The organisation of the monoaminergic and cholinergic systems in the spinal cord

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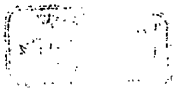
'They begin to tear at the chest, working at the ribs with a handsaw, then using the knife to worry through the great vessels. The doctors are visibly excited, bright as eggs. There shall be a paper in this, societies addressed, circles of illuminati: 'some thoughts, hm, upon the Case of the Late Jm Dyer. An Enquiry into…the Curious and Remarkable…who until his twenty-something year was insensible to…knew not…entirely without sensation…feeling…knowledge of…pain. With proofs, illustrations, exhibits and so forth.'

(Andrew Miller, 'Ingenious Pain')



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Publications arising form this work

Papers:

Stewart, W & Maxwell, D.J. (2000). Morphological evidence for the selective modulation by serotonin of a sub-population of dorsal horn cells which possess the neurokinin-1 receptor. *Eur. J. Neurosci.* **12**, 4583-4588.

Welton, J., Stewart, W., Kerr, R., & Maxwell, D.J. (1999). Differential expression of the muscarinic m2 acetylcholine receptor by small and large motoneurons of the rat spinal cord. *Brain Res.* **817**, 215-219.

Copies of these publications are included in appendix 2.

Abstracts:

Stewart, W & Maxwell, D.J. (2001). 5-HT-immunoreactive terminals selectively target a population of NK-1-immunoreactive neurons in the rat spinal cord. *Scot. Med. J.* **46**, 28.

Stewart, W & Maxwell, D.J. (1999). The acetylcholine m2 muscarinic receptor is expressed by a population of cholinergic dorsal horn neurons in the rat spinal cord. *J Physiol.* **521P**, S120

Stewart, W & Maxwell, D.J. (1999). A population of lamina III/IV neurokinin-1-immunoreactive cells are targeted by serotonin-immunoreactive axons in the rat dorsal horn. *J. Physiol.* **518P**, 166P

Welton, J., Stewart, W., Kerr, R., & Maxwell, D.J. (1998). Differential expression of the muscarinic acetylcholine m2 receptor by large and small motoneurons of the rat lumbar spinal cord. *J. Physiol.* **509P**, 179P.

Stewart, W., Pow, D.V. & Maxwell, D.J. (1997). Evidence for contacts between monoaminergic axons and inhibitory neurons of the superficial dorsal horn in the rat. *J. Physiol.* **505P**, 37P.

List of abbreviations

5-HT	serotonin
ChAT	choline acetyltransferase
CNS	central nervous system
Cy-5	cyanine 5.18
DAB	diaminobenzidine
D-βH	dopamine-β-hydroxylase
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
gly	glycine
G-S	gastrocnemius-soleus
LRSC	lissamine-rhodamine
MOR	μ-opioid receptor
mRNA	messenger ribonucleic acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NeuN	neuronal nuclear protein
NK-1	neurokinin-1 receptor
NOS	nitric oxide synthase
nt	neurotensin
parv	parvalbumin
PB	phosphate buffer
PBS	phosphate buffered saline
PrI	propidium iodide
sst	somatostatin
sst_{2A}	$somatostatin_{2A}$ receptor

Summary

Within the central nervous system, both the monoaminergic and cholinergic systems are recognised as having a significant role in the regulation of nociceptive processing at the level of the spinal dorsal horn producing a depression of the nociceptive signal. Despite this, relatively little is known of the mechanisms and neuronal circuits which are responsible for the action of these systems. In producing their action the descending monoaminergic pathways from the brainstem, which utilise serotonin and noradrenaline may potentially operate via pre- and/or post-synaptic pathways at either direct or indirect sites. For both the monoaminergic and cholinergic systems multiple receptor subtypes exist and the role of each individual subtype is ill understood. In this series of experiments, the relationship of the monoamines to identified neurons of the dorsal horn is first examined. Then, in a separate series of studies the distribution of the muscarinic m2 receptor is examined in the spinal cord with particular reference to the distribution of this receptor in the dorsal horn and the cells which are targeted.

Adult Albino Swiss rats were used throughout. These were anaesthetised with pentobarbatone (1ml of 60mg/ml, i.p.) and perfused prior to removal of mid-lumbar or thoracic spinal cord blocks. These blocks were then sectioned in either the transverse or parasagittal plane then reacted with the primary antibody cocktail required for each protocol. Following this the sections were transferred to solutions of fluorophore-coupled secondary antibodies or, for ultrastructural studies, antibodies coupled to biotin or immunogold. The sections were then processed for analysis using three-colour confocal laser scanning microscopy or electron microscopy.

In sections reacted with serotonin a dense plexus of axons and terminals was identified in the dorsal horn corresponding to the region of lamina I/II$_o$ from which axons emerged which appeared to encircle the somata and proximal dendrites of neurons within lamina III/IV forming 'basket-like' associations. Numerous noradrenergic profiles (revealed with dopamine-β-hydroxylase immunoreactivity) were also noted corresponding to lamina I/II however these did not appear to cluster around the proximal regions of dorsal horn neurons. Examining the relationship of these monoaminergic axons and terminals to specific dorsal horn neuronal subtypes demonstrated few contacts with no evidence of clustering on either excitatory or inhibitory dorsal horn interneurons revealed with antibodies to GABA, glycine, somatostatin, neurotensin, choline acetyltransferase, nitric oxide synthase (NOS) or the μ-opioid receptor. In contrast, cells immunoreactive for the substance P receptor neurokinin-1 (NK-1), located in lamina III/IV and lamina I, received relatively high contact frequencies form serotonin when compared to the dorsal horn interneurons. These contacts were clustered around the proximal dendrites and somata. In addition in approximately 50% of the NK-1-immunoreactive neurons in lamina III/IV the 'basket-like' arrangement of serotonin axons was observed. This correlated with a higher frequency of contacts on these cells. Ultrastructural observations on the relationship of serotonin terminals to GABA-immunoreactive somata showed several close associations however synaptic specialisations were not identified.

In material reacted with the muscarinic m2 acetylcholine receptor immunolabelled profiles were identified in the dorsal horn, ventral horn, intermediate grey and around the central canal in lamina X. In the

dorsal horn approximately 23% of neurons were immunoreactive for the m2 receptor. Examination of the relationship of this receptor to specific dorsal horn neuronal subtypes revealed that some cells immunoreactive for either GABA, NOS, NK-1, parvalbumin or the somatostatin$_{2A}$ receptor were m2 positive with the ratio in part dependant on laminar location. No neurotensin-immunoreactive neurons were m2 receptor-immunoreactive. Throughout the dorsal horn and lamina X 41% of cholinergic neurons were m2 receptor-immunoreactive. In the ventral horn larger rather than smaller motoneurons were found to be receptor positive and these neurons were also noted to receive greater numbers of cholinergic terminals.

These investigations have demonstrated that the cells of the dorsal horn which are NK-1-immunoreactive are unique amongst the neurons studied in receiving significantly greater frequencies of serotonin contacts than the dorsal horn interneurons examined. Furthermore, within lamina III/IV a subpopulation of NK-1-immunoreactive neurons has been identified which are associated with a clustering of serotonergic axons around the proximal dendrites and somata, a relationship which corresponds to a greater number of serotonin contacts on these cells. The neurons of the dorsal horn which are nerokinin-1-immunoreactive are recognised as being almost exclusively tract neurons that project to higher centres associated with nociceptive processing. This identification of a specific relationship between serotonergic profiles and NK-1 neurons of the dorsal horn and the relatively few contacts made by serotonergic and noradrenergic terminals on dorsal horn interneurons suggests a targeted action on projection tract neurons with a more diffuse, perhaps non-synaptic action on local dorsal horn cells.

The expression of the muscarinic m2 acetylcholine receptor on neurons throughout the spinal grey implies a wide role for this receptor in both sensory and motor processing. This somatic expression had not previously been described. Further to this diffuse expression these studies have demonstrated that there is a selective distribution of the receptor amongst particular neuronal subtypes in the dorsal horn and a greater expression of the receptor on larger, presumed α-motoneurons of the ventral horn. This data provides valuable information into the possible roles of this specific cholinergic receptor in the spinal cord.

Chapter 1: The monoaminergic system in the spinal cord

1.1 Introduction

It is commonly accepted that, under certain conditions, one's awareness of an unpleasant or painful stimulus can be suppressed. In man this is most frequently experienced during physical exertion (Henry, 1982) or under extremes of stress such as on the battlefield (Beecher, 1946). This endogenous antinociceptive system would appear in part to originate within the brainstem as demonstrated by Reynolds (1969) who by stimulating in the region of the midbrain was able to perform a laparotomy on unanaesthetised rats. Similarly in man central stimulation in the region of the thalamus can produce analgesic effects (Tsubokawa et al, 1982). The mechanism by which this antinociception arises is clearly of some significance in understanding the process of nociception which may then guide the development of targeted therapies for clinical use. By sectioning the spinal cord, thereby interrupting the pathways to and from the brainstem, a significant reduction in endogenous antinociceptive response is noted suggesting a descending influence on the spinal cord (Berge, 1982). Strongly implicated in this descending antinociceptive pathway are the monoamines in particular serotonin and noradrenaline which originate within nuclei in the brainstem. In regards to these neurotransmitters a great many investigations have provided invaluable information on their roles in antinociception however little is understood of the neural circuitry within the spinal dorsal horn which may produce these effects. This study is directed at identifying the neurons of the spinal dorsal horn in the rat which receive contacts from descending monoaminergic axons

1.2 Origins of Serotonergic Neurons

The serotonin observed at the level of the spinal cord is considered to derive exclusively from descending brain stem efferents. This conclusion has arisen from the marked decrease in serotonin immunoreactivity following transection of the cord (Dahlström & Fuxe, 1965, Magnusson, 1973). In addition there has been a repeated failure to conclusively demonstrate serotonergic somata in the spinal cord though some initial data has suggested monoamine somata at this level. For example, in their study of the monkey spinal cord Lamotte, Johns & de Lanerolle (1982) proposed a population of serotonergic neurons in lamina X at all spinal cord levels using both serotonin immunocytochemistry and the Falck-Hillarp method of formaldehyde induced fluorescence (Falck et al, 1962). However, this observation has not been reliably reproduced in any investigations since. It would seem likely therefore that the group of cells they describe may either be a group of neurons with a similar fluorescence to serotonin using the Falck-Hillarp method (Björklund, Falck & Stenevi, 1970) or they may be cells accumulating rather than synthesising serotonin (Kojima et al, 1983).

In their series of studies investigating the distribution and projections of serotonergic neurons in the brain stem of the cat Taber, Brodal and Walberg(1960) identified eight groups of serotonergic neurons within the brain stem. Dahlström and Fuxe (1965) in their review of the monoaminergic systems of the rat brain stem added to this number by suggesting that in fact nine groups could be identified (groups B1-9). Occasional serotonergic neurons can also be observed in pontine nuclei associated with catecholamine neurons such as the locus coeruleus and subcoeruleus (Leger et al, 1979; Bowker, Westlund &

Coulter, 1981). As with the noradrenergic system this subdivision based on anatomical location has been extended to demonstrate a selective distribution arising from each nucleus .

B1 Group

Serotonin immunofluorescence reveals a cluster of cells in the caudal part of the medulla oblongata in the rat within the nucleus raphe pallidus (Taber et al, 1960; Dahlström & Fuxe, 1965). As with other raphe nuclei this region exhibits a relatively high density of serotonin (Palkovits, Brownstein & Saalvedra, 1974). Initial studies of retrograde cell death following spinal cord transection indicated that cells within the nucleus raphe pallidus had a significant projection to the spinal cord in the cat (Brodal, Taber & Walberg, 1960). This has subsequently been confirmed by the anterograde transport of a variety of tracers which have also revealed that this projection terminates in the ventral horn of rat (Bowker, Westlund & Coulter, 1981; Jones & Light, 1990), cat (Bobillier et al, 1976; Basbaum & Fields, 1979) and primate (Bowker, Westlund & Coulter, 1982). By combining retrograde transport techniques with serotonin immunofluorescence the majority of these cells are confirmed as serotonergic (Bowker, Steinbusch & Coulter, 1981; Bowker, Westlund & Coulter, 1981). In addition to a projection to the ventral horn, the B1 group of cells also contributes a significant projection to the intermediolateral cell column (Loewy & Mckellar, 1981).

B2 Group

The B2 group of cells is located within, and in immediate proximity to, the nucleus raphe obscurus (Taber, Brodal & Walberg, 1960; Dahlström & Fuxe, 1965; Steinbusch, 1981). As with the B1 cell group

retrograde cell death in the cat (Brodal, Taber & Walberg, 1960) and HRP transport studies in rat (Bowker, Steinbusch & Coulter, 1981; Bowker, Westlund & Coulter, 1981) and primate (Bowker, Westlund & Coulter, 1982) have demonstrated a significant projection form this group of cells to the spinal cord. Similarly, the cells of the nucleus raphe obscurus appear to target the ventral horn (Bobillier et al, 1976; Martin, Jordan & Willis, 1978; Basbaum & Fields, 1979) and intermediolateral cell column (Loewy & Mckellar, 1981).

B3 Cell Group

Corresponding to the nucleus raphe magnus, the cells of the B3 group represent the largest collection of serotonergic neurons in the brain stem (Brodal, Taber & Walberg, 1960; Palkovits, Brownstein & Saalvedra, 1974; Steinbusch, 1981). Employing the anterograde transport of tritiated amino acids Basbaum, Clanton and Fields (1978) were able to demonstrate a bilateral projection from the nucleus raphe magnus to the cat spinal cord, in particular, to the regions of the marginal zone, substantia gelatinosa and laminae V, VI and VII. In the corresponding primate investigations a similar projection from the nucleus raphe magnus to laminae I, II and V has been identified (Basbaum, Ralston & Ralston, 1986). This projection gains further support from retrograde transport studies where tracer injected to the lumbar enlargement can subsequently be demonstrated in the nucleus raphe magnus in the cat (Martin, Jordan & Willis, 1978) and in the rat (Bowker, Steinbusch & Coulter, 1981; Bowker, Westlund & Coulter, 1981). A projection to the intermediolateral cell column has also been demonstrated by anterograde HRP transport (Loewy et al, 1981).

That the nucleus raphe magnus contributes a major serotonergic input to the spinal cord is supported by the marked reduction in serotonin-immunoreactive profiles in the cord following a lesion in the nucleus raphe magnus with the loss of immunoreactivity greatest in the dorsal horn (Oliveras et al, 1977). This projection from the nucleus raphe magnus to the spinal cord would appear to be proportionately greater than from any other serotonergic brain stem nucleus (Brodal, Taber & Walberg, 1960). In addition, injection of kainic acid to the region of the nucleus raphe magnus causes an increase in the expression of the proto-oncogene *c-fos* in the dorsal horn, with no *c-fos* expression detected in the ventral horn (Bett & Sandkuhler, 1995). Correspondingly, stimulation of the nucleus raphe magnus results in a detectable release of serotonin at the level of the spinal cord in the rat (Rivot, Chiang & Besson, 1982; Hammond, Tyce & Yaksh, 1985).

It is clear that the serotonergic cells of the medullary raphe nuclei contribute a significant projection to the spinal cord. Indeed quantitative analysis suggests the majority of serotonergic neurons in these nuclei project to the spinal cord (Bowker, Westlund & Coulter, 1981). There is also a degree of selectivity in their projection with the cells of the nucleus raphe pallidus (B1) and nucleus raphe obscuris (B2) primarily projecting to the ventral horn whilst the cells of the nucleus raphe magnus (B3) project to the dorsal horn. All three nuclei project to the intermediolateral cell column.

Group B4

This small group of cells lies close to the midline in the region of the nucleus vestibularis medialis (Dahlström & Fuxe, 1965; Steinbusch,

1981). So far, no evidence of a projection from this region to the spinal cord has been detected (see Bowker, Westlund & Coulter, 1981).

Group B5

The majority of the serotonin-immunoreactive neurons within the B5 group lie within the intermediate part of the nucleus raphe pontis (Taber, Brodal & Walberg, 1960; Dahlstöm & Fuxe, 1965; Steinbusch, 1981). Following a lesion to the spinal cord a few cells within this group undergo cell death (Brodal, Taber & Walberg, 1960). Retrograde transport of HRP confirms the presence of a minor projection from the nucleus raphe pontis to the cord (Martin, Jordan & Willis, 1978; Bowker, Westlund & Coulter, 1981).

Groups B6 and B7

The B7 cell group corresponds to the region of the nucleus raphe dorsalis in the primate (Kapadia, de Lanerolle, & LaMotte, 1985) and the rat (Dahlström & Fuxe, 1965; Steinbusch, 1981) with a caudal extension of cells in the region of the rostral part of the fourth ventricle near the midline (B6 group of Dahlström & Fuxe). Lesioning of the nucleus raphe dorsalis in the cat, rat and guinea pig is not associated with a reduction in the serotonin content of the lumbar enlargement (Oliveras et al, 1977) nor is cell death within the nucleus raphe dorsalis observed on lesioning of the lumbar spinal cord (Brodal, Taber & Walberg,1960). Whilst suggesting that the serotonergic neurons in this region do not project to the spinal cord this observation would appear to be dependant on the region of cord examined, the species and the experimental conditions. Thus, when tracer is injected to the cervical cord in the rat a small number of neurons are identified in the nucleus raphe dorsalis (Bowker, Westlund & Coulter, 1981). In their series of

experiments on the retrograde transport of wheat germ agglutinin conjugated to HRP Mantyh & Peschanski (1982) identified labelled cells in the region of the nucleus raphe dorsalis in the rat following injection of tracer to the lumbar cord. However approximately 40% fewer cells were labelled when compared to the same injection to the cervical cord. This observation of a greater number of cells identified following a cervical as opposed to a lumbar cord injection has also been documented in cat and monkey with the greatest numbers of cells identified in the cat. Again, this projection appears directed at the spinal dorsal horn (Wang & Nakai, 1994).

B8 Group

Serotonergic neurons within the B8 group lie within the nucleus centralis superior and adjacent areas in the brain stem (Steinbusch, 1981). To date, a projection from this region to the spinal cord has not been identified (Brodal, Taber & Walberg, 1960; Bowker, Westlund and Coulter, 1981).

B9 Group

The more diffuse cells of the B9 group are located within the medial lemniscus in the brainstem with occasional, scattered neurons lying dorsal to the medial lemniscus (Dahlström & Fuxe, 1965; Steinbusch, 1981). As with the B7 group of cells, the projection from this group of serotonergic neurons appears to be directed selectively towards the cervical cord with no projection to the lumbar cord (Bowker, Westlund & Coulter, 1981).

Thus though the descending pontine and midbrain serotonergic projection is less extensive there would appear to be a significant projection from groups B5, B7 and B9, those from the latter two directed towards the cervical cord. No descending spinal projection has been reported for groups B6 and B8. Quantitatively it is estimated that less than half of midbrain serotonergic neurons have a spinal projection (Bowker, Westlund & Coulter, 1981).

1.3 Distribution of serotonergic terminals in the spinal grey

Serotonergic terminals are located throughout the spinal grey matter their density varying within the different laminae. In quantitative estimates of the serotonin content in punch specimens sampled from various regions of the spinal cord in a variety of species the highest concentration of serotonin is present in the ventral horn with a correspondingly lower concentration in the dorsal horn. In keeping with this observation, serotonin-immunoreactive profiles in the rat (Steinbusch, 1981; Hadjiconstantinou, 1984) and primate (Kojima et al, 1983) are found in the highest density in the ventral horn where they are closely associated with motor nuclei. The superficial laminae of the dorsal horn are also associated with relatively high serotonin concentrations (Oliveras et al, 1977; Hadjiconstantinou, 1984) with the highest density of serotonin-immunoreactive profiles occurring in the region of lamina I and II_o, few profiles in lamina II_i and copious, diffuse serotonin-immunoreactive profiles in laminae III-VI. This arrangement has been confirmed in rat (Steinbusch, 1981; Maxwell, Leranth & Verhofstad, 1983; Marlier et al, 1991), cat (Ruda & Gobel, 1980; Ruda, Coffield & Steinbusch, 1982; Light, Kavookjian & Petrusz, 1983) and primate (Kojima et al, 1983). As has been discussed, no serotonin-

immunoreactive somata have been confidently identified in the spinal grey in any of the investigations performed to date.

1.4 Ultrastructure of serotonergic profiles in the dorsal horn.

Serotonergic axons within the dorsal horn run in a predominantly rostrocaudal direction with multiple en-passant varicosities along their length (Light, Kavookjian & Petrusz,1983). Initial autoradiographic studies of the fine structure of serotonergic varicosities in the cat medullary dorsal horn identified several ultrastructural subtypes based on varicosity shape and the size, shape and density of vesicles (Ruda & Gobel, 1980). Subsequent immunocytochemical studies in cat (Ruda et al 1982; Light et al, 1983) and rat (Marlier et al, 1991) have shown there to be two types of serotonin varicosity based on morphology that display some variation in frequency throughout the dorsal horn laminae. The commonest varicosity described is found in all laminae, is dome shaped and is associated with numerous flat or oval, agranular vesicles with occasional larger dense core vesicles. Typically these varicosities are associated with a single symmetrical synaptic specialisation (Gray type II synapse) in the cat dorsal horn (Ruda et al, 1982; Light et al, 1983). By contrast, few synapses are noted in the rat dorsal horn (Maxwell, Leranth & Verhofstad, 1983). The second morphological subtype of serotonin varicosity described in the dorsal horn is primarily located in lamina I and lamina II_o. These are typified by large scalloped, or more often oval, varicosities with many large dense core vesicles in addition to smaller agranular vesicles. Again these are associated with symmetrical synapses where present though synaptic specialisations are infrequent when compared with the dome shaped varicosities in both the cat (Ruda et al, 1982;

Light et al, 1983) and the rat (Maxwell, Leranth & Verhofstad, 1983; Marlier et al, 1991).

Serotonin-immunoreactive varicosities as described above are often associated with dendritic profiles with which axo-dendritic synapses can be identified. Similarly axo-somatic contacts have been documented. In contrast, few axo-axonic synapses have been recorded involving serotonin in the dorsal horn (Maxwell, Leranth & Verhofstad, 1983). As noted, synaptic specialisations are more often identified in relation to dome shaped varicosities and are commoner in the cat dorsal horn. It has been shown though that those serotonin-immunoreactive varicosities not associated with a classical synaptic specialisation are often closely opposed to cell bodies or dendrites of neurons in the dorsal horn in both rat and cat (Maxwell, Leranth & Verhofstad, 1983; Ruda et al, 1982; Light et al, 1983; Marlier et al, 1991).

1. 5 SerotonIn receptors in the spinal grey

To date there are some fourteen or more serotonin receptors recognised with seven distinct receptor families identified (Hoyer & Martin, 1997; Martin et al, 1998). These have been termed 5-HT_{1-7} with all being metabotropic save the 5-HT_3 family which are ligand gated. As a greater understanding of these receptors is gained there is a seemingly continuous re-classification and expansion of the sub-types of receptor recognised (see Saxena, DeVries & Villalón, 1998) such that any discussion of the 5-HT receptor distribution is made with the qualification that it represents the information based on the classification at the time of writing. As regards dorsal horn activity and

distribution it is the 5-HT_1, 5-HT_2, 5HT_3 and 5-HT_7 families that are regarded of greatest significance and hence these will be discussed.

5-HT_1 subtype

The 5-HT_1 receptor family can be subdivided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. Of these, autoradiographic studies have identified 1A and 1B subtypes within the spinal grey matter in rat (Pazos & Palacios, 1985; Marlier et al 1991; Laporte et al, 1995) and human (Pazos, Probst & Palacios, 1987a), with the highest density of receptor localised to the superficial dorsal horn laminae. Lesser densities of receptor were identified in the ventral horn, lamina X and the intermediolateral cell column though the gradation with the 1B receptor was less marked than with 1A receptor. Using an anti-idiotypic antibody, which detects 5-HT_{1B}, 5-HT_2 and 5-HT_{2c} receptors, preferential staining in the superficial dorsal horn was again noted in association with dendrites and somata. Rarely was an associated synaptic specialisation seen (Ridet, Tamir & Privat, 1994). Dorsal rhizotomy results in an approximately 20% reduction in 5-HT_{1A} and 5-HT_{1B} receptor binding (Laporte et al, 1995) with a similar reduction following neonatal capsaicin treatment (Daval et al, 1987). Thus, in addition to a somatic and dendritic distribution within the dorsal horn, these receptors are also thought to be present on primary afferent terminals.

5-HT_2 receptor subtype

In contrast with the 5-HT_1 receptor family autoradiographic studies in rat (Marlier et al, 1991; Thor et al, 1993) and human (Pazos, Probst & Palacios, 1987b) have shown the 5-HT_2 receptor subtype to be associated with a greater density of binding in the ventral horn. Little

or no binding has been observed in the dorsal horn. In situ hybridisation for 5-HT_{2A} or 5-HT_{2C} (formerly 1C) receptor mRNA (Pompeiano, Palacios & Mengod, 1994) and immunocytochemistry with a monoclonal antibody to the 5-HT_{2A} receptor (Cornea-Hébert et al, 1999) confirms a greater density in the ventral horn than in the dorsal horn.

5-HT_3 receptor subtype

Binding of the 5-HT_3 receptor is greatest in the superficial layers of the dorsal horn with little in the remainder of the spinal grey (Glaum & Anderson, 1988). As noted for the 5-HT_1 receptors the density of 5-HT_3 binding is significantly reduced on neonatal capsaicin treatment or dorsal rhizotomy thus suggesting a significant expression on primary afferent terminals (Hamon et al, 1989; Kidd et al 1993; Laporte et al, 1995).

5-HT_7 receptor subtype

As with the 5-HT_3 receptor, 5-HT_7 receptor binding is greatest in the superficial dorsal horn with less noted elsewhere (Gustafson et al, 1996).

1.6 Actions of serotonin in the dorsal horn

Effects of brainstem stimulation

The serotonergic system has a significant role in the modulation of sensory information processed at the level of the spinal dorsal horn. This modulatory effect is proposed to originate in brainstem serotonergic neurons located in the dorsal raphe complex and be mediated by serotonergic terminals within the dorsal horn. As might be expected stimulation of the brainstem serotonergic nuclei produces a

measurable rise in serotonin concentration in cerebrospinal fluid around the spinal cord in rats (Hammond, Tyce & Yaksh, 1985), cats (Sorkin, McAdoo & Willis,1993), and primates (Sorkin, McAdoo & Willis, 1992). The observation of a reduced number of dorsal horn cells expressing the immediate early gene *c-fos* in response to a nociceptive stimulus when the dorsolateral funiculus is intact as compared to when it is lesioned suggests this release of serotonin may translate to an influence on the activity of dorsal horn neurons (Liu et al 1997, 1999). In addition, cells immunoreactive for *c-fos* increase in number following stimulation of the raphe nuclei (Bett & Sandkühler, 1995; Sandkühler, 1996). Though over interpretation of observations related to the expression of *c-fos* can be a pitfall, these results would appear to imply both an inhibitory (reduces the number of cells expressing *c-fos* when pathway intact) and an excitatory (increased numbers of dorsal horn *c-fos* cells on brainstem stimulation) influence of descending serotonergic systems on dorsal horn neurons.

Whilst the predominant behavioural and electrophysiological evidence favours an inhibitory action of descending projections on the nociceptive reflexes and dorsal horn cells there have been reports of excitation of cells within the superficial laminae of the dorsal horn following stimulation of the descending tracts (Dubuisson & Wall, 1980). One possible interpretation is that the pathways may operate partly via excitation of local inhibitory dorsal horn interneurons so producing a net inhibition. In addition, the depolarisation of spinal cord primary afferent fibres following raphe nuclei stimulation in the cat suggests brainstem induced antinociception may operate via presynaptic inhibition of nociceptive primary afferent terminals (Proudfit, Larson & Anderson, 1980; Lovick, 1983). In both the

investigations cited it is notable that this brainstem elicited primary afferent depolarisation required both 5-HT and GABA for successful transmission. This may imply co-transmission of GABA with serotonin from terminals containing both serotonin and glutamic acid decarboxylase (Maxwell et al, 1996). As has been discussed the morphological correlate of pre-synaptic inhibition the axo-axonic synapse has not been described for serotonergic terminals in the dorsal horn. An alternative suggestion may be that local dorsal horn GABAergic neurons are involved in the pathway mediating descending inhibition. To further complicate matters a number of transmitters other than serotonin are released in the spinal dorsal horn on raphe stimulation with measurable increases in amino acids such as glutamate, aspartate, glycine and serine being detected (Sorkin, McAdoo & Willis, 1996).

Stimulation of the brainstem increases the threshold for the behavioural response to a nociceptive stimulus (Oliveras et al, 1975; Guilbaud et al, 1977), which in the rat is at least partly mediated by the 5-HT_{1A} receptor (Lin, Peng & Willis, 1996a). The response of individual dorsal horn neurons to nociceptive stimuli is similarly reduced when the brainstem is stimulated (Belcher, Ryall & Schaffner, 1978; Sorkin, McAdoo & Willis, 1993) with 5-HT_{1A} antagonists again reducing the effectiveness of this descending inhibition (Lin, Peng & Willis, 1996a). In addition to the putative role for the 5-HT_{1A} receptor, specific 5-HT_3 receptor antagonists partly inhibit brainstem-induced antinociception thereby suggesting a role for this receptor in descending nociceptive pathways (Peng, Lin & Willis, 1996a). Thus, brainstem-induced antinociception may operate in part through dorsal horn neurons expressing 5-HT_{1A} or 5-HT_3 receptors.

Effects of serotonin

The intrathecal application of serotonin has repeatedly been shown to produce an attenuation in behavioural response to a number of nociceptive stimuli such as paw pressure (Bardin et al, 1997a,b), thermal (Lin, Peng & Willis, 1996; Bardin et al, 1997a; Glaum, Proudfit & Anderson, 1988,1990; Xu, Qiu & Han, 1994) and chemical pain (Bardin et al, 1997a). This attenuation of nociceptive behavioural responses results from an action mediated by specific serotonin receptor subtypes. Among the receptor types implicated is the 5-HT_{1A} subtype. Intrathecal administration of a specific 5-HT_{1A} agonist results in an anti-nociceptive action which is inhibited by 5-HT_{1A} antagonists. These antagonists also produce an inhibition of part of the antinociceptive action of 5-HT (Xu, Qui & Hen, 1994; Lin, Peng & Willis, 1996). Though this pattern of action is the commonest encountered, there have been occasional reports of 5-HT_{1A} receptor agonists having either a facilitatory effect (Crisp et al, 1991) or no effect (Solomon & Gebhart, 1988) on the nociceptive response. Similarly, the results regarding the 5-HT_{1B} receptor have proved inconclusive with evidence of 5-HT_{1B} agonists producing either an increase in nociceptive threshold (Xu, Qui & Hen, 1994) or no effect on nociceptive threshold (Lin, Peng & Willis, 1996). When investigated in behavioural experiments the 5-HT_2 receptor does not appear to be involved in the nociceptive response (Xu, Qui & Hen, 1994). In contrast the 5-HT_3 receptor subtype may have a role in antinociception. As with the 5-HT_1 subtypes the data is mixed with either no effect (Xu, Qui & Hen, 1994; Ali et al, 1996) or an antinociceptive action reported (Glaum, Proudfit & Anderson, 1988, 1990; Bardin et al 1997). To date then the behavioural studies of the

action of serotonin have provided conflicting reports on the roles of specific serotonergic receptor subtypes. A number of possible reasons for this variation in observations exist such as the recognised variations in circuitry that exist between different experimental animals (see section 1.7) and the variable response within the serotonergic system dependant in part on the stimulus employed (Bardin et al 1997a).

Recording the responses of neurons which are located in or originate in the dorsal horn, numerous studies have demonstrated an inhibitory action of serotonin on neurons processing nociceptive information in rat (El-Yassir, Fleetwood-Walker & Mitchell, 1988), cat (Belcher, Ryall & Schaffner, 1978; Headley, Duggan & Griersmith, 1978) and primate (Jordan et al, 1979). As is the case with noradrenaline however, the action of serotonin is not exclusively inhibitory and a facilitatory action on dorsal horn cells has also been observed. In the cat Todd & Millar (1983) reported that 68% of units within laminae I, II and III were excited in response to the ionophoretic application of serotonin. When serotonin is iontophoresed to the substantia gelatinosa and a nociceptive stimulus administered, the effects of serotonin on deeper dorsal horn neurons can be recorded. Under these circumstances though the majority cells in lamina IV or V are inhibited, some are also excited (Headley, Duggan & Griersmith, 1978). In contrast when serotonin was iontophoresed around cells in lamina IV/V, excitation was not observed. Thus, though the bulk of dorsal horn nociceptive neurons are inhibited following the application of serotonin, occasional cells are excited (El-Yassir, Fleetwood-Walker & Mitchell, 1988). This effect is not confined solely to recordings from cells in the dorsal horn with similar responses from spinothalamic tract cells in the thalamus

noted following the administration of serotonin around their origin in the dorsal horn (Jordan et al, 1979).

The responses of a number of projection tracts to serotonin has been extensively investigated and the results have shown a somewhat variable response dependent on the tract under observation and the nature of the stimulus. For example, group II muscle spindle afferent responses conveyed in the spinocervical tract, postsynaptic dorsal column and dorsal spinocerebellar tract neurons in Clarke's column are facilitated by serotonin (Jankowska et al, 1997). Those of the dorsal spinocerebellar tract neurons located in the dorsal horn however were inhibited (Jankowska et al 1995, 1997). This is in contrast to the response to low threshold cutaneous afferents within these tracts where serotonin produces facilitation (Jankowska et al, 1997).

Using receptor specific agonists and antagonists the serotonin receptor subtypes implicated in these responses have been explored. These investigations have suggested both the 5-HT_{1A} and 5-HT_{1B} receptors may be involved in the depressive action of serotonin on dorsal horn cells (El-Yassir, Fleetwood-Walker & Mitchell, 1988; Gjerstad, Tjølsen & Hole, 1997) whilst the 5-HT_2 receptor does not appear to be involved (El-Yassir, Fleetwood-Walker & Mitchell, 1988). A variable response at the 5-HT_3 receptor has been documented with facilitation of nociceptive neurons to a noxious stimulus at low doses of an agonist which becomes inhibitory at higher concentrations (Ali et al, 1996).

As previously noted stimulation of brainstem serotonergic nuclei results in depolarisation of primary afferent fibres. Application of serotonin to *in vitro* slice preparations of rat spinal cord produces a depression of spontaneous dorsal root potentials with a slow depolarisation of primary afferents and depression of transmission between primary afferents and dorsal horn cells (Hentall & Fields, 1983; Lopez-Garcia & King, 1996). To date it has been suggested that this response is mediated via the 5-HT_2 and $GABA_A$ receptor subtypes (Thompson & Wall, 1996). As has been discussed, with no axo-axonic synapses associated with serotonergic terminals in the dorsal horn, this presynaptic action may perhaps be mediated via local dorsal horn GABAergic neurons or by non-synaptic transmission.

1.7 Origins of Noradrenergic Neurons

As in the case of the serotonergic terminals, despite numerous studies of the noradrenergic system no noradrenergic neurons have been identified within the spinal cord, furthermore lesioning of the spinal cord produces a reduction in noradrenergic immunoreactivity caudal to the lesion (Magnusson & Rosengran, 1963). Noradrenergic terminals in the spinal cord therefore are regarded as having a supraspinal origin. In their original topographical and morphological study of the rat CNS, Dahlström and Fuxe (1964) described seven groups of noradrenergic neurons within the brain stem (A1-A7) localised to the medulla (A1-A4) and pons (A5-A7). At the time, they accepted their division was 'partly artificial' and subsequent morphological studies in primates (Garver & Sladek, 1976; Westlund et al 1984) and humans (Farley and Hornykiewicz, 1977) have found the boundaries between cell groups to be less distinct with less adherence to discrete anatomical boundaries. In an extensive review of the central noradrenergic system, Moor and Bloom (1979) proposed the noradrenergic neurons need not be divided beyond the locus coeruleus system (A6 group) and a diffuse lateral tegmental system which included the subcoeruleus (A7 group). Since that review a further population discrete from the lateral tegmental group has subsequently been proposed within the dorsomedial medulla (A2 group; Björklund and Lindvall 1986). Thus three systems of noradrenergic neurons may be described originating within the brain stem. However, the original observations of Dahlström and Fuxe remain in use as a descriptive classification of noradrenergic cell localisation. In addition to these noradrenergic cell groups PNMT-immunocytochemistry reveals three groups of neurons within the medulla, presumed to be adrenergic (Carlton et al, 1989; Hökfelt et al

1974), with high levels of adrenaline detected in these regions by quantification methods (Saavedra et al 1974).

Efferent projections from these noradrenergic nuclei have been traced throughout the central nervous system with projections to the cerebral cortex, cerebellum, hypothalamus, midbrain, medulla and spinal cord all described in rat (Dahlström and Fuxe, 1964; Grzanna and Fritschy 1991; Jones and Moore, 1977; Olsen and Fuxe, 1971; Swanson and Hartmann, 1975) and primate (Garver and Sladek, 1976; Westlund et al 1984). This ubiquitous distribution initially implied that the noradrenergic system had a diffuse influence within the CNS rather than a target specific role (Descarries, Watkins and Lapierre, 1977). However, this original hypothesis has now been revised with the availability of improved electrophysiological and tracer techniques. It is now clear that a high degree of selectivity of projection from the noradrenergic nuclei to the spinal cord and the remaining CNS exists.

A1 and A2 groups

Located within the ventrolateral (A1) and dorsomedial (A2) medulla these cell groups were first suggested to project to the spinal cord following the original Dahlström and Fuxe (1965) studies where they observed an accumulation of catecholamine within somata in these regions, reflected as an increased immunofluorescence, following transection of the spinal cord in rats. Subsequent lesioning studies have demonstrated a projection from the A1 group, and to a lesser extent the A2 group, to the intermediolateral cell column with no projection to the dorsal or ventral horns (Fleetwood-Walker & Coote, 1981). Retrograde tracer studies with injection to the thoracic cord have supported this observation in rat (Satoh et al, 1977), rabbit

(Blessing et al, 1981) and primate (Carlton et al, 1991). Furthermore, stimulation in the thoracic cord produces antidromic responses that can be detected within the A1 group (Fleetwood-Walker, Coote & Gilbey, 1983). The morphological and electrophysiological evidence therefore supports a role for the A1 group in autonomic processing.

