ia afferents in lamina IX of the cat (Fyffe and Light, 1984; Pierce and Mendell, 1993) and rat (Watson and Bazzaz, 2001;

Hughes et al., 2005) spinal cord after injection of HRP or Neurobiotin into identified Ia afferent fibres. In addition, the studies by Fyffe and Light (1984), and Watson and Bazzaz (2001) also examined synapses on Ia afferents in the deep dorsal horn. There are conflicting reports with regard to the number of P boutons that synapse on individual Ia afferents. Firstly, Fyffe and Light found that each Ia bouton in lamina IX was postsynaptic to at least one P bouton, whilst Pierce and Mendell and Hughes et al. stated that 86-87% received axo-axonic contacts from P boutons. In contrast, earlier studies by Ralston and Ralston (1979) and Conradi et al. (1983) stated that fewer than half of the Ia terminals in lamina IX had P boutons associated with them. In the present study, it was concluded that 88% of VGLUT1-/CTb-immunoreactive terminals (which include all proprioceptive afferents in this lamina) had 'GAD65 intense' boutons (putative P boutons) in close contact, and that the number of these varied from 1-10 per VGLUT1-immunoreactive terminal. This data is in good agreement with the results of Pierce and Mendell (1993). Also, proprioceptive afferents (that were immunoreactive for VGLUT1 and CTb) were in contact with an average of 2.8 'GAD65 intense' boutons. This is very close to the value of 2.6-2.7 P boutons in synaptic contact with individual Ia afferent terminals that was previously found using quantitative EM in the cat (Pierce and Mendell, 1993) and rat (Watson and Bazzaz, 2001). It is also similar to the results of Hughes et al. (2005) who found that terminals of identified Ia afferents in lamina IX were in contact with on average 3.6 'GAD65 intense' boutons per afferent terminal. This study also detected 'GAD65 intense' boutons in close contact with VGLUT1/CTb double-labelled terminals in lamina VII, which suggests that P boutons are also found on Ia terminals in this lamina. Preliminary qualitative studies of GAD-immunoreactive boutons that contacted VGLUT1-labelled terminals in the dorsal horn showed that these had no particular pattern of GAD expression, as examples of boutons with relatively high levels of GAD65 were detected as well as boutons with relatively high levels of GAD67. In contrast, GAD-immunoreactive terminals that were in close contact with IB4-labelled terminals in the dorsal

horn of the spinal cord, were more likely to have relatively high levels of GAD65 and lower levels of GAD67 (unpublished observation, Mackie 2005).

EM data from the present study showed that 'GAD65 intense' boutons were in close contact with large unlabelled terminals, that were VGLUT1/CTb immunoreactive, and identified as primary afferent terminals on confocal images. It has previously been noted that synapses between P boutons and Ia afferent terminals do not have a pronounced synaptic specialization (McLaughlin, 1972) and therefore these synapses are generally identified by clustering of synaptic vesicles and a slight increase in the electron density at the limiting membrane (Conradi, 1969c). It was therefore difficult to visualise the synapse between these terminals, as the DAB reaction product was in the presynaptic terminal. In addition, the quality of images obtained from electron microscopy was compromised after the confocal-EM process that has many experimental steps and may affect tissue ultrastructure. Finally, only a low concentration of glutaraldehyde is used in the fixation of tissue being prepared for confocal microscopy, and this may also have affected the quality of EM images obtained in this part of the study, as tissue prepared for EM generally requires a higher concentration of glutaraldehyde in the fixative.

Subsequently, Hughes et al. (2005) followed 38 'GAD65 intense' boutons through serial ultrathin sections and showed that all of these clustered around, and made axo-axonic synapses with large non-immunoreactive terminals that resembled Ia afferents. These synapses showed features that were characteristic of the axo-axonic synapses formed by P boutons on Ia afferents, which lack a pronounced synaptic specialization (Hughes et al., 2005).

P boutons are GABAergic, but are not enriched with glycine (Destombes et al., 1996; Örnung et al., 1996; Watson and Bazzaz, 2001). Mackie et al. (2002) showed that 'GAD65 intense' boutons were never GLYT2 immunoreactive, although most other GABAergic terminals in lamina IX were GLYT2 positive, and presumably glycinergic. This is further evidence that the

'GAD65 intense' population are the P boutons, and that these belong to a different population of cells than those that give rise to the other GABAergic terminals in lamina IX.

4.4.3 Cells of origin of the P boutons

The concentration of each GAD isoform differs between P boutons and other GABAergic terminals in lamina IX and it is therefore likely that 'GAD65 intense' axons belong to a discrete population of GABAergic neurons that have high levels of GAD65 and little or no GAD67 in their axons. It is probable that P boutons form a different functional population from the other GABAergic boutons in the ventral horn, which form axo-somatic and axo-dendritic synapses, and include some of the F boutons that are presynaptic to motoneurons (Destombes et al., 1996; Örnung et al., 1996).

In the developing spinal cord, different classes of ventral horn neurons are generated in distinct positions in the neural tube in response to extracellular signalling, such as the activity of Sonic Hedgehog (Shh). Shh is secreted by the notochord and floor plate, and controls the specification of cell types in the ventral horn (Marti et al., 1995). Four classes of ventral interneuron have been identified in the developing neural tube: V_0, V_1, V_2 and V_3 (Briscoe et al., 2000). Various types of inhibitory interneuron, including Renshaw cells (Sapir et al., 2004) and Ia inhibitory interneurons (Alvarez et al., 2005), appear to originate from V_1 neurons, and these are characterised by the transient expression of the En1 transcription factor during development. Studies of mice, where the synthesis of GFP is driven by En1, have shown that although the majority of GABAergic axons in the ventral horn are GFP labelled, the P boutons are not (Hughes et al., 2005). This suggests that P boutons originate from a different population of neurons than those that give rise to many of the other GABAergic axons in this area.

Mice that express GFP under the control of the GAD65 promoter, GAD65-GFP mice (De Marchis et al., 2004), have been used to investigate the cells of origin of the P boutons after injection of rhodamine labelled biotinylated dextran amine (R-BDA) into lamina IX of an in vitro

spinal cord preparation (Hughes et al., 2005). This study found that although there was extensive retrograde labelling with R-BDA, cells that were also labelled with GFP were present in much smaller numbers, and were only found in the medial aspect of laminae V and VI on the ipsilateral side to the injection. This was not as a result of limited transport of R-BDA as strong labelling of neurons was found up to 3mm rostral and caudal to the injection site. This is evidence that P boutons may be derived from neurons in these laminae on the ipsilateral side at the same rostrocaudal level as the Ia afferent terminals that they innervate (Hughes et al., 2005). In agreement, Jankowska et al. (1981) stated that electrophysiological stimulation of laminae V/VI resulted in PAD of Ia afferents. It is therefore possible that the cells of origin of P boutons are activated directly by group I proprioceptive afferents in a disynaptic circuit, as stimulation of Ia and 1b fibres, that terminate in the medial part of laminae V and VI, where the cells of origin of P boutons are found (Hughes et al., 2005), can evoke PAD in Ia afferents (Rudomin and Schmidt, 1999).

Chapter 5: Changes in GAD-immunoreactivity in the rat dorsal horn after nerve injury.

5.1 Background

Pharmacological studies have shown that GABA (and glycine) have an important role in modulating incoming pain information in the spinal cord (Yaksh, 1989; Hwang and Yaksh, 1997) by suppressing the responses of some dorsal horn neurons to low threshold mechanical stimuli (Sivilotti and Woolf, 1994) therefore ensuring that non-noxious information is not misinterpreted as noxious input. Many studies have used animal models of neuropathic pain to investigate a possible link between partial peripheral nerve injury (a cause of neuropathic pain) and changes in GABA and/or GAD levels in the spinal dorsal horn (Castro-Lopes et al., 1993; Satoh and Omote, 1996; Ibuki et al., 1997; Eaton et al., 1998; Moore et al., 2002; Somers and Clemente, 2002; Polgar et al., 2003). Controversy surrounds these results, with some showing a decrease in GABA/GAD after nerve injury (Castro-Lopes et al., 1993; Ibuki et al., 1997; Eaton et al., 1998; Moore et al., 2002), some finding an increase in either the transmitter itself, or an increase in GABAergic inhibitory tone (Satoh and Omote, 1996; Kontinen et al., 1992) and others stating that there was no detectable change in GABA/GAD after nerve injury (Somers and Clemente, 2002; Polgar et al., 2003) (see chapter 1 for more detail).

In this part of the study, immunocytochemistry, confocal microscopy and image analysis have been used to investigate any changes in the levels of either GAD isoform in laminae I, II or III after CCI and SNT (see chapter 1 for details of animal models).

5.2 Materials and Methods

5.2.1 Animals

Eighteen adult male Sprague Dawley rats were used for this part of the study (275-315g). Seven had CCI of the sciatic nerve, seven had SNT, seven received a sham operation, and four were naïve controls. All procedures were carried out under anaesthesia (1-2% halothane). Initially the biceps femoris was dissected at mid thigh level, to expose the sciatic nerve. For the CCI model, approximately 7mm of the left sciatic nerve was freed from the surrounding tissue and four 4-0 chromic gut sutures were tied loosely around the nerve. These were placed approximately 1mm apart, and were proximal to the trifurcation of the nerve (Bennett and Xie, 1988). Sham operations involved exposure of the left sciatic nerve, without any manipulation. For SNT, the sciatic nerve was ligated at mid-thigh level and a 5mm piece was excised distal to the ligature to prevent regeneration. After CCI, SNT and sham operations, the muscle and skin were closed in two layers using 4.0 Mersilk. Analgesia was administered to animals that had undergone SNT. CCI and sham-operated rats were given no analgesia in case this had any effect on their performance in behavioural testing. All animals were allowed to recover for 24 hours before behavioural testing began.

5.2.2 Behavioural Testing

Behavioural testing to detect thermal hyperalgesia was carried out on all CCI and sham-operated animals on nine occasions: 6, 3, and 1 days before (baseline values) and 1, 4, 6, 8, 11 and 14 days after the procedure. The behavioural testing of these animals was done by a colleague as part of another study (Polgar et al., 2004). The animals were placed in a clear plastic cage with a glass floor and allowed to adapt to their surroundings for 15 minutes before testing began. A Plantar Analgesia Instrument, (Ugo-Basile, Italy; Hargreaves et al., 1988) was used to test the responses of the animals to thermal stimuli. On each occasion, the time taken for withdrawal of

the foot was measured in response to a radiant heat source that was aimed at the plantar surface of the hindpaw. Each testing session involved stimulating each hindpaw five times, with an interval of at least 10 minutes between consecutive stimulation of the same paw. Paws were tested in a random order so that the animals had no expectation of which paw would be tested next. The mean withdrawal latency for each foot was determined. The responses between the ipsilateral and contralateral hindpaws in animals that had undergone CCI and sham surgery were tested for significance for each post-operative time-point with a one-tailed unpaired T-test. Behavioural testing was not performed on animals that had undergone SNT as it is thought that these animals show no signs of neuropathic pain.

5.2.3 Tissue processing and Immunocytochemistry

On the 14th post-operative day, all animals were anaesthetised and perfused with a fixative containing 4% formaldehyde. The fourth lumbar segments were removed and postfixed in the same solution. A notch was made in the ventral horn on the contralateral (right) side of each block to allow the two sides to be distinguished from each other. Vibratome sections (70μm) were cut and processed for immunocytochemical detection of GAD65 and GAD67 as described in chapter 2. The GAD6 and K2 antibodies were used in this part of the study.

5.2.4 Confocal Microscopy and analysis

Four sections from the caudal part of the L4 segment of the spinal cord were used from each animal. The medial part of the dorsal horns from both sides of each section were scanned with the Bio-Rad MRC 1024 confocal microscope (488- and 568-nm lines of the laser, to reveal fluorescein and rhodamine respectively). A single image was captured from both sides of each section through a 20× dry lens, and each optical section was averaged over four scans. Each image covered a field of 619 μm x 464 μm, which ensured that the area of the sciatic nerve in

laminae I-III would be included. This is the area affected by the nerve injury models. To ensure that the two sides of each section were comparable, the laser intensity was always set on the contralateral side and the same laser settings were used when scanning both sides of any given section. The gain of each of the photomultipliers was set so that only a few pixels in each image were equal to the maximum possible pixel luminance value achieved by the laser (a value of 255). This was done since the images corresponding to GAD65- and GAD67- immunoreactivity had a very broad range of pixel intensities, with a few pixels having a high value and the remainder of the pixels having a low value. In a preliminary study, the laser was set to avoid saturation of any of the pixels in the images, but images achieved in this way, were very difficult to analyse, as the pixel range was so small. Lower magnification images were scanned with 4× and 10× objective lenses using light transmitted through a dark field condenser. These images were used to identify laminar boundaries (described further in chapter 2).

