# ORGANISATION OF $\alpha_{2C}$-ADRENERGIC RECEPTORS IN SPINAL PAIN PATHWAYS

by

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# SUMMARY

The $\alpha_{2C}$-subclass of adrenergic receptor mediates some of the antinociceptive actions of noradrenaline in the spinal cord. This receptor is present on axon terminals in the superficial dorsal horn.

A series of double-labelling experiments for confocal microscopy was performed in the rat (Wistar) to investigate the relationship between the $\alpha_{2C}$-adrenergic receptor and each of seventeen chemical markers that label various types of axon terminal in the dorsal horn. Quantitative analysis revealed that $\alpha_{2C}$-adrenergic receptors are neither present on terminals of unmyelinated, myelinated or peptidergic primary afferents nor on descending noradrenergic or serotoninergic terminals, whereas they are present on terminals of spinal origin. $\alpha_{2C}$-adrenergic receptors are predominantly found on axon terminals of excitatory interneuronal populations and also to a lesser extent they are present on terminals of inhibitory interneurons. In addition, the receptor is present on terminals that contain certain peptides, which indicates that subpopulations of interneurons possessing the $\alpha_{2C}$-adrenergic receptor can be differentiated on the basis of their peptidergic content. Electron microscopic analysis revealed that immunoreactivity is predominantly associated with axon terminals that are presynaptic to dendrites while a small proportion of immunoreactive terminals formed axoaxonic synaptic arrangements.

Experimental techniques were combined in order to investigate the relationship of terminals possessing $\alpha_{2C}$-adrenergic receptors with supraspinally projecting neurons. The techniques included retrograde labelling, multiple and sequential-immunolabelling, correlated confocal-electron microscopy and induction of the immediate early gene c-Fos by peripheral noxious stimulation. The findings indicated that axon terminals containing the $\alpha_{2C}$-adrenergic receptor densely innervate spinomedullary neurons that express the substance P receptor, neurokinin-1. The latter terminals are glutamatergic (excitatory) and form synapses with this type of neuron. In addition, a substantial number of neurokinin-1 projection neurons in lamina I that are responsive to peripheral thermal noxious stimulation, i.e. express c-Fos, receives innervation from axon terminals containing $\alpha_{2C}$-adrenergic receptors.

The $\alpha_{2C}$-adrenergic receptor is also present in axon terminals in the lateral spinal nucleus. This nucleus is found in the rat and other rodents and contains projection neurons that are densely innervated by peptidergic varicosities. Double-labelling immunostaining

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experiments showed that $\alpha_{2C}$-adrenergic receptors are present on axon terminals of mainly excitatory interneurons but also of inhibitory interneurons, and frequently contain peptides. Electron microscopy revealed that terminals possessing the receptor are presynaptic to dendrites and somata of neurons in the lateral spinal nucleus. The involvement of lateral spinal nucleus neurons in nociceptive transmission and their relationship with axons that possess $\alpha_{2C}$-adrenergic receptors was investigated. By combining retrograde labelling of projection neurons with induction of c-Fos expression by peripheral noxious stimulation and multiple-immunolabelling, it was possible to identify NK-1 projection neurons in the lateral spinal nucleus that express c-Fos and to determine if such cells receive contacts from terminals possessing the $\alpha_{2C}$ receptor. The results show that neurons in this nucleus are densely innervated by axons that possess the receptor and that a small proportion can be activated by thermal noxious stimulation.

In conclusion, noradrenaline is likely to modulate nociceptive transmission by acting on terminals of interneurons that contain the $\alpha_{2C}$-adrenergic receptor in the superficial dorsal horn and also in the lateral spinal nucleus.

## ACKNOWLEDGEMENTS

## DECLARATION

I certify that the authorship of this thesis is entirely my own and that the work presented in it is substantially my own. Dr. D. J. Maxwell contributed to this work by performing transcardial perfusions and brainstem stereotaxic injections.