A number of non-noradrenergic cells have been detected by retrograde tracer techniques lying within the A1 and A2 cell groups in rat (Westlund et al, 1983) and primate (Westlund et al, 1984). Though several groups have concluded that noradrenergic neurons do project to the spinal cord (Dahlström & Fuxe, 1965; Satoh et al, 1977; Smolen, Glazer and Ross, 1979) there is growing evidence to suggest the spinally projecting neurons emanating from the A1 and A2 groups are not noradrenergic (Blessing et al, 1981; Westlund et al, 1981, 1982, 1984, 1983). The rostral location of spinally projecting neurons within the A1 group (Blessing et al, 1981) does however correspond to the C1 group of adrenergic neurons in the brainstem (Carlton et al, 1989; Hökfelt et al, 1974, Moore and Bloom, 1979, Saavedra et al, 1974). A projection from this group of adrenergic neurons to the spinal cord has been shown using a combined retrograde transport and PNMT-immunocytochemistry technique (Ross et al, 1981).

A4 cell group

Located in the caudal pons in the lateral roof of the fourth ventricle and continuous rostrally with cells within the locus coeruleus (A6 group) this group of noradrenergic cells does not appear to contribute a significant projection to the spinal cord (Dahlström & Fuxe, 1964; Kuypers et al, 1975; Blessing et al 1981; Westlund et al, 1984).

A5 cell group

The A5 group of noradrenergic cells represents a pontine extension of the A1 group with cells located in the ventrolateral brainstem dorsal and lateral to the facial nucleus and the superior olivary complex (Dahlström & Fuxe, 1964; Westlund et al, 1984). Extensive investigation has revealed a significant projection from this group of neurons to the spinal cord. Using a double-labelling, retrograde tracer technique in primates with HRP injection to the cervical enlargement combined with dopamine-β-hydroxylase (D-βH) immunocytochemistry Westlund et al (1984) demonstrated a noradrenergic projection to the cord from this group. This region they estimated to contribute 11.5% of noradrenergic cells projecting to the primate cervical cord. Similar studies have confirmed the presence of this projection from the A5 cell group in rats (Clark, Yoemans & Proudfit, 1991; Fritschy and Grzanna 1990; Grzanna and Fritschy, 1991; Westlund et al 1983).

Injection of tracer to the A5 region reveals a projection to the intermediolateral cell column in rats (Clark and Proudfit, 1993; Loewy, McKellar & Saper, 1979; Loewy et al, 1986). This projection is supported by the observation that HRP injected to the lateral horn in the thoracic cord retrogradely labels cells within the A5 group (Blessing et al, 1981; Fritschy and Grzanna, 1990; Satoh et al, 1977; Westlund et al, 1983). A projection from A5 noradrenergic cells to the dorsal horn laminae IV-VI and to lamina VII has also been identified (Clark and Proudfit, 1993).

A6 cell group (locus coeruleus)

In studying the noradrenergic immunocytochemistry of the brainstem a group of cells located within the nucleus locus coeruleus in the dorsolateral pons is easily distinguishable and represents the densest, most discrete population of noradrenergic neurons in the CNS (Dahlström & Fuxe, 1964;Westlund & Coulter, 1980; Westlund et al, 1984). Retrograde transport has revealed a projection to the spinal cord from this population of neurons in rat (Basbaum & Fields, 1979; Guyenet, 1980; Westlund et al, 1981, 1983; Jones & Yang, 1985; Clark and Proudfit, 1989; Fritschy and Grzanna, 1990), cat (Jones & Moore, 1974; Kuypers & Maisky, 1975; Basbaum & Fields, 1979; Nakazato, 1987) and primate (Hancock and Fougerousse, 1976; Westlund & Coulter, 1980; Westlund et al, 1984). Again Westlund and colleagues (1984) have quantified this projection and estimate the locus coeruleus neurons to account for 29.3% of noradrenergic cells projecting to the cervical cord in primates. Similar studies have estimated the proportion to be higher in the rat at 41% (Westlund et al 1983). However there does appear to be a significant species variation with fewer locus coeruleus cells in rabbit (Blessing et al, 1981) and cat (Stevens, Hodge & Apkarian, 1982; Stevens, Apkarian & Hodge, 1985) contributing to the noradrenergic spinal projection.

In the primate, anterograde transport of tritiated amino acid injected to the region of the locus coeruleus reveals dense terminal labelling in the region of lamina VII-IX and around the central canal (Westlund & Coulter, 1980). Similar findings have been reported for rats (Jones & Yang, 1985). Further evidence in support of a coeruleospinal projection to the ventral horn and lamina X in rats is provided by unilateral or bilateral lesion of the locus coeruleus which results in a

reduction in noradrenaline containing axons in these regions of the spinal cord (Nygren & Olsen, 1977; Commissiong et al, 1978; Clark and Proudfit, 1991). However, though there is agreement on the termination of locus coeruleus noradrenergic fibres within the ventral horn and around lamina X there is some disagreement over the contribution of locus coeruleus neurons to the noradrenergic innervation of the dorsal horn. Injection of tritiated amino acid into the locus coeruleus in rat (Jones and Yang, 1985) and primate (Westlund and Coulter, 1980) has been reported to produce only light to moderate labelling in the dorsal horn suggesting that a major coeruleospinal projection to this region does not exist. However, anterograde transport studies combined with D-βH-immunocytochemistry have indicated a dense projection to the dorsal horn in the rat (Fritschy et al, 1987; Fritschy & Grzanna, 1990). This apparent disagreement may be a result of a variation in projection of locus coeruleus neurons in the different genetic strains of rat used in these studies. This hypothesis has been examined by Proudfit and Clark (1991) comparing the retrograde transport of fluorogold injected to either the dorsal or the ventral horn in two strains of Sprague-Dawley-derived rats. Following this they noted a clear difference in coeruleospinal projection between the two strains used with Harlan-Sprague-Dawley rats (as used by Fritschy et al, 1987) exhibiting a bilateral projection to the spinal cord with more neurons labelled following injections to the dorsal horn than to the ventral horn. In comparison, Sasco-Sprague-Dawley rats were primarily associated with a unilateral projection to the ventral horn with only a light input to the dorsal horn (Clark and Proudfit, 1989). Hence, the differences reported in the projection of locus coeruleus noradrenergic neurons

may in part be a result of variation in the neural circuitry of the substrains of rat employed.

In summary, the locus coeruleus in cat, rat and primate supplies a noradrenergic projection, originating in the ventral and caudal regions of the nucleus, which terminates primarily in the ventral horn, intermediate zone and lamina X. There is however conflicting evidence on the projection of the locus coeruleus to the dorsal horn.

A7 cell group

The A7 group of pontine noradrenergic neurons includes neurons within the nucleus subcoeruleus, the medial and lateral parabrachial nucleus and the Kölliker-Fuse nucleus (Dahlström & Fuxe, 1964). Retrograde transport of HRP injected to the spinal cord in rat (Basbaum & Fields, 1979), cat (Kuypers & Maisky, 1975; Basbaum & Fields, 1979) and primate (Hancock & Fougerousse, 1976) reveals cells within the A7 group. Further, the retrograde transport of an antibody to D-βH (Westlund et al, 1981, 1983, 1984) and double labelling immunohistochemical studies (Blessing et al, 1991; Fritschy & Grzanna, 1990; Westlund et al 1981, 1983, 1984) have identified these cells as noradrenergic.

In quantitative investigations the A7 group has been shown to provide the majority of spinally projecting noradrenergic neurons in both rat (82%; Westlund et al, 1983) and primate (79%; Westlund et al, 1984). Within the A7 group there is some variation in the contribution from each of the subnuclei to the descending projection with, in the primate, the subcoeruleus contributing 50% of spinally projecting noradrenergic neurons, the Kölliker-Fuse nucleus 8% and the parabrachial nuclei 1%

(Westlund et al, 1984). Similar results have been obtained in the rat where the subcoeruleus, Kölliker-Fuse and parabrachial nuclei contribute 45%, 5% and 1% of descending noradrenergic cells (Westlund et al, 1983). In studies of the cat however the contributions to the descending noradrenergic projection from the brain stem nuclei have been shown to be significantly different with the principal descending noradrenergic axons arising from the Kölliker-Fuse nucleus and a relatively minor contribution from subcoeruleus, parabrachial nuclei and the A6 group (Stevens et al 1982, 1985).

Within the A7 group of noradrenergic neurons the subcoeruleus, Kölliker-Fuse and parabrachial nuclei project to specific laminae of the spinal cord. In the rat, anterograde tracing techniques the subcoeruleus has been shown to project to the ventral horn whilst the parabrachial and Kölliker-Fuse nuclei terminate in the superficial dorsal horn (Clark & Proudfit, 1991). Again, this finding is dependant on the strain of rat under study with opposite results obtained for an alternative strain of rat (Sluka & Westlund, 1992). In primates injection of tritiated amino acid to the A7 group produces labelling of the intermediolateral cell column, lamina I, the ventral horn and lamina X (Westlund & Coulter, 1980)

1.8 Distribution of catecholaminergic terminals in the spinal grey

The presence of catecholamines in the spinal cord has been recognised for some time following their identification in specimens taken from the spinal cord (von Euler, 1947; Vogt, 1954; Magnusson, 1973). These investigations however do not give an indication as to the origins of the catecholamines detected in these samples. Using micropunch samples from specific regions of the spinal grey, Zivin et al

(1975) established the distribution of the catecholamines was not uniform across the spinal cord. In this work, they described the concentration of noradrenaline to be greatest in the lateral horn of the thoracic spinal cord with progressively lower concentrations in the ventral then dorsal horns. Little dopamine or adrenaline was identified at the level of the cord. With the development of histofluorescence techniques such as the Falck-Hillarp method (Falck et al, 1962) catecholamine terminals could be identified in the spinal cord (Carlsson et al, 1963; Dahlström & Fuxe, 1964) and with this greater detail on the specific laminar locations of the catecholamines could be derived.

Noradrenaline in the spinal grey

Using immunoreactivity for the noradrenaline synthetic enzyme dopamine-β–hydroxylase (D-βH), noradrenergic terminals can be identified within the spinal grey of the rat (Westlund et al 1983; Proudfit & Clark, 1991; Fritschy & Grzanna, 1990), cat (Westlund et al, 1982; Doyle & Maxwell, 1991, 1993) and primate (Westlund et al,1980, 1984). In each species a similar laminar distribution has been described with terminals associated with motoneurons in the nuclei of the ventral horn (lamina IX of Rexed, 1954), in laminae I, II, IV and VI of the dorsal horn and in lamina X. Heavy labelling is also present in the region of the intermediolateral cell column (Westlund & Coulter, 1980).

1.9 Ultrastructure of catecholamine profiles in the dorsal horn

Catecholamine axons in the dorsal horn display a prominent rostrocaudal orientation with fibres running for some distance and exhibiting multiple varicosities along their length in both the rat (Dahlström & Fuxe, 1964; Fritschy & Grzanna, 1990) and cat (Doyle & Maxwell, 1991). These axons are typically associated with symmetrical synaptic specialisations (Gray type II) in association with dendrites (95% of terminals) or somata (5% of terminals). Axo-axonic synapses are not formed by catecholaminergic terminals (Hagihira et al, 1990; Doyle & Maxwell, 1991). Ultrastructural observations on tissue prepared for tyrosine hydroxylase or dopamine-β-hydroxylase immunoreactivity reveals boutons to be round or oval shaped with predominantly small, irregular, agranular vesicles and occasional dense core vesicles (Doyle & Maxwell, 1991,1993). This suggests these terminals contain not only catecholamines (agranular vesicles, Hökfelt & Ljungdahl, 1972) but may also contain neuropeptides (dense core vesicles, Merighi et al, 1989).

Adrenergic receptors in the spinal grey

A number of adrenergic receptors have been identified (α_{1a}, α_{1b}, α_{1d}, α_{2a}, α_{2b}, α_{2c}, β_1, β_2, β_3). These are G protein-coupled, transmembrane receptors and are employed in a wide variety of biological systems. Of the adrenergic receptors that have been investigated so far, it is the α subset that is of greatest significance in the spinal cord.

α_1-Adrenergic receptors

Autoradiographic investigations using tritiated prazosin in rat (Simmons & Jones, 1988) and cat (Dashwood et al, 1985) have shown α_1 binding sites throughout the spinal grey with a similar binding density in both dorsal and ventral horns. Prazosin is however a non-selective α_1 antagonist therefore, with at least three subtypes of α_1 receptor recognised, more recent investigations have been directed at gaining insight into the distribution of each subtype. This has been achieved using more selective ligands that have revealed the α_{1a} receptor subtype to be the predominant subtype expressed in the rat dorsal and ventral horns with less α_{1b} and little α_{1d} receptor detectable (Wada et al 1996). In situ hybridisation studies whilst confirming the relative densities of the α-adrenoceptor subtypes in the rat suggest the α-adrenergic receptors are predominantly localised to the motor nuclei of the ventral horn (Day et al, 1997; Domyancic & Morilak, 1997). In contrast to this picture in the rat, in situ hybridisation studies in human have identified a significantly different pattern of distribution of α_1-adrenergic receptor subtypes. Here it is the α_{1d}-adrenergic receptor mRNA which predominates with lesser densities of α_{1a} and α_{1b}-receptor mRNA (Smith et al 1999). As in the equivalent studies in the rat these investigators found no convincing evidence of mRNA for the α_1-adrenoceptor subtypes in the dorsal horn. Thus, the available evidence suggests the α_1-adrenoceptors are primarily localised to the ventral horn motor nuclei with a variation in density of each subtype that is species dependant.

α_2-Adrenergic receptors

Autoradiographic investigations have identified α_2-adrenoceptors in the spinal grey with a dense concentration in the region of the substantia gelatinosa and intermediolateral cell column and lower levels in the ventral horn in both rat (Seybold & Elde, 1984; Unnerstall, Kopajtic & Kuhar, 1984; Roudet et al, 1994) and human (Unnerstall, Kopajtic & Kuhar, 1984). Using subtype specific radioligand binding in rat tissue (Uhlen et al, 1997) and in situ hybridisation in human tissue (Smith et al, 1995) the predominant subtype has been identified as the α_{2a}-receptor subtype with little α_{2c} binding and intermediate levels of α_{2b} binding. The availability of specific antibodies to the α_2-adrenergic receptor subtypes has allowed the general observations on the distributions of these receptors to be refined to give detail on which structures within the spinal cord express each subtype. This work has shown α_{2a}-receptor-immunoreactivity in the superficial dorsal horn of the rat (Rosin et al, 1993) in particular on the terminals of substance-P containing primary afferent fibres (Stone et al, 1998). The α_{2C}-receptor is also present in the superficial dorsal horn however this subtype is associated with intrinsic dorsal horn neurons and not found on primary afferent terminals (Stone et al, 1998). In addition, a significant α_{2C}-receptor-immunoreactivity is associated with motor neurons in the ventral horn (Rosin et al, 1996).

1.10 Actions of the catecholamines in the dorsal horn

Effects of brainstem stimulation

Since the early observation that intrathecal injection of adrenaline produced an analgesic response in cats (Weber, 1904) it has been widely recognised that the catecholamines, in particular noradrenaline,

may have a role in antinociception though the mechanism by which the catecholamines produce this anti-nociceptive response remains unclear. At the level of entry of the primary afferent fibres to the spinal dorsal horn no noradrenergic somata have been identified though a dense noradrenergic innervation derived from several brainstem nuclei does exist. In addition, within the dorsal horn specific noradrenergic receptor subtypes have been identified. It is therefore likely that catecholamine induced analgesia involves the interaction of the descending catecholaminergic projection with adrenergic receptors on the nociceptive pathways located in the dorsal horn.

Activation of noradrenergic brainstem nuclei produces a detectable increase in noradrenaline in superficial horn dialysates and cerebrospinal superfusates (Hammond, Tyce & Yaksh, 1985; Abhold & Bowker, 1990). The responses to stimulation of noradrenergic brainstem nuclei however are complex and not uniform between species as may be expected. Locus coeruleus stimulation in the rat produces both behavioural (Segal & Sandberg, 1977; Jones & Gebhart, 1986; West, Yeomans & Proudfit, 1993; Zhang & Zhao, 1994) and electrophysiological (Liu & Zhao, 1992; Zhang & Zhao, 1994) evidence of a reduced nociceptive response. This has been variably attributed to an α_2-adrenoceptor mediated action. In some series the behavioural response was found to be attenuated by specific α_2-antagonists (Jones & Gebhart, 1986; Zhang & Zhao, 1994) whilst in others the efficacy of the α_2-antagonists was dependant on the strain of rat under study (West, Yeomans & Proudfit, 1993). Proudfit and colleagues, elaborating their observations on the differences between two stains of rat in their noradrenergic projections, identified an attenuation of the antinociceptive response to locus

coeruleus stimulation in Harlan-Sprague-Dawley rats when a specific α_2-antagonist was administered which was absent in Sasco-Sprague-Dawley rats (West, Yeomans & Proudfit, 1993). As has been described the locus coeruleus in Harlan-Sprague-Dawley rats projects to the dorsal horn whilst in Sasco-Sprague-Dawley the locus coeruleus projects mainly to the ventral horn (Proudfit & Clark,1991). Observations based on the whole animal's response to a given manipulation are complicated in that not only the sensory response but also motor and autonomic responses are produced all of which interact to give the final reflex. As an example in Wistar rats the antinociceptive motor response of locus coeruleus stimulation is antagonised by the α_2-antagonist yohimbine whilst the response of dorsal horn nociceptive neurons is not (Zhang & Zhao, 1994). Thus the nociceptive reflex of the intact animal to an unpleasant stimulus relies not only on the effects of noradrenaline in the dorsal horn but also in the ventral horn which is at least in part α_2-receptor dependant.

Stimulation of noradrenergic nuclei outside the locus coeruleus in the rat is also associated with an analgesic response. Thus, stimulation in the region of the ventrolateral pontine tegmentum produces a suppression of the thermally induced tail-flick response (Miller & Proudfit, 1990) and reduces the responses of dorsal horn neurons to a nociceptive stimulus (Liu & Zhao, 1992). Both these responses are α_2-adrenergic receptor dependant. In addition, the response of dorsal horn nociceptive neurons to a noxious chemical stimulus (formalin injection to the hindpaw) is reduced on lateral reticular nucleus stimulation, an effect that is mediated at least in part by α_2-adrenergic receptors (Liu & Zhao, 1992; Zhao, Liu & Zhao, 1993).

In the cat, as noted for the rat, stimulation of specific brainstem regions can influence the responses of dorsal horn neurons to a nociceptive stimulus in addition to the behavioural response of the animal. For example, in response to locus coeruleus stimulation both behavioural and electrophysiological responses to a nociceptive stimulus are reduced (Hodge et al, 1983; Zhao & Duggan, 1988). Interestingly this effect was not found to be dependant on noradrenaline as depletion of spinal noradrenaline did not significantly diminish the effect of locus coeruleus stimulation (Hodge et al, 1983) nor did α_2-antagonists inhibit the effect on dorsal horn cells in either rat (Liu & Zhao, 1992; Zhang &Zhao, 1994) or cat (Zhao & Duggan, 1988). Whilst the vast majority of descending locus coeruleus cells in rat (Westlund et al, 1983) and the majority in cat (Stevens et al 1982) contain noradrenaline there is evidence of alternative neurotransmitters within the noradrenergic brainstem nuclei which may be acting as an independent projection or as co-transmitters with the noradrenaline. Within the locus coeruleus, neurons containing enkephalin (Charney et al, 1982), galanin (Holets et al, 1988) and Neuropeptide Y (Holets et al 1988) have been identified and it is entirely feasible that these neurotransmitters may be involved in antinociception at the level of the dorsal horn in addition to noradrenaline.

Stimulation of brainstem noradrenergic centres other than the locus coeruleus in cats is associated primarily with an inhibitory effect on dorsal horn neurons and nociceptive reflexes. Kölliker-Fuse nucleus stimulation for example is associated with a reduction in the response of nociceptive dorsal horn neurons to a noxious stimulus which is noradrenaline dependant (Hodge, Apkarian & Stevens, 1986). Unlike

the situation following locus coeruleus stimulation Kölliker-Fuse nucleus stimulation has also been reported to produce inhibition of non-nociceptive neurons in the dorsal horn (Zhao & Duggan, 1988). As noted previously the Kölliker-Fuse nucleus is the main source of descending noradrenergic neurons to the spinal cord in the cat (Stevens, Hodge & Apkarian, 1982).

Recently the expression of the proto-oncogene *c-fos* has been employed in the investigation of descending projections on nociceptive neurons in the dorsal horn. Using this technique, an increased number of *c-fos*-immunoreactive neurons are noted in the ipsilateral dorsal horn in response to a noxious stimulus on lesioning the dorsolateral funiculus than when the funiculus is intact (Liu et al, 1997, 1999). These results are in support of a descending influence on nociceptive processing in the dorsal horn.

Effects of noradrenaline

Though the stimulation of brainstem noradrenergic nuclei results in a detectable rise in noradrenaline at the level of the dorsal horn part of the response may arise following the release of alternative neurotransmitters either co-released with noradrenaline or via a separate descending projection. It is therefore of value in the study of the actions of the noradrenergic system in nociceptive pathways to study noradrenaline and the noradrenergic agonists/antagonists independently of brainstem stimulation.

When iontophoresed directly around dorsal horn neurons noradrenaline produces a reduction in spontaneous firing and a modality specific, dose dependant reduction in stimulus induced firing

of nociceptive cells in the rat (Reddy & Yaksh, 1980; Miller & Williams, 1989) and cat (Belcher, Ryall & Schaffner, 1978; Headley, Duggan & Griersmith, 1978; Reddy & Yaksh, 1980). In addition to this inhibition of nociceptive cells there have been occasional reports of a less selective effect on cells within lamina III/IV with both nociceptive and non-nociceptive responses being inhibited (Headley, Duggan & Griersmith, 1978). Noradrenergic agonists increase the nociceptive threshold in behavioural experiments (Gorlitz & Frey, 1972; Reddy & Yaksh, 1980;Sagen & Proudfit, 1984). This effect directly correlates with the affinity of the agonist used for α-adrenergic receptors, specifically α_2-adrenergic receptors (Reddy & Yaksh, 1980; Sagen & Proudfit, 1984; Yaksh, 1985) with β-adrenergic agonists having no effect (Reddy & Yaksh, 1980). Additional evidence of the significance of α_2-adrenergic receptors to noradrenaline's action in the dorsal horn follows from the observation that specific adrenergic receptors antagonists inhibit the anti-nociceptive response of either noradrenaline or noradrenergic agonists with an effect directly correlated to α_2-adrenergic receptor affinity (Proudfit & Hammond, 1981; Howe et al, 1983; Sagen & Proudfit, 1984; Peng, Liu & Willis, 1996). The potential therapeutic benefits of this antinociceptive action at the α_2-adrenergic receptor has recently been explored using epidural clonidine as an a analgesice agent (Eisenach, Detweiler & Hood, 1993; Eisenach, DeKock & Klimscha, 1996).

1.11 Morphological basis for the actions of the monoamines in the dorsal horn

From the preceding discussion it can be appreciated that the descending monoaminergic pathways have a selective anti-nociceptive action within the spinal cord dorsal horn. For each of the monoamines implicated in this intrinsic anti-nociceptive system there has been a considerable volume of research directed at the behavioural and electrophysiological characteristics of the response. Data has therefore been gathered with reference to the behavioural and electrophysiological responses to stimulation of the descending tracts in addition to the effects of individual transmitters and their specific receptor subtypes on dorsal horn neurons and ascending tract fibres. However, despite extensive investigation the neural circuitry underlying these responses remains poorly understood.

Whilst innumerable possible pathways could be considered, three main circuits involving the monoamines within the dorsal horn can be outlined. Thus, the monoamines may operate either via direct pre-synaptic inhibition on the primary afferent terminals or via direct post-synaptic inhibition on the ascending tract cells or indirectly via local dorsal horn interneurons which may in turn operate pre- and/or post-synaptically (figure 1.1).

Direct pre-synaptic inhibition (figure 1.1A)

There have been numerous studies of the monoamines in the dorsal horn with no convincing evidence that either catecholaminergic or serotonergic terminals form axo-axonic synapses (the structural correlate of classical pre-synaptic inhibition) within the superficial dorsal horn. However, the monoamines have been shown to influence

the electrophysiological properties of primary afferent fibres as evidenced by an increase in the antidromic threshold or the observation of primary afferent depolarisation. Thus, though a classical direct pre-synaptic connection is unlikely, an alternative mechanism is implied. One consideration is that the monoamines may act via an intervening dorsal horn interneuron, a possibility that will be discussed below. A second is that the monoamines may operate via non-synaptic transmission. In support of this hypothesis the distribution of monoamine terminals places their highest concentration in the superficial dorsal horn and deeper laminae IV-VI, ideally suited to bathe the nociceptive primary afferent terminals located in these regions. In addition, there is evidence that primary afferent fibres express catecholaminergic and serotonergic receptors. It is therefore conceivable that a non-synaptic influence of monoamines on primary afferent terminals could contribute to the antinociceptive actions of the monoamines. Within the current limitations of experimental design however there is insufficient data to be able to confidently evaluate the significance of this pathway.

Direct post-synaptic inhibition (figure 1.1B)

In contrast to the morphological evidence regarding monoaminergic involvement in axo-axonic synapses, axo-somatic and axo-dendritic synapse have been repeatedly observed for both the catecholamines and serotonin in the dorsal horn. In some instances contacts from monoaminergic terminals on identified projection neurons have also been demonstrated. Thus contacts between serotonergic terminals and cells of the dorsal spinocerebellar tract (Jankowska et al, 1995; Maxwell & Jankowska, 1996), postsynaptic dorsal column (Nishikawa et al, 1983), spinomesencephalic tract (Hylden et al, 1986) and

spinothalamic tract (Hylden et al, 1986) have been identified. Contacts between catecholaminergic terminals and cells of the postsynaptic dorsal column (Doyle & Maxwell, 1993) and spinothalamic tract (Westlund et al, 1990) have also been identified. As has been discussed the predominant action of the descending monoaminergic systems in the dorsal horn is inhibition of nociceptive processing. In this case it would be hypothesised that the monoamines act to produce post-synaptic inhibition. This correlates with the presence of α_{2c}-adrenergic and $5HT_{1A}$ receptor suptypes which are concentrated in the dorsal horn.

Indirect action through local dorsal horn neurons (figure 1.1C)

In addition to a direct action either on the primary afferent terminal or the projection neuron, monoamines may also operate via an intervening local dorsal horn neuron (or neurons). In turn these neurons may then operate pre- and/or post-synaptically to influence the transmission of information from primary afferent to projection neuron. This association of serotonergic terminals with intrinsic dorsal horn neurons has been explored in a number of studies. Militec et al (1984) examined the relationship of serotonergic contacts with cells in the superficial laminae of the cat identified by electrophysiological responses and morphological characteristics. Here they found that cells in lamina II, identified as stalked cells by the morphological description of Gobel (1978), received the greatest number of serotonergic contacts whilst marginal and islet cells received relatively few. Serotonin therefore would appear to form contacts predominantly, but not exclusively, with excitatory neurons in the superficial dorsal horn. One possibility therefore in considering the interactions of serotonin with intrinsic dorsal horn neurons to produce inhibition would

be for serotonin to excite inhibitory neurons which in turn may act pre- and/or post-synaptically to produce inhibition. It is proposed that this pathway might operate via the excitatory 5-HT_3 receptor.

Outwith these ultrastructural observations on the monoamines in the dorsal horn limited information is available regarding the dorsal horn cells which may receive monoamine contacts, in particular of their neurotransmitters and hence their possible role in sensory processing. Immunocytochemical observations have shown a limited number of contacts between serotonin terminals and enkephalin- (Glazer & Basbaum, 1976; Miller & Salvatierra, 1998) and neurotensin-immunoreactive (Miller & Salvatierra, 1998) neurons in the superficial dorsal horn. Beyond these reports however, little is known of the cells contacted by the monoamines.

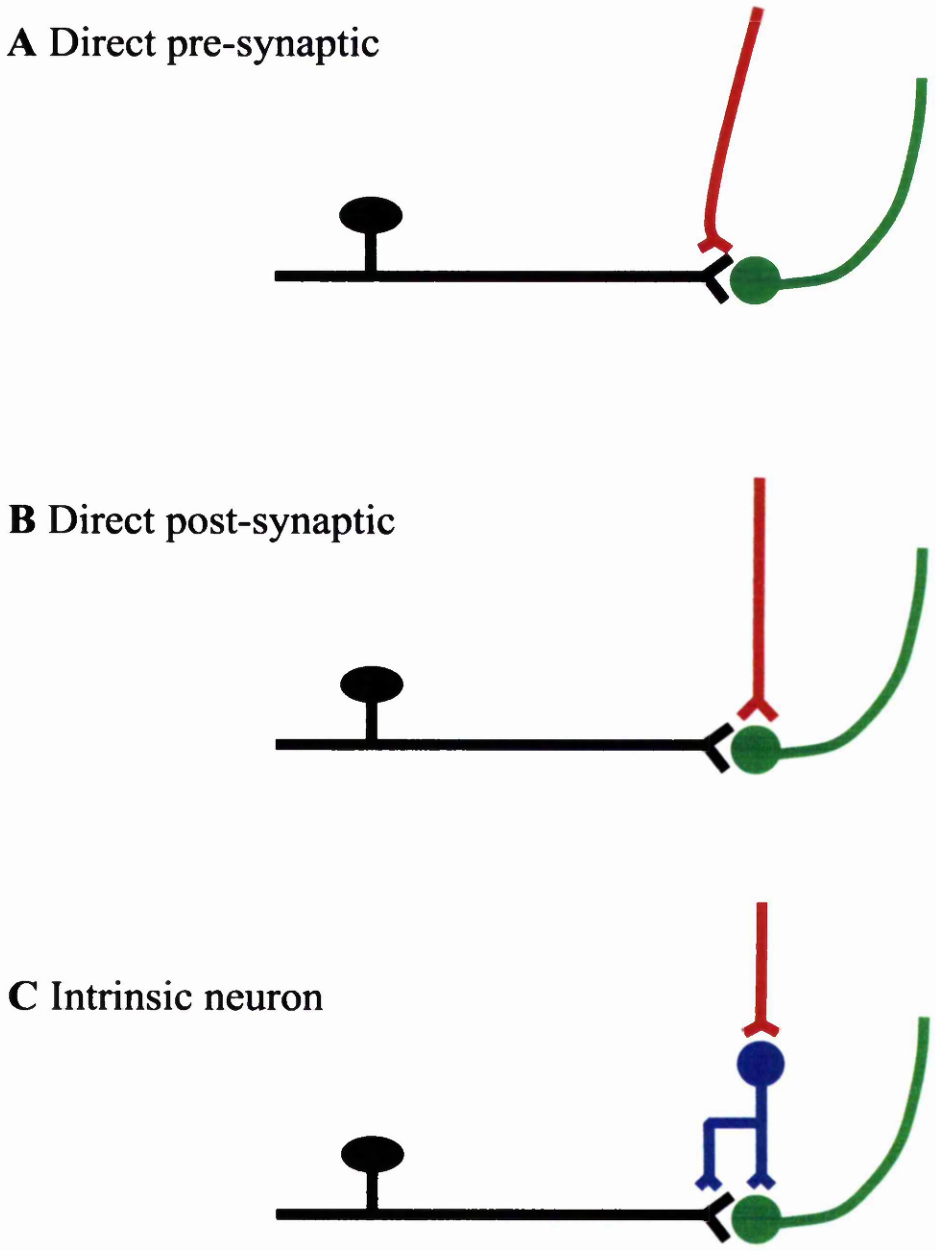


Figure 1.1. The descending monoamines may operate through a number of circuits to produce antinociception in the dorsal horn such as through direct pre-synaptic (A) or post-synaptic (B) contacts or via an indirect action through intrinsic dorsal horn neurons which may in turn act either pre- or post-synaptically (C).

1.12 Neuronal subtypes within the dorsal horn

As has been stated the monoaminergic system may operate either directly or indirectly to influence the transmission of information through the dorsal horn which is likely to be at least partly dependant on local dorsal horn neurons. To gain further insight into the actions of the catecholaminergic and serotonergic systems within the spinal dorsal horn knowledge of the neurons which may be targeted is of value.

γ-Aminobutyric acid (GABA)

Within the central nervous system GABA has a major role as an inhibitory neurotransmitter. Investigations using antibody to either the GABA synthetic enzyme glutamic acid decarboxylase (GAD) or to GABA have revealed numerous immunoreactive profiles within the dorsal horn. Typically GAD-immunoreactive somata are small to medium sized and located in the superficial dorsal laminae I-III, increasing in size in the deeper dorsal laminae (Barber, Vaughn & Roberts, 1982). Similarly, GABA-immunoreactive somata are present throughout the spinal grey, with the exception of lamina IX, with the greatest concentration of somata in the superficial dorsal horn (Carlton & Hayes, 1990). It has been estimated that 28-43% of neurons in laminae I-III of the rat dorsal are GABAergic with the proportion increasing through lamina I to III (Todd & McKenzie, 1989; Todd & Sullivan, 1990). In Golgi stained sections these cells typically have a dendritic architecture in an approximately rostrocaudal orientation within the lamina or with extension into adjacent laminae (Todd & Mckenzie, 1989; Powell & Todd, 1992). Hence, GABA-immunoreactive neurons in the dorsal horn have an appearance typical of islet cells as described by Gobel (1978). In addition to the somatic labelling noted

in GABA prepared material a significant punctate staining within the superficial laminae I-III is present which is less prominent in deeper laminae (Carlton & Hayes, 1990). These profiles correspond to GABAergic terminals which in ultrastructural studies have been described as round with clear synaptic vesicles and occasional dense core vesicles. These studies have noted GABAergic terminals forming the pre-synaptic components of both axodendritic and axosomatic synapses with dorsal horn neurons in addition to forming axoaxonic synapses with unmyelinated primary afferent terminals (Barber et al 1978; Todd & Lochead, 1990). Lamina II GABA-immunoreactive dendrites have also been identified as the pre-synaptic component at dendrodendritic synapses (Mitchell, Spike & Todd, 1993). Synaptic associations between GABAergic profiles and certain projection neurons such as the spinothalamic tract (Carlton et al, 1992; Lekan & Carlton, 1995) and postsynaptic dorsal column cells (Maxwell, Todd & Kerr, 1995) have in addition been reported. Whilst the great majority of GABAergic terminals within the superficial dorsal horn originate from local GABAergic neurons, some terminals arise within the brainstem and represent a descending inhibitory projection from this region (Millhorn et al, 1987; Antal et al, 1996). From this evidence therefore GABAergic neurons display the morphological requirements for a neuron that can act both pre- and post-synaptically within the dorsal horn to influence sensory processing.

GABA has been repeatedly identified as having a role in the inhibition of nociceptive processing at the level of the spinal dorsal horn. Administration of GABA antagonists produces both an increased background activity and stimulus response of nociceptive dorsal horn neurons which may be mediated via the $GABA_A$ receptor subtype

(Peng, Lin & Willis, 1996). Furthermore, in behavioural studies the administration of GABAergic antagonists (Hao, Xu, & Weisenfield-Hallin, 1994), or experimental manoeuvres producing a loss of GABA in the dorsal horn, precipitate an altered perception of non-nociceptive stimuli as nociceptive (allodynia; Ibuki et al, 1997). Inhibition of the responses of nociceptive projection neurons, such as the cells of the spinothalamic tract, to an appropriate stimulus has also been documented for GABA (Lin, Peng & Willis,1996b,c). As noted previously, stimulation in the region of the nucleus raphe magnus can result in the phenomenon of primary afferent depolarisation (Proudfit, Larson & Anderson, 1980; Lovick, 1983). To permit this there must either be a direct or indirect communication between descending tract neurons and primary afferent terminals. The involvement of GABAergic terminals in axo-axonic synapses on primary afferent axons (Todd & Lochead, 1992) and the presence of GABAergic receptors on primary afferent terminals (Désarmenien et al, 1984) therefore would provide the circuitry required for this inhibition. In support of this association the dorsal root potential is reduced (Thompson & Wall, 1996) and primary afferent depolarisation following nucleus raphe magnus stimulation is partly blocked (Lovick, 1983) by the application of GABAergic antagonists.

The identification of GABA as the major inhibitory neurotransmitter in the spinal cord has facilitated the characterisation of neurons under investigation in the dorsal horn as putative inhibitory or excitatory neurons based on their immunoreactivity for GABA. In a series of experiments Todd and co-workers have identified a number of transmitters which co-localise with GABA. Thus dorsal horn neurons containing glycine in lamina I-III (Todd & Sullivan, 1990; Todd, 1991;

Powell & Todd, 1992), choline acetyltransferase in lamina III (Todd, 1991), nitric oxide synthase (Spike, Todd & Johnston, 1993; Laing et al, 1994), neuropeptide Y (Rowan, Todd & Spike, 1993), some enkephalinergic neurons (Todd et al, 1992), the somatostatin$_{2A}$ receptor (Todd, Spike & Polgár, 1993) and the majority of parvalbumin neurons in lamina II and III (Laing et al, 1994), contain GABA. GABA is not present in neurons which contain neurotensin (Todd, Russell & Spike, 1992) or somatostatin (Todd & Spike, 1993) and those that express either the neurokinin-1 receptor (Todd, Spike & Polgár, 1998) or the μ-opioid receptor, MOR-1 (Kemp et al, 1996).

Glycine

The neurotransmitter glycine, with GABA, contributes a significant inhibitory influence on dorsal horn processing. Glycinergic neurons have been identified within the dorsal horn, in particular in the laminae deep to lamina II (Ottersen & Storm-Mathisen, 1987; Todd, 1990). As noted above the glycinergic neurons in the superficial laminae of the dorsal horn colocalise GABA and include a subpopulation of islet cells (Todd & Sullivan, 1990; Todd, 1991; Powell & Todd, 1992). Within laminae I,II and III glycinergic neurons comprise approximately 9, 14 and 30% of the neurons in each lamina respectively (Todd & Sullivan, 1990). The relationship of this transmitter to the transmitters which also colocalise with GABA reveals a subdivision of inhibitory neurons based on which transmitter or transmitters are colocalised with GABA. Thus it has been demonstrated that glycine containing neurons in the superficial dorsal horn often colocalise nitric oxide synthase (Spike, Todd & Johnston, 1993) but do not colocalise choline acetyltransferase (Todd, 1991; Spike, Todd & Johnston, 1993), met-enkephalin (Todd et al, 1992) or neuropeptide Y (Todd & Spike, 1993).