To avoid bias, the person who carried out the analysis was blind to the group from which each section was taken (CCI, SNT, sham-operated or naïve) and which side of the section was ipsilateral or contralateral to the nerve injury. The images scanned with the 4× and 10× lenses were used to draw outlines of the grey-matter for each section. This was done with Neurolucida for Confocal software, as described in chapter 2. The mediolateral width of each dorsal horn was measured and divided by a vertical line down the middle of the drawing to separate the medial and lateral halves. Only the medial part of each dorsal horn was used for analysis, as this includes the sciatic territory. The distribution of myelin bundles, which showed no GAD-immunoreactivity may have differed between the two sides of any given section. The presence of myelin bundles would have affected any comparisons made between the two sides, and they were therefore excluded from the area of the picture that was used for analysis by drawing round them. The resultant drawings were exported from Neurolucida to Adobe Photoshop, where each was

superimposed on the original 20× fluorescent images of that section for GAD65 and GAD67 separately.

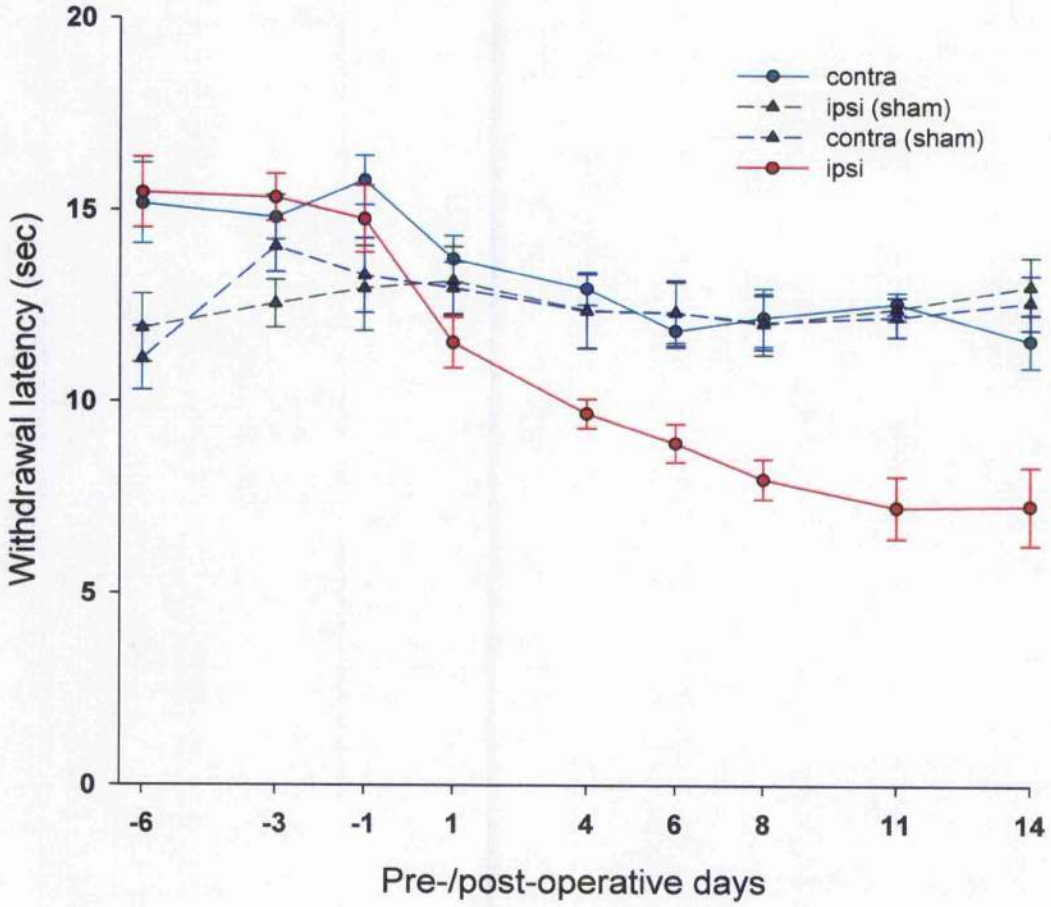
Measurements of immunoreactivity (mean pixel luminance values) for GAD65 and GAD67 were made by using a Zeiss KS400 image analysis system (Kontron Elektronik, Germany). This system calculated the mean pixel luminance values for the medial halves of laminae I, II and III for both sides of every section (excluding the regions occupied by myelin bundles). The values obtained from each lamina on the ipsilateral side were expressed as a percentage of those obtained from the corresponding lamina on the contralateral side. One-way ANOVA was used to determine whether there were any significant differences in these results between the CCI, SNT, sham-operated and naïve groups. Subsequently, Tukey's pairwise test was used post hoc to determine whether these differences were significant ($p<0.05$).

5.3 Results

5.3.1 Behavioural testing

All of the CCI animals displayed alterations in posture similar to those described in other studies that have used this model (Bennett and Xie, 1988; Antal et al., 1990). These animals avoided weight bearing on the affected side whilst at rest and whilst mobile. The affected foot was frequently held in an averted position with the toes plantar-flexed. Sham-operated rats had normal posture and gait. No autotomy or loss of body weight was observed in any of the animals used in this study. Results of the behavioural tests are shown in fig. 5.1.

Fig. 5.1 Withdrawal latencies of CCI and sham-operated animals pre- and post-surgery. The results of behavioural testing for thermal hyperalgesia on the sham-operated animals show that there was no significant change in the mean withdrawal latency of either paw after surgery, when compared to the values obtained before the procedure. In contrast, the mean withdrawal latency calculated for the ipsilateral paw of the CCI-operated rats was lower than that found before surgery, suggesting that the animals had developed signs of thermal hyperalgesia. Statistical analysis using one-tailed unpaired T-test showed that there was a significant difference ($p<0.05$) between the ipsilateral and contralateral sides in CCI animals at 1 day post-surgery. There was also a highly significant difference ($p<0.001$) between the sides in these animals on days 4, 6, 8, 11 and 14 after the procedure.


20
15
10
5
0
Withdrawal latency (sec)
contra
ipsi (sham)
contra (sham)
ipsi
-6
-3
-1
1
4
6
8
11
14
Pre-/post-operative days

5.3.2 Immunocytochemistry and image analysis

In sections from sham-operated and naïve animals, the pattern of GAD65 and GAD67 immunoreactivity closely resembled that which has been previously reported in the dorsal horn of normal rats (Feldblum et al., 1995; Mackie et al., 2003). In sections from animals with CCI (Fig. 5.2) and SNT (Fig. 5.3), immunostaining for GAD65 and GAD67 in the ipsilateral dorsal horn in lamina I-III appeared to be weaker than that found in these laminae on the contralateral side.

Quantitative investigations with image analysis showed that after CCI and SNT, the mean pixel luminance values corresponding to GAD65 calculated for laminae I, II and III on the ipsilateral side of the dorsal horn were slightly less than those calculated for the corresponding laminae of the same section on the contralateral side. This reduction varied from 10-16% (table 5.1). In contrast, the values calculated from the naïve or sham animals were similar on both sides of any given section for each individual lamina. However it only reached significance in one case for lamina II in the SNT model.

Quantitative investigations with image analysis showed that after CCI and SNT, the mean pixel luminance values corresponding to GAD67 calculated for laminae I, II and III on the ipsilateral side of the dorsal horn were less than those calculated for the corresponding laminae of the same section on the contralateral side. This reduction varied from 12-19% for CCI and 35-40% for SNT (table 5.2). In contrast, the values calculated from the naïve or sham animals were similar on both sides of any given section for each individual lamina. However it only reached significance in for lamina III in the CCI model and lamina I-III for the SNT model.

Fig. 5.2 Confocal images show GAD65 and GAD67 on the ipsi- and contra-lateral sides of a section from an animal that had undergone CCI.

The images are all taken from the same Vibratome section, and have had the same adjustments of brightness and contrast made. The strength of immunoreactivity can therefore be compared on both sides of the section for each of the GAD isoforms. Note that there appears to be a slight reduction in GAD65- and a larger reduction of GAD67-immunoreactivity on the ipsilateral side of the section. Images were taken from a single optical section. Scale bar = 100 μm

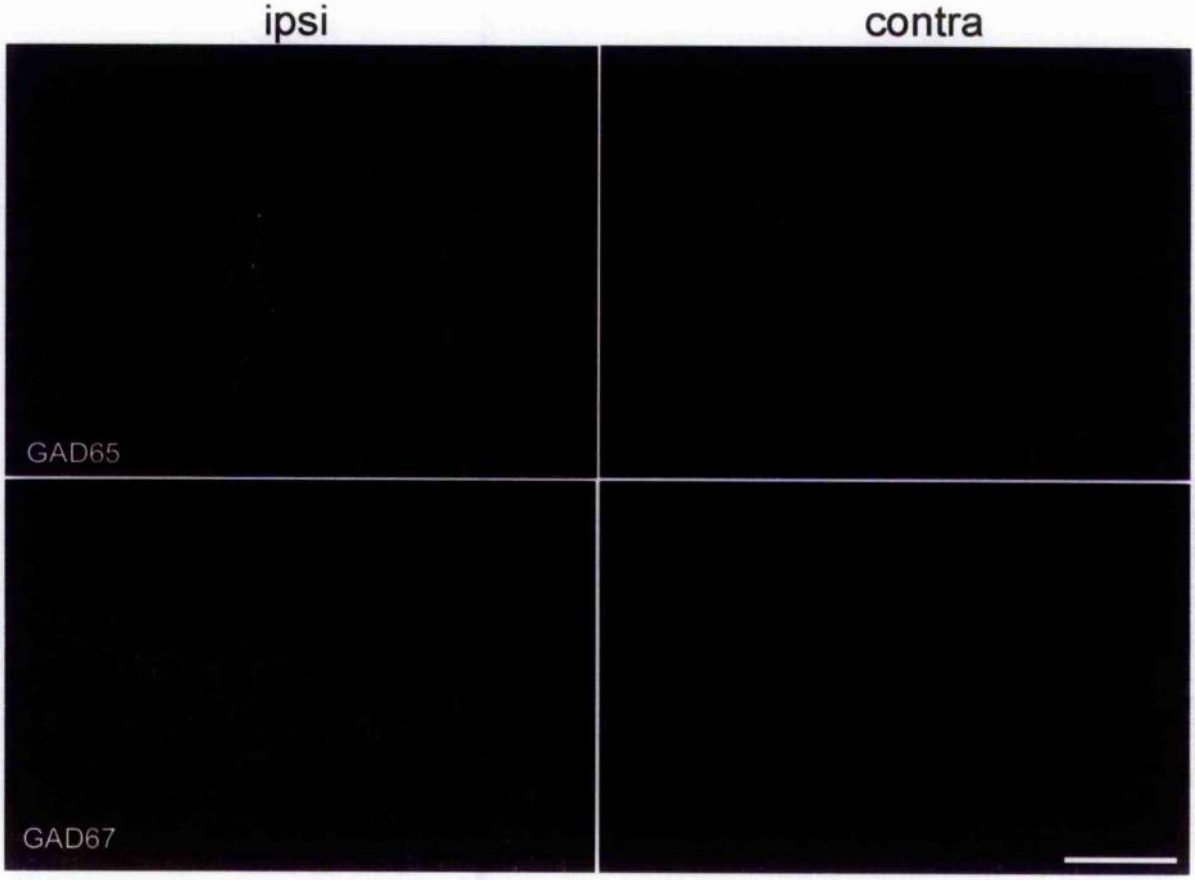
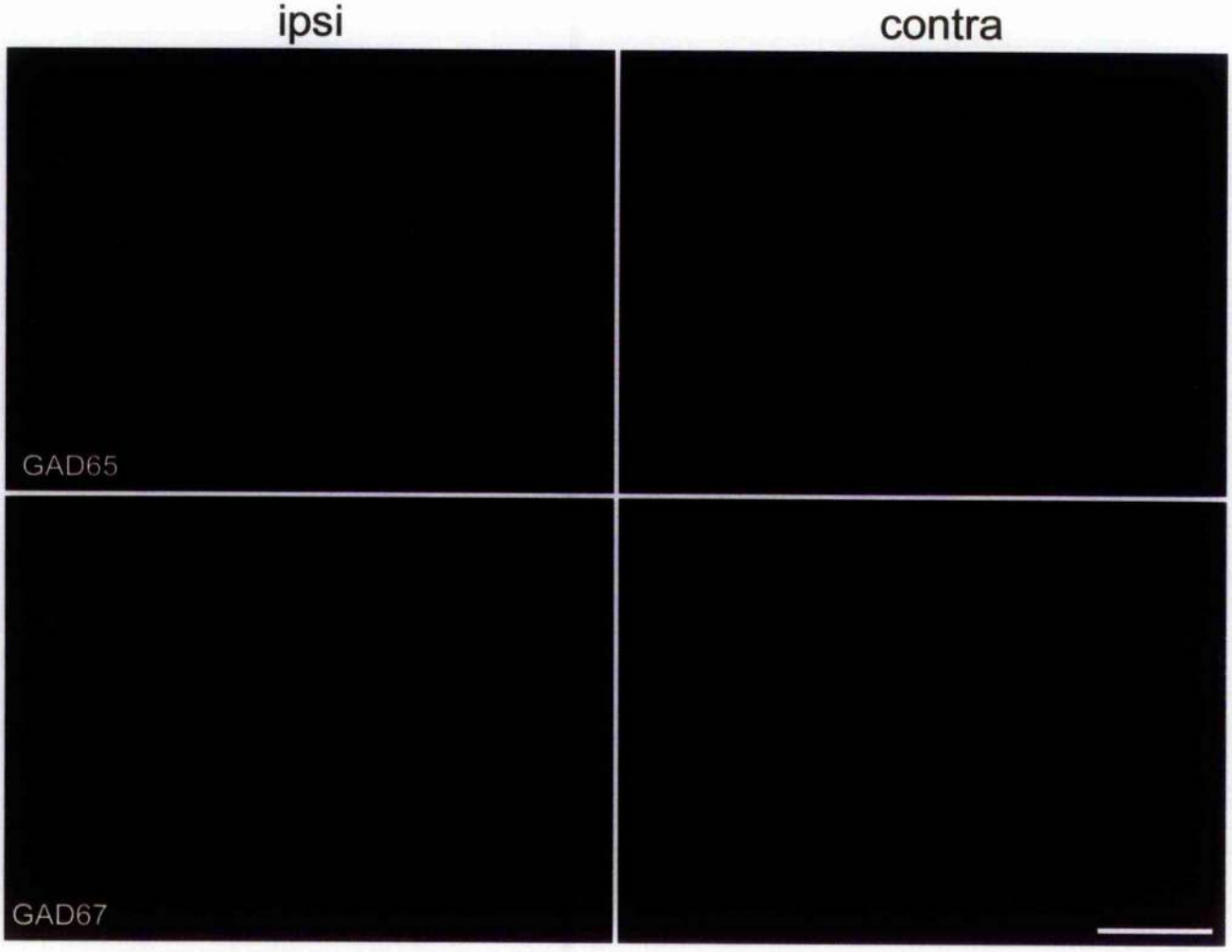