# LIST OF ABBREVIATIONS

(Terms in italic are not defined in the text)

5-HT, serotonin

*ACh, acetylcholine*

AR, adrenergic receptor

*cAMP, cyclic adenosine monophosphate*

CGRP, calcitonin gene-related peptide

*cRNA, complementary ribonucleic acid*

ChAT, choline acetyltransferase

*CNS, central nervous system*

*CSF, cerebrospinal fluid*

CTb, cholera toxin B subunit

CVLM, caudal ventrolateral medulla

CY5, cyanine 5.18

DAB, 3,3'-diaminobenzidine

DβH, dopamine β-hydroxylase

DCV, dense-core vesicle

DRG, dorsal root ganglion

FITC, fluorescein isothiocyanate

*GABA, γ-amino butyric acid*

GAD, glutamic acid decarboxylase

Gly-T2, glycine transporter 2

*HRP, horseradish peroxidase*

IB4, isolectin B4

Ig, immunoglobulin

ip, intraperitoneal

IR, immunoreactive

LC, locus coeruleus

LSN, lateral spinal nucleus

*mRNA, messenger ribonucleic acid*

NE, noradrenaline

NK-1, neurokinin-1 receptor

*NMDA, N-methyl-D-aspartate (receptor)*

NO, nitric oxide

NOS, nitric oxide synthase

PBS, phosphate buffered saline

*PKCγ, protein kinase C-γ*

SD, standard deviation

SP, substance P

TH, tyrosine hydroxylase

VGLUT1, vesicular glutamate transporter 1

VGLUT2, vesicular glutamate transporter 2

VIP, vasoactive intestinal peptide

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*Chapter 6*   **General Discussion**

*Chapter 1*

# General Introduction

Pain is a very versatile sensation, it can be localised or a widespread discomfort, acute or chronic, in an attempt to describe it we talk about piercing, throwing or thumping pain. We even refer to pain as a physical symptom linked to generally unpleasant emotions like frustration, injustice, or rejection. Metaphorically, pain is a universal language that ultimately makes us self-aware of our existence; it could be argued that it represents the survival sense, and that it plays an important role in guiding our actions throughout life. While the meaning and function of pain in human existence is an exciting issue worthwhile to study and debate, it is also self-evident that pain compromises our well being and disrupts our lives to a greater or a lesser extent and for this reason a great concentration of effort is devoted to pain research. Pain research is a vast multidisciplinary field, which focuses on understanding the mechanisms of nociception in order to design effective therapeutic strategies for relieve of pain. Part of this field of research concentrates in the study of the so called endogenous analgesia systems. Indeed the context of the present work is the noradrenergic system, which is one of the major descending analgesia systems, together with the opioidergic and serotoninergic systems.

The three systems are activated by rostral projections from the cortex and hypothalamus, as well as by ascending projections carrying nociceptive or other type of information from caudal structures. All three are known to interact and produce analgesia by spinal mechanisms (reviewed by Willis and Coggeshall, 1991). Evidence for supraspinal antinociception was originally derived from studies in which lesions, electrical stimulation and microinjection of drugs to specific regions of the brain effectively influenced the modulation of pain transmission (Mayer and Price, 1976; Willis et al., 1977; Hayes et al., 1979).

The aim of this introductory chapter is to review the extensive literature up to date available regarding the noradrenergic system. In order to build up a framework, the information has been broken down into sections, which summarise different aspects of the existing evidence for spinal noradrenergic antinociception.

1. Noradrenergic innervation of the spinal cord
2. Effect of noradrenaline on dorsal horn neurons: excitation versus inhibition
3. Mediators and mechanisms of noradrenergic antinociception
4. Functioning of $\alpha_2$-adrenergic receptors
5. Control of noradrenaline release
6. Candidate $\alpha_2$-AR subtypes as mediators of spinal noradrenergic antinociception

# 1. Noradrenergic innervation of the spinal cord.

The noradrenergic system consists of a collection of nuclei which contain cell bodies of neurons that synthesise noradrenaline (NE); such nuclei are found in the brainstem: A1, A2, A3, A4, A5, A6 (also referred to as locus coeruleus, LC) and A7 nuclei (Hillarp et al., 1966; Dahlström, 1971). Anterograde and retrograde tract tracing techniques, frequently combined with immunocytochemical labelling, have been used widely to elucidate the circuitry of the system. Investigations carried out in a variety of species indicated that the pontine nuclei A5, LC and A7 are the major sources of noradrenergic innervation of the spinal cord (Westlund el al., 1981, 1982; Schroder and Skagerberg, 1985; Clark and Proudfit, 1993). Noradrenergic innervation of the spinal cord dorsal horn was proposed to arise principally from the LC nucleus based on investigations in rat, cat, and monkey (Karoum et al., 1980; Westlund et al., 1983, 1984). Nevertheless later studies in the rat gave rise to controversy regarding the specific termination area in the spinal cord corresponding to the different noradrenergic nuclei:

Evidence supports that in the rat, noradrenergic axons from the LC descend within laminae I and II to end in the dorsal horn and intermediate region; while those from A5 and A7 nuclei, descend mainly in the ventral and dorsolateral funiculi to supply motoneurons and autonomic preganglionic neurons (Fritschy and Grzanna, 1990). Additionally this study indicated that the contribution of A5 and A7 to the noradrenergic spinal projection is greater than that of the LC. Contradicting the above studies, noradrenergic neurons located in the pontine A7 nucleus have been reported to constitute the major source of noradrenergic innervation of the spinal cord dorsal horn (Clark and Proudfit, 1991a). The same research group also concluded that LC neurons supply noradrenergic innervation to the intermediate zone and ventral horn and not to the dorsal horn (Proudfit et al., 1990; Clark and Proudfit, 1991b; Proudfit and Clark, 1991).

These inconsistencies could be explained by intraspecies or even substrain differences between the specimens used by the diverse groups. Even though disagreement appears to exist regarding the origin and destination of noradrenergic spinal projections, the observations suggest the presence of a specific descending noradrenergic pathway able to influence the transmission of sensory information at the dorsal horn, while other noradrenergic pathways will be involved in the control of locomotion or autonomic functions by influencing other areas within the spinal cord.

Immunohistochemical investigations in the rat using antiserum against NE identified noradrenergic terminals in laminae I, II and IV-VI of the dorsal horn, as well as in the intermediate region (including the intermediolateral cell column), lamina X and the ventral horn (Dahlström and Fuxe, 1965; Westlund et al., 1983; Mouchet et al., 1992; Rajaofetra et al., 1992). Immunoelectron microscopic studies also in the rat using antiserum against NE showed that noradrenergic terminals principally form axodendritic synapses in the ventral horn and intermediolateral cell column; while in the dorsal horn noradrenergic innervation was suggested to be non-synaptic since no synapses were observed (Rajaofetra et al., 1992). This contrasts with results from electron microscopic investigations in the cat, which used antisera against tyrosine hydroxylase (TH) or dopamine-β-hydroxylase (DβH); these studies reported the presence of TH or DβH-IR axonal boutons forming axodendritic symmetric junctions in the dorsal horn (Doyle and Maxwell, 1991a). Additionally, correlated light microscopy immunohistochemistry showed the presence of the NE markers, TH and DβH, throughout laminae I-IV in the cat (Doyle and Maxwell, 1991b), thus implying a more extensive distribution of the noradrenergic innervation in the dorsal horn than that reported in studies using NE antiserum. In the latter study it was concluded that descending noradrenergic innervation to the cat spinal cord is very widespread while particularly rich in the superficial dorsal horn. Indeed, noradrenergic innervation appears to be more widespread than serotoninergic innervation on the spinal grey matter; as serotoninergic terminals are mainly located in laminae I-II and IX of the rat spinal cord (Steinbusch, 1981). Also, noradrenergic terminals do not colocalise with glutamic acid decarboxylase (GAD) or neuropeptides in the rat spinal cord; and for this reason spinal noradrenergic innervation is considered to be neurochemically exclusive, at least in the rat (Patel et al., 1997).