Punctate staining corresponding to glycinergic axonal profiles can also be identified within the dorsal horn particularly deep to laminae I and II. These terminals have been shown to form the presynaptic component at axodendritic and axosomatic synapses. In addition, these glycinergic terminals form axo-axonic synapses where the post-synaptic component has the characteristics of a primary afferent terminal (Todd, 1990; Todd & Sullivan, 1990; Spike et al, 1997). Functionally glycine has been implicated in the inhibition of nociceptive processing with an increased nociceptive response when glycine antagonists are applied (Peng, Lin & Willis, 1996) and inhibition of spinothalamic tract cells in response to brainstem stimulation that is partly glycine dependant (Lin, Peng & Willis, 1996b,c).

Nitric oxide synthase

Nitric oxide synthase (NOS) serves to catalyse the formation of the neurotransmitter nitric oxide. In addition, antibody to reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase has the same immunoreactive distribution as NOS (Bredt et al, 1991: Laing et al, 1994). Immunoreactivity for NOS or NADPH diaphorase in the spinal cord reveals neurons throughout the dorsal horn with the highest density in laminae II and III and a concentration of punctate staining in lamina II (Bredt et al, 1991; Valtschanoff, Weinberg & Rustioni, 1992; Spike, Todd & Johnston, 1993; Laing et al, 1994). Typically, the neurons identified within the superficial dorsal horn have dendrites orientated in a rostrocaudal direction with occasional recurrent branches (Spike, Todd & Johnston, 1993). Combined immunocytochemical studies have demonstrated that the majority of these neurons co-localise GABA or glycine and GABA and hence they

are presumed inhibitory with the proportion of cells co-localising GABA only increasing in deeper lamina III (Spike, Todd & Johnston, 1993). This study also demonstrated the lamina III choline acetyl transferase neurons co-localise NADPH diaphorase. Nitric oxide is implicated in the modulation of nociceptive information at the level of the dorsal horn with its release at this site implicated in the generation of hyperalgesia and allodynia (Wu et al, 1998).

Parvalbumin

Neurons immunoreactive for the calcium binding protein parvalbumin are present in the superficial dorsal horn distributed throughout lamina II and III (Yamamoto, et al, 1989; Yoshida et al, 1990; Antal et al, 1991; Laing et al, 1994). The great majority of parvalbumin-containing neurons also contain GABA and glycine, and these are therefore presumably inhibitory (Antal et al, 1991; Laing et al, 1994).

Somatostatin and the somatostatin sst_{2a} receptor

Neurons immunoreactive for the neuropeptide somatostatin are predominantly found in lamina II of the superficial dorsal with occasional cells in the deeper dorsal horn (Hunt et al, 1981; Todd & Spike, 1992). These cells do not co-localise GABA and hence are presumed to be excitatory dorsal horn neurons (Todd and Spike, 1993). Several different somatostatin receptors have been identified, of which one, the sst_{2a} receptor, has been identified in the dorsal horn of the rat with immunoreactive neuronal bodies and punctate staining within lamina I and II (Schindler et al, 1997; Todd, Spike & Polgár, 1998). The sst_{2a}-immunoreactive neurons within this region are GABAergic with many also co-localising glycine (Todd, Spike & Polgár, 1998). Hence, it is presumed that this receptor is expressed on

inhibitory neurons in the dorsal horn. In support of a role in nociceptive processing, somatostatin has been shown to have an analgesic action in humans (Sicuteri et al, 1984; Williams et al, 1987). In addition, following its administration a reduction in *c-fos* expression in the trigeminal subnucleus caudalis has been shown in response to a noxious stimulus in the rat (Bereiter, 1997).

μ-opioid receptor (MOR1)

Immunoreactivity for the μ-opioid receptor (MOR1) is typically identified as a dense band of immunostaining within lamina I and II of the dorsal horn in which small neurons, with dendrites orientated in an approximately rostrocaudal direction, are found spanning the border of lamina II with lamina III (Arvidsson et al, 1995; Kemp et al, 1996; Gong et al, 1997; Zhang et al, 1998). Most of these cells are not GABA-immunoreactive and are hence presumed to be excitatory (Kemp et al, 1996). Opioid agonists are recognised as having potent analgesic properties (Martin, 1983) including depression of nociceptive processing in the dorsal horn (Yaksh & Rudy, 1978; Le Bars et al, 1979) and a reduction in *c-fos* expression in the trigeminal circuitry following a noxious stimulus (Bereiter, 1997).

Neurotensin

The response to a noxious stimulus is diminished following the intrathecal application of the peptide neurotensin (Yaksh et al, 1982). Immunoreactivity for this peptide has been described in the superficial dorsal horn with a plexus of fibres in the lamina I/II region and immunoreactive somata deeper in ventral lamina II/III region (Hunt et al, 1981; Seybold & Elde, 1982; Miller & Salvatierra, 1998). These

neurotensin-immunoreactive neurons do not co-localise GABA and hence are regarded as excitatory dorsal horn neurons.

The Neurokinin-1 receptor

The neurokinin-1 receptor serves as the main receptor subtype for the tachykinin peptide substance P (Hershey & Krause, 1990) which is implicated in the processing of nociceptive information at the level of the spinal dorsal horn (Henry, 1976; De Koninck & Henry, 1991; Liu & Sandkuhler, 1995). Immunocytochemical studies have revealed substance P immunoreactivity in small diameter sensory afferents terminating in the region of lamina I/II with a cluster in lamina V and lower density in the remaining dorsal horn (Cuello & Kanazawa, 1978; Ruda, Bennet & Dubner, 1986). Combined electrophysiology and immunocytochemistry has shown these terminals form synaptic associations with nociceptive neurons in the dorsal horn (Ma et al, 1996,1997). Immunoreactivity for the NK-1 receptor has been identified in the dorsal horn with the greatest density of staining in lamina I, light staining in lamina II and intermediate staining in the deeper laminae (Nakaya et al, 1994; Bleazard, Hillan & Morris, 1995; Littlewood et al 1995). Within these regions distinct groups of NK-1 receptor-immunoreactive neurons can be identified located in lamina I with a further population of large neurons located in lamina III/IV which have prominent, dorsally orientated dendrites extending into the more superficial laminae. Very few of these neurons have been found to be GABA-immunoreactive hence they are presumed to be excitatory (Littlewood et al, 1995). There is accumulating evidence that the vast majority of NK-1 receptor positive neurons in lamina I and lamina III/IV represent a population of projection tract neurons to brainstem regions associated with nociceptive processing (Yu et al, 1999; Marshall et al,

1996; Todd, McGill & Shehab, 2000). The neurons described in lamina III/IV have also been shown to receive a selective innervation from substance P containing primary afferent terminals on their proximal and distal dendrites (Naim et al, 1997) with little input from myelinated or non-peptidergic terminals (Naim, Shehab & Todd, 1998; Sakamoto, Spike & Todd, 1999). These two groups of neurons may have differing roles in the processing of nociceptive information in the dorsal horn with lamina I neurons coding for the stimulus intensity whilst lamina III/IV neurons may have a role in the detection of particular nociceptive stimuli (Doyle & Hunt, 1999).

1.13 Objectives of the study

Monoamines in the dorsal horn

As described, little information is available about the neuronal circuitry involving monoaminergic axons and the neurons of the dorsal horn. Previous investigations have identified synaptic contacts between monoaminergic terminals and morphologically identified dorsal horn cells or ascending projection neurons. However the exact nature of the cells contacted, in particular their neurotransmitter substances, has not been clearly identified. Clearly, further information as to the circuitry and transmitters involved in these pathways would be valuable for an improved understanding of monoamine-induced antinociception and the adaptation of and/or development of targeted analgesics. In the following series of experiments the association of monoaminergic terminals with identified dorsal horn cells is examined using a variety of immunocytochemical, imaging and analysis techniques in order that a better understanding of the monoamines and their connections in the dorsal horn might be achieved.

1.14 General Methods

Experimental animals

Adult female Albino-Swiss rats were used throughout these studies. These were obtained immediately prior to perfusion from the colony of The Laboratory of Human Anatomy, University of Glasgow. To minimise the influence of tissue lipofuchsin, in experiments prepared for fluorescence microscopy rats aged less than six months were used. All other animals used were aged between six and twelve months with an average weight of 250g.

Perfusion

Immediately following removal from the colony the animals were deeply anaesthetised by intraperitoneal injection of 1ml of a solution of sodium pentobarbitone (60mg ml^{-1}). Following confirmation of adequate anaesthesia by the loss of paw withdrawal to a painful stimulus (forceps crush), and before cessation of respiration, the thoracic cage was exposed through an anterior incision and a window cut in the thoracic cage. The left ventricle was then located, cannulated and a rinsing solution of warmed mammalian Ringer perfused with the right atrium then opened to allow the perfusate to flow freely. On confirmation of an adequate cannula position and free flow of Ringer the perfusing solution was switched to a solution of warmed fixative which had been prepared immediately prior to perfusion and a total of 1000ml of fixative was perfused under pressure. The optimum perfusion conditions identified in these experiments were such that fixative was introduced to the animal's circulatory system with the minimal possible delay from the onset of dissection (ideally less than one minute). In addition brisk fasciculation of skeletal muscles on introduction of the fixative and adequate

clearing of blood from the liver were regarded as confirmation of fixative perfusion and clearing of the animal respectively. If any of these features was not observed then the fixation of the animal was likely to be suboptimal and the experiment was therefore abandoned at this point and another animal selected for perfusion.

Dissection

Following perfusion the vertebral column was exposed via a posterior skin incision and a dorsal laminectomy performed using blunt ended rongeurs thus revealing the spinal cord with intact dura from sacral to lower thoracic segments. The dura was then carefully opened along its length and individual spinal roots separated, lifted free from the exposed spinal cord and identified with reference to an atlas of rat spinal cord anatomy. The lumbar enlargement was then identified and lumbar segments L3 and L4 removed and placed in a solution of the same fixative as used in the perfusion for a period of postfixation of either 4 or 24 hours depending on the requirements of the antibodies under investigation

Sectioning

Following postfixation the blocks were washed in phosphate buffered saline prior to sectioning into 50μm transverse or parasagital sections on a vibrating microtome (Vibratome, Oxford Instruments). In experiments using transverse sections of spinal cord the sections were now ready to be processed. Where the protocol required that parasagital sections be used each block was first bisected through the dorsal median fissure in the midsagital plane. The two hemi-blocks then obtained were mounted on the cut mid-sagittal face, sectioned through their entire extent from lateral to medial and gathered in a

Petrie dish containing PBS. These sections were then viewed using a binocular dissecting microscope and those containing the substantia gelatinosa selected for further processing. This technique permitted the maximum number of sections containing superficial dorsal horn to be processed from each animal.

Processing for confocal microscopy

The sections obtained following the above processing were now placed in a solution of 50% ethanol for 30 minutes before being transferred to 10% normal donkey serum in PBS for one hour to bind free antibody sites. These two steps served to reduce the effect of background staining so improving staining quality. Following this the sections were transferred to the primary antibody solution. In each case the combinations of primary antibodies included in the primary antibody solutions were such as to permit identification of three types of immunofluorescence in each tissue section. For each of the following protocols the primary antibodies chosen were such as to include antibodies to reveal monoaminergic profiles using rat or rabbit anti-serotonin antibodies (both at 1:200 dilution) and mouse anti-dopamine β-hydroxylase (1:500) together with a third antibody specific to a particular dorsal horn neuronal subtype. Thus in any one section the relationship of the monoamine-immunoreactive profiles to dorsal horn neurones could be examined. Primary antibody solutions were prepared in phosphate buffered saline containing 1% normal donkey serum and 0.1% Triton X-100. The sections were then incubated in the primary antibody cocktail for an incubation time dependant on the antibodies under investigation and the plane of the Vibratome sections being studied. After incubation in the primary antibody solutions the sections were rinsed with PBS and transferred to solutions containing

fluorophores coupled to secondary antibodies specific to the primary antibody species such that each antibody species included in the primary antibody cocktail could be identified separately using three-colour laser scanning confocal microscopy. To permit this, secondary antibodies raised in donkey were chosen which were coupled to the fluorophores fluorescein isothiocyanate (FITC), cyanine 5.18 (Cy-5) or lissamine rhodamine (LRSC; all Jackson Immunoresearch) with each antibody diluted 1:100 in PBS containing 0.1% Triton X-100 (no blocking serum). After a 3-hour incubation in the secondary antibody solutions the sections were again rinsed, wet mounted with anti-fade medium (Vectashield, Vector Laboratories Inc.) and stored at -20°C until analysed.

Control Experiments

In each case the specificity and staining characteristics of the antibodies under investigation have been extensively described previously. As additional controls in this series of experiments, for each protocol sections were collected and processed exactly as for experimental sections however with the omission of one of the primary antibodies under study. The tissue was then processed exactly as for the experimental tissue including addition of secondary antibody and the tissue examined with the confocal microscope. In each case the control did not show any discernable specific fluorescence for the omitted antibody.

Confocal Microscopy

Material prepared for immunofluorescence studies was examined using a three colour confocal laser-scanning microscope (BioRad MRC 1024) equipped with a krypton-argon laser producing excitation

wavelengths of 488, 568, and 647nm and mounted on a Nikon Optiphot with stage motor. These wavelengths optimally excite FITC, LRSC and Cy-5 respectively hence allowing each to be identified in the sections under examination. All of the elements of this scanning apparatus are coordinated through a PC running BioRad designed software allowing the functions of the microscope to be controlled 'on-screen' via the PC. Thus, once a field of view has been selected under direct epifluorescence the microscope can then be driven via the PC. Each of the emission channels can be selected and scanned either individually, thus minimising bleedthrough, or all three channels can be scanned at the same time and assigned a separate pseudocolour to aid differentiation. In addition the stage motor can be used to step through the section in set intervals in the z-plane. These functions therefore permit a desired field to be scanned in a series of steps in the z-plane with the resultant single or multi-channel images stored for later analysis. The images can then be analysed as single optical channels or combined to give a three-colour image for each optical section. In addition the series, or stack, of images in the z-plane can be merged to provide a single image through the profile of interest. These manipulations can either be performed through the BioRad supplied software or by using a freely available software package Confocal Assistant (distributed via the internet).

Antibodies

Appendix 1 lists the antibodies used in this series of experiments, their dilutions and commercial sources.

1.15 Specific Methods

A. The relationship of the monoamines serotonin and noradrenaline to intrinsic dorsal horn neurons

Tissue from three experimental animals was prepared for each of the experimental conditions under study with the process of anaesthesia, perfusion and dissection as described in the general methods. For the purposes of these studies 4% formaldehyde in 0.1M phosphate buffer (pH 7.6) was used as the fixative solution. The mid-lumbar blocks obtained were then sectioned either in the transverse plane (for somatic labelling studies) or the parasagittal plane (for dendritic labelling studies). The sections were then incubated in 50% ethanol followed by 10% normal donkey serum prior to incubation in the primary antibody cocktails. As outlined in the general methods, each primary antibody cocktail included antibodies to serotonin and D-βH together with a third primary antibody to the specific dorsal horn neuronal subtype under investigation. In the studies where monoamine contacts on somatic profiles were the subject of the investigation either rabbit anti-GABA (1:1000), rat anti-glycine (1:5000), rat anti-neurotensin (1:1000) or rat anti-somatostatin (1:100) antibodies were included as the third antibody in the cocktail. To provide more extensive dendritic labelling a second series of experiments were performed using either goat anti ChAT (1:100), rabbit anti-MOR1 (1:1000) or sheep anti-NOS (1:2000). All primary antibody dilutions were in PBS with 0.3% Triton X-100 and 1% normal donkey serum (bovine serum albumin with GABA and Glycine antibodies). Following a 48-hour incubation at room temperature the sections were rinsed then transferred to solutions containing species specific secondary antibodies coupled to fluorophores for a period of 3 to 4 hours. The

sections were then rinsed, wet mounted with anti-fade medium and stored at -20°C until analysed

Scanning and Analysis

Somatic labelling

Under direct epifluorescence five sections through the dorsal horn from each animal were examined in their entirety from medial to lateral extent and all immunoreactive somatic profiles identified. As they were identified series of images taken sequentially for each fluorophore were recorded through the immunoreactive profile with an oil immersion objective lens of X60 at an optical zoom of X1 at intervals of 0.5μm in the z-plane. This process was repeated for all immunofluorescent somatic profiles that could be clearly identified within each dorsal horn. The individual images for each optical section were then examined and the presence or absence of monoamine contacts on immunoreactive somata recorded. Throughout this series of experiments a contact between two immunofluorescent channels was regarded as being present when the two colour channels were in direct apposition with no pixel of separation between them.

Somatic and dendritic labelling

Where the antibody under investigation permitted more extensive dendritic and somatic labelling parasagittal vibratome sections were used. From these four immunoreactive cells from each animal were selected at random, scanned and analysed. Using a x40 oil immersion objective lens with an optical zoom of 1.5, single sequential images were collected for each of the fluorescent channels at 1μm intervals in the z-plane through the entire immunoreactive profile within the optical field of view. This process was then repeated for overlapping fields

until the complete immunoreactive profile within the 50μm section had been recorded.

Once gathered, the images were used to trace the outline of the immunoreactive profile and to map onto the outline any monoamine contacts. This was achieved using a 3D neuron-tracing package, NeuroLucida for Confocal (MicroBrightField, Inc), with a dedicated analysis package, NeuroExplorer. Each reconstruction was generated by first merging the three individual optical images for each individual z-interval using Confocal Assistant to provide a series of three-colour images through the cell. The stack of optical images was then imported into the NeuroLucida program where the series of images could then be scrolled through one by one. If necessary, areas of particular interest could then be magnified for closer inspection within NeuroLucida. Each immunoreactive cell was now traced and all associated monoamine-immunoreactive contacts plotted on the outline. This process was repeated for each overlapping field until the entire profile had been reconstructed. The resultant image and its associated datafile were then used to calculate the total number of contacts on each cell and to perform a modified Sholl analysis (Sholl, 1953) for monoamine contact densities calculated within a series of shells increasing in steps of 25μm radii centred on the cell body. The reconstructed images and contact density data obtained were then used to compare individual cells of a particular type and to permit different cell subtypes to be compared.

Statistical analysis

Where the monoamine contact densities were compared between two different populations of immunoreactive dorsal horn neurons the data were tested for statistical significance using the Mann-Whitney 'U'-test for non parametric data.

B. Ultrastructural analysis of monoamine contacts and inhibitory dorsal horn neurons

Adult female Albino-Swiss rats of between six and twelve months of age were anaesthetised, perfused with fixative solution containing 1% glutaraldehyde and 1% formaldehyde in phosphate buffered saline and mid-lumbar spinal cord blocks were removed and postfixed overnight. Transverse Vibratome sections (50μm thick) were then collected and placed in a 50% solution of alcohol for 30 minutes. The sections were then rinsed with PBS and excess aldehyde groups adsorbed by incubation in a 1% solution of sodium borohydride for 30 minutes. This step permits better antibody penetration in addition to reducing background staining. Following this, the sections were rinsed extensively in PBS with a minimum of 9 PBS changes over a 90 minute period. Background staining was then further reduced by a one-hour incubation in blocking serum consisting of 10% normal goat serum in PBS. Next, the sections were rinsed and the primary antibody solution added. In each case the primary antibody solutions contained antibody to serotonin (rat anti-5-HT; 1:2000) together with antibodies to mouse anti-GABA (1:500) diluted in PBS with 1% normal goat serum. Following 48hours incubation at 7°C the sections were rinsed and placed in a solution containing biotinylated anti-rat antibody (1:200) with an anti-mouse antibody coupled to 1nm gold particles (1:200) overnight. The sections were then rinsed and the gold particles intensified using a silver intensification reagent (IntenSE, Sigma Chemical Co., England). The sections were now reacted to reveal biotin binding using the avidin-biotin-peroxidase complex technique (ABC technique). This involved an overnight incubation in a fresh solution of avidin-biotin-complex (Vector Laboratories Ltd, England)

followed by a further rinse with PBS then PB. The sections were now placed in a solution containing hydrogen peroxide plus 3,3'-diaminobenzidine diluted in phosphate buffer for a period of approximately 4 minutes. During this time the reaction was monitored constantly. Following the DAB reaction, the sections were rinsed further and processed for viewing on the electron microscope.

Firstly, the sections were postfixed in 1% osmium for 30 minutes then en bloc stained using a saturated solution of uranyl acetate in 70% acetone (30 minutes) prior to dehydration through a series of acetone rinses (90% for 10 minutes; 100% for 3x10 minute rinses) ending in a 1:1 solution of acetone and Durcupan (1hour). From the acetone/Durcupan solution the sections were then transferred to freshly prepared Durcupan overnight. The sections were then flat embedded in resin between two acetate foils and polymerised at 60°C for a minimum of 24 hours. Once this was complete, the sections were examined under light microscopy and those with the optimal staining chosen for further processing. These were then attached to resin blocks, trimmed to the area of interest and serial ultrathin sections collected on Formvar coated copper grids. The sections were now ready for viewing with the electron microscope.

Electron Microscope

The sections prepared as described above were examined using a Phillips CM100 electron microscope with stage driver and goniometer attached to a frame grabber system to allow digital images to be viewed and captured by a linked PC and monitor. Also running on the PC was a package that permitted the X-Y coordinates of any point on a section to be recorded with reference to two user specified reference

points. This could then be used to drive the stage remotely and relocate any field of interest in serial sections thus allowing one point to be followed through several ultrathin sections. Images were captured on an integral camera for printing at a later stage.

C. The relationship of serotonin and noradrenaline to neurokinin-1-immunoreactive dorsal horn neurons

Methods

Seven adult female Albino-Swiss rats were anaesthetised, perfused and dissected as has been described. Following a four hour postfixation period the fourth lumbar spinal cord blocks were sectioned in the parasagittal plane and those sections which included the substantia gelatinosa were selected for further processing. These sections were then incubated in 50% ethanol and blocked with 10% normal donkey serum as in the general methods. Following this the sections were transferred to the primary antibody solution containing rabbit anti-serotonin (1:200), mouse anti-D-βH (1:500) and guinea-pig anti-NK-1 (1:1000) for a two day incubation period. The sections were then rinsed and placed in secondary antibody solution containing fluorophore conjugated antibodies for a three-hour incubation. The sections were then rinsed, wet mounted with Vectashield and stored at -20°C until required.

Scanning and analysis

Immunoreactive profiles were first identified under direct epifluorescence with a X20 objective then scanned in their entirety within the vibratome section using a X40 oil immersion objective with an optical zoom of X1.5 as in the previous protocol. Relatively few lamina III/IV NK-1-immunoreactive profiles are normally present within one vibratome section. Hence, to provide an adequate sample for analysis all lamina III/IV neurons that included the soma and dendrites within a vibratome section from each of the spinal cord sections available for analysis were scanned to permit suitable analysis to be performed. Each NK-1-immunoreactive cell was scanned in its entirety

in overlapping optical fields and the resultant image files used to reconstruct the cell and associated contacts in 3D using the NeuroLucida software. The datafiles thus generated were then used to compare the distribution densities of the monoamine contacts on the NK-1-immunoreactive cells.

1.16 Results

A. Monoamine immunoreactivity in the spinal cord

Using these experimental protocols the serotonin-immunoreactivity observed was consistent with previous reports for the rat spinal cord. Numerous serotonin-immunoreactive axons running in a rostrocaudal direction with multiple en-passant varicosities along their length were found within lamina I and outer lamina II. In the deeper laminae of the dorsal horn the serotonergic profiles were less frequent, particularly in inner lamina II, though immunoreactive profiles were still present. Occasional immunoreactive axons were seen to course out of the plexus in lamina $I/II_{o,}$ through lamina II_I and into the region of lamina III/IV forming a distinct 'basket like' arrangement around presumed neuronal somata and proximal dendrites. This arrangement is illustrated in figure 1.2 which shows the serotonin-immunoreactivity in a parasagittal section of the rat mid-lumbar superficial dorsal horn. The dense band of immunoreactivity corresponding to the region of lamina I/II_o can be appreciated with a representative 'basket-like' formation of serotonergic axons forming around a proximal dendrite and cell body of an unidentified neuron in lamina III. The highest density of immunoreactive profiles outside the dorsal horn was associated with the motor nuclei of the ventral horn and in the region of lamina X with sparse serotonin immunoreactivity in the intervening spinal grey. The penetration of the rabbit anti-serotonin antibody was such that the immunofluorescent signal was effectively uniform through the thickness of the section. However, with the rat anti-serotonin the penetration was less satisfactory with the immunofluorescent signal diminishing with increasing distance from the surface of the section beyond 10-15μm. Where possible therefore the rabbit anti-5-HT was used.

The distribution of dopamine-β-hydroxylase-immunoreactive profiles corresponded to previous descriptions. The greatest density of labelling was observed in axons and terminals within the motor nuclei of the ventral horn. Dense D-βH-immunoreactivity was also present in laminae I/II of the dorsal horn where immunoreactive axons running in a rostrocaudal direction were present, again showing multiple en-passant swellings. Relatively little D-βH-immunoreactivity was present in lamina III with intermediate levels in the deeper dorsal horn laminae IV/VI and lamina X. No serotonin or D-βH-immunoreactive somata were identified in any of the tissue sections examined during the course of these experiments.

B. The association of the monoamines with inhibitory dorsal horn neurons

Using antibodies produced specifically for use with formalin fixed material GABA- and glycine-immunoreactive profiles were revealed in a distribution corresponding to previous reports. A dense band of GABA-immunoreactivity was present in laminae I-III of the dorsal horn consisting of both punctate staining, representing GABAergic axon terminals, and somatic labelling. Occasionally an extension of the immunoreactive label to the proximal few microns of a large dendrite was noted, however no detail on dendritic architecture beyond the first few micrometres could be derived. GABA-immunoreactive somata were found throughout the superficial dorsal horn with lowest frequency in lamina I and highest in lamina III.

Glycine-immunoreactive profiles were similarly located in the superficial dorsal horn with both somatic and punctate labelling present. In general the density of labelling was less than in material prepared for GABA-immunoreactivity. This relatively low density of punctate labelling permitted somatic profiles to be more clearly identified. In a similar manner to GABA-immunoreactive somata, occasional immunoreactivity of the proximal dendrites was observed. As noted by previous investigators few glycinergic neurons were present in lamina I with the frequency of immunoreactive somata increasing in the deeper lamina, the highest density being observed in lamina III.

On no occasion was a basket like arrangement of serotonin-immunoreactive axons identified in relation to either GABA- or glycine-immunoreactive profiles. A total of 151 GABA-immunoreactive and 307 glycine-immunoreactive neurons from lamina I-III were examined for monoaminergic contacts from material prepared from three experimental animals (15 sections in total). Serotonin-immunoreactive profiles formed contacts with 24.5% (37 out of 151 neurons) of GABAergic and 27.7% (85 out of 307 neurons) of glycinergic somata within laminae I-III (figure1.3). No differences were observed in the proportions of neurons in lamina II or III which received contacts from serotonin-immunoreactive terminals. Thus 25.6% and 24.2% of GABAergic neurons and 21.1% and 28.7% of glycinergic neurons in lamina II and III respectively were associated with serotonin contacts. Lamina I GABAergic and glycinergic neurons were too few to allow meaningful data to be derived.

Figure 1.4A illustrates the association of serotonin-immunoreactive terminals to GABA- or Glycine-immunoreactive somata. In the majority of cases no contacts were present on either cell type. Where contacts were formed these were few in number (1 to 4 per cell) and not associated with a 'basket-like' arrangement of serotonin-immunoreactive terminals.

Noradrenergic axons formed contacts with few inhibitory neurons in the superficial dorsal horn. Thus D-βH-immunoreactive profiles were identified in relation to 13.2% (20 out of 151) GABAergic neurons and 16.9% (52 out of 307) glycinergic neurons. This corresponded to 10.9% of lamina II and 17.7% of lamina III GABAergic neurons receiving noradrenergic contacts and 14.9% of lamina II and 17.7% of lamina III glycinergic neurons. Figure 1.4B illustrates the frequency of association of monoaminergic contacts with GABA or glycinergic neurons.

C. The association of the monoamines with excitatory dorsal horn neurons

Neurons revealed with either neurotensin or somatostatin-immunoreactivity were observed in a distribution as previously described for these antibodies. Neurotensin labelled somata were present throughout the superficial dorsal horn in laminae I-III with processes predominantly localised to the marginal zone. Somatostatin-immunoreactive somata were noted in lamina II with immunoreactive varicosities present in this lamina and in lamina I.

Serotonin-immunoreactive profiles contacted 42.6% (49 out of 115) of neurotensin-immunoreactive somata and 39.4% (87 out of 221) of

somatostatin-immunoreactive neurons (figure 1.3). The proportion of lamina III neurotensin-immunoreactive neurons which received serotonergic contacts was greater than for neurons in lamina II (53.7% and 32.8% respectively). As with the GABA- and glycine-immunoreactive neurons no 'basket like' arrangement of serotonergic profiles was observed in association with somatostatin- or neurotensin-immunoreactive neurons. Figure 1.4A illustrates the association of the monoaminergic contacts with neurotensin and somatostatin-immunoreactive somata. As was noted for the inhibitory neurons where contacts were observed these were few in number for both neurotensin (1 to 4 contacts per cell) and somatostatin (1 to 3 contacts per cell) neurons.

As was observed with the inhibitory neurons few D-βH-immunoreactive terminals were found in association with either the neurotensin- or somatostatin-immunoreactive somata: 18.2% and 8.6% of cells respectively were identified as receiving contacts. The frequency of noradrenergic contacts on these neurons is illustrated in figure1.4B.

D. The association of the monoamines with somatic and dendritic profiles of selected dorsal horn neurons

Though the antibodies used in the previous experiments revealed large numbers of somatic profiles, the distribution of immunoreactivity revealed little of the associated dendritic architecture. It is of value in understanding the relationships of the monoamines to dorsal horn cells to identify not only somatic profiles but also any associated dendritic arbor. As discussed this pattern of immunoreactivity has been observed with antibodies to choline acetyltransferase (ChAT), nitric oxide synthase (NOS) and the μ-opioid receptor (MOR1). The association of these neurons with GABA has also been investigated with ChAT and NOS cells co-localising GABA and therefore presumed inhibitory. MOR1 cells do not contain GABA and are presumed excitatory. Each of the neuronal subtypes revealed with these antibodies have a dendritic architecture that is orientated in a predominantly rostrocaudal direction. Hence in a parasagittal section of the dorsal horn the maximum extent of the neuron can be examined. In total 13 (4,4,5) ChAT cells, 12 (6,3,3) MOR1 cell, 8 (3,3,2) lamina I, 10 (3,3,4) lamina II and 10 (3,3,4) lamina III NOS cells were examined from three experimental animals (figures in brackets- number of cells from each animal).

Choline acetyltransferase-immunoreactive profiles were observed in a distribution that corresponded to the descriptions in previous published work. Immunoreactive somata were located in the dorsal horn in lamina III, IV and V with dendrites orientated in a predominantly rostrocaudal direction often emerging form a cell body with a long axis running dorsoventral. Punctate staining, representing cholinergic axon terminals, was also observed throughout the superficial dorsal horn

with a particularly dense plexus spanning the border of inner lamina II with lamina III. This distribution is illustrated in the montage reconstruction of a ChAT-immunoreactive neuron in figure 1.5B. These neurons, located in lamina III, were of moderate size (mean diameter 14μm, range 8-20μm) with dendrites which could be traced to some distance from the cell body in a single vibratome section. Frequently medium to small diameter ChAT-immunoreactive dendrites appeared to orientate such as to enter the dense ChAT-immunoreactive plexus. On occasion neurons were noted where a dendrite initially coursed in a ventral direction deeper into the dorsal horn before turning back on itself to terminate in the region of this plexus.

Nitric oxide synthase-immunoreactive profiles were present throughout the superficial dorsal horn with a plexus of terminals in the region of lamina II and somata of moderate size (mean diameter 14μm, range 4 to 20μm) located in lamina I, II and III. From these, dendrites running in a rostrocaudal direction were observed that remained within their lamina of origin with occasional extension to neighbouring laminae (figure 1.7). Immunoreactivity for the μ-opioid receptor was observed predominantly in a region corresponding to laminae I and II with small sized MOR1 somata (mean 8μm diameter, range 6 to 12μm) localised to a region in the inner part of lamina II at the boundary with lamina III Occasional somata were also noted in lamina III. From these somata dendrites radiated in a rostrocaudal orientation that frequently divided to generate branches which ran back towards the cell body. Typically the dendrites observed had many dendritic spines and remained within the band of MOR1-immunoreactivity in inner lamina II. On one occasion, an immunoreactive axon was observed emerging from the

somata which could be followed to some distance form the cell body into lamina III (figure1.9).

On no occasion in examining the relationship of the monoamines to ChAT- (figure 1.6), NOS- (figure 1.8) or MOR1-immunoreactive somata (figure 1.10) and their associated dendrites was a 'basket-like' arrangement of serotonin-immunoreactive identified in association with these cells. However, occasional monoamine contacts were present distributed in a seemingly sporadic manner on these cells. The number of serotonin-immunoreactive contacts identified ranged from 4 to 33 per ChAT cell (mean packing density 0.37 contacts/100 μm^2), 2 to 12 per MOR1 (mean packing density 0.42 contacts/100 $\mu m^{2)}$ cell and 0 to 23 per NOS cell (mean packing density 0.3 contacts/100 μm^2). Little variation in the frequency of these contacts along the length of dendritic tree was observed (figure 1.11A). Thus, though 5-HT-immunoreactive profiles were associated with ChAT-, MOR1- and NOS-immunoreactive cells, they were few in number and did not display a 'basket-like' concentration around somata and proximal dendrites. There was no significant difference in the frequency of serotonin contacts observed on each of these cells (figure 1.19).

Noradrenergic axons formed few contacts on each of the cells observed (0-3 contacts per ChAT cell, 0-3 contacts per MOR1 cell and 0-11 contacts per NOS cell). As observed for the serotonin contacts on these cells the contacts appeared to be sporadic with no discernable pattern of distribution (figure 1.11B).

E. Ultrastructural relationship of serotonin-immunoreactive profiles with inhibitory dorsal horn neurons.

Within the material prepared GABA-immunoreactive somata and medium to large sized dendrites were easily distinguishable by a heavier accumulation of silver intensified gold particles within their substance than the background gold particle density. Despite extensive observations of 15 GABA-immunoreactive somata in material prepared from three separate experimental animals, few serotonin-immunoreactive profiles were identified in close proximity to immunoreactive somata. On no occasion did the monoamine terminal form a synaptic relationship with the GABA-immunoreactive cell body. In each case the identified terminal was followed through consecutive ultrathin sections through the entire extent of the varicosity. In three instances though the varicosity lay in close proximity to the somatic membrane, an extension of the membrane of a surrounding glial cell intervened between the terminal and the cell body (figure 1.12).

F. The association of the monoamines with Neurokinin-1-immunoreactive neurons in the dorsal horn

Immunoreactivity for the NK-1 receptor in these studies corresponded to previous descriptions. Frequent, relatively large immunoreactive neurons (mean 18μm diameter, range 8-24 μm) were present in lamina I. Typically these neurons had dendrites which radiated in an approximately rostrocaudal direction for some distance within lamina I (figure 1.16). Occasionally these lamina I cells were noted to have dendrites that, following a short rostrocaudal course, descended into the region of lamina II. In addition to these superficial cells, a significant number of cells were noted in the deeper dorsal horn in the region of lamina III/IV. As with the lamina I NK-1-immunoreactive neurons these were of large diameter (mean 20μm diameter, range 12 to 30μm) however the dendrites of these neurons were typically orientated in a dorsoventral manner with a small number of large diameter dendrites projecting into laminae I and II (figure 1.13). Occasional cells with a dendrite which initially followed a rostrocaudal orientation were noted however in the sections where these dendrites could be followed to their termination they were found ultimately to turn dorsally and terminate in the more superficial laminae.

Laminae III/IV NK-1 neurons and the monoamines.

Parasagittal sections from seven experimental animals were analysed yielding a total of 33 NK-1-immunoreactive somata located in laminae III/IV with dendrites which could be followed into the superficial dorsal horn laminae. In all cases monoamine contacts were identified in association with these cells. The total number of serotonergic contacts ranged from 9 to 165 contacts per cell (mean packing density 0.63

contacts per 100μm^2) with between 1-36 D-βH contacts present per cell (mean packing density 0.12 contacts100μm^2). The frequency of these contacts is illustrated in figure 1.17A where the numbers of contacts per 100μm length of dendrite are plotted at increasing distances form the soma. As illustrated there is a tendency for 5-HT contacts to cluster around the proximal dendrites of these neurons whereas the NA contacts were relatively sparse and sporadically distributed across the dendritic tree.

Observation of these cells by direct epifluorescence and in merged series of confocal images revealed two distinct populations of NK-1-immunoreactive lamina III/IV neurons based on their association with serotonin-immunoreactive axons and terminals. In the first group of cells serotonergic axons and terminals are intimately woven around the proximal dendrites and somata of NK-1-immunoreactive neurons producing the 'basket like' distribution of serotonin-immunoreactive profiles as observed in dorsal horn sections prepared for serotonin-immunoreactivity. Figure 1.13A illustrates this arrangement for an NK-1-immunoreactive cell from lamina III. The second population of cells were not associated with this 'basket like' arrangement of serotonergic axons though they did receive sporadic contacts from 5-HT-immunoreactive terminals (figure 1.13B). Of the 33 cells analysed, 16 were associated with a 'basket-like' arrangement of serotonergic axons, 17 were not. The adjacent cells illustrated in figure 1.13, which were reconstructed using the NeuroLucida program, demonstrate each of these patterns of distribution. These cells were present within the same tissue section at a similar depth in the tissue, hence the observed difference in distribution is not a result of a difference in immunostaining or antibody penetration.