ipsi
contra
GAD65
GAD67

Fig. 5.3 Confocal images show GAD65 and GAD67 on the ipsi- and contralateral sides of a section from an animal that had undergone SNT.

The images are all taken from the same Vibratome section, and have had the same adjustments of brightness and contrast made. The strength of immunoreactivity can therefore be compared on both sides of the section for each of the GAD isoforms. Note that there appears to be a slight reduction in GAD65- and a larger reduction of GAD67-immunoreactivity on the ipsilateral side of the section. Images were taken from a single optical section. Scale bar = 100μm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GAD67

Table 5.1 Ipsilateral mean pixel luminance values for each lamina expressed as a percentage of the mean value found for the same lamina on the contralateral side (GAD65)

Procedure	Lamina I (GAD65)	Lamina II (GAD65)	Lamina III (GAD65)
Naïve	99.54 (88.9 – 105)	100.65 (90.7- 112.0)	100.44 (90.3 – 111.7)
Sham	99.73 (81.6 -115.3)	100.911 (91.9 -112.0)	97.83 (87.5 –115.6)
CCI	89.85 (81.1 – 96.1)	89.86 (80.8 – 96.0)	89.56 (81.0 – 100.0)
SNT	89.74 (78.8 – 107.2)	83.78 * (72.2 – 96.0)	85.72 (73.7 – 99.1)

- significant difference from values measured in sham-operated and naïve animals

The numbers in brackets represent the range of values calculated in the four animals from each model. Significant differences regarding GAD65 were found between the values calculated for lamina II in SNT animals and the values calculated for naïve and sham-operated animals in the same lamina. In contrast, no significant differences were detected between the values calculated for sham-operated animals when compared with values calculated for naïve animals in laminae I-III.

Table 5.2 Ipsilateral mean pixel luminance values for each lamina expressed as a percentage of the mean value found for the same lamina on the contralateral side (GAD67).

Procedure	Lamina I (GAD67)	Lamina II (GAD67)	Lamina III (GAD67)
Naïve	98.84 (86.1 – 112.6)	100.56 (90.4 – 110.8)	100.57 (93.2 – 108.1)
Sham	100.85 (90.9 – 116.2)	98.08 (94.2 – 106.3)	97.14 (86.9 – 107.9)
CCI	87.74 (75.8 – 95.6)	86.18 (73.8 – 98.1)	80.61 * (73.5 – 87.7)
SNT	64.67 * (39.4 – 75.4)	59.69 * (38.2 – 75.2)	58.45 * (37.5 – 75.3)

* significant difference from values measured in sham-operated and naïve animals

The numbers in brackets represent the range of values calculated in the four animals from each model. Significant differences were also found between the values calculated for GAD67 in lamina III of the CCI animals and the same lamina of the naïve, sham-operated and SNT animals. Finally, regarding GAD67, significant differences were found between the values calculated for laminae I, II and III of SNT animals when compared to those calculated in the corresponding laminae in all of the other groups of animals. In contrast, no significant differences were detected between values calculated for sham-operated animals when compared with values calculated for naïve animals.

5.4 Discussion

In this study, a reduction in immunoreactivity was detected for GAD65 and GAD67 in laminae I, II and III of the ipsilateral dorsal horn of rats after partial and complete nerve injury. No difference in staining was noted for GAD65 or GAD67 between the two sides of sections from sham-operated and naïve animals. This conclusion was made by comparing the mean pixel luminance value of the ipsilateral dorsal horn to that measured for the contralateral side of spinal cord sections from CCI and SNT animals. However, statistical analysis of the data calculated for GAD65 showed that this reduction only reached significance in one case for lamina II in the SNT model. Statistical analysis of the data calculated for GAD67 showed that this reduction only reached significance in for lamina III in the CCI model and lamina I-III for the SNT model. (Tables 5.1 and 5.2). The results from behavioural testing confirmed that the CCI animals exhibited signs of neuropathic pain. Sham-operated animals had similar withdrawal latencies before and after the procedure. This shows that the surgical manipulation of the skin and muscle that occurred when the animals underwent the procedure had no detectable effect on their behaviour post-surgery. In addition, immunocytochemistry showed that GAD levels on the ipsilateral sides of sections from these animals was similar to those found on the contralateral side after surgical intervention, which suggests that there has been no loss of GAD65 or GAD67.

5.4.1 Technical Considerations

The K2 antibody used to detect GAD67 immunoreactivity shows very weak cross-reactivity with GAD65 (Esclapez et al., 1994, Hughes et al., 2005) and therefore it is theoretically possible that K2 detected a decrease in GAD65 immunoreactivity rather than a decrease in GAD67 in this part of the study. However, in a study by Mackie et al. (2003) in which the K2 antibody was used to investigate GAD65 and GAD67 in axonal boutons, cross-reactivity of the K2 antibody with GAD65 was noted as minor (see chapter 3). Also, the reduction in GAD67 was

substantially more than the reduction in GAD65 detected for the SNT group. It is therefore unlikely that cross-reactivity of the K2 antibody with GAD65 would account for the reduction in GAD67 that was detected.

In this study, the ratio of the mean pixel luminance values of the ipsilateral and contralateral sides of each section were calculated and these values were used to establish if there was a reduction in GAD levels in the ipsilateral side (when compared to the contralateral side) after nerve injury. To eliminate any coincidental differences between the two sides of any section, scans were obtained from the same focal depth on either side of the section, and the same laser settings were used for both sides of each individual section. Also myelin bundles were excluded from the area which pixel luminance measurements were made from. This was done since the size and number of myelin bundles differed substantially between the two sides of any given section. Also, myelin bundles on the ipsilateral side of sections taken from animals with nerve injury, may have reduced in size as a result of the nerve injury, since they consist of myelinated primary afferents that pass ventrally from the dorsal columns. Including these may therefore have introduced a bias to measurements that were made. This method of analysis assumes that there is no change in GAD levels on the contralateral side after nerve injury. It is possible that there was an increase in GAD levels on the contralateral side to the injuries, rather than a reduction on the ipsilateral side, but this is not consistent with the results of the majority of previous studies that have been carried out. Also, when the staining for GAD65 and GAD67 on contralateral sides of operated animals was compared with that from sections of spinal cord from control animals, the level of staining appeared to be similar for each of the isoforms. Also, in agreement with this, many studies that have investigated changes in GABA or GAD levels after nerve injury have suggested that this is restricted to the ipsilateral side (Castro-Lopes et al., 1993; Satoh and Omote, 1996; Moore et al., 2002) or is more pronounced on this side (Ibuki et al., 1997, Eaton et al., 1998).

It became apparent late in the study that although the black level offset on the Lasersharp 2000 acquisition programme (Bio-Rad Cell Science Division, Hemel Hempstead, UK) used with the confocal microscope was set at zero, this was not reflected in the actual offset of the images provided. As a result, pixels with a low numerical value were assigned a value of zero. This occurred with all of the images captured using this confocal software, and so comparisons can still be made, although these comparisons are non-linear.

5.4.2 Studies of GABA and neuropathic pain

The present study describes a reduction in GAD levels in the spinal dorsal horn after partial peripheral nerve injury and complete nerve transection. Other studies have suggested that the density of GABAergic cells also decrease in this area after peripheral nerve injury (Castro-Lopes et al., 1993; Ibuki et al., 1997; Eaton et al., 1998). The majority of studies have examined changes in the dorsal horn after partial peripheral nerve injury, and two such studies by Ibuki et al., 1997 and Eaton et al., 1998 have described a dramatic bilateral decrease in GABAergic cell numbers after CCI, although this was more severe on the side ipsilateral to the nerve injury. Interestingly, the time course of this decline in numbers matched that of the behavioural signs detected, but the allodynia and hyperalgesia only affected the ipsilateral side (Ibuki et al., 1997; Eaton et al., 1998). The authors suggested that, although decreased GABA levels did contribute to the underlying mechanisms of neuropathic pain, there might have been other factors that also influenced the onset. As the loss of GABA-immunoreactivity showed some signs of recovery after a prolonged survival time in the study by Ibuki et al., it was suggested that at least some of the initial GABA loss after injury resulted from the down-regulation of GABA synthesis in the surviving neurons, and that normal synthesis was reinstated after a short period of time in these neurons (Ibuki et al., 1997). In contrast to these two reports, stereological studies by Polgar et al. in 2003 and 2004 suggested that there is no loss of GABAergic neurons after CCI in rats,

although animals developed behavioural signs of thermal hyperalgesia. This study used stereological analysis of semi-thin sections that were reacted with GABA and glycine antibodies. They found that there was no reduction in the percentage of neurons in lamina I, II or III that were immunoreactive with either antibody after CCI (Polgar et al., 2003). Subsequently, the same authors carried out an analysis of the packing density of neurons in laminae I-III with an antibody against NeuN (a marker for neurons) and the optical dissector method, and showed that there was no apparent reduction in neurons in laminae I-III after CCI. These combined results suggest that there is no loss of GABAergic neurons after CCI in rats.

It is probable that the dramatic loss of GABA-immunoreactivity described in the studies by Ibuki et al. (1997) and Eaton et al. (1998) is attributable to poor retention of GABA after fixation. Rapid fixation with glutaraldehyde-containing fixatives is generally required for immunocytochemical detection of GABA, as the two aldehyde groups can bind free amino acids to nearby proteins in the tissue, and therefore prevent their loss through diffusion, which is otherwise very rapid. The study by Ibuki et al. (1997) used formaldehyde rather than glutaraldehyde, and therefore the retention of GABA would be expected to be poor. The GABA immunostaining presented in unoperated animals in the Eaton study is much weaker than previously reported by other studies in the rat and in other animals (Magoul et al., 1987; Todd and Sullivan, 1990; Carlton and Hayes, 1990; Maxwell et al., 1991). The immunostaining in the study by Ibuki et al. (1997) and that of Eaton et al. (1998) was uneven, and therefore difficult to interpret. Also, a modest loss of GABA from these neurons may have been sufficient to take them below the detection threshold. This is unlikely to be the case in the study by Polgar et al., where glutaraldehyde fixation of tissue was used in conjunction with the more sensitive post-embedding method (Polgar et al., 2003; 2004). It is therefore likely that there is no significant loss of GABAergic neurons after peripheral nerve injury. In 2002, a study by Moore et al. stated that there was apoptosis of neurons after another type of nerve injury (SNI). Apoptosis was

demonstrated in this study by combining the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL; a marker of apoptosis) with immunostaining for NeuN. However, a recent study by Polgar et al. (2005) disagreed. In contrast, the study by Polgar et al. (2005) stereologically analysed neurons in laminae I-III of the rat dorsal horn. The authors also used the TUNEL-staining method and immunocytochemical detection of cleaved (activated) caspase-3 in spinal cord sections obtained from SNI animals. Four weeks post-surgery, there was no reduction in the density of neurons on the ipsilateral side of operated animals, compared to that found on the contralateral side, or in sham-operated or naïve rats. In addition, 1 week after the procedure, apoptotic neurons were not detected in the spinal dorsal horn, as virtually all of the TUNEL- positive cells were labelled with the antibody against Iba-1, a marker for microglia, while none were NeuN-positive. This suggested that the apoptosis involved microglia rather than neurons. As all SNI animals showed signs of tactile allodynia in the ipsilateral hind paw, the study concluded that neuronal apoptosis in the spinal dorsal horn is not essential for the development of neuropathic pain (Polgar et al., 2005).