Since two patterns of association of serotonergic axons with lamina III/IV NK-1 cells were observed, it was of interest to identify whether this correlates with any difference in contact frequency. This was achieved by comparing serotonin contact frequencies at increasing distances form the cell body in the two populations of cells. This analysis revealed that the basket associated group received a significantly greater frequency of 5-HT contacts on proximal dendrites (defined as those within a 100μm radius of the soma) than cells not associated with a basket arrangement of serotonergic axons (mean number of contacts per 100μm±SD = 13±5.8 and 5±2.9, respectively; $p<0.001$, Mann-Whitney *U*-test). At distances greater than 100μm from the cell body there is no significant difference in the frequency of contacts between the two cell groups (figure 1.15B).

Lamina I NK-1 neurons and the monoamines.

Twelve lamina I NK-1-immunoreactive neurons from 4 experimental animals were analysed. As with the lamina III/IV neurons all were associated with serotonin contacts ranging from 8 to 174 contacts (mean packing density 0.6 contacts per 100μm^2). Dopamine-β-hydroxylase contacts were however uncommon with only 0 to 12 contacts per cell (figure1.16). As noted for the deeper dorsal horn NK-1 neurons the 5-HT-immunoreactive contacts tended to be clustered around the proximal dendrites whilst the D-βH contacts were sporadically distributed across the dendritic profile (figure 1.18). Unlike the cells of lamina III/IV the lamina I NK-1-immunoreactive cells did not display any clear morphological division in respect to the arrangement of serotonergic axons around the soma and proximal dendrites with no 'basket like' associations seen. Occasional serotonergic axons were

noted which ran parallel to NK-1 somata or dendrites for a distance forming several en-passant contacts (figure 1.17). However, where these were present they did not correlate with any significant difference in contact frequency on these cells as compared to those lamina I cells which did not have a serotonin axon running in parallel.

Comparison of serotonin contact frequencies on different types of dorsal horn cells.

From the above data lamina III/IV neurokinin-1 receptor immunoreactive neurons are associated with two different distributions of serotonin axons on proximal dendrites which correlates with a difference in the frequency of contacts that can be identified on the proximal dendrites of these profiles. Furthermore, in both the lamina I and lamina III/IV populations of NK-1-immunoreactive neurons there is a tendency for serotonin contacts to cluster around the proximal dendrites which was not observed in the studies of ChAT-, NOS- or MOR1-immunoreactive neurons. In figure 1.19 the serotonin contact densities are plotted for the lamina III/IV neurons (basket and non-basket cells), ChAT neurons, MOR1 neurons and lamina III NOS neurons. This demonstrates the higher frequency of 5-HT contacts associated with the proximal dendrite (within a 100μm radius of the soma) on these NK-1 neurons with respect to the other populations of cells examined. This is true even for the non-basket NK-1 neurons where on the proximal 100μm the mean number of contacts was 5 ± 2.9 compared to 1.9 ± 1.3 on ChAT neurons, 1.8 ± 1 on MOR1 neurons or 2 ± 1.5 on lamina III NOS neurons ($p<0.01$ in all cases; mean number of contacts $\pm$ SD; Mann-Whitney *U*-test). A similar clustering of serotonin contacts around the proximal dendrites of lamina I NK-1 neurons was noted. The frequency of contacts on these proximal dendrites was

significantly greater than on lamina I NOS cells (8±5.8 and 1.2±1.3 contacts per 100μm respectively in the first 100 μm). Thus, uniquely for the cells examined in this study, the NK-1 receptor-immunoreactive neurons in the dorsal horn are associated with greater numbers of serotonergic contacts on proximal dendrites than any other neuron examined. For all cells studied the frequency of D-βH contacts was relatively low.

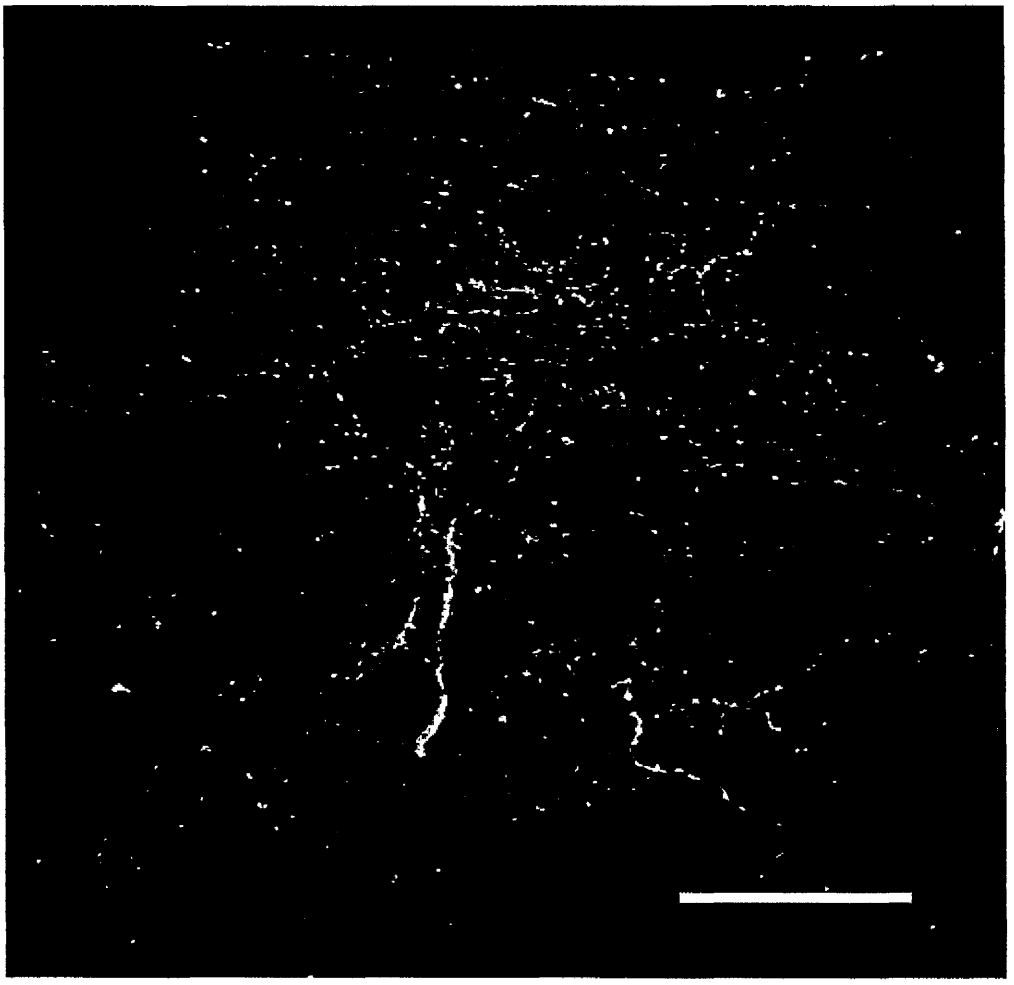


Figure 1.2. Serotonin immunoreactivity in the superficial dorsal horn of the rat lumbar spinal cord. This projected image composed of 21 single images in the z-plane taken at 1μm intervals demonstrates the distribution of serotonin-immunoreactive profiles in the superficial dorsal horn. A dense band of immunoreactivity corresponding to rostrocaudally orientated axons and associated terminals in the lamina I/II_o region is present with a lower density of profiles in the deeper lamina. In addition to the rostrocaudal axons a 'basket-like' formation of serotonin-immunoreactive axons is present formed by axons extending out of the lamina II region and encircling an unidentified cell body and it's associated proximal dendrite within lamina III (scale bar 50μm).

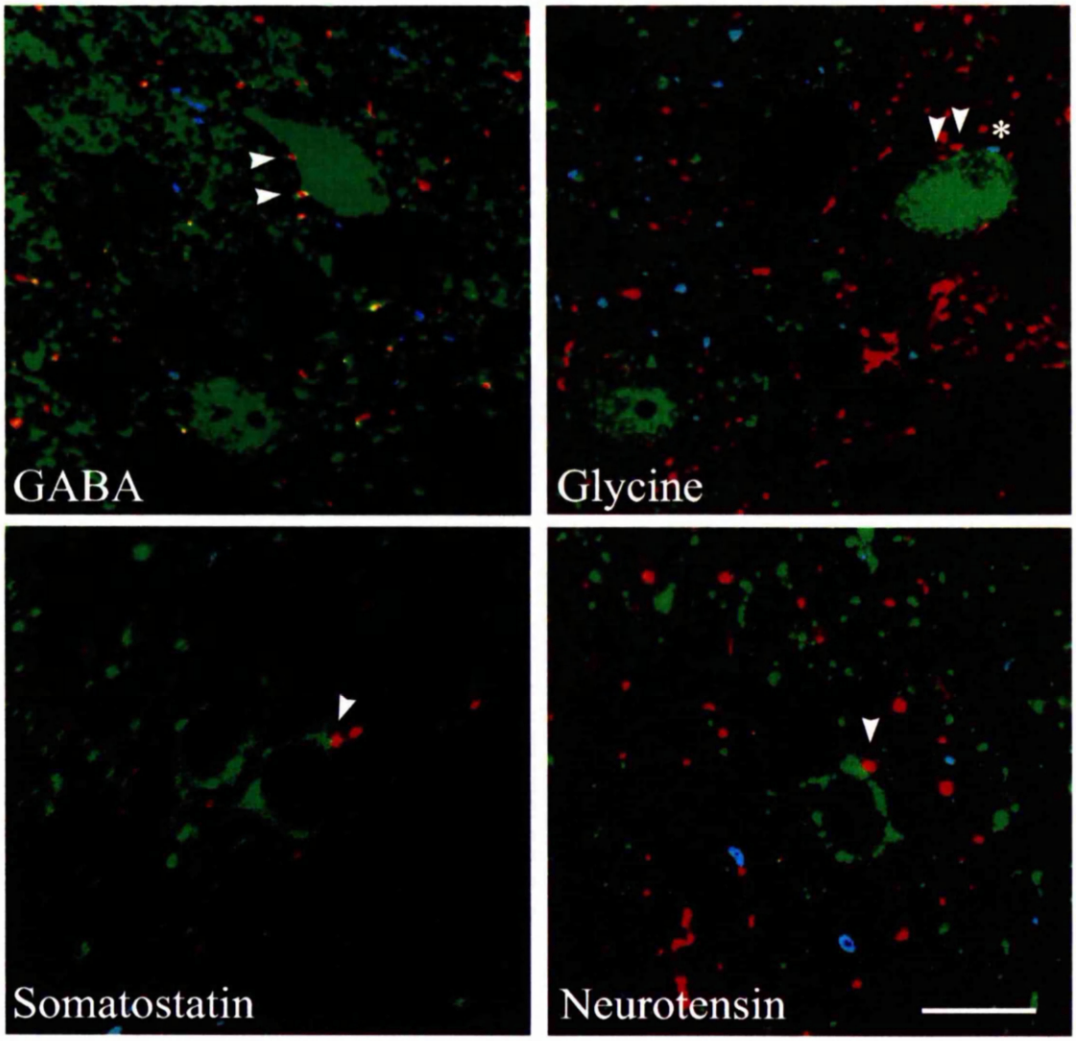


Figure 1.3. A proportion of each of the four dorsal horn cell types examined in transverse sections received somatic contacts from monoaminergic terminals. This series of confocal images, created from single optical sections, illustrates examples of serotonergic contacts observed in relation to these cells (arrowheads). Noradrenergic contacts (asterisk) were infrequent (green - neurotransmitter; red - 5-HT; blue - D-βH; scale bar 10μm).

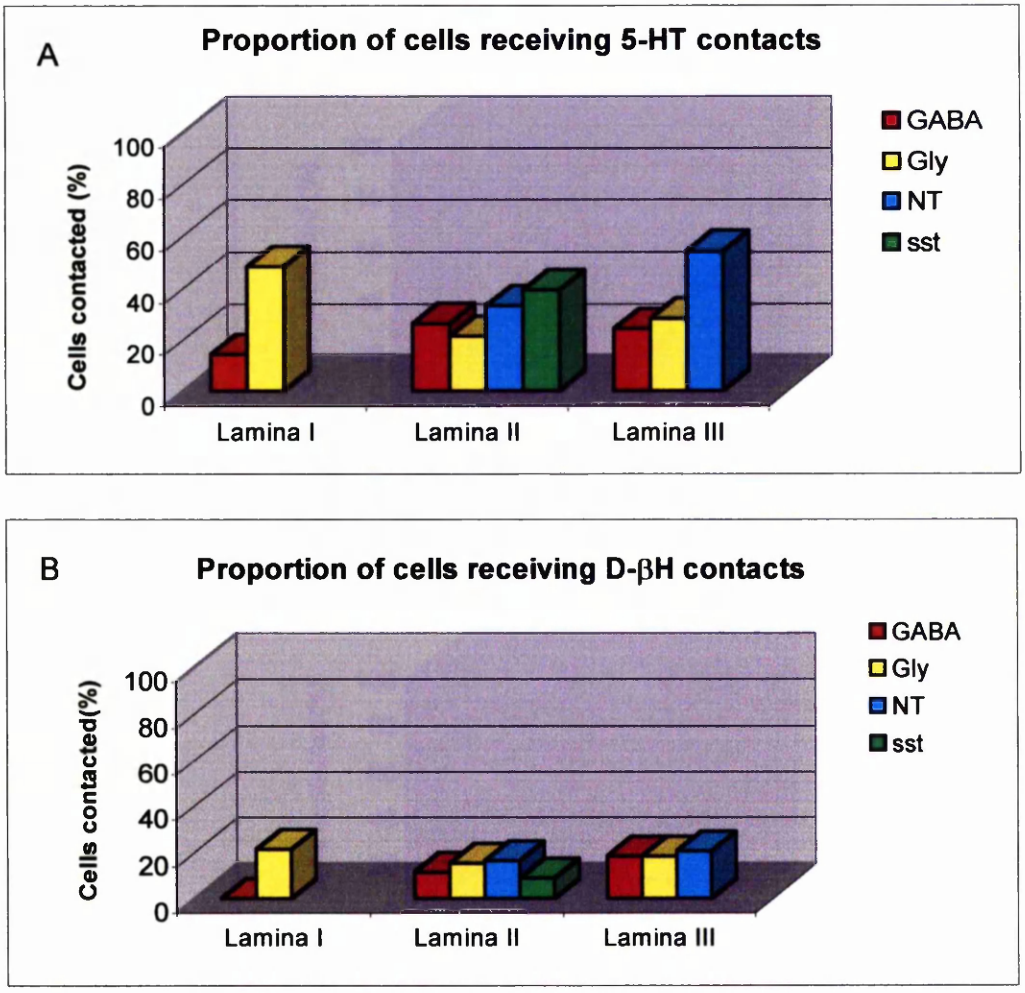


Figure 1.4. Serotonin contacts were identified on a proportion of all cell types examined (A). Few lamina I GABA or Gly neurons were encountered hence the interpretation of the data for this lamina is limited. In lamina II examples of all three cell types were encountered with a similar proportion of cells for each subtype associated with serotonergic contacts. In lamina III a greater proportion of neurotensin than GABA or glycine neurons received 5-HT contacts. For all cell types examined the frequency of contacts from D-βH-immunoreactive terminals was low (B).

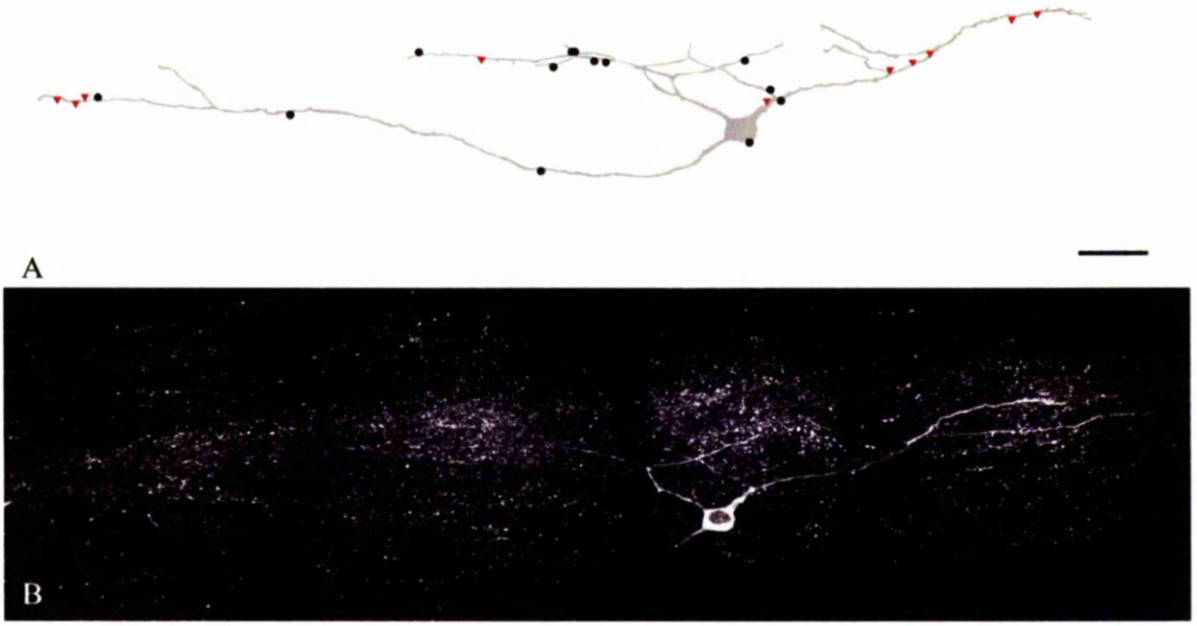


Figure 1.5. Choline acetyltransferase neurons were found within the lamina III region with dendrites which coursed in an approximately rostrocaudal direction Occasional cells were observed where a dendrite which initially followed a ventral course deeper into the dorsal horn before turning back on itself to enter the region of the plexus of ChAT-immunoreactive terminals spanning the lamina II/III border.. Using the individual images for each field of view and with the aid of the NeuroLucida package these cells were traced and monoamine contacts plotted on the resultant image. An example of this technique is illustrated in image A for the cell in the montage image B. As can be appreciated, though monoamine contacts are present these are few and distributed across the entire dendritic profile (circles - 5-HT contacts; triangles D-βH contacts; scale bar 50μm).

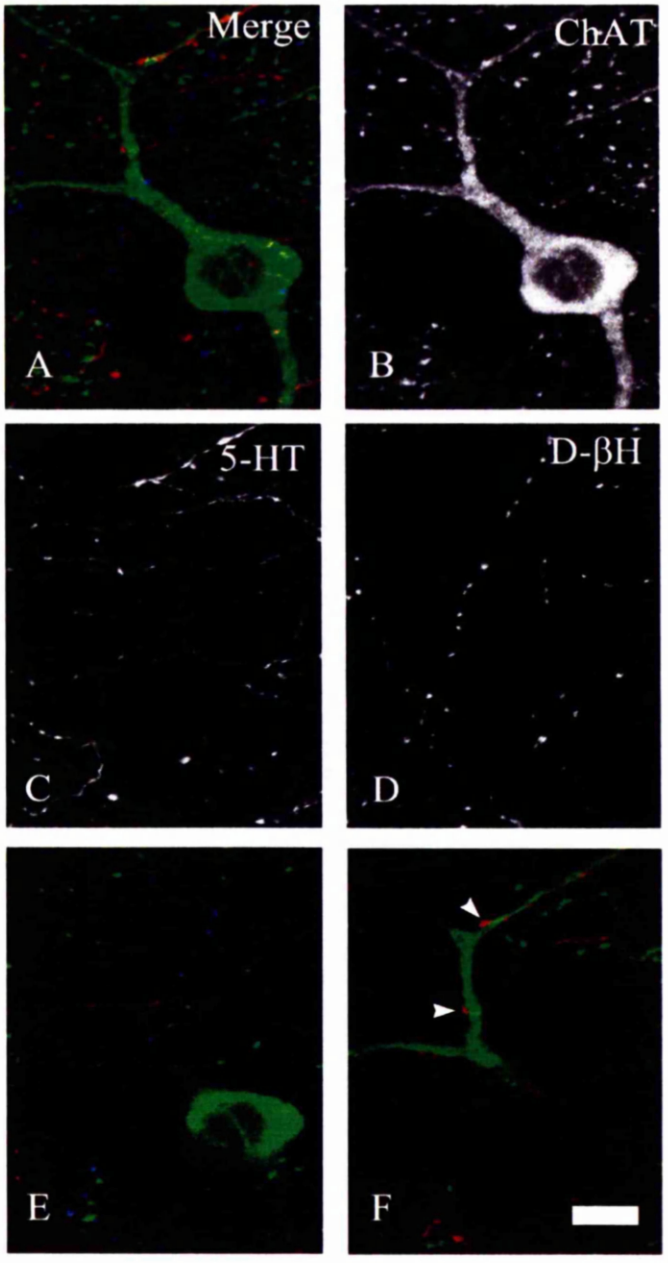


Figure 1.6. At a higher magnification the relationship of the monoamine terminals to the ChAT-immunoreactive neurons can be appreciated. As can be seen from the merged series of several images through the cell body (A), no basket-like clustering of 5-HT terminals around the soma and proximal dendrites is observed. This is confirmed by examining each of the colour channels individually where no clear association between 5-HT (C) or D-βH (D) terminals and the ChAT profile exists. Plates E and F show single merged optical images through the soma and a proximal dendrite. Two serotonergic contacts are present on the dendrite in F (arrowheads; scale bar 10μm).

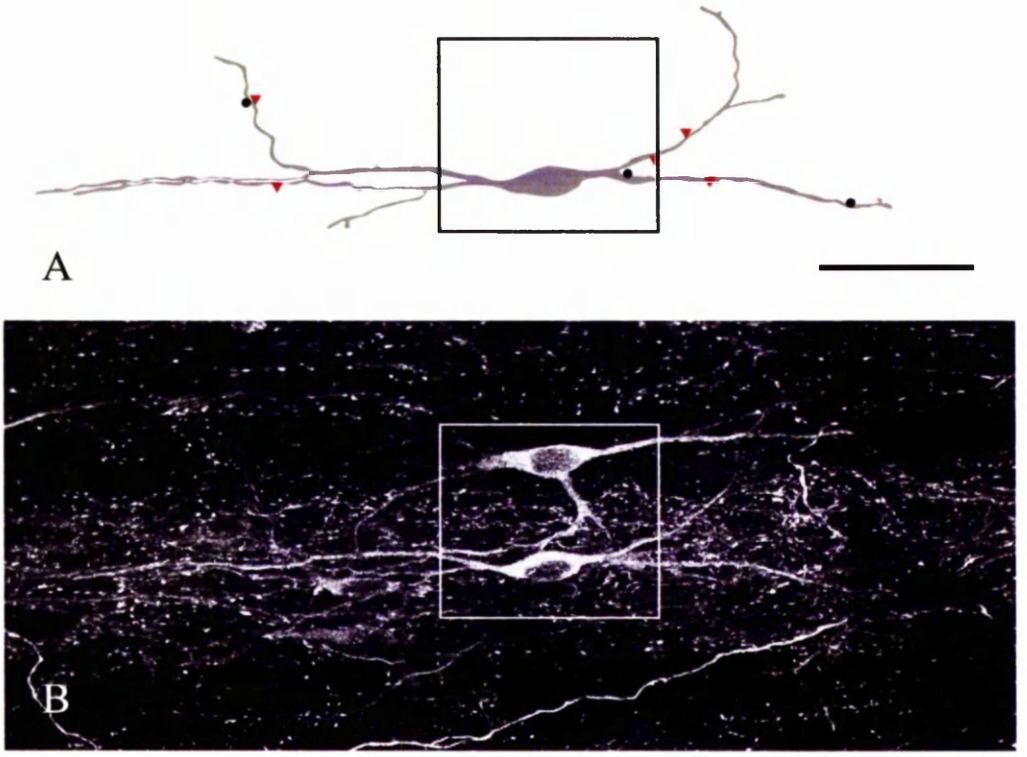


Figure 1.7. NOS-immunoreactive neurons were identified throughout lamina I-III with a plexus of terminals in the region of lamina II as shown in the montage image of two cells located in lamina II (B). Typically, these neurons had dendrites which were orientated in a rostrocaudal direction. Tracing the cells and plotting monoamine contacts on this outline demonstrates the relative paucity of contacts associated with these cells (A; circles - 5-HT contacts; triangles - D-βH contacts; scale bar 50μm).

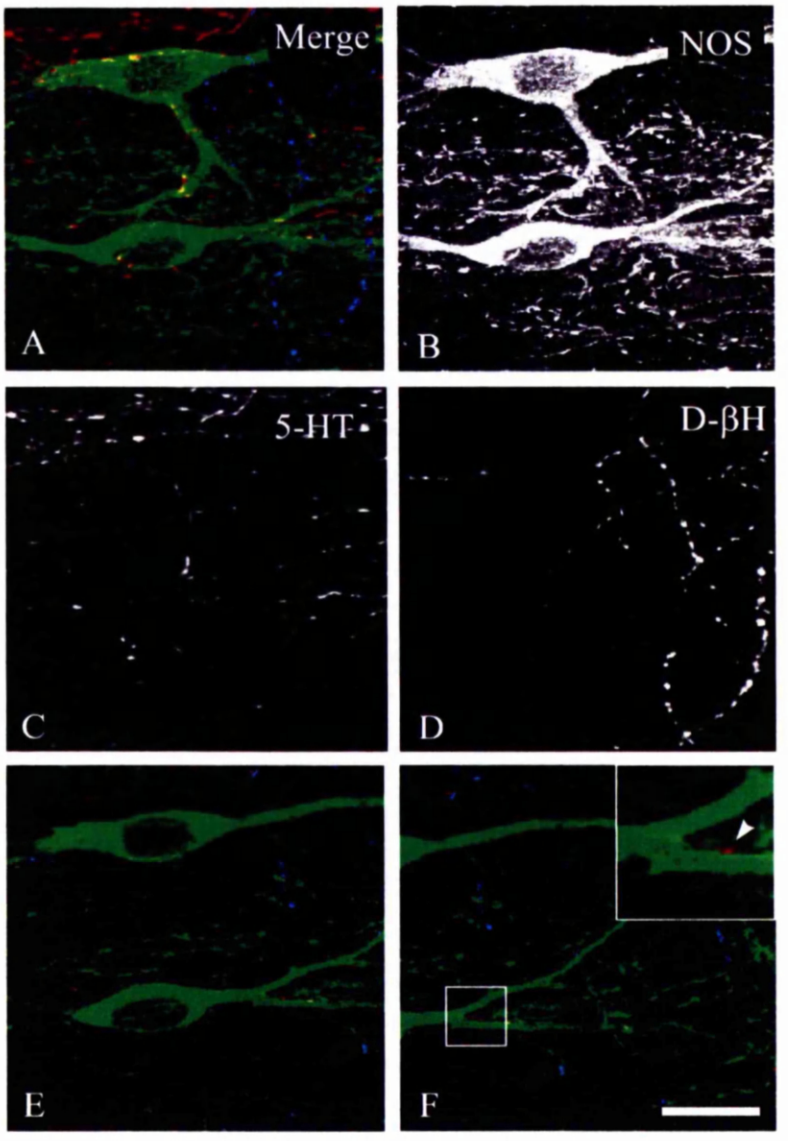


Figure 1.8. In this series of higher magnification, projected images of the cell in figure 1.7 no clear association between NOS- (B), 5-HT- (C) or D-βH-immunoreactive (D) profiles can be identified. This is confirmed in the single optical sections through the cell body (E) and proximal dendrite (F) where a single 5-HT contact is present on the proximal dendrite (arrowhead; scale bar 10μm).

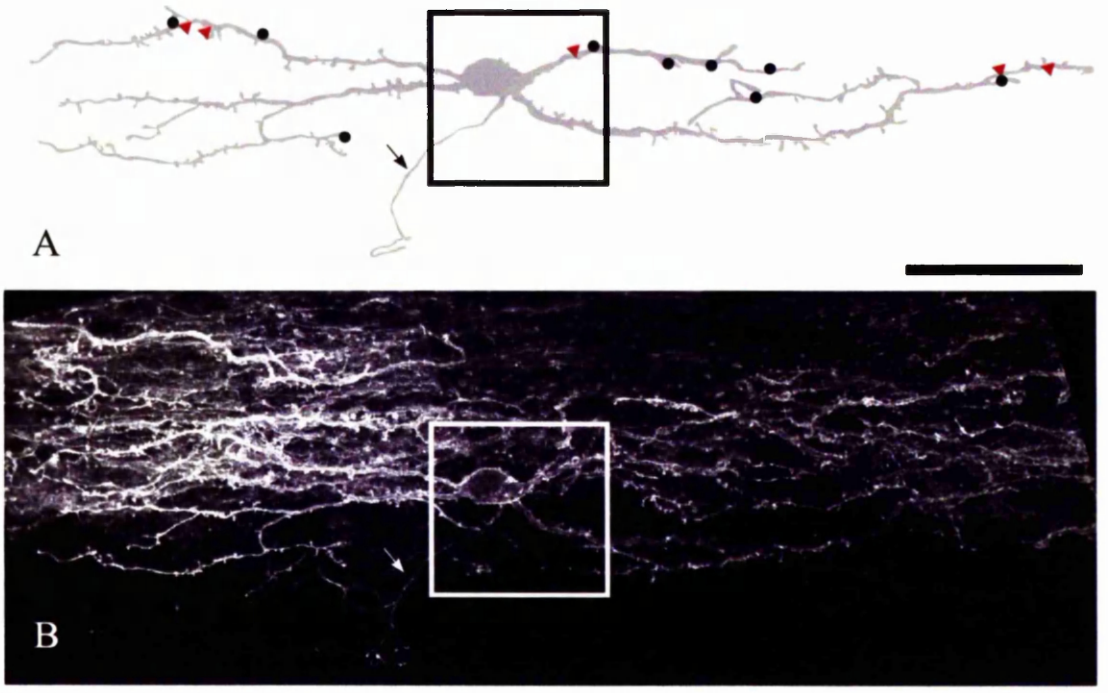


Figure 1.9. Montage reconstruction of a typical MOR1-immunoreactive neuron located at the margin of lamina II/III (B). As is common in MOR1-immunoreactive neurons in the dorsal horn dendrites were orientated in a rostrocaudal direction and had numerous dendritic spines. Recurrent dendritic branches were also commonly noted. In addition, the cell illustrated here displays another common feature of MOR1-immunoreactivity with axonal labelling evident (arrow). As with the ChAT- and NOS-immunoreactive neurons sparse monoamine contacts were scattered across the dendritic profile (A; circles - 5-HT; triangles - D-βH; scale bar 50μm).

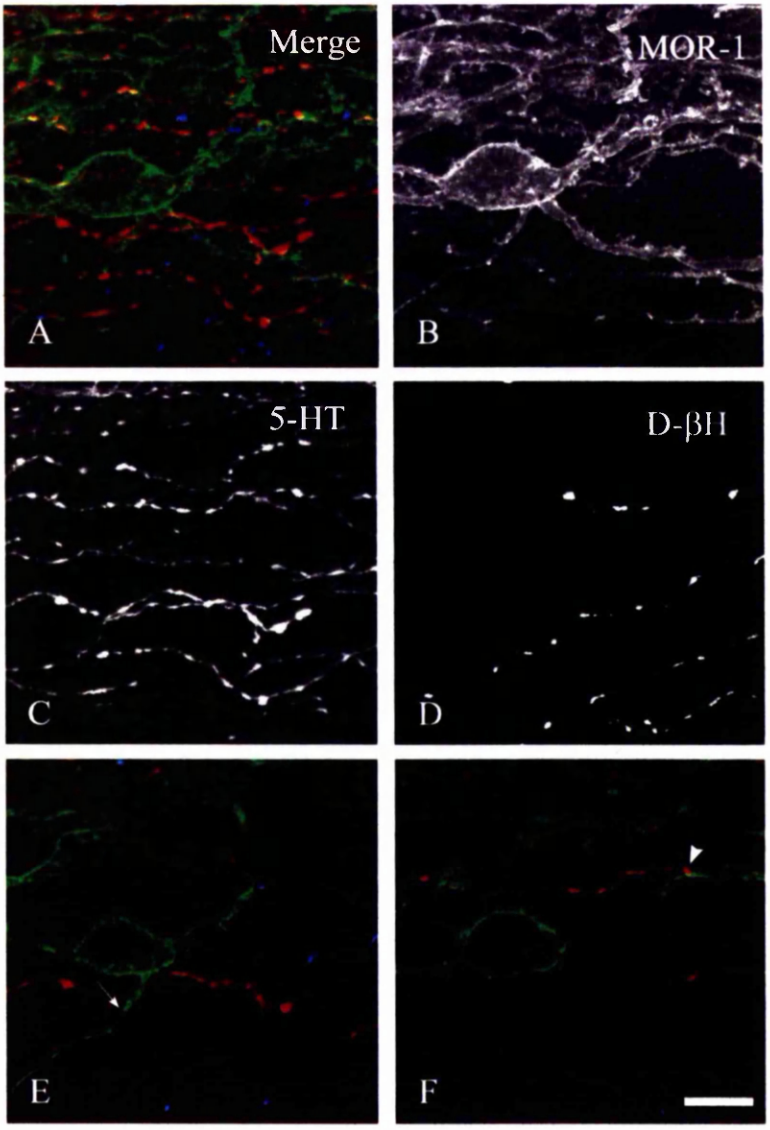
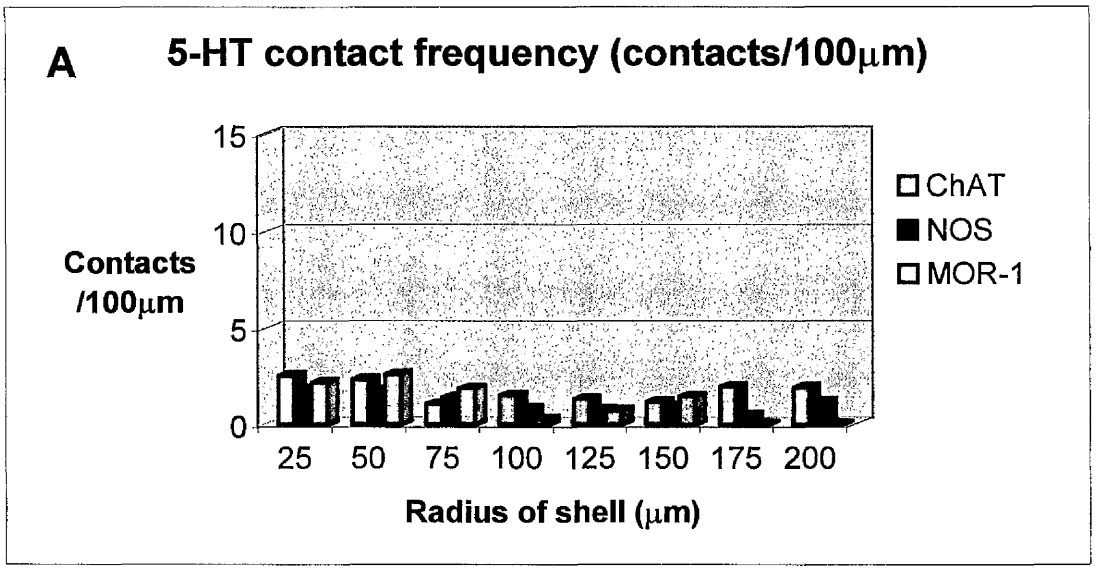


Figure 1.10. This series of projected confocal images through the MOR1 cell in figure 1.09 illustrates the relationship of the monoamines to MOR1 cells (A,B,C,D). No basket like formation of serotonergic axons with these neurons was identified though occasional monoamine contacts were present distributed in a sporadic manner across the dendritic profile. One such 5-HT contact is illustrated in the single merged optical section in image F (arrowhead). A feature of MOR1-immunoreactivity in the dorsal horn is the appearance of axonal staining in some cells (E; arrow - axon; scale bar 10μm).



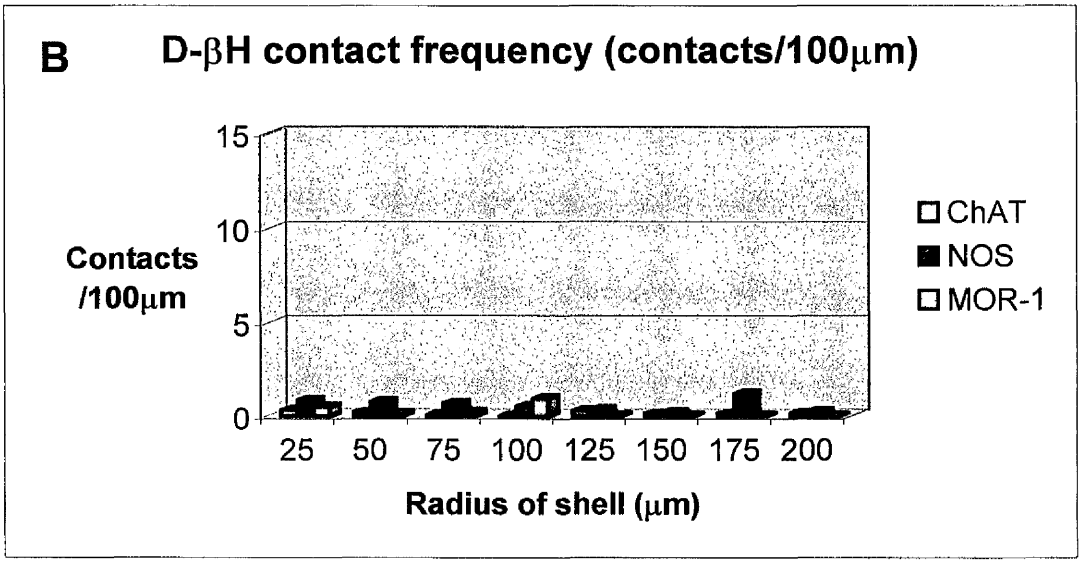


Figure 1.11. Sholl analysis of the distribution of serotonin (5-HT; A) and noradrenaline (D-βH; B) contacts on ChAT-, NOS- and MOR1-immunoreactive neurons. Contact frequencies are expressed per 100μm length of dendrite within concentric shells of increasing radii in 25μm steps, centred on the cell body. The mean 5-HT contact frequencies for the three cell types were similar across the dendritic profile with few contacts present showing no clear pattern of distribution (A). Noradrenergic contacts were uniformly infrequent in each of the cells examined and as with 5-HT showed no particular distribution pattern (B).

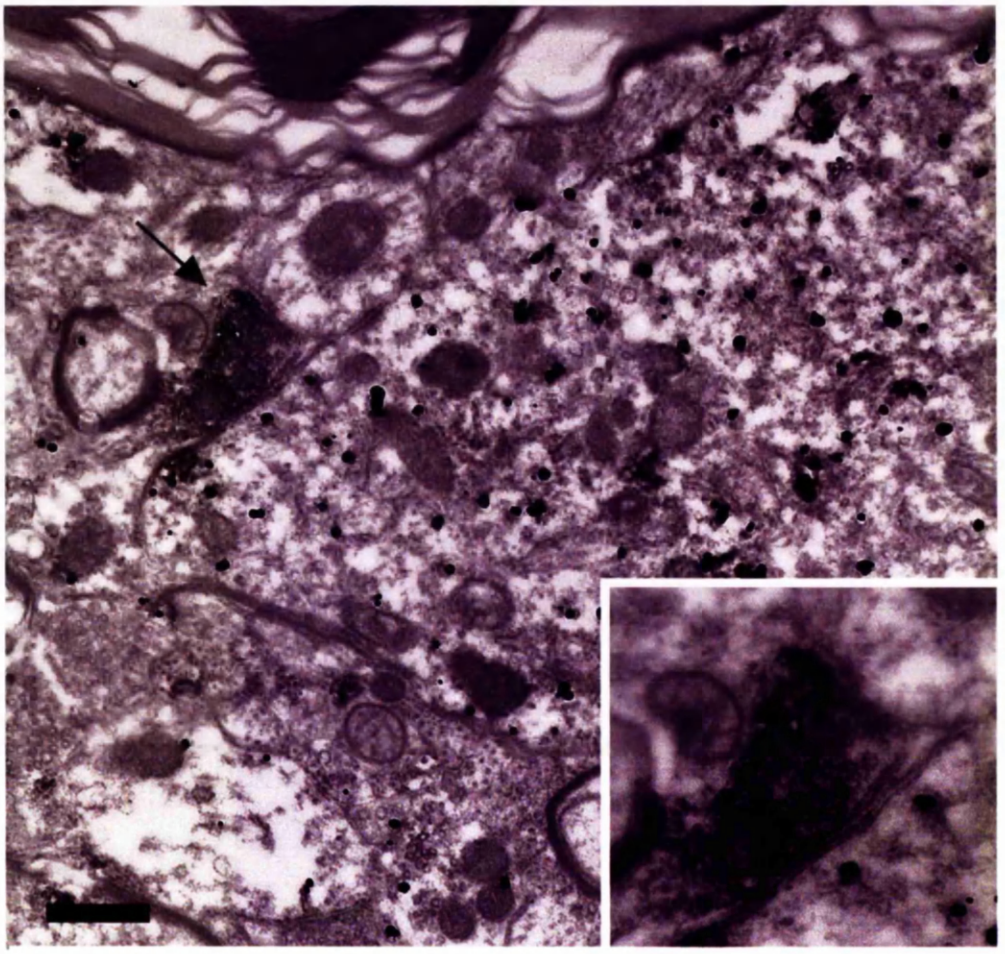


Figure 1.12. In the ultrastructural observations on the association of serotnin-immunoreactive profiles with GABAergic somata, no synaptic contacts were observed. However, on several occasions DAB labelled serotonergic boutons (arrow) were encounterd which formed close associations with immunogold labelled GABAergic somata as illustrated above. In each case a glial process was identified interposed between the bouton and somatic membranes (scale bar 0.5μm).

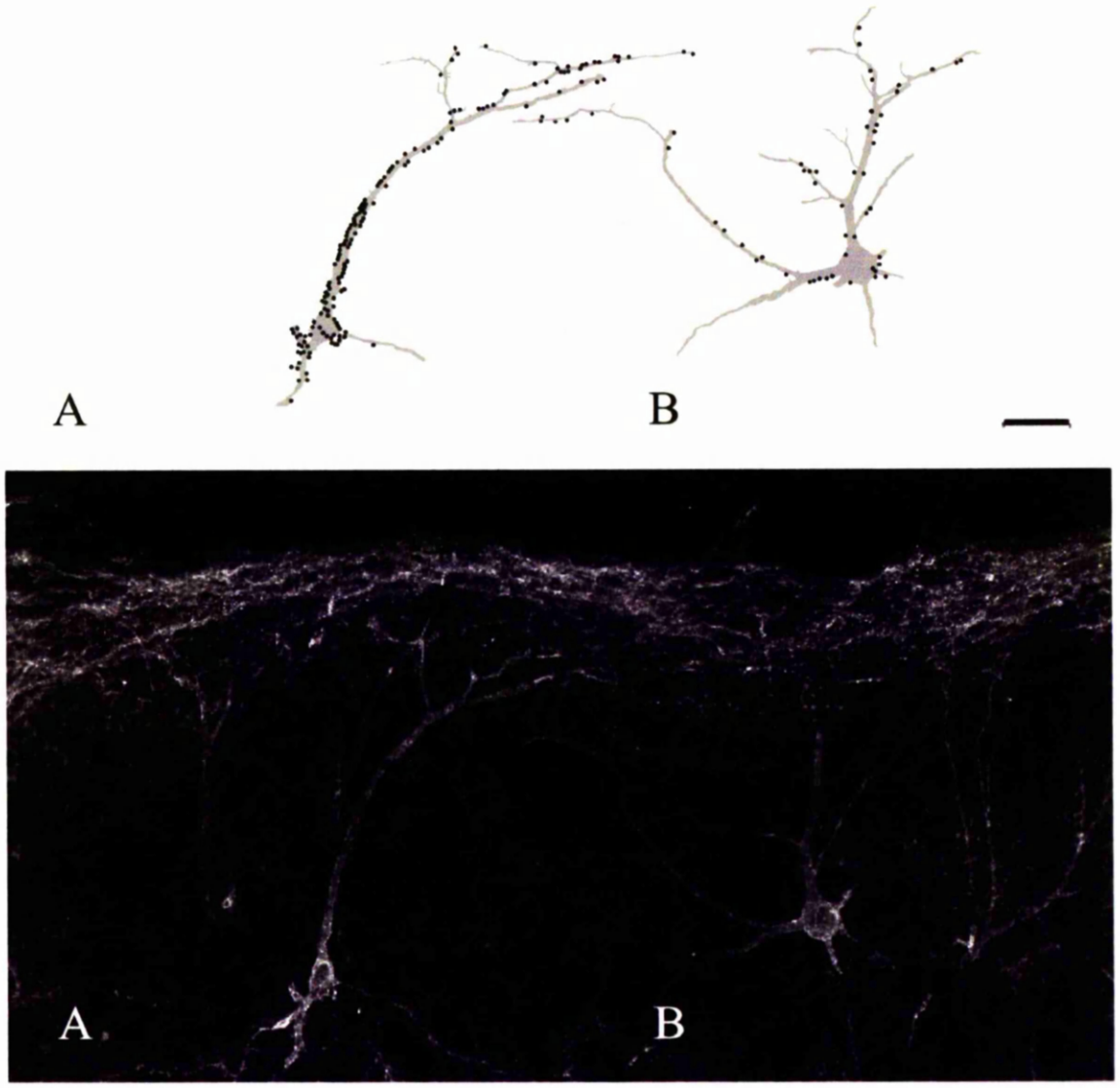


Figure 1.13. Reconstructions and images of two NK-1-immunoreactive cells with cell bodies in lamina IV (A and B) which lie adjacent to each other in the same spinal cord section. The upper images show the 5-HT contacts associated with these cells plotted on outlines created using the NeuroLucida package. Cell A was associated with a basket-like arrangement of 5-HT-immunoreactive axons and terminals whereas cell B was not. Note the very large numbers of contacts associated with the dorsally directed proximal dendrite and the soma of cell A. The lower image shows a montage image of these two cells created from series of projected confocal images from multiple overlapping fields (scale bar 50μm).

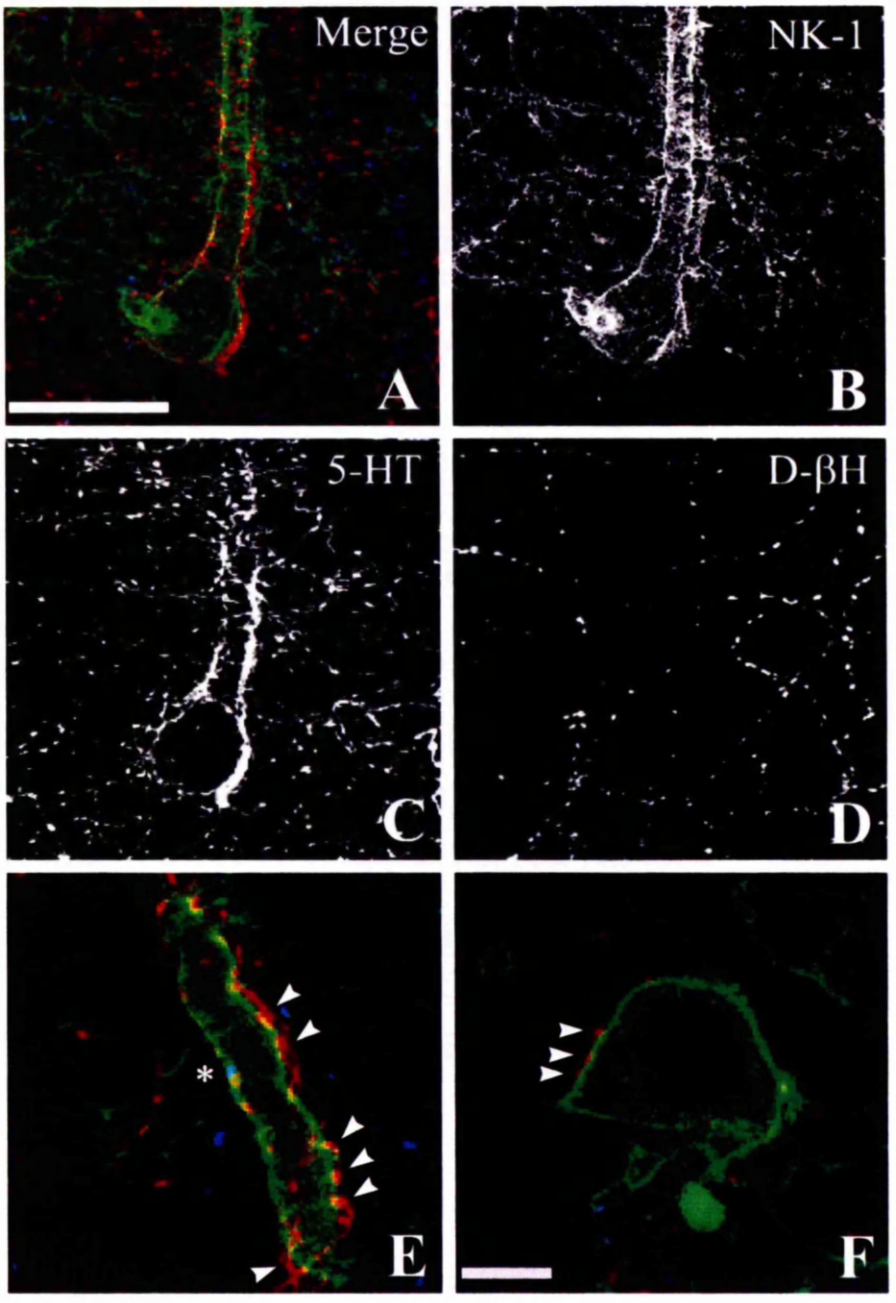


Figure 1.14. (A-D) A series of projected confocal microscope images through the soma and proximal dendrite of an NK-1-immunoreactive neuron located in lamina III showing the presence of a basket-like arrangement of 5-HT axons around the soma and proximal dendrite (A and C). Noradrenergic axons do not appear to form any particular pattern of association (D). Examining these associations in three-colour, merged images in single optical sections (E and F; images from cell A figure 1.13) this relationship is found to correspond to a high frequency of serotonergic contacts on the proximal dendrite and soma of these cells (arrowheads). A single noradrenergic contact is present on the proximal dendrite (asterisk; green - NK-1, red - 5-HT, blue - D-βH; scale bar A-D 25μm; scale bar E & F 10μm).

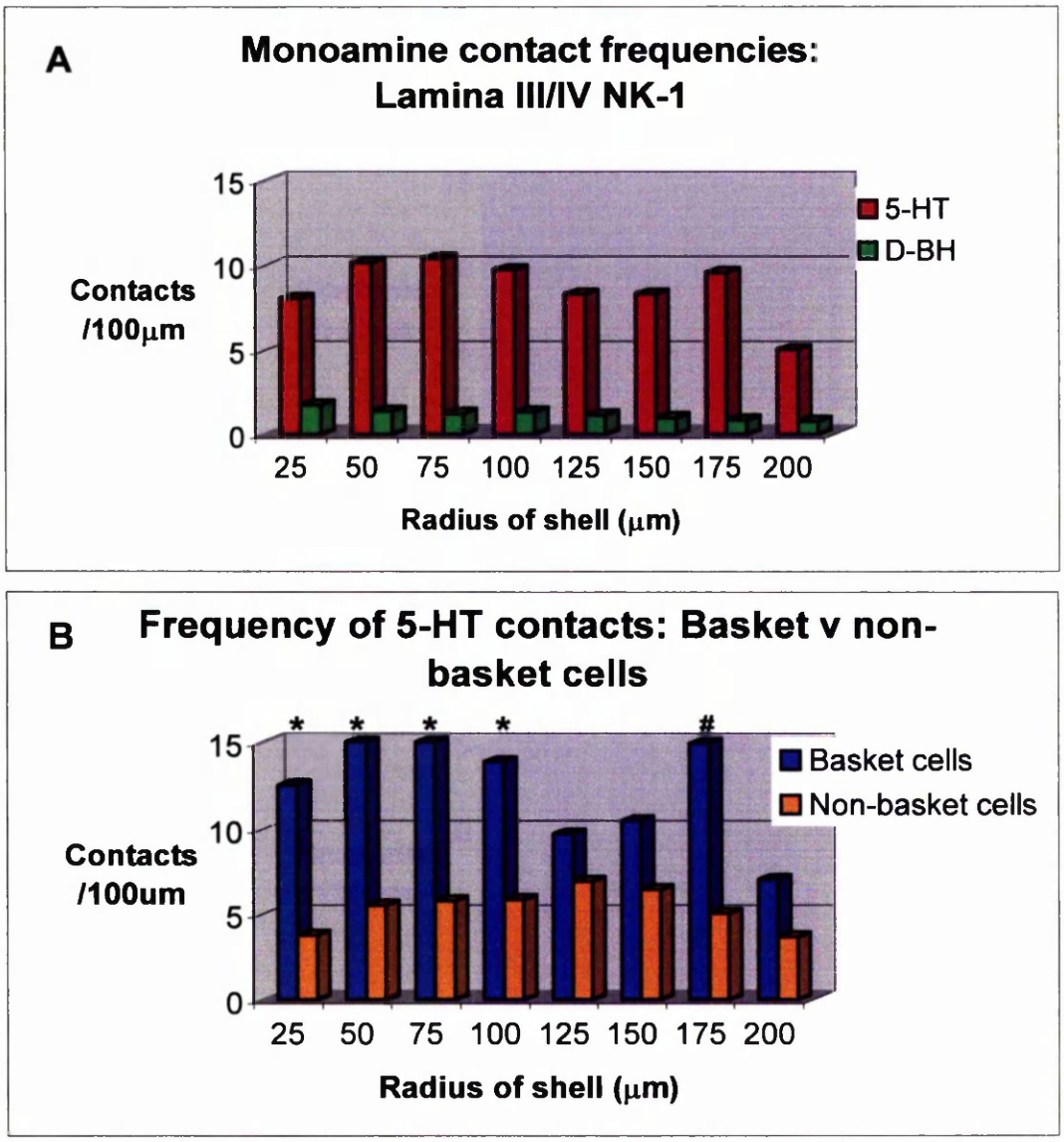


Figure 1.15. Sholl analysis of the distribution of serotonergic (5-HT) and noradrenergic (D-βH) contacts on lamina III/IV NK-1-immunoreactive cells. In each case the data presented is for mean contact frequencies expressed per 100μm unit lengths of dendrite within shells with radii increasing in 25μm increments centred on the soma. Serotonergic contacts on these cells were more frequently identified than noradrenergic contacts which were sparse. Furthermore, serotonergic contacts tended to be concentrated around the proximal dendrites whilst noradrenergic contacts were evenly distributed (A). Serotonergic axons were noted to form basket-like arrangements around the proximal dendrite and cell body of 16 out of the 33 cells examined. When the serotonin contacts on these two groups of cells was compared (B) a significantly greater frequency of 5-HT contacts was identified on the proximal dendrites of the basket-associated group (basket cells) than the cells not associated with baskets (non-basket cells). At distances greater than 100μm from the cell body there was no significant difference between the two groups (*$p<0.0001$; # - not significant; Mann-Whitney *U*-test).

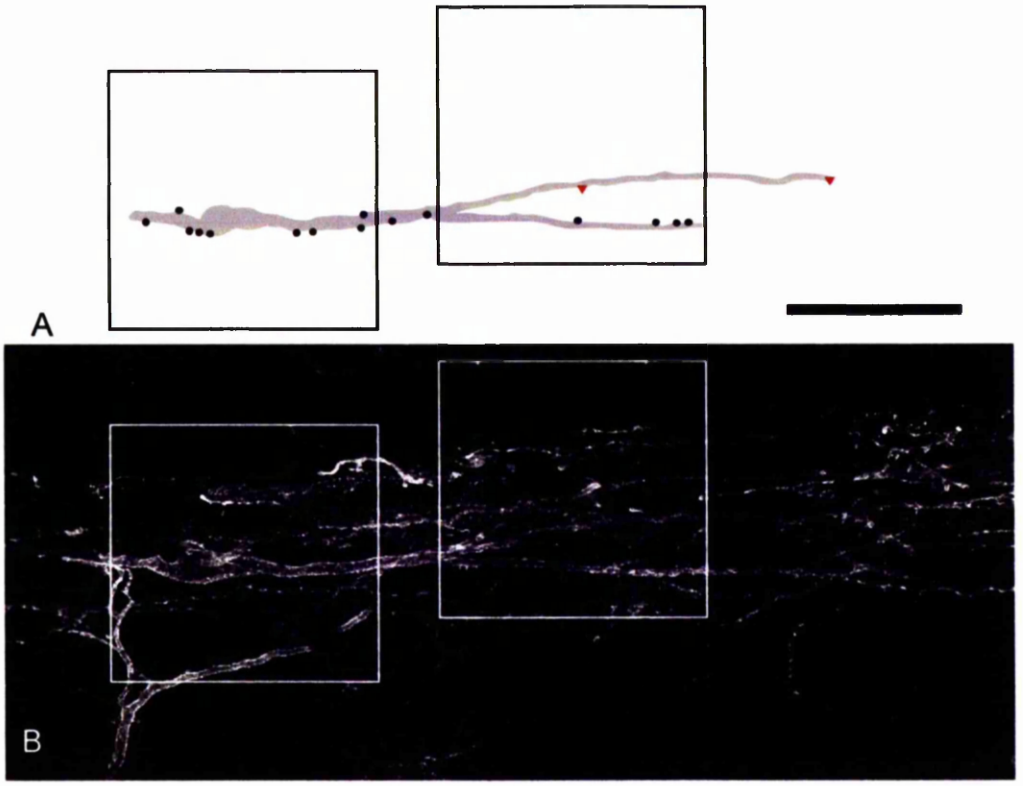


Figure 1.16. Reconstruction (A) and images (B) of an NK-1-immunoreactive neuron located in lamina I. Typically these cells had dendrites orientated in a rostrocaudal direction and were associated with frequent 5-HT contacts (circles). Few noradrenergic contacts were observed on these cells (triangles; scale bar 50μm).

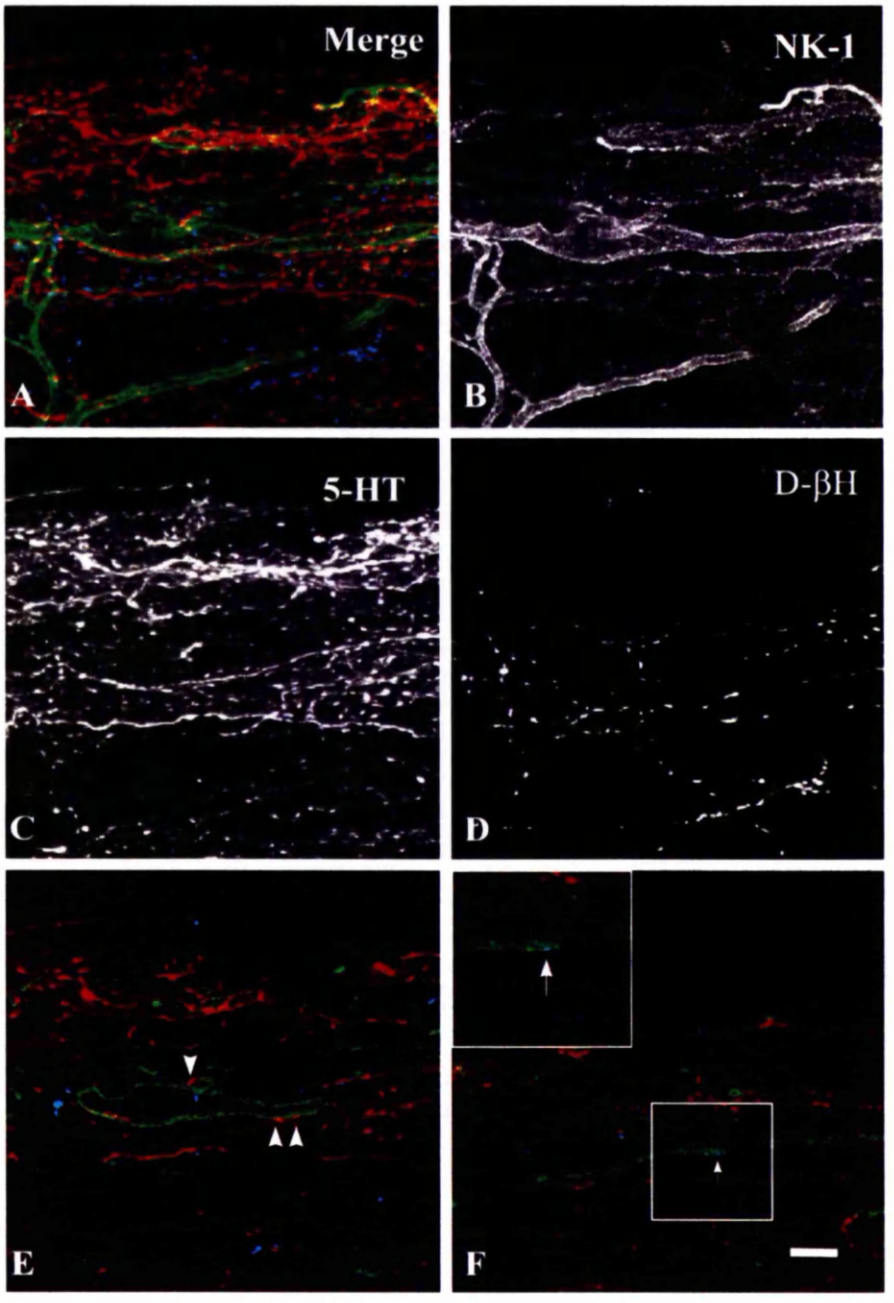


Figure 1.17. Higher magnification images of the fields illustrated in figure 1.16 A & B. Though serotonin contacts are frequent on the lamina I NK-1 neurons, they do not form the 'basket-like' arrangements as identified on a proportion of the deeper NK-1 neurons (A, C). As is indicated in the single optical section in image E the 5-HT axons, orientated in the same rostrocaudal plane as the NK-1 neurons, formed en-passant contacts with the NK-1 cell bodies and proximal dendrites (arrowheads). Noradrenergic contacts were, as in the other cell subtypes examined, infrequent (F; arrow - D-βH contact; scale bar 10μm).

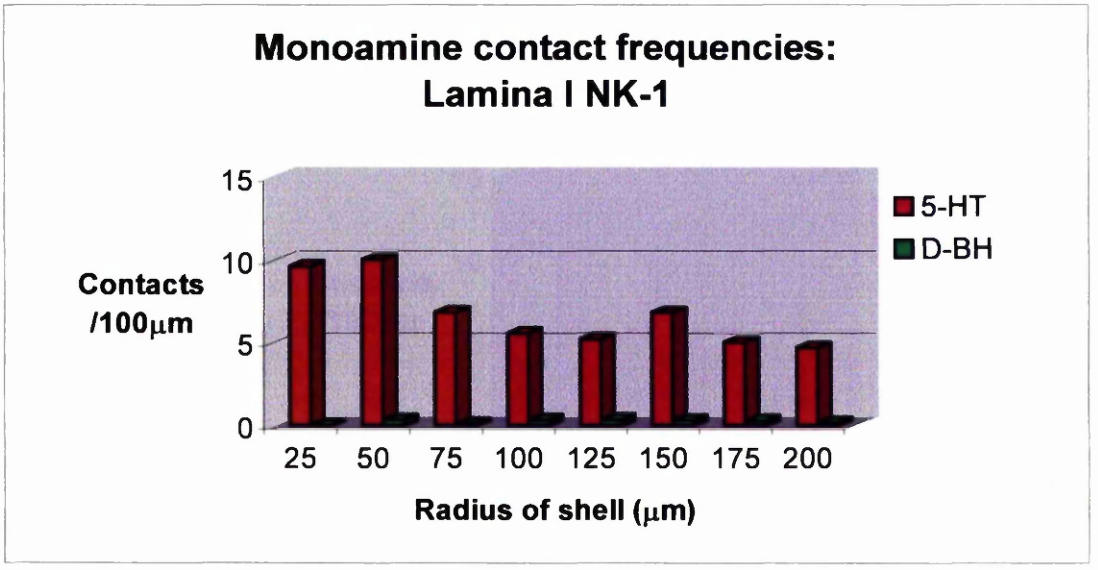


Figure 1.18. Serotonin-immunoreactive profiles formed relatively frequent contacts with lamina I NK-1-immunoreactive neurons when compared to noradrenergic profiles. In addition 5-HT contacts were more commonly associated with the proximal dendrites of these cells with the frequency of contact decreasing with increasing distance from the cell body.

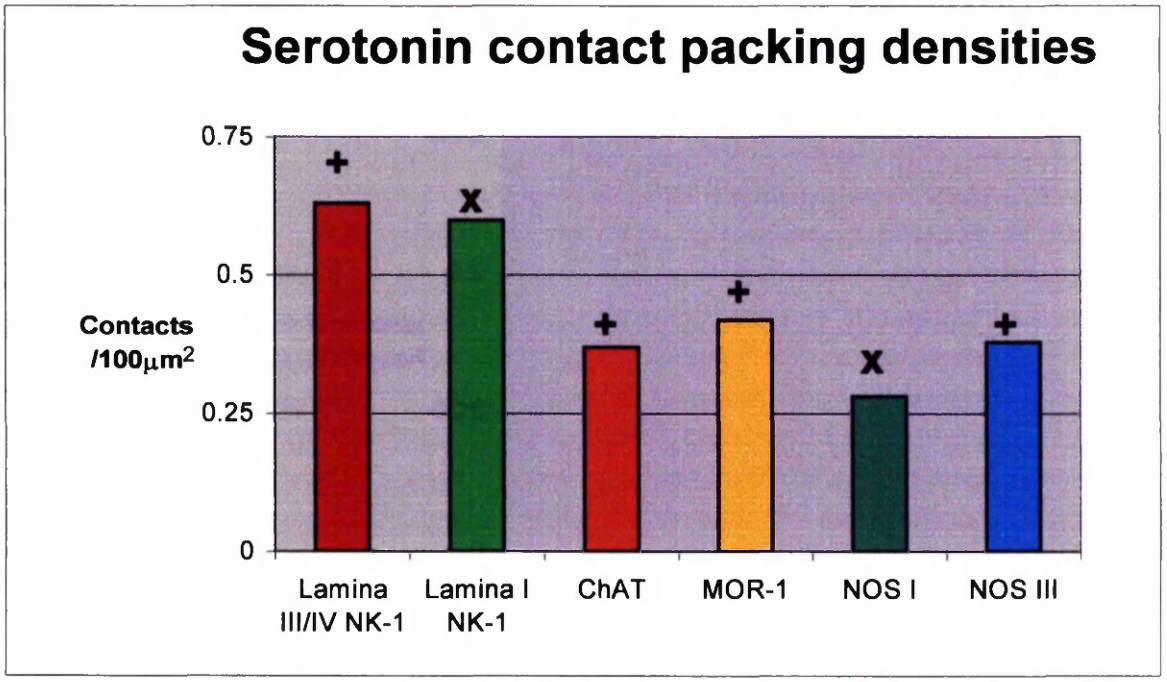


Figure 1.19. Neurokinin-1-immunoreactive neurons appear unique amongst the subtypes of neurons examined in receiving a relatively high frequency of serotonergic contacts when compared to non-NK-1 dorsal horn cells. Thus the lamina III/IV NK-1 neurons had a significantly greater serotonin contact density than the ChAT, MOR1 and lamina III NOS neurons (NOS III) which were examined in this study (+ = $p<0.001$; Mann-Whitney *U*-test). Similarly the lamina I NK-1 neurons received greater frequencies of 5-HT contacts than the lamina I NOS neurons (NOS I; x = $p<0.001$; Mann-Whitney *U*-test). There was no significant difference in contact frequencies between the lamina I NK-1 neurons and lamina III/IV NK-1 neurons.

1.17 Discussion

Methodoiogicai considerations

For each of the antibodies employed in this study the antibody staining and distribution conformed to previous descriptions. In particular the antibodies to GABA and glycine produced specifically for use in formalin fixed material (Pow, Wright & Vaney, 1995) provided a staining pattern that corresponded to that reported in previous studies (Todd & Sullivan, 1990). The resolution afforded by the confocal microscope permitted highly detailed analysis of the association between monoamine terminals and dorsal horn neurons and, when combined with the NeuroLucida package, the analysis could be extended to provide a full 3-dimensional analysis of the relationships of the profiles within the vibratome sections. The combination of confocal microscopy with 3-dimensional reconstruction and analysis used in this study is one that has not previously been applied to the investigation of dorsal horn neuronal circuitry. Clearly the ability to relate profiles in 3-planes and analyse this relationship with minimal difficulty adds to the data that can be generated and conclusions that can be drawn from a single investigation. Previous investigations have been limited by the resolution of light (Miletic et al, 1984) and fluorescence microscopy (Miller & Salvatiera, 1998) or a small sample of neurons identified on morphological features rather than immunocytochemical features (Marlier et al, 1991). Furthermore, the ability to generate datasets including volume and surface area of selected profiles extends the potential for analysis.

Distribution of monoamine contacts in the dorsal horn

The principal finding of this study is that the descending serotonergic system preferentially forms large numbers of contacts with neurokinin-1 receptor immunoreactive neurons in lamina I and laminae III/IV of the spinal dorsal horn. Furthermore, within this population of cells there is a subpopulation (approximately 50%) of the NK-1 receptor-immunoreactive neurons in lamina III/IV which receive large numbers of contacts from 5-HT axons. This large number of contacts has been shown to correlate with serotonergic axons clustered around the somata and proximal dendrites of these NK-1 cells. In contrast 5-HT terminals formed few contacts with either excitatory (neurotensin, somatostatin, μ-opioid receptor) or inhibitory (γ-aminobutyric acid, glycine, choline acetyltransferase, nitric oxide synthase) dorsal horn local circuit cells. Noradrenergic terminals were few in number and distributed evenly along the dendritic tree for all cell types examined. These observations suggest that the serotonergic system in the dorsal horn preferentially targets a subpopulation of neurons which almost all project to supraspinal regions associated with nociceptive processing (Yu et al, 1999; Todd, McGill & Shehab, 2000).

Previous investigations have recorded serotonergic contacts associated with neurons in the cat dorsal horn of a similar frequency to the non-NK-1 neurons in the present investigation (Nishikawa et al, 1983; Miletic et al, 1984). Similarly, serotonergic contacts have been more frequently observed in association with dendritic than somatic profiles (Ruda et al, 1982; Light et al, 1983; Maxwell, Leranth & Verhofstad, 1983; Hylden et al, 1986; Marlier et al, 1991). Attempts to further characterise the targets for these serotonergic terminals either by morphological characteristics or immunocytochemical profiles have

identified both inhibitory and excitatory neurons receiving contacts from serotonergic terminals (Glazer & Basbaum, 1976; Miletic et al, 1984; Miller & Salvatiera, 1998) with some suggestion that there is a preferential innervation of inhibitory neurons (Militec et al, 1984). Whilst confirming that a proportion of excitatory and inhibitory local circuit cells receive serotonergic contacts this study has failed to identify a preferential innervation of inhibitory dorsal horn cells. Indeed, the reverse appears the case for somatic contacts where a greater proportion of excitatory neurons received somatic serotonin contacts than was the case for inhibitory neurons. In the work of Militec et al (1984) examining dorsal horn neurons in lamina I and II far greater numbers of serotonergic contacts per cell in each of the subtypes they studied were identified. This may arise due to the greater spatial resolution afforded by confocal microscopy when compared to light microscopy for identifying appositions. Hence, the higher absolute numbers of contacts observed in their study may have arisen as a result of an overestimate of the number of appositions. In addition, the neurons analysed in this study were identified as specific subpopulations based on their immunoreactivity. Militec and co-workers examined neurons based on their morphology. This study has shown that serotonergic terminals have the capacity to preferentially target subpopulations of cells within the dorsal horn. Thus in the small sample of neurons studied in the previous work a number of different neurons may have been included in each population so influencing the data. It would seem unlikely this could have such a significant effect as to produce such a high number of contacts as they observed however as in the corresponding laminae and neurons in this study contact numbers of the range they report were only found in relation to the NK-1 neurons.

In the ultrastructural observations in this study, no synaptic contacts were identified between serotonergic terminals and the somata of inhibitory neurons revealed by GABA-immunoreactivity, though occasional close appositions were noted. Previous ultrastructural observations on serotonergic profiles in the dorsal horn have recorded few or no axosomatic synaptic associations in the superficial dorsal horn (Ruda & Gobel, 1980; Ruda, Coffield & Steinbusch, 1982; Light, KavooKjian & Petrusz, 1983; Maxwell, Leranth & Verhofstad, 1983). Thus, the failure to identify synapses formed by serotonergic terminals on GABA-immunoreactive somata is in keeping with previous studies and suggests at least some of the few contacts observed with confocal microscopy may be non-synaptic.

Serotonergic contacts on projection tract neurons have also previously been described (see introduction for references), though no attempt had been made to identify the receptor characteristics of these neurons. In the present work the observation of a distinct population of neurons with a significantly higher frequency of serotonin contacts than any of the other neurons examined is one which had previously only been described with lamina I projection neurons (Hylden et al, 1995). In the present study the neurons of lamina I and Lamina III/IV which possess the substance P receptor neurokinin-1 have been shown to receive a greater frequency of serotonergic contacts than any of the other cell types examined in this study. Furthermore, approximately 50% of the lamina III/IV neurokinin-1 receptor-immunoreactive neurons were associated with clusters of serotonergic axons around their somata and proximal dendrites. This observation corresponded to a higher frequency of serotonergic contacts on these cells than those without this clustering. The arrangement of

serotonergic axons clustering around neuronal profiles observed here has not previously been described for serotonin in the dorsal horn. A distinction between two populations of cells based on the number of serotonergic contacts each receives has previously been made in the cat by Hylden et al (1986) who described two populations of lamina I projection neurons in this way. In the present study no clear separation of lamina I NK-1 receptor neurons could be made. It is conceivable that the NK-1 cells examined in this investigation could correspond to the more densely innervated population described in the cat by Hylden and co-workers. However, in this study cells were observed with contact numbers from 8 to 174 per cell. It is likely then that if a distinction between sparsely and densely innervated neurons exists in lamina I of the rat dorsal horn, NK-1 neurons would be included in both groups. Alternatively in the rat a clear distinction between groups of neurons based on their serotonin innervation may not exist within lamina I.

The observation of a distinct configuration of serotonergic axons in this investigation which formed 'basket-like' structures around proximal dendrites and somata of cells in the region of lamina III/IV led to the investigation of which cells may be associated with such a distribution of axons and whether this corresponded to a higher frequency of serotonergic contacts. Given the location of these profiles and their outline traced by the serotonergic axons the population of neurons which most closely resembled this description were the lamina III/IV neurokinin-1 receptor-immunoreactive neurons described by Todd and co-workers (see Naim et al, 1997). This hypothesis has been confirmed in the present study where two populations of lamina III/IV cells immunoreactive for the NK-1 receptor have been identified based

on their relationship to serotonergic axons and a correspondingly higher frequency of serotonergic contacts. This division would appear to be valid as examples of both types of neuron were present in material from each of the animals perfused and could be identified within the same section. Hence it is unlikely this observation resulted from an artefact of processing. Todd & colleagues (2000) have shown that almost all of the neurokinin-1 cells in lamina III/IV of the rat dorsal horn project to brainstem centres involved in nociceptive processing. The densely innervated NK-1 cells identified in this investigation are therefore likely to represent a subgroup of projection neurons. Previous investigations into the relationship of serotonergic terminals to projection neurons have observed a wide range of contact frequencies on labelled cells (Nishikawa et al, 1983; Hylden et al, 1986; Jankowska et al, 1995). These investigations did not however identify any subgrouping of the projection cells based on frequency of serotonergic contacts. This may suggest that the current observation is of a unique population of cells in the dorsal horn laminae III/IV which are NK-1 receptor-immunoreactive and receive a dense serotonergic innervation. Alternatively, the previous investigations may have included examples of these cells though their presence would have been obscured in the mixed sample of neurons examined in these papers. By selecting a relatively large population of cells and focussing on one specific receptor subtype in the present study the distinction between the two populations could be appreciated.