In 1993, Castro-Lopes et al. described a decrease in GABA levels in the spinal dorsal horn after complete SNT. They found an ipsilateral decrease in GABAergic cell numbers and a reduction in the intensity of immunostaining in lamina II after SNT as early as two weeks post-injury, although this change was more pronounced after 3-4 weeks. GABA-immunoreactive cell numbers on the ipsilateral side were 93.7% of those found on the contralateral side 2 weeks after injury, 83.8% (at 3 weeks) and 72.5% (after 4 weeks) (Castro-Lopes et al., 1993). The authors believed that the reduction in GABA detected in their study was the result of diminished sensory input (after deafferentation). This reduction in GABA and inhibitory transmission detected after SNT also appears to be unrelated to cell loss, as a study by Coggeshall et al. (2001) and found that there was no reduction in cell numbers in laminae I-III after complete SNT used a stereological method for counting cells.

5.4.3 GAD65 and GAD67 levels in the dorsal horn of neuropathic rats

Moore et al. (2002) have investigated changes in GABAergic inhibition in the superficial dorsal horn of rats after CCI, SNI and SNT. Part of the study examined GAD65- and GAD67-immunoreactivity and protein levels in CCI and SNT models by using immunohistochemistry with the GAD6 and K2 antibodies, and Western blotting, respectively. Western blotting showed that dorsal horn levels of GAD65 protein decreased by 20-40% in a time dependent manner in the ipsilateral dorsal horn of CCI and SNI animals. Immunohistochemistry revealed a generalised reduction in GAD65 immunoreactivity in the ipsilateral dorsal horn (laminae I-IV) after CCI and SNI. The authors stated that GAD67 levels were generally unaffected by partial peripheral nerve injury, although some loss occurred after CCI. The present study used a quantitative approach with immunocytochemistry and image analysis to examine in detail any changes in GAD-immunoreactivity that occurred after partial peripheral nerve injury or complete SNT, in individual laminae of the rat spinal cord. In agreement with Moore et al. (2002), qualitative analysis showed a reduction in GAD65- and GAD67-immunoreactivity in all laminae of the ipsilateral dorsal horn in the CCI model when compared to that found on the contralateral side. Quantitative analysis however showed that there was a significant reduction in GAD65- and GAD67-immunoreactivity in lamina II of SNT animals. A decrease in GAD67-immunoreactivity was also detected in lamina III of CCI animals and laminae I and III of SNT animals. It is possible that the remaining results in this study would have been significant if the sample size had been increased. Moore et al. found a 20-40% reduction in GAD65 protein in laminae I-IV, whilst the current study found that there was a 10-20% reduction in GAD65-immunofluorescence in laminae I, II and III. The present study detected a change in GAD67-immunoreactivity relatively similar to the level of reduction found in GAD65-immunoreactivity, unlike in the study by Moore et al. (2002). This current study found that there was also a reduction in immunoreactivity for both GAD isoforms after SNT, and that the reduction in GAD67-immunoreactivity was more

pronounced than that calculated in sections from CCI animals (based on mean pixel luminance values). No data were presented from immunocytochemical or Western blot studies on GAD levels after SNT in the study by Moore et al. (2002).

Moore et al. (2002) also recorded evoked inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs, respectively) in lamina II neurons as part of their study. They found that although fast excitatory transmission remained intact after all three nerve injuries, $GABA_A$ receptor-mediated IPSCs were substantially reduced after both partial nerve injuries when compared with measurements made in naïve rats and animals that had undergone SNT. In addition, although most IPSCs recorded in naïve animals had both $GABA_A$ and glycine-mediated components, this was not the case after partial nerve injury. After CCI and SNI (but not SNT) the kinetics of the IPSCs shifted towards that which resembled purely glycinergic currents. Since, there was a reduction in $GABA_A$ receptor-mediated IPSCs in conjunction with reduced GAD65-immunoreactivity and protein levels in sections from CCI animals, the authors suggested that the reduction in GAD had resulted in reduced presynaptic GABA levels, that had effectively reduced inhibitory transmission. Our data however shows that there was also a reduction in GAD65- and GAD67- immunoreactivity on the ipsilateral sides of spinal cord sections from SNT animals, although Moore et al. did not find any reduction in $GABA_A$ receptor-mediated IPSCs in this model.

It is possible to conclude that a decrease in GAD65 and GAD67 in the spinal dorsal horn does not result in a reduction in GABA-mediated inhibitory transmission in neuropathic animals. Although behavioural signs of neuropathic pain occur in conjunction with decreased GAD65- and GAD67- immunoreactivity in the CCI model (this study) and a reduction in $GABA_A$ receptor-mediated IPSCs (Moore et al., 2002), decreased GAD65- and GAD67- immunoreactivity also occurs in the SNT animals (this study) without any reduction in $GABA_A$ receptor-mediated IPSCs (Moore et al., 2002).

General conclusions

Investigation 1.

It appears that there is a different pattern of GAD-immunoreactivity in the spinal cord, than in the brain. In several brain regions, GAD65-immunoreactivity is predominantly found in cell bodies, and GAD67-immunoreactivity is mainly found in axon terminals. In contrast, this study found no GAD65-immunoreactive cell bodies, and few GAD67-immunoreactive cells. Immunostaining for both GAD isoforms was found in axonal boutons in the dorsal and ventral horns of the rat spinal cord, and interestingly, a high proportion of boutons in the ventral horn had relatively higher levels of GAD67-immunoreactivity. Although all axonal boutons were immunoreactive for both GAD isoforms, the GAD67 antibody (K2) used in this part of the study (and in investigation 4) is reported to cross-react slightly with GAD65. After the completion of this part of the study, and investigation 4, another GAD67 antibody (monoclonal) became available commercially that shows no cross-reactivity with GAD65. This antibody was used for investigation 3. Part of this investigation compared the two GAD67 antibodies and showed that some of the boutons in the ventral horn that were GAD67-immunoreactive with the K2 antibody were not immunoreactive with the monoclonal antibody. This suggests that a small proportion of the GAD-immunoreactive terminals in the ventral horn do not express GAD67. These include the P boutons.

Investigation 3

The main finding of this part of the study was that the 'GAD65 intense' boutons described in investigation 1 are the P boutons. These boutons differ from the majority of GAD-immunoreactive boutons in the ventral horn as these have relatively high levels of GAD65 and relatively low levels of GAD67. The opposite is true of the majority of GAD-immunoreactive terminals in this region of the spinal cord (shown in investigation 1), and this suggests that these terminals belong to a different population of GABAergic neurons.

Investigation 4

This part of the study found that, after SNT, there was a significant reduction in GAD65-immunoreactivity in lamina II. Also, a significant decrease in GAD67-immunoreactivity was detected in lamina III of CCI animals and laminae I, II and III of SNT animals. The combined results of this part of the present study, and that of a study by Moore et al. (2002) suggest that a decrease in GAD65 and GAD67 in the spinal dorsal horn after nerve injury does not result in a reduction in GABA-mediated inhibitory transmission in neuropathic animals. There may therefore be a compensatory mechanism available that involves up-regulation of the unaffected GAD isoform. Perhaps this occurs in the axonal boutons in the dorsal horn that showed relatively equal levels of the two GAD isoforms (as described in investigation 1).

Reference List

Alvarez FJ, Kavookjian AM, Light AR (1992) Synaptic Interactions Between GABA-Immunoreactive Profiles and the Terminals of Functionally Defined Myelinated Nociceptors in the Monkey and Cat Spinal Cord. Journal of Neuroscience 12: 2901-2917.

Alvarez FJ, Dewey DE, Harrington DA, Fyffe RE (1997) Cell-type specific organization of glycine receptor clusters in the mammalian spinal cord. Journal of Comparative Neurology 379: 150-170.

Alvarez FJ, Jonas PC, Sapir T, Hartley R, Berrocal C, Geiman EJ, Todd AJ, Goulding M (2005) Postnatal Phenotype and Localization of Spinal Cord V1 Derived Interneurons. The Journal of Comparative Neurology 493: 177-192.

Andersen P, Eccles JC, Loyning Y, Voorhoeve PE (1963) Strychnine-Resistant Inhibition in the Brain. Nature 200: 843-845.

Antal M, Freund TF, Polgar E (1990) Calcium-binding proteins, parvalbumin- and calbindin-D 28k-immunoreactive neurons in the rat spinal cord and dorsal root ganglia: a light and electron microscopic study. Journal of Comparative Neurology 295: 467-484.

Antal M, Polgar E, Chalmers J, Minson JB, Llewellynsmith I, Heizmann CW, Somogyi P (1991) Different Populations of Parvalbumin-D28K-Immunoreactive and Calbindin-D28K-Immunoreactive Neurons Contain GABA and Accumulate H-3 D-Aspartate in the Dorsal Horn of the Rat Spinal Cord. Journal of Comparative Neurology 314: 114-124.

Antal M, Petko M, Polgar E, Heizmann CW, StormMathisen J (1996) Direct evidence of an extensive GABAergic innervation of the spinal dorsal horn by fibres descending from the rostral ventromedial medulla. Neuroscience 73: 509-518.

Apud JA, Tappaz ML, Celotti F, Negricesi P, Masotto C, Racagni G (1984) Biochemical and Immunochemical Studies on the GABAergic System in the Rat Fallopian Tube and Ovary. Journal of Neurochemistry 43: 120-125.

Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Ji FY, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K (1996) Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. Biochemical and Biophysical Research Communications 229: 891-895.

Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K (1997) Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67kDa isoform of glutamic acid decarboxylase. Proceedings of the National Academy of Sciences of the United States of America 94: 6496-6499.

Averill S, Mcmahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical Localization of TrkA Receptors in Chemically Identified Subgroups of Adult Rat Sensory Neurons. European Journal of Neuroscience 7: 1484-1494.

Bao L, Wang HF, Cai HJ, Tong YG, Jin SX, Lu YJ, 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. European Journal of Neuroscience 16: 175-185.

Barber RP, Vaughn JE, Saito K, Mclaughlin BJ, Roberts E (1978) GABAergic Terminals Are Presynaptic to Primary Afferent Terminals in Substantia Gelatinosa of Rat Spinal Cord. Brain Research 141: 35-55.

Barber RP, Vaughn JE, Roberts E (1982) The cytoarchitecture of GABAergic neurons in rat spinal cord. Brain Research 238: 305-328.

Battaglioli G, Martin DL (1990) Stimulation of Synaptosomal Gamma Aminobutyric Acid Synthesis by Glutamate and Glutamine. Journal of Neurochemistry 54: 1179-1187.

Beal JA, Cooper MH (1978) Neurons in Gelatinosal Complex (Lamina II and Lamina III) of Monkey (Macaca-Mulatta) - Golgi Study. Journal of Comparative Neurology 179: 89-122.

Beal JA, Nandia KN, Knight DS (1989) Characterization of long ascending tract projection neurons and non-tract neurons in the superficial dorsal horn (SDH). In Processing of sensory information in the Superficial Dorsal Horn of the Spinal Cord. Cervero F, Bennett GJ, Headley PM (eds.) pp.181-197. Plenum, New York.

Bennett DLH, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, Mcmahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. Journal of Neuroscience 18: 3059-3072.

Bennett GJ, Xie YK (1988) A Peripheral Mononeuropathy in Rat That Produces Disorders of Pain Sensation Like Those Seen in Man. Pain 33: 87-107.

Bennett GJ (1997) Neuropathic Pain: An Overview. In Borsook D (ed.). Molecular Neurobiology of Pain, Progress in Pain Research and Management, Vol. 9. IASP Press, Seattle. USA.

Bessou P, Perl ER, Schmitt LA (1969) Response of Cutaneous Sensory Units with Unmyelinated Fibers to Noxious Stimuli. Journal of Neurophysiology 32: 1025-1043.

Bohlhalter S, Weinmann O, Mohler H, Fritschy J-M (1996) Laminar Compartmentalization of $GABA_A$-Receptor Subtypes in the Spinal Cord I. An immunohistochemical study. The Journal of Neuroscience 16: 283-297.

Boue-Grabot E, Roudbaraki M, Bascles L, Tramu G, Bloch B, Garret M (1998) Expression of GABA receptor rho subunits in rat brain. Journal of Neurochemistry 70: 899-907.

Bowery NG (1993) $GABA_B$ receptor pharmacology. Annual Review of Pharmacological Toxicology 33: 109-147.

Bowker RM, Westlund KN, Sullivan MC, Coulter JD (1982) Organization of Descending Serotonergic Projections to the Spinal Cord. Progress in Brain Research 57: 239-265.

Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101: 435-445.

Broman J, Anderson S, Ottersen OP (1993) Enrichment of glutamate-like immunoreactivity in primary afferent terminals throughout the spinal cord dorsal horn. European Journal of Neuroscience 5: 1050-1061.

Broman J, Adahl F (1994) Evidence for Vesicular Storage of Glutamate in Primary Afferent Terminals. Neuroreport 5: 1801-1804.

Brown AG, Fyffe RE, Rose PK, Snow PJ (1981) Spinal cord collaterals from axons of type II slowly adapting units in the cat. Journal of Physiology 316: 469-480.

Brune W, Weber RG, Saul B, von Knebel DM, Grond-Ginsbach C, Kellerman K, Meinck HM, Becker CM (1996) A GLRA1 null mutation in recessive hyperekplexia challenges the functional role of glycine receptors. American journal of human genetics 58: 989-997.

Bu DF, Erlander MG, Hitz BC, Tillakaratne NJK, Kaufman DL, Wagnermcpherson CB, Evans GA, Tobin AJ (1992) Two Human Glutamate Decarboxylases, 65kDa GAD and 67kDa GAD, Are Each Encoded by A Single Gene. Proceedings of the National Academy of Sciences of the United States of America 89: 2115-2119.

Bu DF, Tobin AJ (1994) The Exon-Intron Organization of the Genes (GAD1 and GAD2) Encoding two Human Glutamate Decarboxylases (GAD (67) and GAD(65)) Suggests That They Derive from A Common Ancestral GAD. Genomics 21: 222-228.

Burgess PR, Perl ER (1967) Myelinated Afferent Fibres Responding Specifically to Noxious Stimulation of Skin. Journal of Physiology London 190: 541-&.

Burke RE, Walmsley B, Hodgson JA (1979) HRP anatomy of group Ia afferent contacts on alpha motoneurones. Brain Research 160: 347-352.

Carlton SM, Hayes ES (1990) Light microscopic and ultrastructural analysis of GABA-immunoreactive profiles in the monkey spinal cord. Journal of Comparative Neurology 300: 162-182.

Castrolopes JM, Tavares I, Coimbra A (1993) GABA Decreases in the Spinal Cord Dorsal Horn After Peripheral Neurectomy. Brain Research 620: 287-291.

Cervero F, Iggo A (1980) The Substantia Gelatinosa of the Spinal Cord - A Critical Review. Brain 103: 717-772.

Chang YC, Gottlieb DI (1988) Characterization of the Proteins Purified with Monoclonal-Antibodies to Glutamic Acid Decarboxylase. Journal of Neuroscience 8: 2123-2130.

Choi SY, Morales B, Lee HK, Kirkwood A (2002) Absence of long-term depression in the visual cortex of glutamic acid decarboxylase-65 knock-out mice. Journal of Neuroscience 22: 5271-5276.

Coggeshall RE, Lekan HA, White FA, Woolf CJ (2001) A-fiber sensory input induces neuronal cell death in the dorsal horn of the adult rat spinal cord. Journal of Comparative Neurology 435: 276-282.

Condie BG, Bain G, Gottlieb DI, Capecchi MR (1997) Cleft palate in mice with a targeted mutation in the gamma-aminobutyric acid-producing enzyme glutamic acid decarboxylase 67. Proceedings of the National Academy of Sciences of the United States of America 94: 11451-11455.

Conradi S (1969a) Ultrastructure and Distribution of Neuronal and Glial Elements on Motoneuron Surface in Lumbosacral Spinal Cord of Adult Cat. Acta Physiologica Scandinavica 5-49.

Conradi S (1969b) Ultrastructure and Distribution of Neuronal and Glial Elements on Surface of Proximal Part of Motoneuron Dendrite, As Analyzed by Serial Sections. Acta Physiologica Scandinavica 49-84.

Conradi S (1969c) Ultrastructure of Dorsal Root Boutons on Lumbosacral Motoneurons of Adult Cat, As Revealed by Dorsal Root Section. Acta Physiologica Scandinavica 85-&.

Conradi S, Cullheim S, Gollvik L, Kellerth JO (1983) Electron Microscopic Observations on the Synaptic Contacts of Group Ia Muscle Spindle Afferents in the Cat Lumbosacral Spinal Cord. Brain Research 265: 31-39.

Cortes R, Ceccatelli S, Schalling M, Hokfelt T (1990) Differential Effects of Intracerebroventricular Colchicine Administration on the Expression of Messenger RNAs for Neuropeptides and Neurotransmitter Enzymes, with Special Emphasis on Galanin - An In situ Hybridization Study. Synapse 6: 369-391.

Coull JAM, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424: 938-942.

Craig AD (1995) Distribution of Brain Stem Projections from Spinal Lamina I Neurons in the Cat and the Monkey. Journal of Comparative Neurology 361: 225-248.

Crawford JM, Curtis DRVoorhoeve PE, Wilson VJ (1963) Strychnine and Cortical Inhibition. Nature 200: 845-846.

Cullheim S, Kellerth JO (1981) Two Kinds of Recurrent Inhibition of Cat Spinal Alpha Motoneurones As Differentiated Pharmacologically. Journal of Physiology-London 312: 209-224.

Curtis DR, Hosli L, Johnston GA, Johnston IH (1968a) Hyperpolarization of Spinal Motoneurones by Glycine and Related Amino Acids. Experimental Brain Research 5: 235-258.

Curtis DR, Hosli L, Johnston GA (1968b) A pharmacological study of the depression of spinal neurones by glycine and related amino acids. Experimental Brain Research 6:1-18.

Curtis DR, Duggan AW, Johnston GA (1969) Glycine, Strychnine, Picrotoxin and Spinal Inhibition. Brain Research 14: 759-&.

Curtis DR, Duggan AW, Johnston GA (1971) Specificity of Strychnine As A Glycine Antagonist in Mammalian Spinal Cord. Experimental Brain Research 12: 547-557.

Curtis DR, Duggan AW, Felix D, Johnston GA (1971) Bicuculline, An Antagonist of GABA and Synaptic Inhibition in Spinal Cord of Cat. Brain Research 32: 69-&.

Curtis DR, Lodge D, Bornstein JC, Peet MJ (1981) Selective effects of (-)-baclofen on spinal synaptic transmission in the cat. Experimental Brain Research 42: 158-170.

Curtis DR, Gynther BD, Malik R (1986) A Pharmacological Study of Group I Muscle Afferent Terminals and Synaptic Excitation in the Intermediate Nucleus and Clarkes Column of the Cat Spinal Cord. Experimental Brain Research 64: 105-113.

d'Incamps BL, Destombes J, Thiesson D, Hellio R, Lasserre X, Kouchtir-Devanne N, Jami L, Zytnicki D (1998) Indications for GABA-immunoreactive axo-axonic contacts on the intraspinal arborization of a Ib fiber in cat: A confocal microscope study. Journal of Neuroscience 18: 10030-10036.

Danik M, Cassoly E, Manseau F, Sotty F, Mouginot D, Williams S (2005) Frequent Coexpression of the Vesicular Glutamte Transporter 1 and 2 Genes, As Well As Coexpression With Genes for Choline Acetyltransferase or Glutamic Acid Decarboxylase in Neurons of Rat Brain. Journal of Neuroscience 81:506-521.

De Marchis S, Temoney S, Erdelyi F, Bovetti S, Bovolin P, Szabo G, Puche AC (2004) GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors. European Journal of Neuroscience 20: 1307-1317.

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

Destombes J, HorcholleBossavit G, Simon M, Thiesson D (1996) GABA-like immunoreactive terminals on lumbar motoneurons of the adult cat. A quantitative ultrastructural study. Neuroscience Research 24: 123-130.

Devor M, Raber P (1990) Heritability of Symptoms in An Experimental Model of Neuropathic Pain. Pain 42: 51-67.

Diaz-Ruiz A, Vergara P, Perez-Severiano F, Segovia J, Guizar-Sahagun G, Ibarra A, Rios C (2005) Cyclosporin A inhibits constitutive nitric oxide synthase activity and neuronal and endothelial nitric oxide synthase expressions after spinal cord injury in rats. Neurochemical Research 30: 245-251.

Dolphin AC, Scott RH (1990) Activation of Calcium Channel Currents in Rat Sensory Neurons by Large Depolarizations: Effect of Guanine Nucleotides and (-)-Baclofen. European Journal of Neuroscience 2: 104-108.

Drew CA, Johnston GAR, Weatherby RP (1984) Bicuculline-Insensitive GABA Receptors - Studies on the Binding of (-)-Baclofen to Rat Cerebellar Membranes. Neuroscience Letters 52: 317-321.

Dumoulin A, Alonso G, Privat A, Feldblum S (1996) Biphasic response of spinal GABAergic neurons after a lumbar rhizotomy in the adult rat. European Journal of Neuroscience 8: 2553-2563.

Dumoulin A, Triller A, Dieudonne S (2001) IPSC kinetics at identified GABAergic and mixed GABAergic and glycinergic synapses onto cerebellar Golgi cells. Journal of Neuroscience 21: 6045-6057.

Dun NJ, Dun SL, Wu SY, Forstermann U, Schmidt HH, Tseng LF (1993) Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. Neuroscience 54: 845-857.

Durkin MM, Smith KE, Borden LA, Weinshank RL, Branchek TA, Gustafson EL (1995) Localization of Messenger-RNAs Encoding three GABA Transporters in Rat Brain - An In Situ Hybridization Study. Molecular Brain Research 33: 7-21.

Eaton MJ, Plunkett JA, Karmally S, Martinez MA, 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. Journal of Chemical Neuroanatomy 16: 57-72.

Eccles JC, Eccles RM, Magni F (1961) Central inhibitory action attributable to presynaptic depolarization produced by muscle afferent volleys. Journal of Physiology 159: 147-166.

Eccles JC, Schmidt RF, Willis WD (1962) Presynaptic inhibition of the spinal monosynaptic reflex pathway. Journal of Physiology 161: 282-297.

Eccles JC, Willis WD, Schmidt R (1963) Pharmacological Studies on Presynaptic Inhibition. Journal of Physiology London 168: 500-&.

Eccles RM, Lundberg A (1959) Supraspinal control of interneurones mediating spinal reflexes. Journal of Physiology 147: 565-584.

Edelhoff S, Grubin CE, Karlsen AE, Adler DA, Foster D, Disteche CM, Lernmark A (1993) Mapping of Glutamic Acid Decarboxylase (GAD) Genes. Genomics 17: 93-97.

Eguibar JR, Quevedo J, Jimenez I, Rudomin P (1994) Selective Cortical Control of Information Flow Through Different Intraspinal Collaterals of the Same Muscle Afferent Fiber. Brain Research 643: 328-333.

Enz R, Brandstatter JH, Hartveit E, Wassle H, Bormann J (1995) Expression of GABA Receptor Rho-1 and Rho-2 Subunits in the Retina and Brain of the Rat. European Journal of Neuroscience 7: 1495-1501.

Erlander MG, Tillakaratne NJK, Feldblum S, Patel N, Tobin AJ (1991) Two Genes Encode Distinct Glutamate Decarboxylases. Neuron 7: 91-100.

Erlander MG, Tobin AJ (1991) The Structural and Functional-Heterogeneity of Glutamic Acid Decarboxylase - A Review. Neurochemical Research 16: 215-226.

Esclapez M, Tillakaratne NJK, Tobin AJ, Houser CR (1993) Comparative Localization of Messenger-RNAs Encoding two Forms of Glutamic-Acid Decarboxylase with Nonradioactive Insitu Hybridization Methods. Journal of Comparative Neurology 331: 339-362.

Esclapez M, Tillakaratne NJK, Kaufman DL, Tobin AJ, Houser CR (1994) Comparative Localization of two Forms of Glutamic-Acid Decarboxylase and Their Messenger-RNAs in Rat Brain Supports the Concept of Functional Differences Between the Forms. Journal of Neuroscience 14: 1834-1855.

Feldblum S, Dumoulin A, Sandillon P, Privat A (1993) Plasticity of the Spinal GABAergic System in the Deafferented Rat Spinal Cord. Journal of Neurochemistry 61: S133.

Feldblum S, Dumoulin A, Anoal M, Sandillon F, Privat A (1995) Comparative distribution of GAD (65) and GAD(67) mRNAs and proteins in the rat spinal cord supports a differential regulation of these two glutamate decarboxylases in vivo. Journal of Neuroscience Research 42: 742-757.

Feldblum S, Anoal M, Lapsher S, Dumoulin A, Privat A (1998) Partial deafferentation of the developing rat spinal cord delays the spontaneous repression of GAD (67) mRNAs in spinal cells. Perspectives on Developmental Neurobiology 5: 131&.

Fleming A, Todd AJ (1994) Thyrotropin-Releasing Hormone-Like and GABA-Like Immunoreactivity Coexist in Neurons in the Dorsal Horn of the Rat Spinal Cord. Brain Research 638: 347-351.

Fonnum F (1968) Distribution of Glutamate Decarboxylase and Aspartate Transaminase in Subcellular Fractions of Rat and Guinea-Pig Brain. Biochemical Journal 106: 401-412.

Frank K, Fuortes MGF (1957) Presynaptic and Postsynaptic Inhibition of Monosynaptic Reflexes. Federation Proceedings 16: 39-40.