A common feature of all cells examined in this study was the relative paucity of noradrenergic contacts and their sporadic distribution across the immunoreactive profiles with both somatic and dendritic contacts observed. In no case was a population of cells identified where the

frequency of noradrenergic contacts appeared higher than in comparable neurons. For all the cell types examined the total number of noradrenergic contacts ranged from 0 to a maximum of 36. This observation is consistent with previous studies on the noradrenergic innervation of the dorsal horn where few contacts on dorsal horn cells have been described (Doyle & Maxwell, 1993). The present estimate is likely to be a reasonable representation of the distribution of noradrenergic terminals in the dorsal horn. Ultrastructural observations have previously identified noradrenergic synapses to be commonest on medium sized dendrites (described as 0.5-2μm diameter) though also present on large and small dendrites (Doyle & Maxwell, 1991). In the current study dendrites of this diameter were included in the analysis hence significant numbers of noradrenergic contacts should not have been omitted. A higher frequency of contacts on these medium sized neurons was not observed in this case. This may reflect the possibility that the present study cannot differentiate between close appositions and synaptic contacts. Hence, an overestimate of the number of contacts on large and small sized dendrites is conceivable. In the work of Doyle & Maxwell the number of contacts were based on true synaptic contacts.

Functional considerations

Clearly from the present data it can be seen that neither the serotonergic nor noradrenergic systems form large numbers of contacts with dorsal horn local circuit neurons. However, neurons in lamina I and lamina III/IV immunoreactive for the neurokinin-1 receptor, almost all of which are projection tract neurons, in contrast do receive relatively large numbers of serotonergic contacts. Thus, the descending serotonergic system would appear to selectively target projection tract neurons in the dorsal horn. As has been discussed, the descending monoaminergic systems have a selective role in antinociception at the level of the dorsal horn thus the findings of the present study must be interpreted in light of this action and the previous investigations of the monoaminergic pathways.

Monoamines and intrinsic dorsal horn cells

In the modulation of nociceptive information within the superficial dorsal horn one of the many possible theoretical circuits requires the monoamines to act via local dorsal horn neurons which in turn may operate either pre- or post-synaptically to mediate inhibition. Contacts between monoaminergic terminals and dorsal horn local circuit neurons have previously been described (Glazer & Basbaum, 1976; Hoffert et al, 1983; Miletic et al, 1984; Miller & Salvatierra, 1998). In this investigation examining the somatic and dendritic profiles of a wide variety of inhibitory and excitatory dorsal horn neurons, monoamine contacts were found to be relatively infrequent and distributed across the entire immunoreactive profile. At first inspection this may imply a minimal influence of monoaminergic terminals on these neurons. Contradicting this assertion however is the substantial evidence form behavioural and electrophysiological studies to suggest

that the monoaminergic systems, at least in part, produce their antinociceptive action via alternative neurotransmitters which are located in the neurons of the dorsal horn. For example, in the response of the dorsal horn to serotonin or its agonists there is evidence of an involvement of a variety of transmitters located within neurons of the dorsal horn. The inhibitory neurotransmitters GABA and glycine have both been demonstrated to produce an antinociceptive action at the level of the dorsal horn (Willcockson et al, 1984) which can be blocked by selective antagonist agents. Administration of selective GABA antagonists can also produce a partial inhibition of the of the antinociceptive effects of brainstem stimulation (Lin, Peng & Willis, 1994) as well as a partial inhibition of the antinociceptive responses to 5-HT (Yang et al, 1998) or a specific 5-HT_3 receptor agonist (Alhaider, Lei & Wilcox, 1991). In addition, serotonergic contacts have previously been reported on enkephalin containing neurons which in the dorsal horn are thought to represent a subpopulation of GABAergic neurons (Glazer & Basbaum, 1976; Miller & Salvatierra, 1998). In turn dorsal horn neurons utilising GABA as a transmitter have been demonstrated to form axo-axonic synapses on unmyelinated primary afferent terminals (Todd & Lochead, 1990) and to form contacts with projection neurons within nociceptive pathways (Lekan & Carlton, 1995). These neurons would therefore appear to be ideally placed as interneurons in a model neural circuit of the dorsal horn between the descending monoaminergic axons and the nociceptive pathways of the dorsal horn permitting both pre- and post-synaptic inhibition. It is conceivable that the few contacts between monoaminergic terminals and inhibitory neurons described in the present study may be sufficient for this pathway to operate. Of particular importance to the proposed interaction of these neurons is

the failure to demonstrate classical synaptic associations between serotonergic axon terminals and GABAergic somata suggesting that if this pathway is of significance it is likely to operate via non-synaptic transmission.

The significance of non-synaptic or volume transmission in the CNS has been debated for some considerable time (Golgi, 1891) with a recently developing interest in light of the expanding ultrastructural observations regarding putative neural circuits in the CNS (see Zoli et al, 1998). The proposal is that classical synapses need not be a prerequisite for intercellular communication. Instead, in addition to this mechanism, neurons may communicate via non-synaptic means through transmitter-receptor interactions where the transmitter is present at a sufficient concentration within the bathing extracellular fluid with the associated receptor expressed on the membrane of the target neuron, not necessarily within the synaptic cleft. Within the dorsal horn, noradrenaline (Rajaofetra, et al, 1992; Ridet et al, 1993) and serotonin (Maxwell, Leranth & Verhofstad, 1983; Ridet et al, 1993) typically form few classical synapses, with non-junctional varicosities being frequent. Evidence from several locations within the CNS suggests that the monoamines may indeed operate in part through non-synaptic transmission with serotonin release in regions of the CNS where synaptic specialisations are few corresponding with a high density of high affinity serotonin receptors (Bunin & Wightman, 1998). In the dorsal horn several receptor subtypes which have been identified, in particular 5-HT_{1A} (Marlier et al1991b) and 5-HT_3 (Glaum & Anderson, 1988) receptors are found in high concentration. Of these the 5-HT_{1A} receptor subtype has a high affinity for serotonin whilst the 5-HT_3 receptors have a relatively low affinity (Hoyer, 1990). In the

model of monoaminergic circuitry in the dorsal horn, it would be proposed that serotonin would excite inhibitory dorsal horn neurons, an action via the 5-HT_3 receptor. From the data presented in this study the action of serotonin on these neurons might be expected to operate via volume transmission (figure 1.20C). From the foregoing discussion however the expectation of the involvement of the low affinity 5-HT_3 receptor does not satisfactorily correlate with volume transmission. Clearly this association, in particular the monoamine receptor subtypes expressed by the dorsal horn local circuit neurons, requires further investigation.

Monoamines and dorsal horn neurokinin-1 receptor-immunoreactive neurons.

In the superficial dorsal horn the neuropeptide substance P is localised to small diameter primary afferent fibres which convey nociceptive information (Lawson, Crepps & Perl, 1997) and produce excitation of nociceptive neurons (Henry, 1976; Liu & Sandkühler, 1995). The corresponding receptor for this peptide, the NK-1 receptor, is in turn located on a population of neurons in lamina I and lamina III/IV which almost all project to brainstem regions involved with nociceptive processing (Todd, McGill & Shehab, 2000). This study has shown that in comparison to the intrinsic dorsal horn neurons studied, the NK-1 receptor-immunoreactive neurons of both lamina I and lamina III/IV are targeted by large numbers of serotonin-immunoreactive terminals with approximately half of the lamina III/IV neurons receiving a particularly high density of contacts. Thus, it would appear that the serotonergic system preferentially targets this population of nociceptive, projection tract neurons within the dorsal horn. Preliminary ultrastructural studies suggest that, as with intrinsic dorsal horn neurons, synaptic contacts

between serotonin terminals and neurokinin-1 receptor-immunoreactive profiles are infrequent though close appositions are noted (Maxwell, unpublished observations). However, the high density of serotonergic terminals associated with these neurons is likely to be of functional significance delivering a preferentially high concentration of serotonin to the extracellular fluid surrounding these profiles which by volume transmission is proposed to result in inhibition of these cells. In particular the high density of serotonergic terminals around the proximal dendrites and somata of the basket-associated cells would be expected to deliver an even higher concentration of serotonin at these sites. As has been established the neurokinin-1 cells of the dorsal horn represent a population of nociceptive projection tract neurons thus these serotonin terminals are directed towards the postsynaptic inhibition of these cells. It may be expected based on the density of specific receptor subtypes and the proposed direct inhibition that these cells would express the 5-HT_{1A} receptor subtype which has a relatively high affinity for serotonin and so is suited to non-synaptic transmission (figure1.20A).

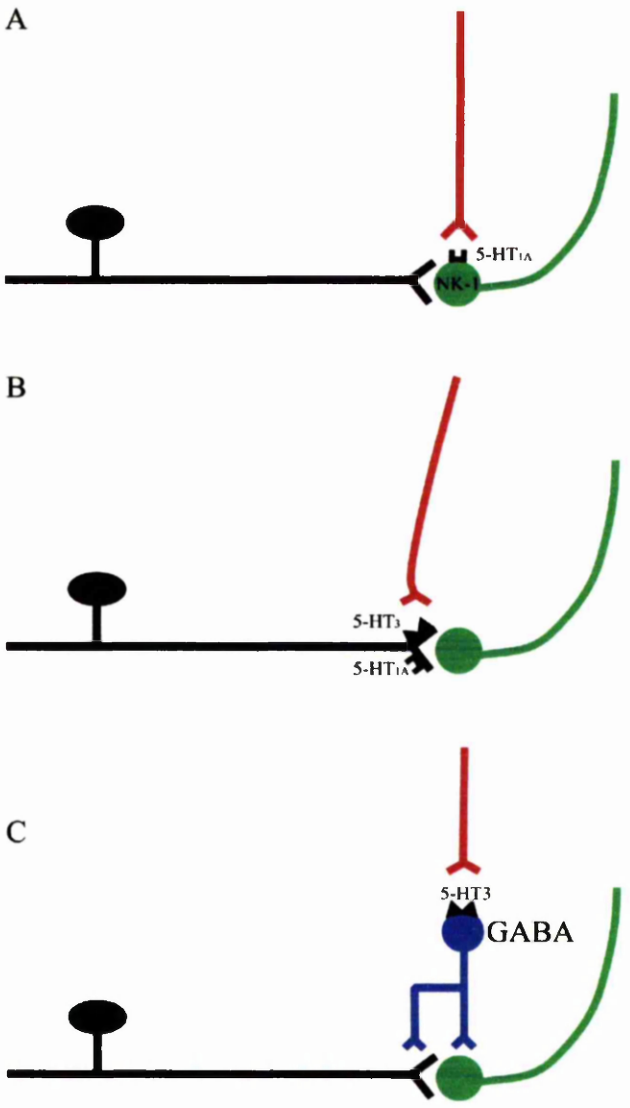


Figure 1.20. Proposal for the neural circuitry involving serotonin and nociceptive cells of the dorsal horn. From the data presented serotonin terminals (red) would appear to preferentially target NK-1 neurons (green) in lamina I and lamina III/IV of the dorsal horn which are also the targets for nociceptive primary afferent terminals (black). In mediating the inhibitory action of serotonin on these projection neurons it would seem, of the receptor subtypes present within the dorsal horn, the 5-HT_{1A} would be expected to be of importance to this pathway (A). In addition to this direct post-synaptic action, an action via the excitation of inhibitory dorsal horn neurons (blue) is also proposed (B). With the low frequency of contacts which have been identified on these cells this may operate in part via a volume transmission mechanism at the 5-HT_3 receptor subtype. Finally, though there is no morphological evidence to support a synaptic influence at the primary afferent terminal, 5-HT may still operate via volume transmission to produce pre-synaptic inhibition in this pathway. Evidence would suggest the presence of both 5-HT_{1A} and 5-HT_3 receptor subtypes on these terminals.

Chapter 2: The Cholinergic System in the Spinal Cord

2.1 The cholinergic system in the spinal cord

Activation of the cholinergic system in the spinal cord has a key role in a number of pathways producing antinociception (Iwamoto & Marion, 1993; Naguib & Yaksh, 1994; Abram & Winne, 1995), a rise in blood pressure (Feldman, Terry & Buccafusco, 1996), depolarisation of motoneurons (Zieglgänsberger & Reiter, 1974) and generation of programmed motor responses (Cowley & Schmidt, 1994). In addition, therapeutic agents have been identified acting via cholinergic receptors that have potential clinical applications in pain control (Swedberg et al, 1997). Despite this, relatively little is known about the chollnergic system in the spinal cord or the cholinergic receptor subtypes that may be of greatest value for therapeutic development.

2.2 Cholinergic profiles in the spinal cord

The distribution of cholinergic profiles in the spinal cord has been explored in a number of immunohistochemical studies using antibodies to the acetylcholine synthetic enzyme choline acetyltransferase. This technique is of particular value as immunoreactivity for this enzyme can be detected in somata, terminal boutons and medium to large sized dendrites of cholinergic neurons therefore allowing a great deal of the architecture of these ceils to be studied within one preparation. As may be expected from the involvement of acetylcholine in sensory, motor and autonomic pathways immunoreactive profiles are found throughout the spinal grey matter. Cholinergic neurons can therefore be identified in the motor nuclei of the ventral horn throughout the rostrocaudal extent of the spinal cord, in lamina X around the central canal, in the intermediate grey particularly in relation to autonomic nuclei and in the dorsal horn where they are concentrated in laminae III, IV and V. A similar distribution of immunoreactivity has been

described in rat (Barber et al, 1984; Borges & Iverson, 1986; Ichikawa et al, 1997), cat (Aquilonius, Eckernas & Gillberg, 1981; Kimura et al, 1981) and man (Aquilonius, Eckernas & Gillberg, 1981; Gillberg et al, 1982; Schafer et al, 1995).

2.3 Cholinergic ventral horn cells

Within the ventral horn choline acetyltransferase immunoreactivity is associated with both large and small motoneurons which correspond to the cholinergic efferent neurons innervating the skeletal muscles (Schafer et al, 1995). In addition to these choline acetyltransferase immunoreactive somata identified within the motor columns their dendrites can also be identified forming distinct bundles of immunoreactive profiles coursing throughout the ventral horn between the motor columns (Barber et al, 1984). Many of these cholinergic ventral horn neurons also receive synaptic contacts from cholinergic axons which may partly arise as recurrent collateral branches (Nagy, Yamamoto & Jordan, 1993; Schafer, Eiden and Weihe, 1998). At the ultrastructural level the synapses between cholinergic terminals and the ventral horn motoneurons exhibit a specific structural specialisation, the so called C-terminal, which has a postsynaptic specialisation formed by a subsurface cistern of endoplasmic reticulum separated from the plasma membrane by a relatively constant 10μm. The exact functional importance of this structure is unclear though it has been suggested it may perhaps be involved in calcium regulation at the synaptic junction. There is a strong association between the C-terminal and choline acetyltransferase immunoreactivity and as such it has been proposed as a morphological correlate for cholinergic terminals in this location (Nagy, Yamamoto & Jordan, 1993; Li et al, 1995).

2.4 Cholinergic dorsal horn cells

As noted above, choline acetyltransferase immunoreactivity reveals cholinergic neurons within laminae III, IV and V of the dorsal horn. These neurons give rise to dendrites running in a predominantly rostrocaudal direction with many extending into more superficial laminae and to some distance from the cell body (Barber et al, 1984; Todd, 1991). Combined immunocytochemistry for cholinergic profiles with antibodies to other neurotransmitters has revealed that the cholinergic dorsal horn cells contain GABA hence they are presumed to be inhibitory (Todd, 1991; Laing et al, 1994).

In addition to immunoreactive somata a dense region of punctate immunoreactivity is present at the inner lamina II border with lamina III representing a plexus of cholinergic terminals (Ribeiro-da-Silva & Cuello, 1990; Todd, 1991). Whilst there is evidence of a descending cholinergic projection from brainstem nuclei (Bowker et al, 1983) it is thought that the bulk of the terminals in this plexus originate from spinal cholinergic neurons as little immunoreactivity is lost following lesioning of the descending projection fibres (Kanazawa, et al, 1979). An origin in primary afferent terminals is also regarded as unlikely as choline acetyltransferase has not been identified in dorsal root ganglia of the rat (Barber et al, 1984). Ultrastructural studies of the cholinergic terminals within lamina II_i and lamina III have shown them to form predominantly symmetrical synaptic contacts with dendritic structures. Cholinergic terminals are also present in lamina I and lamina II_o though in a lower density than around the lamina II/III border and more often associated with symmetrical axo-axonic synapses (Ribeiro-da-Silva & Cuello, 1990). Many of the primary afferent sensory axons within the superficial dorsal horn terminate as structures termed glomeruli which

have been subdivided into two distinct types based on morphological criteria. In type I glomeruli the central axon is thought to derive from unmyelinated primary afferents whilst in type II the central axon is thought to derive from myelinated primary afferents (Ribiero-da-Silva & Coimbra, 1982). Cholinergic dendrites have been observed postsynaptic to both type I and type II synaptic glomeruli, in addition cholinergic axons form symmetrical axo-axonic synapses with the central axons of each type of glomerulus. Cholinergic axon terminals have also been identified pre-synaptic to dorsal horn dendrites, again forming symmetrical synapses (Ribeiro-da-Silva & Cuello, 1990). Thus from this arrangement of contacts primary afferent activity may influence cholinergic neurons within the dorsal horn via axodendritic associations. Cholinergic dorsal horn neurons in turn may result in both pre- and post-synaptic inhibition of information processed in the dorsal horn (primarily nociceptive information) via axo-axonic and axo-dendritic synapses.

2.5 Cholinergic neurons in the intermediate grey

Throughout the rostrocaudal extent of the spinal cord small to intermediate sized cholinergic neurons can be identified in the intermediate grey matter. In segments of the spinal cord associated with the origins of autonomic efferents larger cholinergic neurons, representing preganglionic autonomic neurons, are interspersed among these small to intermediate sized cholinergic cells (Barber et al 1984; Borges & Iversen, 1986). Lamina X has been noted to be particularly rich in small cholinergic neurons and dendritic profiles. From this region dendritic bundles extend through the surrounding grey matter to the dorsal, ventral and intermediate grey (Borges & Iversen, 1986).

2.6 Cholinergic receptors in the spinal cord

Application of acetylcholine to neurons within the spinal cord has been revealed to be associated with both muscarinic and nicotinic receptor specific response. Muscarinic receptor associated responses in the spinal cord are associated with a variety of responses including antinociception (Yaksh, Dirkson & Harty, 1985; Naguib & Yaksh, 1994; Abram & Winne, 1995; Eisenach, 1999), hypertension (Buccafusco, 1996) and both excitation and inhibition of motor neurons (Kurihara et al, 1993). Similarly nicotinic receptor responses have been shown to produce antinociception (Damaj et al, 1998; Marubio et al, 1999; Pan, Chen & Eisenach, 1999) and hypertension (Khan, Taylor & Yaksh, 1994).

Both muscarinic and nicotinic receptors have been identified within the spinal cord. Autoradiographic studies have localised muscarinic receptors throughout the spinal grey with highest densities in laminae II and III of the dorsal horn, lamina X and lamina IX with similar distributions in rat, cat and man (Kayaaip & Neff, 1980; Gillberg & Aquilonius, 1985; Gillberg, d'Argy and Aquilonius, 1988). The pathological process which underlies the debilitating condition amyotrophic lateral sclerosis produces a loss of neurons in the spinal motor nuclei and has been shown to result in a significant reduction in binding of muscarinic receptors in the ventral horn suggesting their presence on motor neurons (Gillberg & Aquilonius, 1985).

To date five distinct muscarinic receptor subtypes have been identified by molecular techniques, confirmed pharmacologically and denoted m1-m5 (Caulfield, 1993). Each of the receptors m1 to m4 have been

identified immunocytochemically within dorsal root ganglion with m2 and m4 receptors localised to small to medium sized neurons whilst m1 and m3 receptors do not show any particular distribution (Bernardini, Levey & Augusti-Tocco, 1999). Autoradiography for the m2 receptor has shown that it is distributed throughout the dorsai and ventral horns whilst the m3 receptor is predominantly localised to iaminae I to III. The m4 receptor is less specifically localised but is, nevertheless, present in the spinal cord. The m1 receptor has not been detected in the spinal cord by this technique (Höglund & Baghdoyan, 1997). These investigations are restricted in the conclusions that can be drawn as there is significant cross-reactivity within the agents used for the receptor subtypes. Conclusions on each of the receptor distributions have therefore been based on a knowledge of the kinetics of each receptor and using this information, together with varying the incubation times of the labelled agonist with or without presaturation of the other subtypes, to derive this data on receptor distributions. Though this approach does provide valuable data on general distributions of the muscarinic receptors it is likely to lead to significant and unavoidable errors as a result of overlap in labelling between the receptor subtypes. As specific antibodies to the muscarinic receptor subtypes become more widely available immunocytochemical techniques can be added to the observations which already exist so allowing a greater understanding of the distribution of muscarinic receptors in the spinal cord to be gained.

This approach has been employed in investigating the distribution of the m2 receptor subtype in the rat spinal cord. Mice lacking the m2 receptor demonstrate a significant reduction in antinociception and motor responses on administration of a non-specific muscarinic

agonist suggesting a role for this receptor in nociceptive processing and motor control (Gomeza et al, 1999). In pharmacological studies the role of this receptor in nociception has been debated with either no role (Naguib & Yaksh, 1997; Honda et al, 2000) or an antinociceptive effect, possibly through a pre-synaptic action (Bleazard & Morris, 1993) suggested. Recently the distribution of immunoreactivity for a specific m2 receptor antibody in the spinal cord has been investigated Yung & Lo (1997). Here m2-immunoreactive profiles were found to be abundant in the superficial laminae of the dorsal horn particularly in relation to punctate structures with no somatic labelling noted. In the ventral horn m2-immunoreactive profiles in apposition to presumed large motoneuron cell bodies were present. The absence of somatic staining in these experiments is of interest and at seeming variance to previous results using autoradiographic detection of the m2 receptor where it had been suggested that motoneurons do express the m2 receptor (Vilaro et al, 1992). Recently the m2-receptor has also been identified immunohistochemically on small diameter dorsal root ganglion cells (Haberberger, Henrich, Courad & Kummer, 1999).

Nicotinic cholinergic receptors have been reported to be significant in nociceptive and autonomic reflexes and, though present in lower concentrations than muscarinic receptors, nicotinic receptors have been demonstrated in high concentration in the superficial dorsal horn by autoradiography (Gillberg & Aquilonius, 1985; Gilberg, d'Argy & Aquilonius, 1988) and immunohistochemistry (Swanson et al, 1987). In addition receptor is also expressed on dorsal root ganglion cells (Polz-Tejra, Hunt & Schmidt, 1980). Nicotinic receptors are also present in the intermediate and ventral grey of the spinal cord (Swanson et al, 1987; Gilberg, d'Argy & Aquilonius, 1988). Administration of a nicotinic

receptor agonist has been shown to produce an increase in the threshold for a response to nociceptive stimuli (Lawand, Lu & Westlund, 1999) which is lost in mice lacking the nicotinic receptor (Marubio et al, 1999).

As described above the muscarinic m2 receptor is thought to be of some significance to responses throughout the spinal cord and in particular to the nociceptive pathway. Little detailed information is currently available on the cells which express this receptor and hence the circuits that it is involved in. In the following studies the distribution of the m2 receptor within the spinal cord is examined with particular attention to the cells within the superficial dorsal horn which express this receptor. In addition the relationship of the receptor to the cholinergic cells of the spinal cord is examined in detail.

2.7 Methods

Experimental animals

Adult female Albino-Swiss rats of less than six months of age were used throughout. The procedures for anaesthesia, perfusion and fixation were as described in the general methods section in chapter1 with 4% formaldehyde in phosphate buffer used as the fixative.

A. Distribution of m2-immunoreactive cells within the dorsal horn

Following an overnight postfixation period, mid lumbar spinal cord blocks from three animals were sectioned into 50μm transverse vibratome sections, incubated with 50% ethanol then transferred to a blocking solution of 10% normal donkey serum. The sections were then placed in the primary antibody cocktail containing rat anti-m2 (1:500) and mouse anti-NeuN (1:500), diluted in PBS with 1% normal donkey serum and 0.3% Triton X-100. Following an overnight incubation in primaries the sections were rinsed with PBS and transferred to the secondary antibody solutions containing FITC-anti-rat (1:100) and Cy-5-anti-mouse (1:100) in PBS with 0.3% Triton X-100 for a two hour incubation. The sections were then stained for propidium iodide by incubating in a solution of propidium iodide (1:100) with RNAase (1:100) in PBS for 30 minutes at 35°C. They were then rinsed, mounted with Vectashield and stored until analysed. Propidium iodide is excited at 568nm and has an emission spectrum distinct from those of FITC and Cy-5. Hence, using the three-colour confocal microscope NeuN, m2 receptor and propidium iodide stained profiles could be distinguished in a single tissue section.

Scanning and Analysis

Two complete dorsal horn sections from each of the three animals were examined. Overlapping series of 15 consecutive images at 1μm intervals in the z-plane were gathered for each dorsal horn beginning from the first 'full-face' image scanned from the surface of the section. Each series of sections were scanned using a x40 oil immersion objective lens. To scan each of the dorsal horn sections in their entirety between 25 and 35 overlapping series of images were therefore required. The series of images obtained were then viewed and a representative sample of dorsal horn neurons, identified by their double labeliing with NeuN and propidium iodide, examined for the presence or absence of m2 receptor-immunoreactivity. The sampled neurons were then plotted on an outline of the dorsal horn traced with a standard graphics package and their m2 receptor-immunoreactivity noted on this diagram. Data from each animal could then be pooled on a composite plot of the dorsal horn of the fourth lumbar segment.

Modified Optical Dissector Technique

In the quantitative analysis of the proportion of neurons within the dorsal horn expressing the muscarinic m2 receptor a modification of the optical dissector technique was employed to avoid selection bias towards larger immunoreactive somata (Coggeshall, 1992). This bias arises as a single optical section through a tissue which contains cells of varying diameters will result in a greater chance of a large cell being sampled than a small cell. Unless this bias is taken into consideration in the sampling method a disproportionate sample of large cells will be included in the data. To be confident of minimising the influence of this bias a method must be employed to counter this tendency to sample

large cells. One method of achieving an unbiased sample is by employing the optical dissector technique.

As applied to the current investigation two of the series of images acquired as described above were selected, the first as the 'reference section' and the second the 'look-up section'. The separation between these two images was such that there was no possibility of a neuronal nucleus lying entirely between the two images. For this series of experiments the separation between these two optical sections was 7μm with the reference section being at 5μm in the series of images and the look-up section being at 12μm. These images were then compared with cells selected for analysis only if their nuclei were present in the reference section and absent from the look-up section. By this sampling scheme, bias towards large cells should be excluded and a representative sample of superficial dorsal horn cells obtained.

In this series of experiments the analysis was performed by taking the series of images through the tissue section for the NeuN-immunoreactivity, merging these and saving the subsequent merged image as a single reference file. Single optical sections representing reference and look-up images were then selected as described above at 5 and 12μm through the stack of images. These images were then merged selecting a different pseudocolour (green or red) for the reference and the look-up images. The resultant merged image therefore allowed identification of nuclei within the reference and look-up sections in different colours. Any nucleus present in the reference section and in the look-up section was then automatically attributed a pseudocolour representing the merging of the two images (see figure 2.1). These cells were then excluded form analysis. Cells which were

included in the sample using this method were then followed through serial images with the m2 receptor and NeuN images merged. The presence or absence of receptor for each of the cells was then noted.

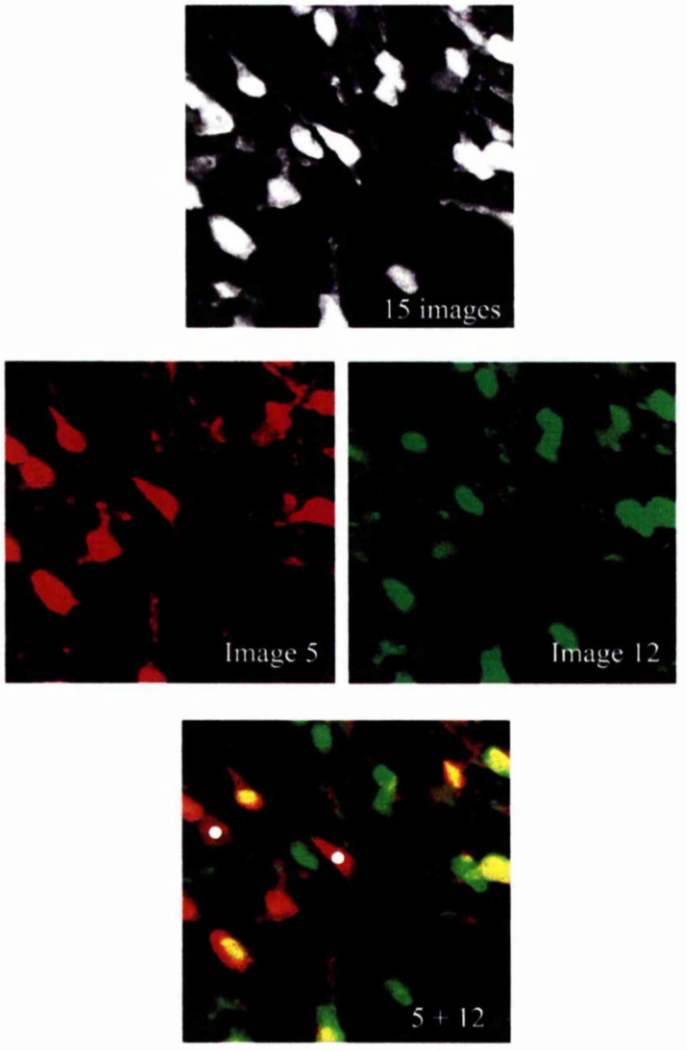


Figure 2.1. In the modified optical dissector technique, as applied in this series of experiments, a series of 15 images were gathered at 1μm intervals in the z-plane for NeuN immunoreactivity . Of these, the 5th (image 5) was then selected as the reference image and the 12th (image 12) as the look-up image. These images were then merged using Confocal Assistant with each being assigned a separate colour (5 + 12). Any nucleus present in both reference and look-up section appeared in the resultant image with a pseudocolour assigned by the software representing the merging of the two separate colours. These cells were excluded from further analysis with only those cells present in the reference and not the look-up sections included (circles).

B. Quantitative analysis of the subtypes of dorsal horn neurons expressing the m2-receptor

Transverse vibratome sections from three animals for each protocol were collected as has been described in the previous section. Following incubation in ethanol and blocking serum these were then placed in primary antibody solutions containing PBS with 1% NDS plus 0.3% triton X-100 and antibody to the m2 receptor (rat anti-m2; 1:500). To this either sheep anti-NOS (1:2000), mouse anti-parvalbumin (1:100), rabbit anti-neurotensin (1:1000), guinea-pig anti-sst_{2A} (1:2000), guinea-pig anti NK-1 (1:1000) or rabbit anti-GABA (1:1000) was added as antibody to a population of dorsal horn cells of interest. The sections were incubated in this primary antibody solution for one day following which they were rinsed, transferred to the secondary solutions containing the appropriate fluorophore coupled antibodies for 3 hours, rinsed, wet mounted and stored until required.

Analysis

A total of nine dorsal horn sections was analysed for each of the primary antibody combinations (three dorsal horns form each animal). In each case the entire thickness of the vibratome section was scanned using the confocal microscope from the first complete optical field to the last in 2μm z-steps using a X40 oil immersion objective lens. Series of images were gathered through the medio-lateral extent of laminae I-III with each optical field scanned overlapping such that the entire superficial dorsal horn was sampled. The series of images obtained was then examined in its entirety and all immunoreactive dorsal horn cells identified. These cells were then followed through the series of optical sections and examined for the presence or absence of

m2 receptor-immunoreactivity and the details noted on an outline of the dorsal horn. Data was therefore obtained on the m2 receptor status and location for each immunoreactive dorsal horn cell in the vibratome section. By transferring this data to a composite drawing of a standard L4 dorsal horn section, the results for each of the separate dorsal horns could then be pooled.

C. The relationship of the m2 receptor to choline acetyltransferase-immunoreactive neurons in the dorsal horn and lamina X.

Fourth lumbar spinal cord blocks from four experimental animals were sectioned in the transverse plane and placed in 50% ethanol then 10% NDS as has been described. The sections were then incubated for 24 hours in a primary antibody solution containing rat anti-m2 (1:500) and goat anti-ChAT (1:100) diluted in PBS with 1% NDS and 0.3% Triton X-100. Following this the sections were transferred to secondary antibody solutions containing FITC anti-rat and LRSC anti-goat (both 1:100) in PBS for 3 hours before being mounted with anti-fade medium and stored. In two separate animals one week prior to perfusion the animal was pre-injected with 250μl of a 2mg/ml solution of fluorogold (i.p.) to act as a retrograde tracer of autonomic efferents so staining lateral horn cells. The eighth thoracic and fourth lumbar spinal cord blocks were then collected, sectioned and processed as has been described above with the addition of rabbit anti-fluorogold antibody (1:5000) to the primary antibody solution which was in turn revealed with Cy-5 anti-rabbit included in the secondary antibody solution.

Analysis

Four sections from each of the animals were examined. First, a series of overlapping images through the entire thickness of the tissue section was gathered at a low magnification (X20 objective) for ChAT-immunofluorescence. These images were then used to generate a montage image of the ChAT-immunoreactive profiles in the cord section. ChAT-immunoreactive neurons are sparse in the dorsal horn, thus the montage generated as described above clearly showed each of the ChAT-immunoreactive neurons in the cord section. Using this montage for reference, each of the neurons was then re-scanned for both ChAT and m2-immunoreactivity using a X40 oil immersion objective with an optical zoom of 1.5. The cells were then closely examined for the presence of m2-immunoreactivity and this information recorded on the original montage image. For the mid-lumbar sections obtained from the fluorogold pre-injected animals sections from the region corresponding to the intermediolateral grey were also examined for the presence or absence of fluorogold staining. The thoracic sections from these animals were examined to confirm that the fluorogold injection had been successful and that labelled presynaptic sympathetic motor neurons could be clearly identified.

D. The relationship of the m2 receptor to small and large motoneurons of the ventral horn.

Motoneurons of the gastrocnemius-soleus (G-S) group were retrogradely labelled in three adult female Albino-Swiss rats, under Halothane anaesthesia, by an intramuscular injection of fluorogold (100μl of a 2mg/ml solution). After a seven day survival the animals were anesthetised, perfused with 4% formaldehyde in phosphate

buffer and the 4th and 5th lumbar segments were removed. These were then postfixed for 4 hours prior to sectioning in the transverse plane as has been described. The sections were then incubated in 50% ethanol followed by 10% NDS for 1 hour prior to transferring them to the primary antibody cocktail for a two day incubation at room temperature. The antibody cocktail consisted of rabbit anti-fluorogold (1:5000), goat anti-ChAT (1:100) and rat anti-m2 receptor (1:1000) in PBS with 1% NDS plus 0.3% Triton X-100. The sections were then rinsed and placed in solution containing fluorophore coupled antisera which consisted of FITC anti-rat, LRSC anti-goat and Cy-5 anti-rabbit antibodies (all 1:100) in PBS with 0.3% triton X-100. Following a two-hour incubation the sections were rinsed, mounted on glass slides with anti-fade medium and stored at -20°C until required.

Analysis

Sections were examined using the confocal microscope to determine the relationship between the G-S motoneurons, the receptor and ChAT-immunoreactive terminals. Series of six sequential optical sections at 1μm intervals were then gathered from ventral horn nuclei which contained Fluorogold-labelled cells using a X20 objective lens. Cells with obvious nucleoli were chosen for analysis. Each cell was now numbered and examined for the presence or absence of m2 receptor-immunoreactivity with a subjective estimate of the intensity of receptor immunoreactivity recorded. In addition the number of cholinergic contacts on each cell was counted and averaged for each of the six optical sections. Finally, diameters of cell bodies were measured with a Kontron KS 400 image analysis system and expressed as diameters of an equivalent circle thus permitting the

circumference of each cell to be estimated and the number of cholinergic contacts expressed per micron of circumference.

2.8 Results

A. The distribution of the acetylcholine m2 muscarinic receptor in spinal cord.

Muscarinic m2 receptor-immunoreactivity was present throughout the grey matter of the spinal cord (figure 2.2). A high density of labelling was present within the superficial laminae of the dorsal horn (lamina I and II). Much of this labelling was noted to be punctate though a significant number of clearly labelled somatic profiles was present with labelling uniformly present around the cell membrane which occasionally extended to the proximal dendrites. Deeper laminae of the dorsal horn displayed a lower density of staining with both punctate and somatic labelling present. In the thoracic cord a small number of neurons in the region of the intermediolateral cell column were noted which were immunoreactive for the muscarinic m2 receptor and showed immunoreactivity for fluorogold following retrograde transport of the tracer from an intraperitoneal injection (figure 2.3). Around the central canal in the region of lamina X numerous medium to large sized neurons were present. Coursing from this region and from the region of the lateral grey a number of m2 receptor-immunoreactive profiles with the appearance of large axons or dendrites are present linking to the motor nuclei of the ventral horn. Within the ventral horn motor nuclei numerous medium to large sized neurons are noted which display strong receptor immunoreactivity.

B. The distribution of muscarinic m2 receptor-immunoreactive somata in the dorsal horn.

Six complete sections of dorsal horn were sampled from three experimental animals (two from each animal) and analysed using the modified dissector method. In total 285/1246 (22.9%) dorsal horn neurons sampled were immunoreactive for the m2-receptor. These cells were distributed throughout the superficial and deep dorsal horn laminae though the proportion of positive cells in each laminar region increased from the superficial to deep laminae (figure 2.4). All of the cells sampled were NeuN-immunoreactive and hence assumed to be neuronal. The lowest proportion of m2-immunoreactive neurons identified corresponded to those located in lamina I where only 9.3% (10/107) of neurons sampled expressed the receptor. The highest proportion of m2-receptor-immunoreactive neurons was identified in the deeper dorsal horn in laminae IV-VI where 28.6% (172/601) of neurons were receptor positive whilst 19.1% of lamina II/III (103/538) cells were immunoreactive for the receptor (figure 2.5).

As has been noted m2-receptor-immunoreactive neurons are also present around the central canal in the region of lamina X. With the same form of analysis the proportion of neurons in lamina X that displayed m2 receptor-immunoreactivity was calculated. Of the neurons sampled 23.8% (15/63) were immunoreactive for the receptor.