Franklin IK, Wollheim CB (2004) Commentary- GABA in the endocrine pancreas: Its putative role as an islet cell paracrine-signalling molecule. Journal of General Physiology 123: 185-190.

Fu TC, Schomburg ED (1974) Electrophysiological investigation of the projection of secondary muscle spindle afferents in the cat spinal cord. Acta Physiologica Scandinavica 91: 314-329.

Fyffe REW (1979) Morphology of Group-II Muscle Afferent Fiber Collaterals. Journal of Physiology London 296: 39-40.

Fyffe REW, Light AR (1984) The Ultrastructure of Group Ia Afferent Fiber Synapses in the Lumbosacral Spinal Cord of the Cat. Brain Research 300: 201-209.

Game CJA, Lodge D (1975) Pharmacology of Inhibition of Dorsal Horn Neurons by Impulses in Myelinated Cutaneous Afferents in Cat. Experimental Brain Research 23: 75-84.

Gaspary HL, Wang WG, Richerson GB (1998) Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. Journal of Neurophysiology 80: 270-281.

Geiman EJ, Zheng W, Fritschy JM, Alvarez FJ (2002) Glycine and GABA (A) receptor subunits on Renshaw cells: Relationship with presynaptic neurotransmitters and postsynaptic gephyrin clusters. Journal of Comparative Neurology 444: 275-289.

Gobel S (1978) Golgi Studies of Neurons in Layer 1 of Dorsal Horn of Medulla (Trigeminal-Nucleus-Caudalis). Journal of Comparative Neurology 180: 375-393.

Gracely RH, Lynch SA, Bennett GJ (1992) Painful Neuropathy- Altered Central Processing Maintained Dynamically by Peripheral Input. Pain 51: 175-194.

Gray EG (1962) A Morphological Basis for Presynaptic Inhibition. Nature 193: 82-&.

Grudt TJ, Perl ER (2002) Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. Journal of Physiology London 540: 189-207.

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.

Harkness DH, Brownfield MS (1986) A Thyrotropin-Releasing Hormone-Containing System in the Rat Dorsal Horn Separate from Serotonin. Brain Research 384: 323-333.

Harrison PJ, Jankowska E (1989) Primary Afferent Depolarization of Central Terminals of Group II Muscle Afferents in the Cat Spinal Cord. Journal of Physiology London 411: 71-83.

Hendrickson AE, Tillakaratne NJK, Mehra RD, Esclapez M, Erickson A, Vician L, Tobin AJ (1994) Differential Localization of two Glutamic-Acid Decarboxylases (GAD65 and GAD67) in Adult Monkey Visual Cortex. Journal of Comparative Neurology 343: 566-581.

Herbison AE, Simonian SX, Norris PJ, Emson PC (1996) Relationship of neuronal nitric oxide synthase immunoreactivity to GnRH neurons in the ovariectomized and intact female rat. Journal of Neuroendocrinology 8(1):73-82.

Hodgson AJ, Penke B, Erdei A, Chubb IW, Somogyi P (1985) Antisera to gamma-aminobutyric acid. I. Production and characterization using a new model system. Journal of Histochemical Cytochemistry 33: 229-239.

Holstege JC (1991) Ultrastructural Evidence for GABAergic Brain Stem Projections to Spinal Motoneurons in the Rat. Journal of Neuroscience 11: 159-167.

Hughes DI, Scott DT, Todd AJ, Riddell JS (2003) Lack of evidence for sprouting of Aβ afferents into the superficial laminae of the spinal cord dorsal horn after nerve section. Journal of Neuroscience 23: 9491-9499.

Hughes DI, Mackie M, Nagy GG, Riddell JS, Maxwell DJ, Szabo G, Erdelyi F, Veress G, Szucs P, Antal M, Todd AJ (2005) P boutons in lamina IX of the rodent spinal cord express high levels of glutamic acid decarboxylase-65 and originate from cells in deep medial dorsal horn. Proceedings of the National Academy of Sciences of the United States of America 102: 9038-9043.

Hunt CC, Kuffler SW (1951) Further Study of Efferent Small Nerve Fibres to Mammalian Muscle Spindles - Multiple Spindle Innervation and Activity During Contraction. Journal of Physiology London 113: 283-297.

Hunt CC, Kuffler SW (1951) Further study of efferent small nerve fibers to mammalian muscle spindles; multiple spindle innervation and activity during contraction. Journal of Physiology 113: 283-297.

Hunt SP, Kelly JS, Emson PC, Kimmel JR, Miller RJ, Wu JY (1981) An Immunohistochemical Study of Neuronal Populations Containing Neuropeptides Or Gamma-Aminobutyrate Within the Superficial Layers of the Rat Dorsal Horn. Neuroscience 6: 1883-1898.

Hwang JH, Yaksh TL (1997) The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. Pain 70: 15-22.

Hylden JLK, Hayashi H, Dubner R, Bennett GJ (1986) Physiology and Morphology of the Lamina I Spinomesencephalic Projection. Journal of Comparative Neurology 247: 505-515.

Ibuki T, Hama AT, Wang XT, Pappas GD, 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.

Ikegaki N, Saito N, Hashima M, Tanaka C (1994) Production of Specific Antibodies Against GABA Transporter Subtypes (GAT1, GAT2, GAT3) and Their Application to Immunocytochemistry. Molecular Brain Research 26: 47-54.

Jankowska E, Lindstrom S (1972) Morphology of interneurones mediating Ia reciprocal inhibition of motoneurones in the spinal cord of the cat. Journal of physiology226: 805-823.

Jankowska E, Mccrea D, Rudomin P, Sykova E (1981) Observations on Neuronal Pathways Subserving Primary Afferent Depolarization. Journal of Neurophysiology 46: 506-516.

Jankowska E, Riddell JS (1995) Interneurones mediating presynaptic inhibition of group II muscle afferents in the cat spinal cord. Journal of physiology 483 (Pt 2): 461-471.

Ji FY, Kanbara N, Obata K (1999) GABA and histogenesis in fetal and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase. Neuroscience Research 33: 187-194.

Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? Trends in Neurosciences 26: 696-705.

Johnston GA (1994) $GABA_C$ receptors. Progressions in Brain Research 100: 61-65.

Johnston GAR (1976) Physiologic pharmacology of GABA and its agonists in the vertebrate nervous system. In Roberts E, Chase TN, Towers DB (eds), GABA in Nervous System Function. Raven Press, New York, pp. 395-411.

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

Jursky F, Tamura S, Tamura A, Mandiyan S, Nelson H, Nelson N (1994) Structure, Function and Brain Localization of Neurotransmitter Transporters. Journal of Experimental Biology 196: 283-295.

Jursky F, Nelson N (1996) Developmental expression of GABA transporters GAT1 and GAT4 suggests involvement in brain maturation. Journal of Neurochemistry 67: 857-867.

Karlsten R, Gordh T (1997) How do drugs relieve neurogenic pain? Drugs & Aging 11: 398-412.

Kash SF, Johnson RS, Tecott LH, Noebels JL, Mayfield RD, Hanahan D, Baekkeskov S (1997) Epilepsy in mice deficient in the 65kDa isoform of glutamic acid decarboxylase. Proceedings of the National Academy of Sciences of the United States of America 94: 14060-14065.

Kaufman DL, Houser CR, Tobin AJ (1991) Two Forms of the Gamma-Aminobutyric-Acid Synthetic Enzyme Glutamate Decarboxylase Have Distinct Intraneuronal Distributions and Cofactor Interactions. Journal of Neurochemistry 56: 720-723.

Kellerth JO, Szumski AJ (1966) Effects of picrotoxin on stretch-activated postsynaptic inhibitions in spinal motoneurones. Acta Physiologica Scandinavica 66: 146-156.

Kellerth JO, Berthold CH, Conradi S (1979) Electron-Microscopic Studies of Serially Sectioned Cat Spinal Alpha-Motoneurons 3. Moto-Neurons Innervating Fast-Twitch (Type-Fr) Units of the Gastrocnemius-Muscle. Journal of Comparative Neurology 184: 755-767.

Kim SH, Chung JM (1992) An Experimental Model for Peripheral Neuropathy Produced by Segmental Spinal Nerve Ligation in the Rat. Pain 50: 355-363.

Kirkwood PA, Sears TA (1974) Monosynaptic excitation of motoneurones from secondary endings of muscle spindles. Nature 252: 243-244.

Kirsch J, Betz H (1993) Widespread Expression of Gephyrin, A Putative Glycine Receptor Tubulin Linker Protein, in Rat-Brain. Brain Research 621: 301-310.

Kiyama H, Emson PC (1991) Colchicine-Induced Expression of Proneurotensin Messenger RNA in Rat Striatum and Hypothalamus. Molecular Brain Research 9: 353-358.

Kleinbergerdoron N, Kanner BI (1994) Molecular Properties of GABA Transporters. Journal of Neurochemistry 63: S38.

Kontinen VK, Stanfa LC, Basu A, Dickenson AH (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.

Kvamme E, Torgner IA, Roberg B (1991) Evidence Indicating That Pig Renal Phosphate-Activated Glutaminase Has A Functionally Predominant External Localization in the Inner Mitochondrial-Membrane. Journal of Biological Chemistry 266: 13185-13192.

Laing I, Todd AJ, Heizmann CW, Schmidt HHHW (1994) Subpopulations of GABAergic Neurons in Laminae I-III of Rat Spinal Dorsal Horn Defined by Coexistence with Classical Transmitters, Peptides, Nitric-Oxide Synthase Or Parvalbumin. Neuroscience 61: 123-132.

LaMotte CC, Kapadia SE, Shapiro CM (1991) Central projections of the sciatic, saphenous, median, and ulnar nerves of the rat demonstrated by transganglionic transport of choleragenoid-HRP (B-HRP) and wheat germ agglutinin-HRP (WGA-HRP). Journal of Comparative Neurology 311: 546-562.

Lawson SN, Crepps BA, Perl ER (1997) Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. Journal of Physiology London 505: 177-191.

Levy RA (1977) Role of GABA in Primary Afferent Depolarization. Progress in Neurobiology 9: 211-267.

Lewis PR, Navaratnam V (1970) Histochemical demonstration of cholinergic elements in the ventral horn of the rat spinal cord. Journal of physiology210: 81P-82P.

Light AR, Perl ER (1979) Spinal Termination of Functionally Identified Primary Afferent Neurons with Slowly Conducting Myelinated Fibers. Journal of Comparative Neurology 186: 133-150.

Light AR, Trevino DL, Perl ER (1979) Morphological Features of Functionally Defined Neurons in the Marginal Zone and Substantia Gelatinosa of the Spinal Dorsal Horn. Journal of Comparative Neurology 186: 151-171.

Lima D, Coimbra A (1986) A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. Journal of Comparative Neurology 244: 53-71.

Ling LJ, Honda T, Shimada Y, Ozaki N, Shiraishi Y, Sugiura Y (2003) Central projection of unmyelinated (C) primary afferent fibers from gastrocnemius muscle in the guinea pig. Journal of Comparative Neurology 461: 140-150.

Liu CN, Wall PD, Ben Dor E, Michaelis M, Amir R, Devor M (2000) Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Llewellynsmith IJ, Minson JB (1992) Complete Penetration of Antibodies Into Vibratome Sections After Glutaraldehyde Fixation and Ethanol Treatment- Light and Electron-Microscopy for Neuropeptides. Journal of Histochemistry

Lloyd DPC (1943) Conduction and synaptic transmission of the reflex response to stretch in spinal cats. Journal of Neurophysiology 6: 317-326.

Lloyd DPC (1946) Facilitation and Inhibition of Spinal Motoneurons. Journal of Neurophysiology 9: 421-438.

Lynn B, Carpenter SE (1982) Primary Afferent Units from the Hairy Skin of the Rat Hindlimb. Brain Research 238: 29-43.

Mackie M, Hughes DI, Maxwell DJ, Tillakaratne NJK, Todd AJ (2003) Distribution and co-localisation of glutamate decarboxylase isoforms in the rat spinal cord. Neuroscience 119: 461-472.

Magoul R, Onteniente B, Geffard M, Calas A (1987) Anatomical Distribution and Ultrastructural Organization of the GABAergic System in the Rat Spinal Cord - An Immunocytochemical Study Using Anti-GABA Antibodies. Neuroscience 20: 1001-1009.

Malcangio M, Bowery NG (1996) GABA and its receptors in the spinal cord. Trends in Pharmacological Science 17: 457-462.

Marti E, Bumcrot DA, Takada R, Mcmahon AP (1995) Requirement of 19K Form of Sonic Hedgehog for Induction of Distinct Ventral Cell Types in CNS Explants. Nature 375: 322-325.

Martin DL, Rimvall K (1993) Regulation of Gamma-Aminobutyric-Acid Synthesis in the Brain. Journal of Neurochemistry 60: 395-407.

Matsushita M (1969) Some aspects of interneuronal connections cat's spinal gray matter. Journal of Comparative Neurology 136: 57-80.

Matthews PB (1963) The response of de-efferented muscle spindle receptors to stretching at different velocities. Journal of Physiology168: 660-678.