C. Quantitative analysis of the subtypes of dorsal horn neurons expressing the m2-receptor

The above data demonstrates that the muscarinic m2 receptor is present on a significant proportion of neurons in the superficial dorsal horn, particularly in the lamina II/III region where a number of neuronal subtypes have been described. Examining the relationship of this receptor to specific neuronal subtypes in this region reveals that the proportion of cells expressing the receptor is not uniform amongst those cells examined (figure 2.6). One thousand and ninety-four GABA-immunoreactive neurons were identified throughout laminae I to III of which 34% displayed immunoreactivity for the m2 receptor with little variation in proportion of positive cells between the laminae. The distribution of the GABA-immunoreactive cells from the nine dorsal horn sections which were analysed is illustrated in figure 2.7. This illustration serves to demonstrate the uniform distribution of cells which were double immunofluorescent for GABA and the m2 receptor. A similar proportion of NOS-immunoreactive neurons was identified as m2 receptor-immunoreactive (28%; figure 2.8). In contrast however there was a clear variation in the proportion of cells which were positive within each of the laminae with 4%, 20% and 55% of NOS-immunoreactive neurons m2 receptor-immunoreactive in lamina I, II and III respectively (figure 2.6). Furthermore, within lamina III it is the deeper placed NOS cells which tended to express the m2 receptor (figure 2.8). A correlation between cell location and m2 receptor-immunoreactivity was also noted for parvalbumin-immunoreactive neurons (figure 2.9). Of the 125 parvalbumin neurons identified 8% (14/125) were identified as m2 receptor positive with the proportion remaining constant through lamina II and III (figure 2.6). The parvalbumin cells which were sampled were distributed evenly across

the mediolateral extent of the dorsal horn around the border of lamina II with lamina III. However, m2 receptor-immunoreactive neurons were found almost exclusively amongst the cells at the medial extent of this region with all but two of these cells identified here. The two cells not at this medial location were identified at the extreme lateral margin of the dorsal horn grey matter with no m2 positive parvalbumin neurons noted between these two groups. Of the 456 sst_{2A}-immunoreactive neurons examined, 115 (25%) were found to co-express the m2 receptor with a greater proportion of the lamina II immunoreactive neurons positive than lamina I neurons (27% and 7% respectively; figure 2.10).

Thus of the presumed inhibitory dorsal horn neurons examined, with the exception of parvalbumin-immunoreactive neurons, between 25 and 30% are m2 receptor-immunoreactive. This contrasts with neurotensin-immunoreactive neurons which are presumed excitatory. Of the 151 neurotensin-immunoreactive neurons sampled from laminae I to III none expressed the m2 receptor (figure 2.11). Low m2 receptor-immunoreactivity was also noted with lamina I NK-1 receptor-immunoreactive neurons of which only 12% (15/122) were m2 receptor positive. In contrast the proportion of the deeper dorsal horn NK-1 receptor-immunoreactive neurons which expressed the m2 receptor was higher with 42% (36/86) immunoreactive (figure 2.12).

D. The relationship of the m2 receptor to choline acetyltransferase-immunoreactive neurons in the dorsai horn and lamina X.

As previously described choline acetyltransferase neurons were identified throughout the deeper dorsal horn laminae (III-VI) and around the central canal in the region of lamina X. In total, 41% (101/246) of ChAT-immunoreactive neurons in the dorsal horn and lamina X were immunoreactive for the muscarinic m2 receptor. The proportion of cells immunoreactive for the receptor was similar in lamina III (46%) and lamina X (44%) with fewer cells in lamina IV-VI immunoreactive (37%; figure 2.14B). Figure 2.13 illustrates the distribution of ChAT-immunoreactive neurons in one of the dorsal horn sections examined with an example of an m2 receptor-immunoreactive neuron whilst in figure 2.14A a composite drawing of all the ChAT-immunoreactive neurons has been generated showing the distribution of the receptor positive and receptor negative neurons in the nine dorsal horn sections examined. This composite illustration highlights the variation in proportion of receptor positive neurons dependant on their location within the respective laminae that is not immediately apparent in single dorsal horn sections. Within lamina III-V and lamina X the receptor positive cholinergic neurons have a uniform distribution which mirrors that of the receptor negative cells. In lateral lamina VI however the composite plot reveals a focus of cholinergic neurons which show a high proportion of m2 receptor-immunoreactivity. The nature of these neurons is not immediately apparent though it is conceivable that they may correspond to a caudal extension of the intermediolateral cell column in the animals under investigation. In the same animals as used for the initial observation of the distribution of m2 receptor neurons which had received an intraperitoneal injection of

fluorogold the tracer was examined for in the region of this cluster of m2 positive cholinergic neurons. Confirming previous studies on the extent of the neurons of the intermediolateral cell column in the rat, no fluorogold neurons were identified in any dorsal horn section examined from the mid-lumbar spinal cord (figure 2.15).

E. The relationship of the m2 receptor to small and large motoneurons of the ventral horn.

Typically, retrogradely labelled neurons of the gastrocnemius-soleus group were found in the ventral horn of the fourth lumbar segment in a column located in the dorsal part of the lateral motor nucleus. This distribution corresponds to that identified by previous investigators (Nicolopolous-Stournaras & Iles, 1983; Peyronnard et al, 1986). A total of 153 motoneurons (50, 70 and 33 from each of the three animals) was examined for the presence of m2 receptor-immunoreactivity and cholinergic contacts. Figure 2.17A shows the frequency of receptor-immunoreactivity in relation to cell diameter. With increasing cell diameter the proportion of cells which were receptor immunoreactive increased such that cells with diameters greater than 40μm were almost always m2 receptor immunoreactive (99/100 cells). These cells characteristically displayed intense immunoreactivity for the m2 receptor which was evenly distributed along the surface of the cell and extended along the proximal dendrites (figure 2.16). In contrast to these large cells, the proportion of smaller cells (less than 35μm in diameter) which were receptor-immunoreactive was lower (60%) with immunoreactivity for the receptor being relatively weak if present (figure 2.16). This difference correlated with the frequency of cholinergic terminals identified in apposition with these groups of cells. Typically the larger cells were associated with numerous cholinergic

terminals whilst smaller cells received few or no cholinergic contacts (figure 2.17B).

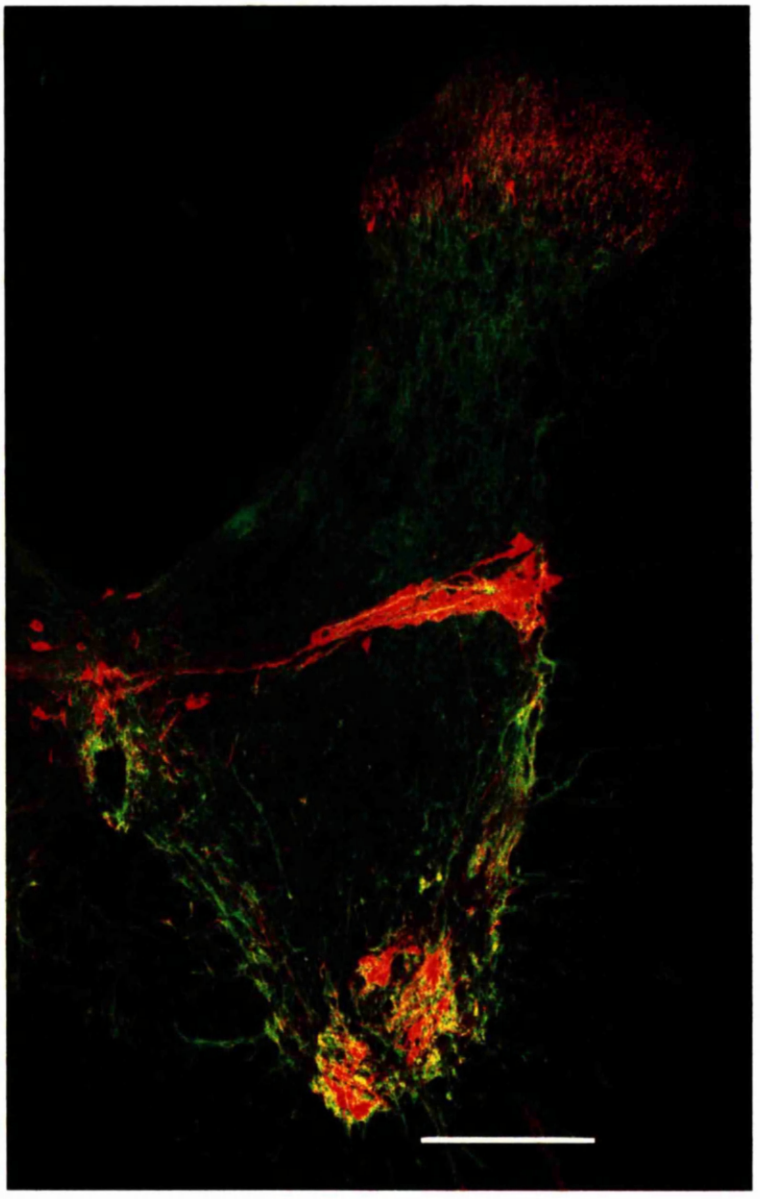


Figure 2.2. The distribution of the muscarinic m2 receptor (green) and choline acetyltransferase profiles (red) in the rat thoracic spinal cord. This montage image, created from multiple merged, projected two-colour images of a section from the eighth thoracic spinal cord block, demonstrates clearly the distribution of m2 receptor- and ChAT-immunoreactivity. The m2 receptor is present on profiles throughout the spinal grey. In the dorsal horn there is a relatively high density of labelling within lamina I and lamina II though staining is also noted in the deeper laminae with both punctate and somatic labelling identified. M2 receptor-immunoreactivity is also present in high density in the motor nuclei of the ventral horn, lamina X and in the region of the intermediolateral cell column. Interconnecting each of these areas were numerous immunoreactive profiles consistent with large axons or dendrites. ChAT-immunoreactive profiles corresponded to previous descriptions with scattered neurons in the deeper dorsal horn, the intermediolateral cell column, lamina X and the motor nuclei of the ventral horn (scale bar 250μm).

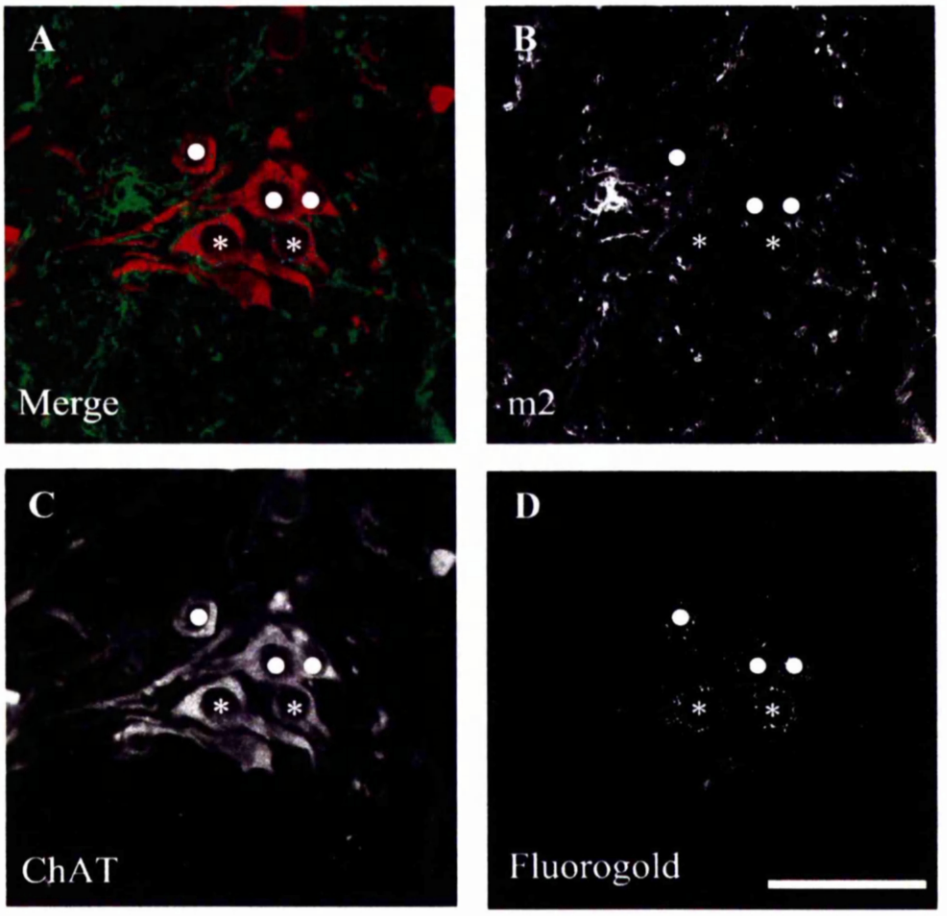


Figure 2.3. In the region of the intermediolateral cell column a proportion of ChAT-immunoreactive neurons (red in A) which were also m2 receptor-immunoreactive (green in A) neurons were identified. Following an intraperitoneal injection of fluorogold these cells were immunoreactive for fluorogold (blue in A) and hence thought to be autonomic efferent neurons (asterisk - m2 receptor positive cell; circle - m2 receptor negative cell; single optical sections; scale bar 50μm).

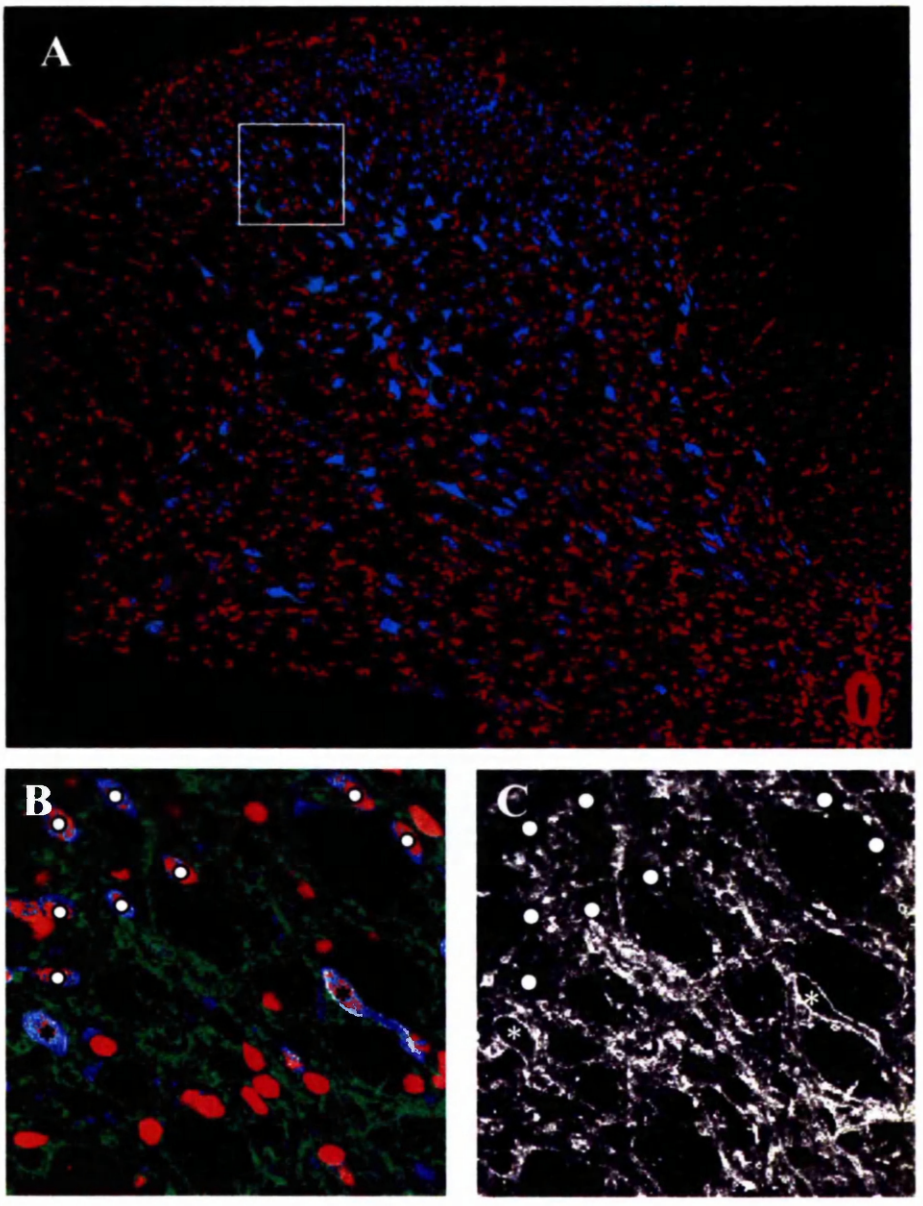


Figure 2.4. Double labelled propidium iodide (red) and NeuN-immunoreactive (blue) profiles, corresponding to neurons, were identified throughout the dorsal horn as illustrated in the montage image reconstructed from the projected, merged images through one of the dorsal horns used in the analysis (A). As illustrated in the single optical sections in images B & C, immunoreactivity for the muscarinic m2 receptor (green; C) is present on a proportion of these cells (asterisks) associated with a uniform expression over the cell body and proximal dendrites. Non-m2 receptor-immunoreactive neurons are also present (circles)

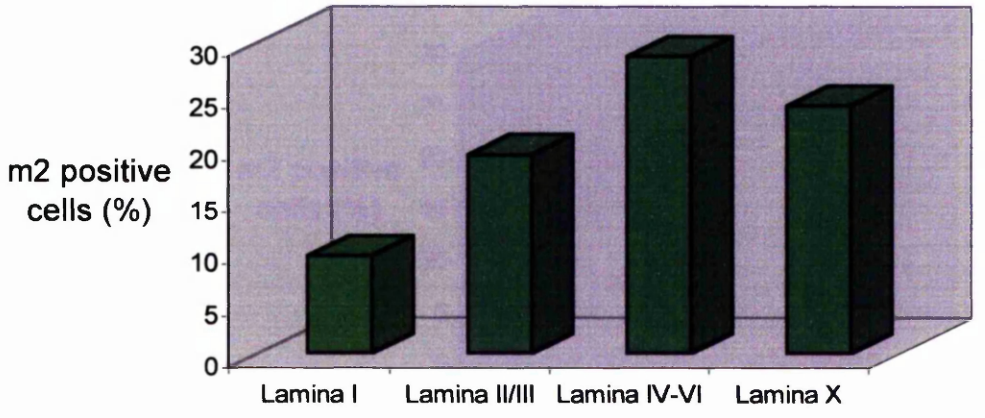


Figure 2.5. Cells immunoreactive for the muscarinic m2 receptor were identified in all laminae of the lumbar dorsal horn and lamina X. Of the cells sampled from the dorsal horn, 22.9% were m2 receptor-immunoreactive however the proportion of immunoreactive cells varied between the laminae with the cells of the deeper laminae (IV-VI) more commonly receptor positive. A similar proportion of the neurons in the region of lamina X were receptor positive (23.8%) as in the dorsal horn.

Cell Type	Location	m2 +ve	Total	% positive
GABA	**lamina I**	**23**	**72**	**32**
	lamina II	**192**	**616**	**31**
	lamina III	**158**	**406**	**39**
NOS	lamina I	1	27	4
	lamina II	68	337	20
	lamina III	67	122	55
parv	**lamina II**	**6**	**76**	**8**
	lamina III	**5**	**60**	**8**
sst2a	lamina I	3	45	7
	lamina II	112	411	27
neurotensin	**lamina I**	**0**	**7**	**0**
	lamina II	**0**	**72**	**0**
	lamina III	**0**	**72**	**0**
NK-1	lamina I	15	122	12
	lamina III/IV	36	86	42

Figure 2.6. Frequency of m2 receptor expression on different subtypes of cells in the dorsal horn related to laminar location.

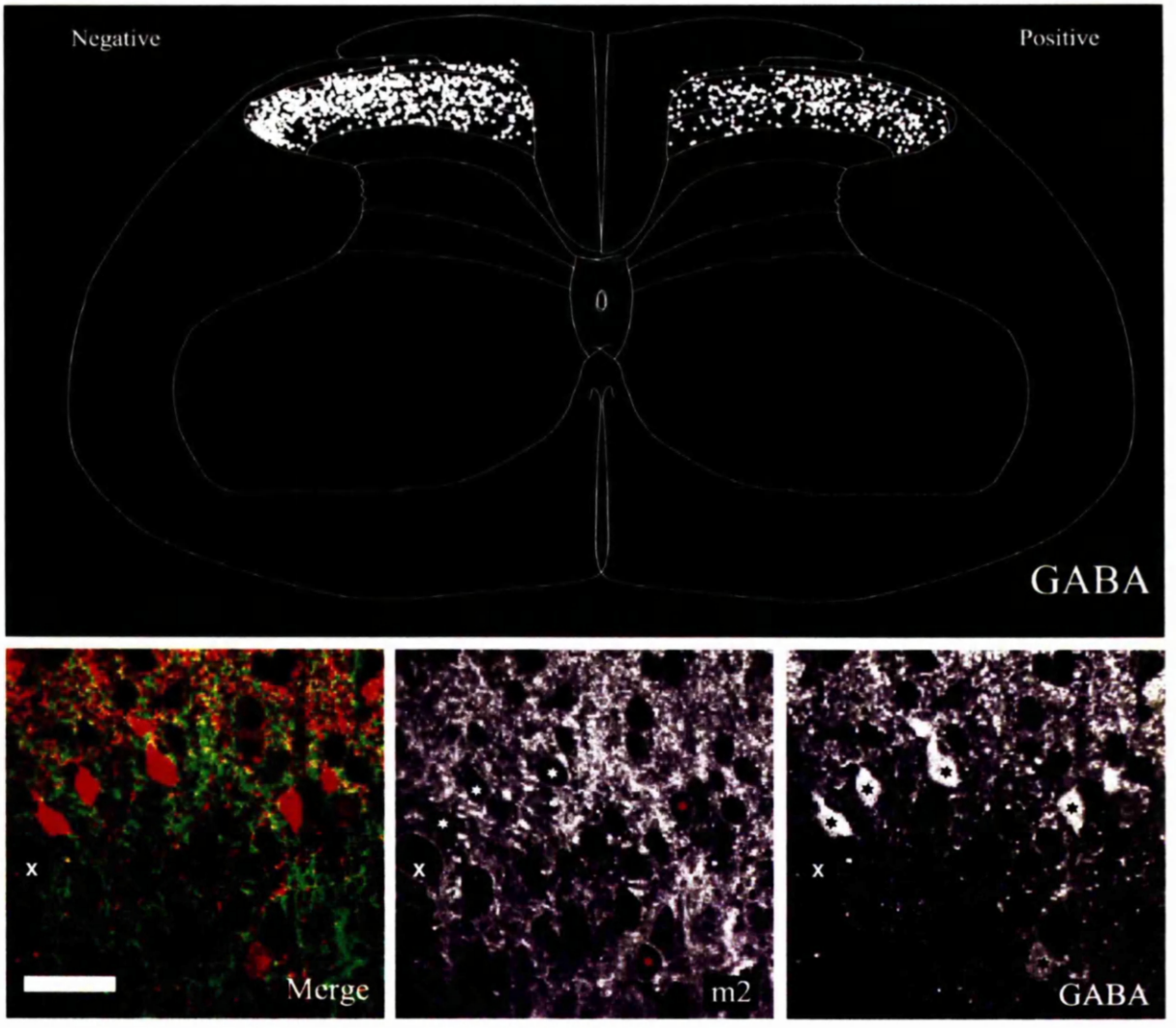


Figure 2.7. Composite image illustrating the distribution of the 1094 GABA-immunoreactive neurons sampled in this study and their relationship to the muscarinic m2-receptor. GABA-immunoreactive neurons which expressed the m2 receptor were identified throughout the superficial horn and corresponded to between 31% and 39% of the GABA-immunoreactive neurons within each lamina. This relationship is illustrated in the confocal images where the GABA-immunoreactivity (red) and m2 receptor-immunoreactive in a single optical section from lamina II is shown. Both m2 receptor positive (red stars) and m2 receptor negative (white stars) GABA neurons can be clearly identified. In addition, an m2 receptor-immunoreactive neuron which was not GABA-immunoreactive is noted (X; scale bar 25μm)

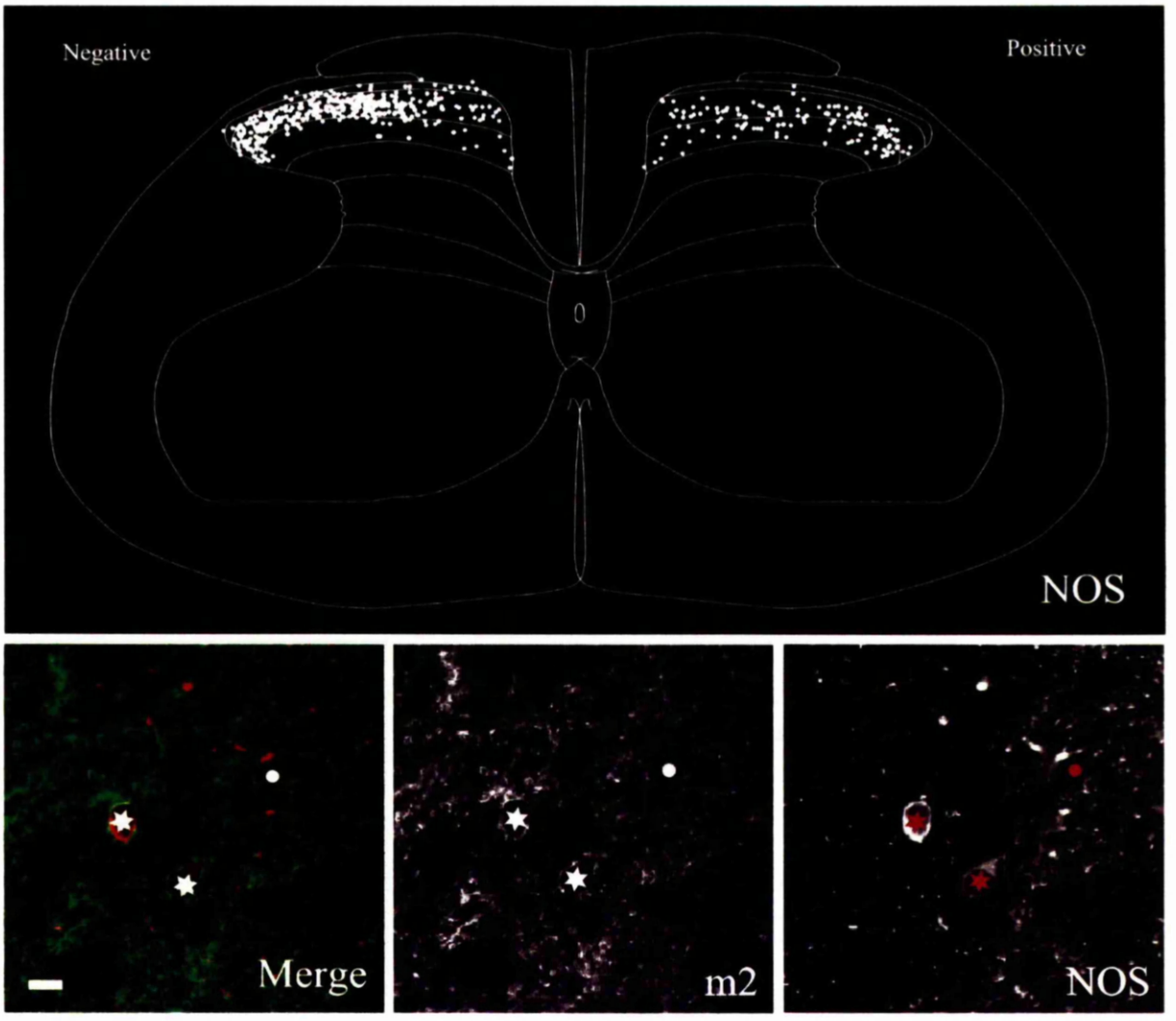


Figure 2.8. Composite image showing the locations of NOS-immunoreactive cells which were m2 receptor positive or negative in the superficial dorsal horn. As illustrated m2-receptor immunoreactive NOS cells were present in each of the laminae I-III. However the proportion of m2 receptor positive cells increased from lamina I to III such that though only 4% of lamina I cells were immunoreactive for the receptor this figure was 55% in lamina III. The confocal images illustrate for a single optical section the relationship of this receptor to two NOS-immunoreactive neurons in lamina III (stars). An m2 receptor-immunoreactive cell which was not NOS-immunoreactive is also noted (circle; scale bar 10μm).

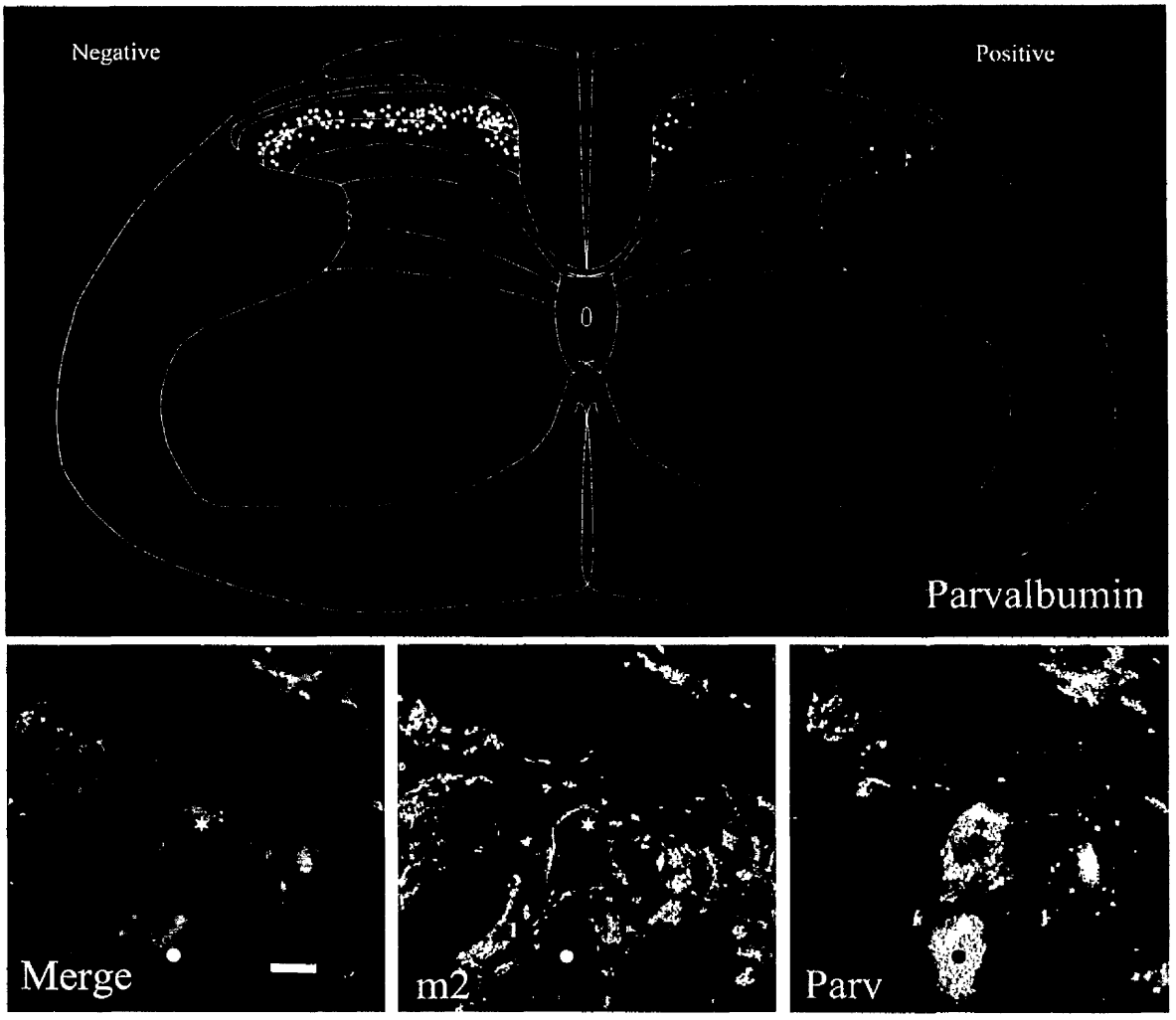


Figure 2.9. Immunoreactivity for the m2 receptor was infrequent in relation to parvalbumin-immunoreactive cells in the superficial dorsal horn as illustrated in the composite image showing m2 receptor positive and m2 receptor negative parvalbumin neurons in the superficial dorsal horn. Those parvalbumin neurons which were m2 receptor-immunoreactive were located at the medial or lateral extremes of the dorsal horn. The series of confocal images illustrates in a single optical section the relationship of the m2 receptor to a parvalbumin neuron (star). An m2 receptor negative parvalbumin-immunoreactive neuron is also noted (circle; scale bar 25μm).

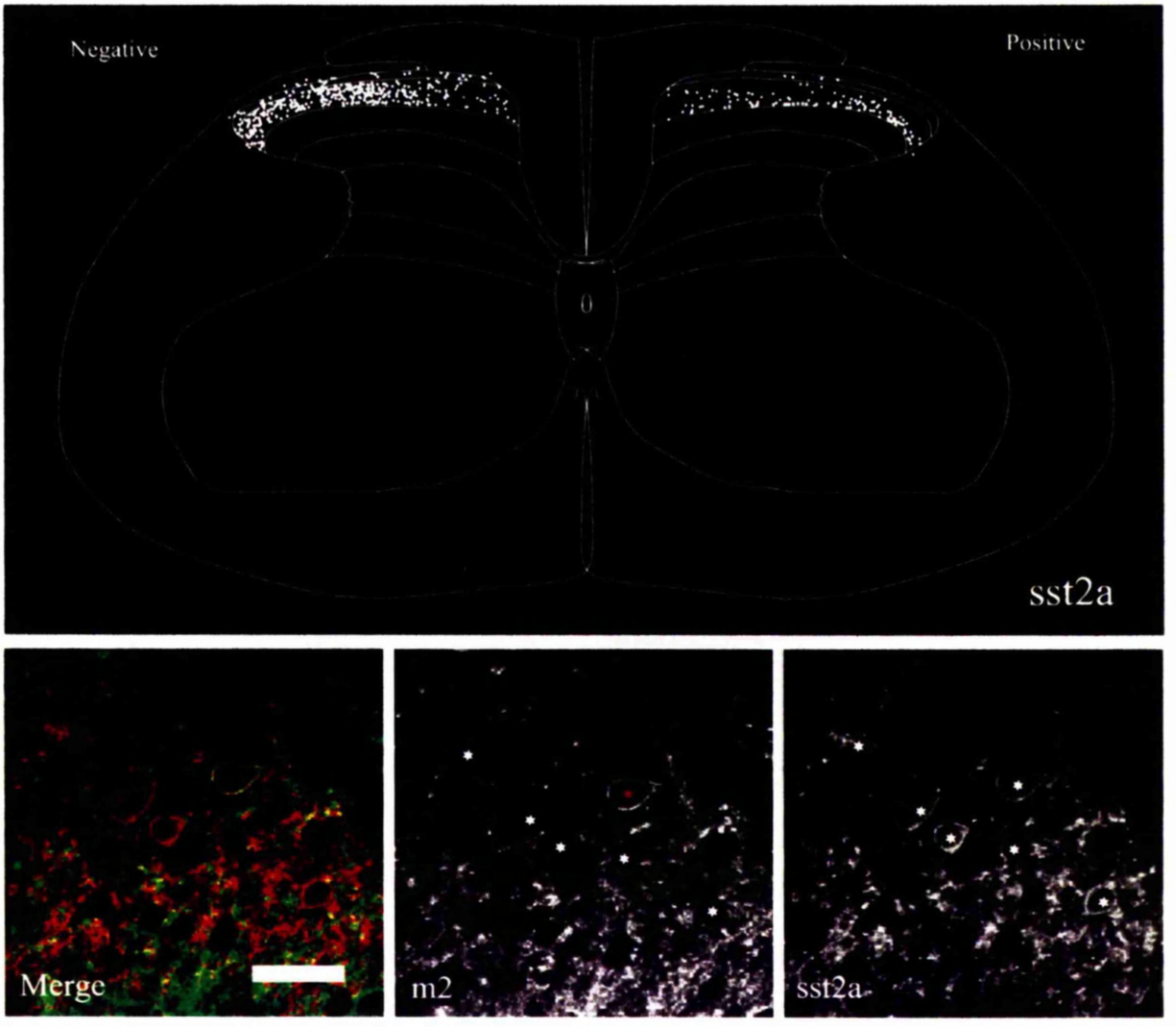


Figure 2.10. Immunoreactivity for the m2 receptor was more commonly identified in association with sst_{2A}-immunoreactive neurons in lamina II than lamina I as illustrated in the composite image showing the locations of all m2 receptor positive and negative sst_{2A} neurons sampled. Several sst_{2A}-immunoreactive neurons (stars) are present in the single optical sections showing m2- and sst_{2A}-immunoreactivity in lamina II of the dorsal horn. Of these a single sst_{2A} neuron is m2 receptor-immunoreactive (red star; scale bar 25μm).