Maxwell DJ, Noble R (1987) Relationships Between Hair Follicle Afferent Terminations and Glutamic Acid Decarboxylase-Containing Boutons in the Cats Spinal Cord. Brain Research 408: 308-312.

Maxwell DJ, Christie WM, Short AD, Brown AG (1990) Direct Observations of Synapses Between GABA-Immunoreactive Boutons and Muscle Afferent Terminals in Lamina VI of the Cats Spinal Cord. Brain Research 530: 215-222.

Maxwell DJ, Christie WM, Short AD, Brown AG (1991) Direct observations of synapses between GABA-immunoreactive boutons and identified spinocervical tract neurons in the cat's spinal cord. Journal of Comparative Neurology 307: 375-392.

Maxwell DJ, Riddell JS (1999) Axoaxonic synapses on terminals of group II muscle spindle afferent axons in the spinal cord of the cat. European Journal of Neuroscience 11: 2151-2159.

Maxwell L, Maxwell DJ, Neilson M, Kerr R (1996) A confocal microscopic survey of serotoninergic axons in the lumbar spinal cord of the rat: Co-localization with glutamate decarboxylase and neuropeptides. Neuroscience 75: 471-480.

Mclaughlin BJ (1972) The fine structure of neurons and synapses in the motor nuclei of the cat spinal cord. Journal of Comparative Neurology 144: 429-460.

Mclaughlin BJ, Barber R, Saito K, Roberts E, Wu JY (1975) Immunocytochemical Localization of Glutamate Decarboxylase in Rat Spinal Cord. Journal of Comparative Neurology 164: 305-321.

Meeley MP, Martin DL (1983) Inactivation of Brain Glutamate Decarboxylase and the Effects of Adenosine 5'-Triphosphate and Inorganic-Phosphate. Cellular and Molecular Neurobiology 3: 39-54.

Melzack R, Wall PD (1965) Pain Mechanisms - A New Theory. Science 150: 971-&.

Mendell LM, Henneman E (1971) Terminals of single Ia fibers: location, density, and distribution within a pool of 300 homonymous motoneurons. Journal of Neurophysiology 34: 171-187.

Miller LP, Walters JR, Martin DL (1977) Postmortem Changes Implicate Adenine Nucleotides and Pyridoxal-5'-Phosphate in Regulation of Brain Glutamate Decarboxylase. Nature 266: 847-848.

Miller LP, Walters JR (1979) Effects of Depolarization on Cofactor Regulation of Glutamic Acid Decarboxylase in Substantia Nigra Synaptosomes. Journal of Neurochemistry 33: 533-539.

Molander C, Xu Q, Grant G (1984) The Cytoarchitectonic Organization of the Spinal Cord in the Rat 1. The Lower Thoracic and Lumbosacral Cord. Journal of Comparative Neurology 230: 133-141.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. Journal of Neuroscience 22: 6724-6731.

Mulder AH, Snyder SH (1974) Potassium-Induced Release of Amino Acids from Cerebral Cortex and Spinal Cord Slices of Rat. Brain Research 76: 297-308.

Naim M, Spike RC, Watt C, Shehab SAS, Todd AJ (1997) Cells in laminae III and IV of the Rt Spinal Cord that Possess the Neurokinin-1 Receptor and Have Dorsally Directed Dendrites Receive a Major Synaptic Input from Tachykinin-Containing Primary Afferents. The Journal of Neuroscience 17: 5536-5548.

Neal MJ, Pickles HG (1969) Uptake of 14C glycine by spinal cord. Nature 222: 679-680.

Nusser Z, Roberts JDB, Baude A, Richards JG, Sieghart W, Somogyi P (1995) Immunocytochemical Localization of the Alpha-1 and Beta-2/3 Subunits of the GABA(A) Receptor in Relation to Specific GABAergic Synapses in the Dentate Gyrus. European Journal of Neuroscience 7: 630-646.

O'Brien JA, Berger AJ (1999) Cotransmission of GABA and glycine to brain stem motoneurons. Journal of Neurophysiology 82: 1638-1641.

Obrien C, Woolf CJ, Fitzgerald M, Lindsay RM, Molander C (1989) Differences in the Chemical Expression of Rat Primary Afferent Neurons Which Innervate Skin, Muscle Or Joint. Neuroscience 32: 493-502.

Ochoa J, Torebjork HE, Culp WJ, Schady W (1982) Abnormal spontaneous activity in single sensory nerve fibers in humans. Muscle Nerve 5: S74-S77.

Ogurusu T, Taira H, Shingai R (1995) Identification of GABA(A) Receptor Subunits in Rat Retina- Cloning of the Rat GABA(A) Receptor Rho(2)-Subunit cDNA. Journal of Neurochemistry 65: 964-968.

Ogurusu T, Eguchi G, Shingai R (1997) Localization of gamma-aminobutyric acid (GABA) receptor rho 3 subunit in rat retina. Neuroreport 8: 925-927.

Örnung G, Shupliakov O, Linda H, Ottersen OP, StormMathisen J, Ulfhake B, Cullheim S (1996) Qualitative and quantitative analysis of glycine- and GABA-immunoreactive nerve terminals on motoneuron cell bodies in the cat spinal cord: A postembedding electron microscopic study. Journal of Comparative Neurology 365: 413-426.

Otsuka M, Iversen LL, Hall ZW, Kravitz EA (1966) Release of Gamma-Aminobutyric Acid from Inhibitory Nerves of Lobster. Proceedings of the National Academy of Sciences of the United States of America 56: 1110-1115.

Ottersen OP, StormMathisen J (1987) Distribution of Inhibitory Amino Acid Neurons in the Cerebellum with Some Observations on the Spinal Cord - An Immunocytochemical Study with Antisera Against Fixed GABA, Glycine, Taurine, and Beta-Alanine. Journal of Mind and Behavior 8: 503-518.

Paxinos G, Watson C (1986) The rat brain in stereotaxic Coordinates. 2nd edn. Academic. London.

Persson H, Pelto-Huikko M, Metsis M, Soder O, Brene S, Skog S, Hokfelt T, Ritzen EM (1990) Expression of the neurotransmitter-synthesizing enzyme glutamic acid decarboxylase in male germ cells. Molecular Cell Biology 10: 4701-4711.

Pierce JP, Mendell LM (1993) Quantitative Ultrastructure of Ia Boutons in the Ventral Horn - Scaling and Positional Relationships. Journal of Neuroscience 13: 4748-4763.

Polc P, Mohler H, Haefely W (1974) Effect of Diazepam on Spinal Cord Activities - Possible Sites and Mechanisms of Action. Naunyn-Schmiedebergs Archives of Pharmacology 284: 319-337.

Polgar E, Shehab SAS, Watt C, Todd AJ (1999) GABAergic neurons that contain neuropeptide Y selectively target cells with the neurokinin 1 receptor in laminae III and IV of the rat spinal cord. Journal of Neuroscience 19: 2637-2646.

Polgar E, Hughes DI, Riddell JS, Maxwell DJ, Puskar Z, Todd AJ (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.

Polgar E, Gray S, Riddell JS, Todd AJ (2004) Lack of evidence for significant neuronal loss in laminae I-III of the spinal dorsal horn of the rat in the chronic constriction injury model. Pain 111: 144-150.

Polgar E, Hughes DI, Arham AZ, Todd AJ (2005) Loss of neurons from laminae I-III of the spinal dorsal horn is not required for development of tactile allodynia in the spared nerve injury model of neuropathic pain. Journal of Neuroscience 25: 6658-6666.

Price DD, Hayashi H, Dubner R, Ruda MA (1979) Functional Relationships Between Neurons of Marginal and Substantia Gelatinosa Layers of Primate Dorsal Horn. Journal of Neurophysiology 42: 1590-1608.

Price GW, Wilkin GP, Turnbull MJ Bowery NG (1984) Are baclofen-sensitive $GABA_B$ Receptors present on primary afferent terminals of the spinal cord? Nature 307: 71-74.

Price TJ, Carvero F, de Koninck Y (2005) Role of Cation-Chloride-Cotransporters (CCC) in Pain and Hyperalgesia. Current Top Medical Chemistry 5:547-555.

Proudlock F, Spike RC, Todd AJ (1993) Immunocytochemical Study of Somatostatin, Neurotensin, GABA, and Glycine in Rat Spinal Dorsal Horn. Journal of Comparative Neurology 327: 289-297.

Puskar Z, Polgar E, Todd AJ (2001) A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord. Neuroscience 102: 167-176.

Radian R, Ottersen OP, StormMathisen J, Castel M, Kanner BI (1990) Immunocytochemical Localization of the GABA Transporter in Rat Brain. Journal of Neuroscience 10: 1319-1330.

Ralston HJ (1965) Structure of Substantia Gelatinosa of Cat Spinal Cord. Journal of Physiology London 179: 22-&.

Ralston HJ, Ralston DD (1979) Identification of Dorsal Root Synaptic Terminals on Monkey Ventral Horn Cells by Electron Microscopic Autoradiography. Journal of Neurocytology 8: 151-166.

Renshaw B (1940) Activity in the simplest spinal reflex pathways. Journal of Neurophysiology 3: 373-387.

Rethelyi M, Light AR, Perl ER (1982) Synaptic Complexes Formed by Functionally Defined Primary Afferent Units with Fine Myelinated Fibers. Journal of Comparative Neurology 207: 381-393.

Rexed B (1952) The Cytoarchitectonic Organization of the Spinal Cord in the Cat. Journal of Comparative Neurology 96: 415-&.

Rexed B (1954) A Cytoarchitectonic Atlas of the Spinal Cord in the Cat. Journal of Comparative Neurology 100: 297-379.

Ribeiro-da-Silva A, Coimbra A (1982) Two Types of Synaptic Glomeruli and Their Distribution in Laminae I-III of the Rat Spinal Cord. Journal of Comparative Neurology 209: 176-186.

Ribeiro-da-Silva A, Castrolopes JM, Coimbra A (1986) Distribution of Glomeruli with Fluoride-Resistant Acid-Phosphatase (FRAP)-Containing Terminals in the Substantia Gelatinosa of the Rat. Brain Research 377: 323-329.

Rimvall K, Martin DL (1992) Increased Intracellular Gamma-Aminobutyric-Acid Selectively Lowers the Level of the Larger of two Glutamate Decarboxylase Proteins in Cultured GABAergic Neurons from Rat Cerebral Cortex. Journal of Neurochemistry 58: 158-166.

Rimvall K, Sheikh SN, Martin DL (1993) Effects of Increased Gamma-Aminobutyric-Acid Levels on GAD67 Protein and Messenger-RNA Levels in Rat Cerebral Cortex. Journal of Neurochemistry 60: 714-720.

Rimvall K, Martin DL (1994) The Level of GAD(67) Protein Is Highly Sensitive to Small Increases in Intraneuronal Gamma-Aminobutyric-Acid Levels. Journal of Neurochemistry 62: 1375-1381.

Roberts E, Frankel S (1950) Gamma-Aminobutyric Acid in Brain - Its Formation from Glutamic Acid. Journal of Biological Chemistry 187: 55-63.

Roberts E, Frankel S (1951) Glutamic Acid Decarboxylase in Brain. Federation Proceedings 10: 237.

Roberts E, Bregoff HM (1953) Transamination of Gamma-Aminobutyric Acid and Beta-Alanine in Brain and Liver. Journal of Biological Chemistry 201: 393-398.

Roberts PJ, Gupta HK, Shargill NS (1978) The interaction of baclofen (beta-(4-chlorophenyl)GABA) with GABA systems in rat brain: evidence for a releasing action. Brain Research 155: 209-212.

Robertson B, Grant G (1989) Immunocytochemical evidence for the localization of the GM1 ganglioside in carbonic anhydrase-containing and RT 97-immunoreactive rat primary sensory neurons. Journal of Neurocytology 18: 77-86.

Rowan S, Todd AJ, Spike RC (1993) Evidence That Neuropeptide-Y Is Present in GABAergic Neurons in the Superficial Dorsal Horn of the Rat Spinal Cord. Neuroscience 53: 537-545.

Rozzo A, Armellin M, Franzot J, Chiaruttini C, Nistri A, Tongiorgi E (2002) Expression and dendritic mRNA localization of GABA(C) receptor rho 1 and rho 2 subunits in developing rat brain and spinal cord. European Journal of Neuroscience 15: 1747-1758.

Rudomin P (1990) Presynaptic Inhibition of Muscle-Spindle and Tendon Organ Afferents in the Mammalian Spinal Cord. Trends in Neurosciences 13: 499-505.

Rudomin P, Jimenez I, Quevedo J, Solodkin M (1990) Pharmacologic analysis of inhibition produced by last order intermediate nucleus interneurons mediating nonreciprocal inhibition of motoneurons in cat spinal cord. Journal of Neurophysiology 63: 147-160.

Rudomin P, Schmidt RF (1999) Presynaptic inhibition in the vertebrate spinal cord revisited. Experimental Brain Research 129: 1-37.

Sakatini K, Chesler M, Hassan AZ (1991) GABA-sensitivity of dorsal horn axons: an in vitro comparison between adult and neonatal rat spinal cords. Brain Research 542: 273-279.

Salganicoff. L, Derobert. ED (1965) Subcellular Distribution of Enzymes of Glutamic Acid Glutamine and Gamma-Aminobutyric Acid Cycles in Rat Brain. Journal of Neurochemistry 12: 287-&.

Sapir T, Geiman EJ, Wang Z, Velasquez T, Mitsui S, Yoshihara Y, Frank E, Alvarez FJ, Goulding M (2004) Pax6 and Engrailed 1 regulate two distinct aspects of Renshaw cell development. Journal of Neuroscience 24: 1255-1264.

Sapir T, Geiman EJ, Wang Z, Velasquez T, Mitsui S, Yoshihara Y, Frank E, Alvarez FJ, Goulding M (2004) Pax6 and engrailed 1 regulate two distinct aspects of renshaw cell development. Journal of Neuroscience 24: 1255-1264.

Satoh O, Omote K (1996) Roles of monoaminergic, glycinergic and GABAergic inhibitory systems in the spinal cord in rats with peripheral mononeuropathy. Brain Research 728: 27-36.

Schneider SP (1992) Functional properties and axon terminations of interneurons in laminae III-V of the mammalian spinal dorsal horn in vitro. Journal of Neurophysiology 68: 1746-1759.

Schneider SP, Fyffe REW (1992) Involvement of GABA and Glycine in Recurrent Inhibition of Spinal Motoneurons. Journal of Neurophysiology 68: 397-406.

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.

Shank RP, Aprison MH (1970) Metabolism In Vivo of Glycine and Serine in Eight Areas of Rat Central Nervous System. Journal of Neurochemistry 17: 1461-&.

Shehab SAS, Spike RC, Todd AJ (2004) Do central terminals of intact myelinated primary afferents sprout into the superficial dorsal horn of rat spinal cord after injury to a neighboring peripheral nerve? Journal of Comparative Neurology 474: 427-437.

Simmons DR, Spike RC, Todd AJ (1995) Galanin Is Contained in GABAergic Neurons in the Rat Spinal Dorsal Horn. Neuroscience Letters 187: 119-122.

Sivilotti L, Woolf CJ (1994) The Contribution of GABA(A) and Glycine Receptors to Central Sensitization - Disinhibition and Touch-Evoked Allodynia in the Spinal Cord. Journal of Neurophysiology 72: 169-179.

Sloviter RS, Dichter MA, Rachinsky TL, Dean E, Goodman JH, Sollas AL, Martin DL (1996) Basal expression and induction of glutamate decarboxylase and GABA in excitatory granule cells of the rat and monkey hippocampal dentate gyrus. Journal of Comparative Neurology 373: 593-618.

Soghomonian JJ, Chesselet MF (1992) Effects of Nigrostriatal Lesions on the Levels of Messenger-RNAs Encoding two Isoforms of Glutamate Decarboxylase in the Globus Pallidus and Entopeduncular Nucleus of the Rat. Synapse 11: 124-133.

Soghomonian JJ, Martin DL (1998) Two isoforms of glutamate decarboxylase: why? Trends in Pharmacological Sciences 19: 500-505.

Solimena M, Decamilli P (1991) Autoimmunity to Glutamic-Acid Decarboxylase (GAD) in Stiff Man Syndrome and Insulin-Dependent Diabetes Mellitus. Trends in Neurosciences 14: 452-457.

Somers DL, Clemente FR (2002) Dorsal horn synaptosomal content of aspartate, glutamate, glycine and GABA are differentially altered following chronic constriction injury to the rat sciatic nerve. Neuroscience Letters 323: 171-174.

Somogyi P, Hodgson AJ, Chubb IW, Penke B, Erdei A (1985) Antisera to Gamma-Aminobutyric Acid .2. Immunocytochemical Application to the Central Nervous System. Journal of Histochemistry and Cytochemistry 33: 240-248.

Somogyi J (2006) Functional significance of co-localisation of GABA and gluttamate in nerve terminals: a hypothesis. Current topics in Medical Chemistry 6: 969-973.

Spike RC, Todd AJ (1992) Ultrastructural and Immunocytochemical Study of Lamina II Islet Cells in Rat Spinal Dorsal Horn. Journal of Comparative Neurology 323: 359-369.

Spike RC, Todd AJ, Johnston HM (1993) Coexistence of NADPH- Diaphorase with GABA, Glycine, and Acetylcholine in Rat Spinal Cord. Journal of Comparative Neurology 335: 320-333.

Spike RC, Watt C, Zafra F, Todd AJ (1997) An ultrastructural study of the glycine transporter GLYT2 and its association with glycine in the superficial laminae of the rat spinal dorsal horn. Neuroscience 77: 543-551.

StormMathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug FMS, Ottersen OP (1983) First Visualization of Glutamate and GABA in Neurons by Immunocytochemistry. Nature 301: 517-520.

Stuart GJ, Redman SJ (1992) The Role of GABA-A and GABA-B Receptors in Presynaptic Inhibition of Ia EPSPs in Cat Spinal Motoneurons. Journal of Physiology London 447: 675-692.

Sugimoto T, Bennett GJ, Kajander KC (1990) Transsynaptic Degeneration in the Superficial Dorsal Horn After Sciatic Nerve Injury - Effects of A Chronic Constriction Injury, Transection, and Strychnine. Pain 42: 205-213.

Sugiura Y, Lee CL, Perl ER (1986) Central Projections of Identified, Unmyelinated (C) Afferent Fibers Innervating Mammalian Skin. Science 234: 358-361.

Sutherland FI, Bannatyne BA, Kerr R, Riddell JS, Maxwell DJ (2002) Inhibitory amino acid transmitters associated with axons in presynaptic apposition to cutaneous primary afferent axons in the cat spinal cord. Journal of Comparative Neurology 452: 154-162.

Taal W, Holstege JC (1994) GABA and Glycine Frequently Colocalize in Terminals on Cat Spinal Motoneurons. Neuroreport 5: 2225-2228.

Tal M, Wall PD, Devor M (1999) Myelinated afferent fiber types that become spontaneously active and mechanosensitive following nerve transection in the rat. Brain Research 824: 218-223.

Tillakaratne NJK, Mouria M, Ziv NB, Roy RR, Edgerton VR, Tobin AJ (2000) Increased expression of glutamate decarboxylase (GAD(67)) in feline lumbar spinal cord after complete thoracic spinal cord transection. Journal of Neuroscience Research 60: 219-230.

Todd AJ, Lewis SG (1986) The Morphology of Golgi-Stained Neurons in Lamina II of the Rat Spinal Cord. Journal of Anatomy 149: 113-119.

Todd AJ, Mckenzie J (1989) GABA-immunoreactive Neurons in the Dorsal Horn of the Rat Spinal Cord. Neuroscience 31: 799-806.

Todd AJ, Sullivan AC (1990) Light Microscope Study of the Coexistence of GABA-Like and Glycine-Like Immunoreactivities in the Spinal Cord of the Rat. Journal of Comparative Neurology 296: 496-505.

Todd AJ (1991) Immunohistochemical Evidence That Acetylcholine and Glycine Exist in Different Populations of GABAergic Neurons in Lamina III of Rat Spinal Dorsal Horn. Neuroscience 44: 741-746.

Todd AJ, Spike RC, Russell G, Johnston HM (1992) Immunohistochemical Evidence That Met-Enkephalin and GABA Coexist in Some Neurons in Rat Dorsal Horn. Brain Research 584: 149-156.

Todd AJ, Spike RC (1993) The Localization of Classical Transmitters and Neuropeptides Within Neurons in Laminae I-III of the Mammalian Spinal Dorsal Horn. Progress in Neurobiology 41: 609-645.

Todd AJ, Spike RC, Chong D, Neilson M (1995) The Relationship Between Glycine and Gephyrin in Synapses of the Rat Spinal Cord. European Journal of Neuroscience 7: 1-11.

Todd AJ (1996) GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. European Journal of Neuroscience 8: 2492-2498.

Todd AJ, Watt C, Spike RC, Sieghart W (1996) Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. Journal of Neuroscience 16: 974-982.

Todd, AJ (1997) A method for combining confocal and electron microscopic examination of sections processed for double- or triple-labelling immunocytochemistry. Journal of neuroscience 73: 149-157.

Todd AJ, Spike RC, Polgar E (1998) A quantitative study of neurons which express neurokinin-1 or somatostatin sst(2a) receptor in rat spinal dorsal horn. Neuroscience 85: 459-473.

Todd AJ, Puskar Z, Spike RC, Hughes C, Watt C, Forrest L (2002) Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance P-containing afferents and respond to noxious stimulation. Journal of Neuroscience 22: 4103-4113.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ (2003) The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. European Journal of Neuroscience 17: 13-27.

Todd AJ, Koerber HR (2005) Neuroanatomical substrates of spinal nociception. In Wall and Melzack's Textbook of Pain. 5th Ed. McMahon SB, Koltzenburg M (eds.). Churchill Livingstone, Edinburgh. Pp.73-90.

Tong YG, Wang HF, Ju G, Grant G, Hokfelt T, Zhang X (1999) Increased uptake and transport of cholera toxin B subunit in dorsal root ganglion neurons after peripheral axotomy: Possible implications for sensory sprouting. Journal of Comparative Neurology 404: 143-158.

Towers S, Princivalle A, Billinton A, Edmunds BB, Urban L, Castro-Lopes J, Bowery NG (2000) European Journal of Neuroscience 12:3201-3210.

Tuchscherer MM, Seybold VS (1989) A Quantitative Study of the Coexistence of Peptides in Varicosities Within the Superficial Laminae of the Dorsal Horn of the Rat Spinal Cord. Journal of Neuroscience 9: 195-205.

Uchizono K (1965) Characteristics of Excitatory and Inhibitory Synapses in Central Nervous System of Cat. Nature 207: 642-&.

Valtschanoff JG, Weinberg RJ, Rustioni A, Schmidt HHHW (1992) Nitric Oxide Synthase and GABA Colocalize in Lamina II of Rat Spinal Cord. Neuroscience Letters 148: 6-10.

Vandenpol AN, Gorcs T (1988) Glycine and Glycine Receptor Immunoreactivity in Brain and Spinal Cord. Journal of Neuroscience 8: 472-492.

Varoqui H, Schafer MKH, Zhu HM, Weihe E, Erickson JD (2002) Identification of the differentiation-associated Na+/P-I transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. Journal of Neuroscience 22: 142-155.

Watson AHD, Bazzaz AA (2001) GABA and glycine-like immunoreactivity at axoaxonic synapses on Ia muscle afferent terminals in the spinal cord of the rat. Journal of Comparative Neurology 433: 335-348.

Wilson PR, Yaksh TL (1978) Baclofen Is Antinociceptive in Spinal Intrathecal Space of Animals. European Journal of Pharmacology 51: 323-330.

Wingo WJ, Awapara J (1950) Decarboxylation of L-Glutamic Acid by Brain. Journal of Biological Chemistry 187: 267-271.

Woolf CJ (1983) Evidence for A Central Component of Post-Injury Pain Hypersensitivity. Nature 306: 686-688.

Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral Nerve Injury Triggers Central Sprouting of Myelinated Afferents. Nature 355: 75-78.

Woolf CJ, Shortland P, Reynolds M, Ridings J, Doubell T, Coggeshall RE (1995) Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. Journal of Comparative Neurology 360: 121-134.

Woolf CJ, Mannion RJ (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353: 1959-1964.

Wu JY, Su YYT, Lam DMK, Brandon C, Denger L (1979) Purification and Regulation of GAD. Brain Research Bulletin 4: 687.

Yaksh TL (1989) Behavioral and Autonomic Correlates of the Tactile Evoked Allodynia Produced by Spinal Glycine Inhibition - Effects of Modulatory Receptor Systems and Excitatory Amino Acid Antagonists. Pain 37: 111-123.

Yasumi M, Sato K, Shimada S, Nishimura M, Tohyama M (1997) Regional distribution of GABA transporter 1 (GAT1) mRNA in the rat brain: Comparison with glutamic acid decarboxylase(67) (GAD(67)) mRNA localization. Molecular Brain Research 44: 205-218.

Yoshimura M, Nishi S (1995) Primary Afferent-Evoked Glycine-Mediated and GABA-Mediated IPSPs in Substantia Gelatinosa Neurons in the Rat Spinal Cord In-Vitro. Journal of Physiology London 482: 29-38.

Zafra F, Gomeza J, Olivares L, Aragon C, Gimenez C (1995) Regional Distribution and Developmental Variation of the Glycine Transporters GLYT1 and GLYT2 in the Rat CNS. European Journal of Neuroscience 7: 1342-1352.

Zhang DX, Pan ZH, Zhang XH, Brideau AD, Lipton SA (1995) Cloning of Gamma-Aminobutyric Acid Type C Receptor Subunit in Rat Retina with a Methionine Residue Critical for Picrotoxinin Channel Block. Proceedings of the National Academy of Sciences of the United States of America 92: 11756-11760.