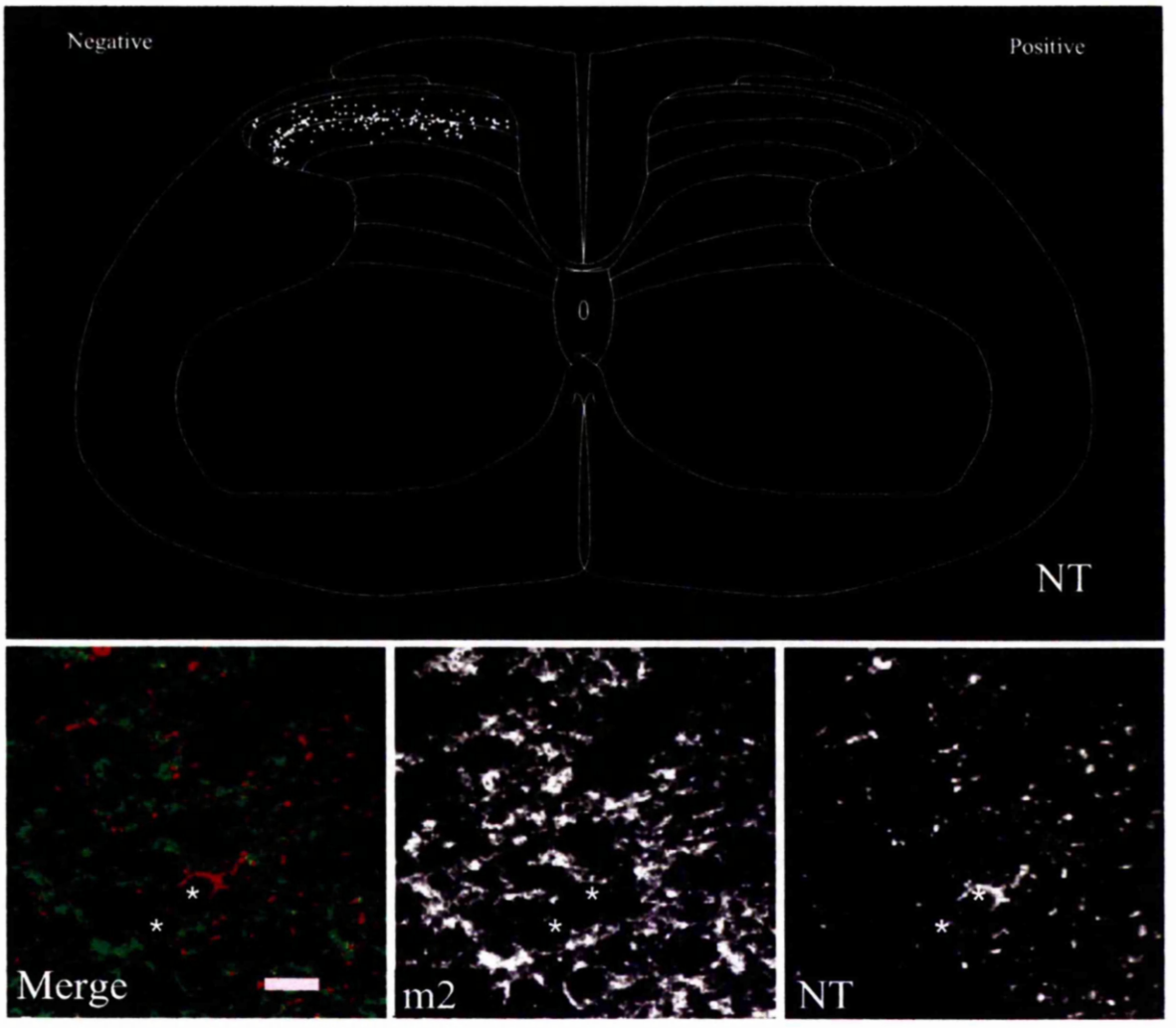


Figure 2.11. Composite image illustrating the locations of m2 receptor negative, neurotensin-immunoreactive neurons in the superficial dorsal horn. A total of 151 neurotensin-immunoreactive neurons was sampled with no m2 receptor-immunoreactive cells identified. Examples of two neurotensin cells located in lamina II are shown in the single optical sections (scale bar 10μm).

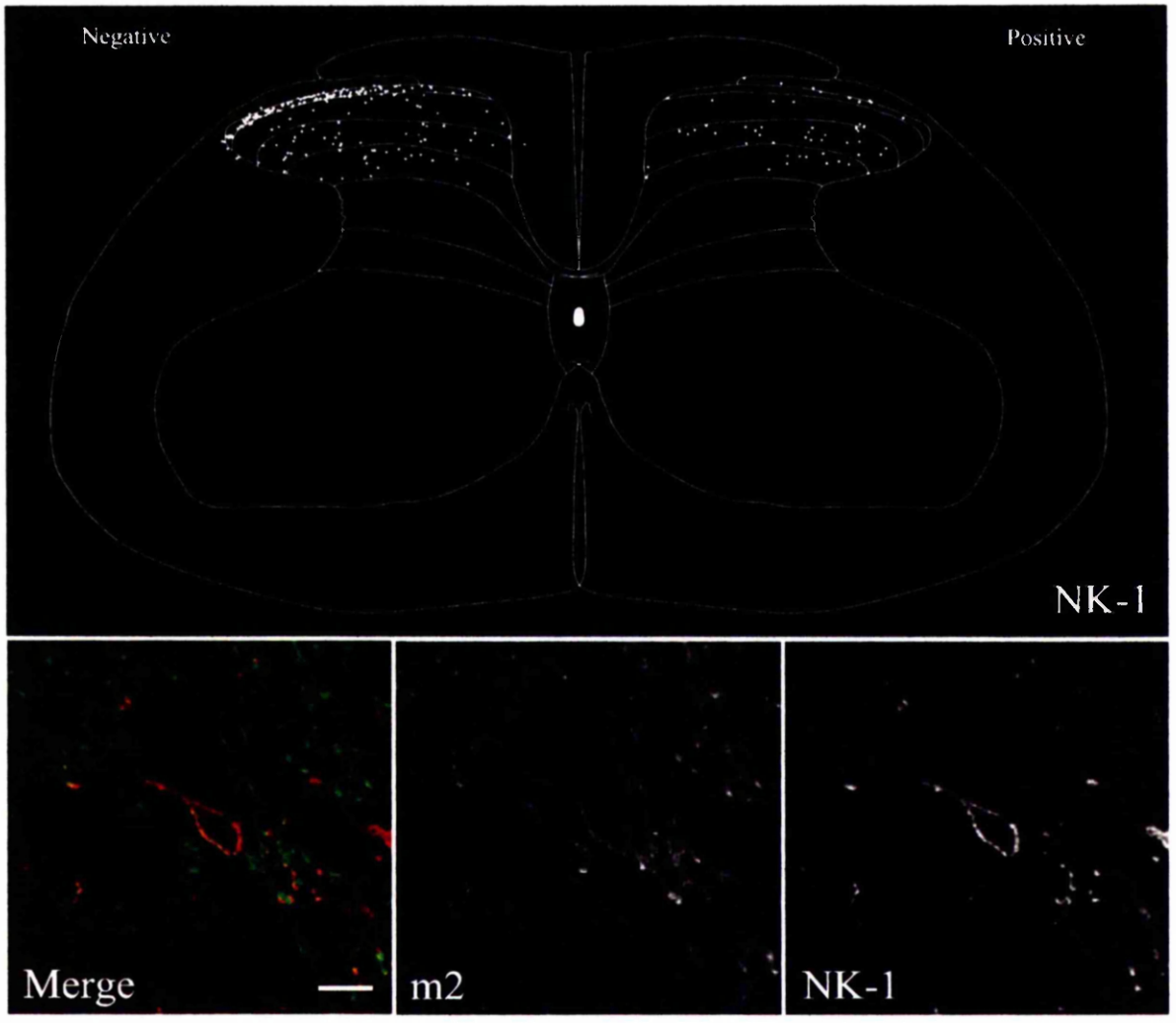


Figure 2.12. Composite image illustrating the pooled data on the locations of m2 receptor positive and m2 receptor negative neurokinin-1-immunoreactive neurons in the dorsal horn. Few lamina I NK-1 neurons (12%) were noted to also be m2 receptor immunoreactive. However in the deeper laminae (III-IV) the frequency of m2 receptor positive neurons was found to be greater (42%). The single optical section confocal images illustrate an NK-1 neuron in lamina III which was also m2 receptor-immunoreactive (scale bar 25μm).

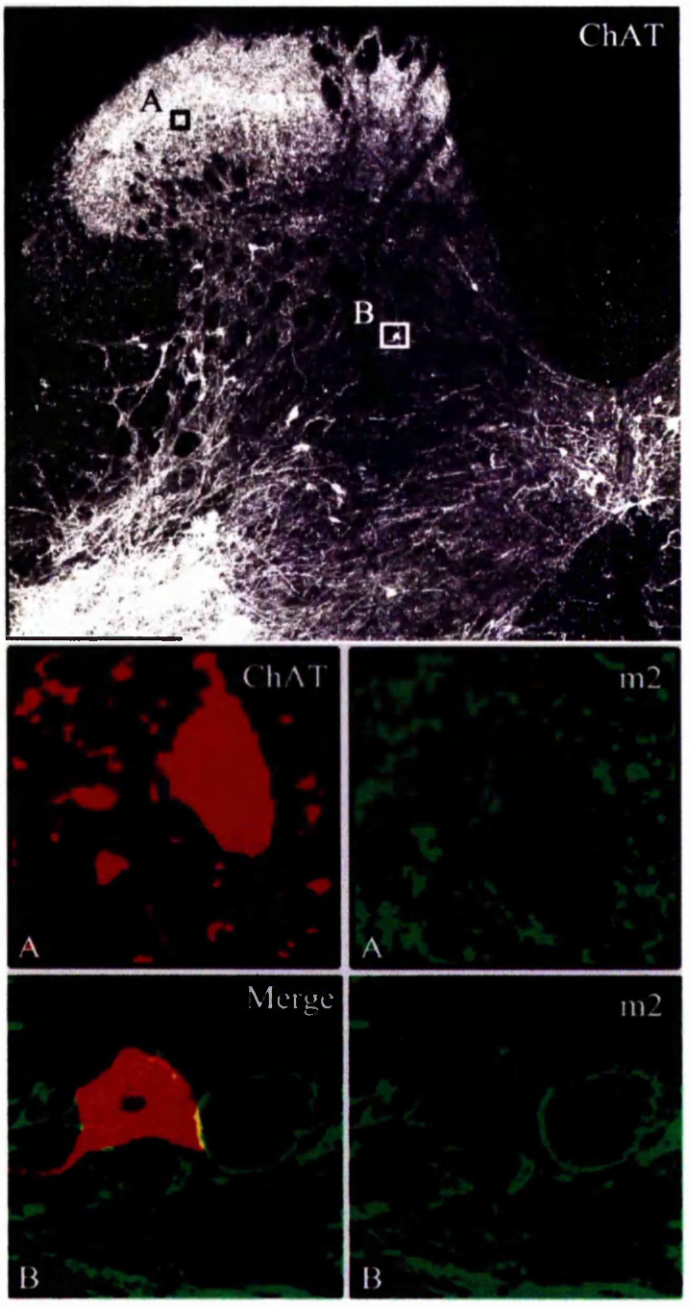


Figure 2.13. In this montage image constructed form multiple merged images through overlapping optical fields the choline acetyltransferase-immunoreactivity in the dorsal horn and lamina X is illustrated. A dense plexus of ChAT-immunoreactive profiles was confirmed around the border of lamina II and III with immunoreactive somata scattered throughout the deeper laminae of the dorsal horn (laminae III-VI). In addition, numerous ChAT-immunoreactive somata were identified around the central canal in lamina X. Many of the ChAT-immunoreactive somata also expressed the m2-receptor. In the fields corresponding to area A and B in the montage image, single optical sections are presented illustrating an m2 positive lamina III neuron and an m2 negative lamina V neuron adjacent to an m2 positive neuron which is not ChAT-immunoreactive.

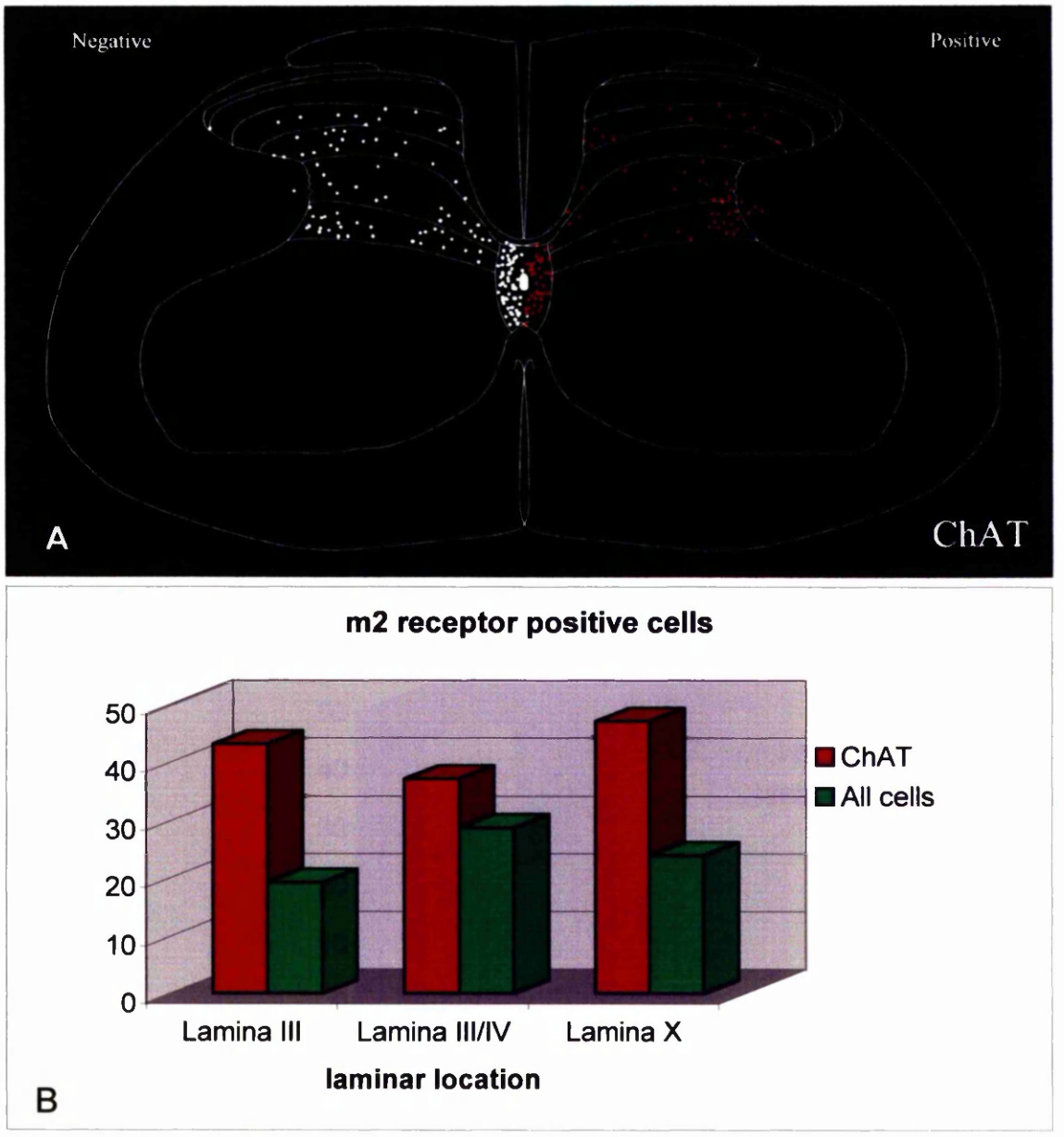


Figure 2.14. Muscarinic m2 receptor-immunoreactive ChAT cells were identified throughout the dorsal horn laminae III-VI and lamina X as illustrated in this composite image showing the locations of m2 positive and m2 negative ChAT cells (A). A relatively high density of m2 receptor-immunoreactive neurons was identified in the region of lateral lamina VI in a location which might suggest a caudal extension of the intermediolateral cell column. However following an intraperitoneal injection of fluorogold ChAT neurons in this location do not show immunoreactivity for fluorogold and therefore are not thought to be autonomic efferent neurons (figure 2.15). Choline acetyltransferase-immunoreactive neurons were frequently found to express the m2 receptor with similar proportions of cells in lamina III (46%) and lamina X (44%) m2 receptor positive whilst fewer lamina IV-VI cells were receptor positive (37%). This data is illustrated in B with the equivalent data for all neurons at each location included for comparison.

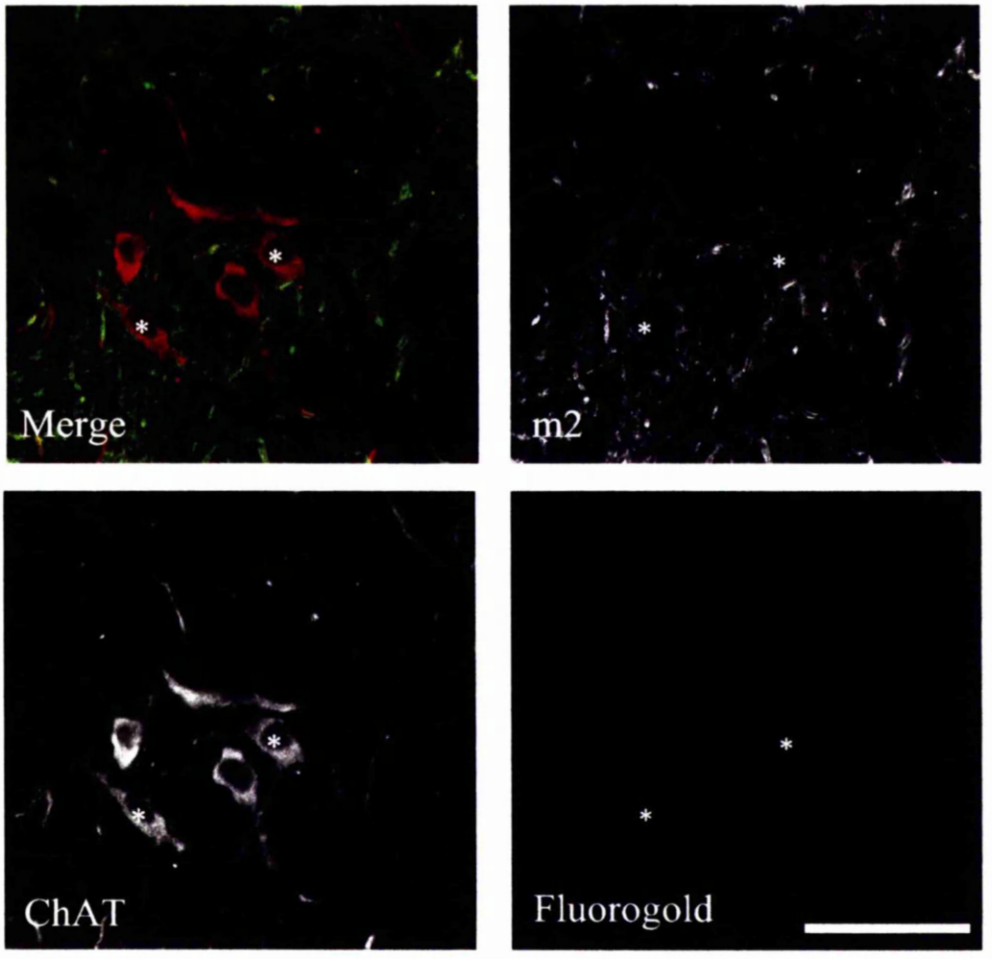


Figure 2.15. As illustrated in figure 2.14A a cluster of ChAT-immunoreactive neurons which express the m2 receptor is located in the region of lateral lamina VI. It is conceivable that these neurons may represent a caudal extension of the intermediolateral cell column in these animals. To explore this possibility animals were injected with the retrograde tracer fluorogold intraperitoneally. Sections were then processed for m2-, ChAT and fluorogold-immunoreactivity to identify labelled cells. As illustrated in this series of single optical sections through a cluster of ChAT-immunoreactive neurons in this region no fluorogold-immunoreactivity was identified in relation to these cells. The cluster of neurons identified as m2 receptor-immunoreactive therefore would not appear to be autonomic efferent neurons (asterisk - m2 receptor-immunoreactive cell; scale bar 50μm).

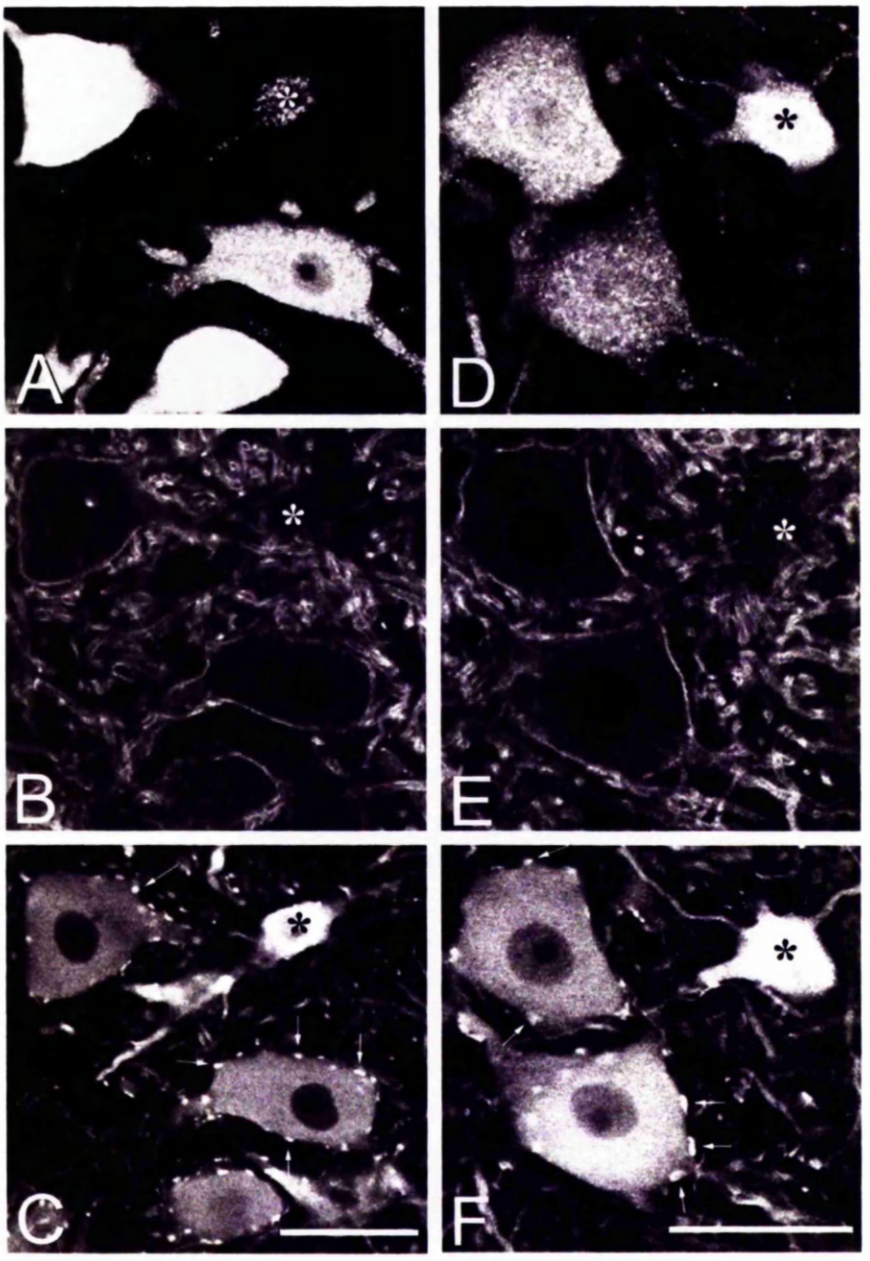


Figure 2.16. Confocal microscope images of immunofluorescence for Fluorogold (A,D), the m2 receptor (B,E) and choline acetyltransferase (C,F) associated with two groups of motoneurons in the ventral horn. A, B and C show large motoneurons which were immunoreactive for the m2 receptor (B) and a small cell which is not labelled (asterisk). Note that immunoreactivity for the receptor is associated particularly with plasma membranes and is present on numerous dendrites which were often also labelled for ChAT (C). D, E and F show further examples of a group of motoneurons. Large motoneurons are positive for the receptor (E), but on this occasion, a small cell is also weakly positive (asterisk). The arrows in C and F indicate some of the appositions made by cholinergic axons with large motoneurons. Note that in B and E these axons do not exhibit immunoreactivity for the m2 receptor (scale bar - 50μm).

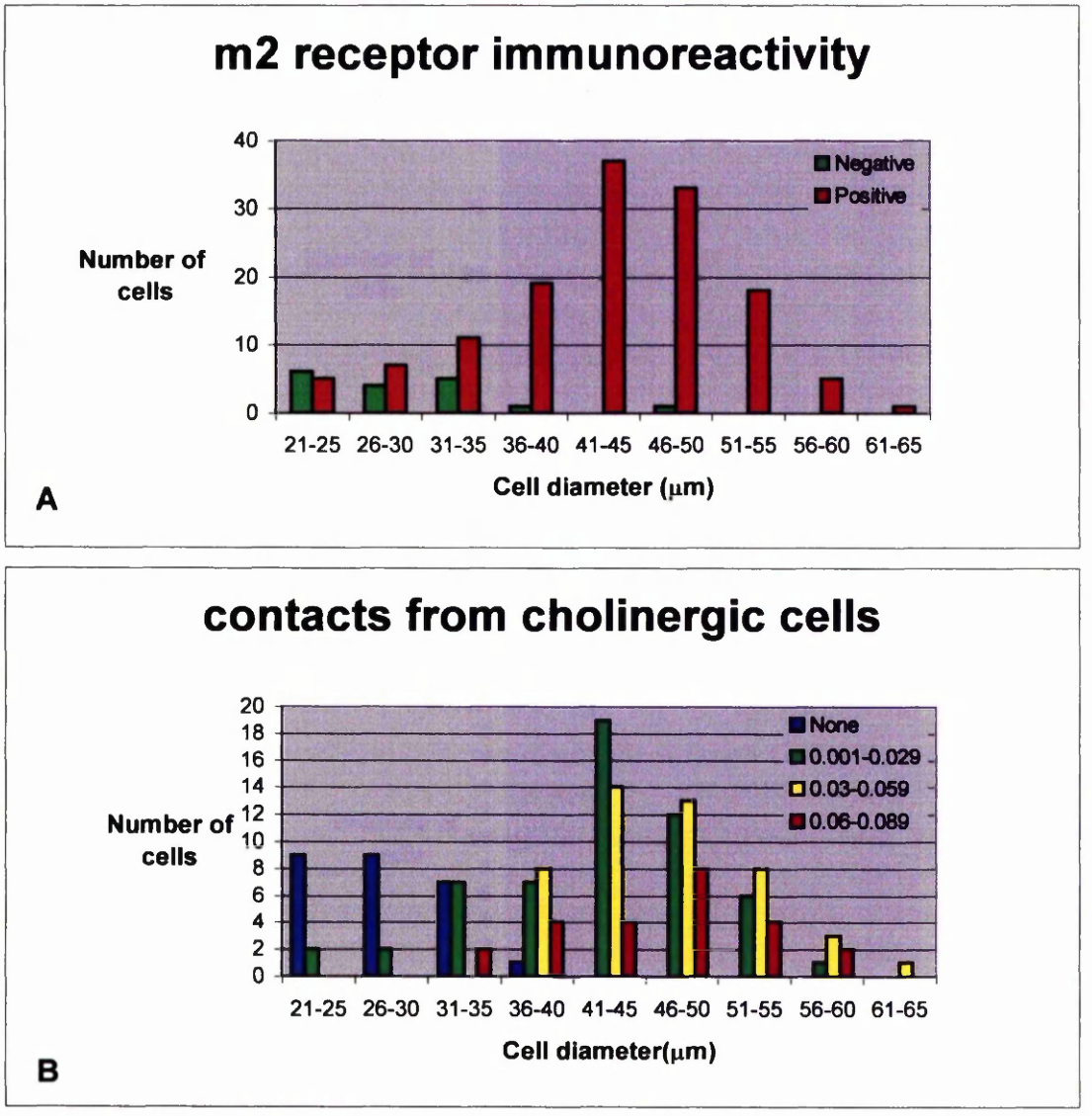


Figure 2.17. (A) Histogram illustrating the relationship between cell diameter and immunoreactivity for the m2 receptor. Almost all immunonegative cells are small (less than 40μm diameter). (B) Relationship between the numbers of cholinergic contacts (expressed as numbers of contacts per μm cell circumference) and cell diameter. Small cells receive few or no contacts.

2.9 Discussion

The muscarinic m2 acetylcholine receptor in the dorsal horn.

The principal finding of this study is that the acetylcholine m2 muscarinic receptor is present on a significant proportion (22.9%) of neurons in the lumbar dorsal horn and that there is a demonstrable selectivity of expression of the receptor on different neuronal subtypes. This first observation contradicts that of Yung and Lo (1997) where in a similar investigation of the distribution of m2 receptor-immunoreactivity in the rat spinal cord they failed to identify neuronal profiles expressing the receptor. Though these studies were similar in design, there are some methodological differences that may explain these varying observations. In both studies the receptor antibody used was a rat monoclonal antibody to the muscarinic m2 receptor obtained form the same source (Chemicon) though the dilution required in the present experiment for satisfactory staining was 1:500 as opposed to 1:2000 in the Young & Lo study. It is unlikely however that this could account for the complete absence of somatic staining in their study. Neither is the fixative used likely to have had a major influence. In their investigation the fixative used included 0.1% glutaraldehyde. During the preparatory work for this investigation the antibody was tested with fixatives which included glutaraldehyde with no discernable loss in staining pattern. The strain of rat used in either study is different with Young & Lo employing Sprague-Dawley rats as opposed to the Albino-Swiss animals used here. It is reasonable to assume that as for other neuronal systems (see the variation in the monoaminergic system for example; Proudfit & Clark,1991) the cholinergic system may show variation both between species and between strains. Thus it is conceivable that the variation may arise as a result of a true difference in the cholinergic circuitry of Sprague-Dawley versus Albino-Swiss

rats. It is more likely however that the observed difference is as a result of the superior spatial resolution afforded with confocal microscopy and the ability of this technique to reveal a field in a single optical plane minimising the influence of surrounding immunoreactive profiles. This imaging technique therefore allows immunoreactive profiles to be studied with minimal interference from surrounding immunoreactivity.

Cholinergic mediated analgesia involves the acetylcholine muscarinic receptors, in particular the muscarinic m2 receptor (Bleazard & Morris, 1993; Iwamoto & Marion, 1993; Gomeza et al, 1999). Little is known however of the neural circuitry involving these receptors within the dorsal horn. In this study it has been demonstrated that immunoreactivity for the m2 receptor can be detected on the cell bodies of a significant proportion of neurons within the dorsal horn. Furthermore the receptor is expressed on a variable proportion of the neuronal subtypes which were examined with greater numbers of neurons in the deeper laminae receptor positive than in lamina I. This distribution corresponds to that of the cholinergic innervation of the dorsal horn where numerous cholinergic terminals form a dense plexus spanning the inner lamina II/ lamina III border (Todd, 1991). None of the neurons in the superficial dorsal horn immunoreactive for the excitatory neurotransmitter neurotensin were m2 receptor-immunoreactive. This contrasts with the presumed inhibitory neurons examined which (with the exception of parvalbumin-immunoreactive neurons) were commonly m2 receptor-immunoreactive. This was most evident for the NOS-immunoreactive neurons of the deeper lamina III where the majority (55%) of neurons showed receptor-immunoreactivity. This group of NOS-immunoreactive neurons

frequently co-localise GABA (Spike, Todd & Johnston, 1993). Thus, this investigation suggests the m2-receptor preferentially targets the inhibitory local circuit neurons in the dorsal horn. The muscarinic m2 receptor is recognised to have a role in mediating antinociception in the dorsal horn which is presumed to arise through a lowering of cAMP levels in the target cell resulting in an inhibitory response (Richards, 1991). Cholinergic mediation of antinociception has also been described as operating via the facilitation of the release of inhibitory neurotransmitters in the superficial dorsal horn such as GABA (Baba et al, 1998) and nitric oxide (Xu et al, 2000). However, the expression of this receptor on inhibitory cells implies that acetylcholine would inhibit rather than facilitate these neurons. Therefore, to explain acetylcholine's facilitation of the release of inhibitory neurotransmitters alternative, non-m2 receptors must also be expressed by these cells (Baba et al, 1998). The action of acetylcholine on the neurons of the superficial dorsal horn therefore appears complex and likely to involve multiple cholinergic receptors, the overall effect being to reduce nociceptive transmission in the dorsal horn.

As discussed in the previous chapter, neurons in lamina I and lamina III/IV of the dorsal horn which are immunoreactive for the substance P receptor neurokinin-1 respond to nociceptive stimuli and almost all project to brainstem regions associated with nociceptive processing (Todd, McGill & Shehab, 2000). This study has shown that a proportion of these neurons are immunoreactive for the muscarinic m2 receptor hence suggesting that, via this receptor, acetylcholine may act to inhibit nociceptive transmission in ascending neurons.

Much of the muscarinic receptor mediated antinociception in the dorsal horn is likely to arise form the cholinergic neurons which are located in this region as, though a descending cholinergic system has been described (Bowker et al, 1983), transection of these descending tracts has little influence on the effect of muscarinic antagonists on nociceptive responses (Zhou & Gebhart, 1992). In the present study the distribution of cholinergic neurons in the dorsal horn confirmed the results of previous investigations (Barber et al, 1984). Of these neurons a significant proportion were found to be immunoreactive for the muscarinic m2 receptor, particularly the cells of lamina III. Thus, it would appear that a proportion of the cholinergic neurons in the dorsal horn are likely to be subject to an inhibitory influence from acetylcholine operating through the m2 receptor. The source of this cholinergic inhibition is unclear though a release from terminals of local dorsal horn neurons providing a feedback loop in the nociceptive pathway is entirely feasible.

Intriguingly a population of cholinergic neurons which were commonly m2 receptor-immunoreactive was identified in the lateral part of the deep dorsal horn. This cluster of cells is in a corresponding location to the intermediolateral cell column of the upper lumbar and thoracic spinal cords. However, as would be expected from their mid-lumbar origin, these cells failed to show immunoreactivity for fluorogold following an intraperitoneal injection of this tracer substance and so would not appear to be autonomic efferents. It is possible that these cells correspond to a specific group of cholinergic dorsal horn interneurons that as yet have not been functionally identified.

In addition to examining the cholinergic cells of the dorsal horn this study also considered m2 receptor expression of the cholinergic neurons of lamina X. As with the dorsal horn neurons a significant number were identified as m2 receptor-immunoreactive. The neurons of this region are known to give rise to an extensive dendritic arbor permeating the extent of the spinal grey matter (Borges & Iverson, 1986). This region is also known to include a population of neurons, many of which are choline acetyltransferase-immunoreactive, which are active during programmed motor activity (Carr et al, 1995; Huang et al, 2000). The expression of the m2 receptor on many of these neurons again suggests that these cells are subjected to an inhibitory influence mediated by acetylcholine which may serve as a feedback control mechanism.

The muscarinic m2 acetylcholine receptor in the ventral horn.

This study has demonstrated the presence of immunoreactivity for the muscarinic m2 acetylcholine receptor on motoneuron cell bodies and dendrites in the rat spinal motor nuclei. Furthermore a differential expression has been identified between the large and small neurons of these motor nuclei where most large neurons, which have also been shown to receive relatively high densities of cholinergic terminals, express the receptor whilst smaller neurons frequently do not.

The distribution of the m2 receptor as demonstrated by the highly sensitive and specific techniques of immunocytochemistry and confocal microscopy in the present investigation confirms the observations based on in situ hybridisation studies in which m2 receptor has been shown to be associated with motoneurons (Vilaro et al, 1992). These observations are however at variance with those of

Yung and Lo (1997) in their immunocytochemical investigation of the m2 receptor in the rat. In their study they reported 'no m2 immunoreactivity has been found in the perikarya of motor neurons' in addition to a conclusion that much of the immunoreactivity which they had observed was related to axonal profiles as opposed to the dendritic labelling reported in this study. In addition they report that no ChAT-immunoreactive terminals were present in the ventral horn. This is at variance to previously reported data (see Nagy, Yamamoto & Jordan, 1993). It is likely that, as discussed above, these differences may in part be explained by the superior spatial resolution and minimisation of the interference of background immunoreactivity that the protocol used in this investigation affords.

The identification of a higher density of cholinergic terminals on larger motoneurons of the ventral horn when compared to smaller motoneurons confirms previous observations (Barber et al, 1984; Borges & Iverson, 1986). Corresponding to this variation in density of cholinergic terminals on motoneurons this study has also demonstrated a parallel differential distribution of the m2 receptor on small and large motoneurons. It has been suggested that there is a difference in size within the motoneuron groups of the rat ventral horn with the γ-motoneurons typically smaller (less than 35μm) than the α-motoneurons. Thus from the present data it would appear that there is a preferential innervation of α-motoneurons by cholinergic terminals and that the acetylcholine released may act on these neurons through the m2 muscarinic receptor. In contrast γ-motoneurons receive few cholinergic terminals and so the influence of acetylcholine on these cells may be expected to be less significant.

In studying the effects of acetylcholine on neonatal rat spinal motoneurons Kurihara et al (1993) described both an excitatory response and an inhibitory response thought to be mediated by muscarinic m3 and m2 receptors respectively. Thus the expression of two different muscarinic receptors by these neurons would appear to produce opposing responses to one transmitter substance. In addition the present investigation has shown that immunoreactivity for the m2 receptor is diffusely expressed across the dendritic and somatic membrane and therefore not solely localised to synaptic specialisations. This distribution favours a diffuse, non-synaptic mode of transmission which may be associated with fine tuning the cells' response rather than a rapid synaptic response. The origins of the cholinergic terminals identified in this study on these motoneurons remains unclear though it would appear that at least some represent recurrent collateral branches from local motoneurons (Cullheim, Kellerth & Conradi, 1977). Hence the complex circuitry involving these terminals and the muscarinic receptors is presumed to permit fine tuning of motor responses within the motor nuclei.

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Appendix 1: Antibodies used in these studies

Primary antibody	Species	Dilution	Source
5-HT	rat	1:200	Eugene Tech
5-HT	rat	1:2000 (e)	Eugene Tech
5-HT	rabbit	1:200	Affiniti
ChAT	goat	1:100	Chemicon
D-βH	mouse	1:500	Affiniti
Fluorogold	rabbit	1:5000	Chemicon
GABA	rabbit	1:1000	Dr DV Pow
GABA	mouse	1:500 (e)	Affiniti
Glycine	rat	1:5000	Dr DV Pow
m2 receptor	rat	1:500	Chemicon
MOR1	rabbit	1:1000	Gramsch
NeuN	mouse	1:500	Chemicon
Neurotensin	rabbit	1:1000	Peninsula
NK-1	guinea-pig	1:1000	Affiniti
NOS	sheep	1:2000	Dr PC Emson
Parvalbumin	Mouse	1:100	Sigma
Somatostatin	rabbit	1:100	Peninsula
sst_{2A}	guinea-pig	1:2000	Gramsch

e - dilution used for ultrastructural studies.