## 2. Effect of noradrenaline on dorsal horn neurons: excitation *versus* inhibition

The release of NE in the spinal cord leads to antinociception. Stimulation of the sciatic nerve, dorsolateral funiculus, medulla and certain supraspinal regions such as the LC or A7 noradrenergic nuclei, or others like the periaqueductal grey or reticular formation, can cause release of NE in the spinal cord which produces inhibition of dorsal horn neurons and results in antinocieption (Tyce and Yaksh, 1981; Hammond et al., 1985; Abhold and Bowker, 1990). Intrathecal administration of NE mimics the latter stimulation-

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induced antinociceptive effects, further supporting that the descending noradrenergic system contributes to spinal antinociception (Yaksh, 1985). These two lines of evidence together with the fact that noradrenergic terminations are present in those laminae where nociceptive neurons (laminae I and IV-VI) and nociceptive primary afferents also exist (laminae I-II) (Christensen and Perl, 1970; Menétrey et al., 1977; Light and Perl, 1979b; Sugiura et al., 1986; Alvarez et al., 1993), suggest that spinal NE release can potentially be responsible -at least partly- for descending tonic inhibition of spinal nociception at two different levels, i.e. primary afferent and spinal neuron level.

However, disagreement exists about the effect of NE on dorsal horn neurons. Ionophoretic application of NE to the dorsal horn has been reported to produce not only inhibitory but also excitatory effects. Application of NE to the dorsal horn leads generally to inhibition of the background activity of the cells and their response to excitatory amino acids (Biscoe et al., 1966; Belcher et al., 1978; Headley et al., 1978; Fleetwood-Walker et al., 1985; Davies and Quinlan, 1985; Howe and Zieglgänsberger, 1987). Apparently controversial observations regarding the effect of NE on cells from superficial *versus* deep laminae of the dorsal horn, as well as according to the response characteristics of the cells. On one hand, NE has been indicated to inhibit low-threshold and wide dynamic range cells in superficial laminae and to excite proprioceptive cells in deep laminae (Howe and Zieglgänsberger, 1987); but on the other hand NE has been reported to excite low-threshold cells in superficial laminae, suggested to be interneurons, and to inhibit high-threshold cells in lamina I and wide dynamic range cells in the deep dorsal horn (Todd and Millar, 1983; Millar and Williams, 1989). Indeed these results are not necessarily contradictory and could be complementary, if we consider that the recordings can correspond to different populations of neurons within the same area.

## 3. Mediators and mechanisms of noradrenergic antinociception

In the CNS, investigations on $\alpha$-adrenergic receptors (ARs) have shown that NE induces excitation acting on $\alpha_1$-ARs, while NE induces inhibition acting on $\alpha_2$-ARs. (DeBernardis et al. 1986; Bylund et al., 1994; Arnsten et al., 1998). Based on this line of evidence and previous observations, Peng and coworkers (1996) proposed a model of noradrenergic antinociception (Fig. 1.1, adapted from Peng et al., 1996). In this model $\alpha_1$-ARs are present on inhibitory interneurons of the superficial dorsal horn; and $\alpha_2$-ARs are

located on deep dorsal horn neurons which have ascending supraspinal projections. The inhibitory interneuron in the superficial dorsal horn is excited by NE acting on $\alpha_1$-ARs which induces the synaptic release of GABA and/or glycine, that in turn inhibit the deep dorsal horn neuron. The deep projection neuron is also directly inhibited by NE through $\alpha_2$-ARs.

This paradigm fits with the proposal by Millar and Williams (1989) that NE induces excitation of low-threshold inhibitory interneurons, which synapse on high-threshold and wide dynamic range neurons. However the latter model does not agree with some of the current evidence. The effect of NE on substantia gelatinosa neurons was studied in a slice preparation. Intracellular recording showed that NE has a predominant hyperpolarising effect in the superficial dorsal horn (lamina II), which is mediated by $\alpha_2$-ARs since hyperpolarisation was only reversed by $\alpha_2$-AR antagonists (North and Yoshimura, 1984). A high concentration of binding sites was shown in lamina II by autoradiography after application of [$^3$H]-clonidine, i.e. an antinociceptive $\alpha_2$-AR agonist; such sites are considered to represent $\alpha_2$-AR binding sites (Bouchenafa and Livingstone, 1987). NE-induced antinociceptive effects can be reproduced by $\alpha_2$-AR agonists, and reversed by $\alpha_2$-AR antagonists, while $\beta$ or $\alpha_1$-AR agonists and antagonists do not mimic or reverse NE-antinociceptive effects respectively (Howe et al., 1983; Yaksh, 1985; Proudfit, 1988). These facts suggest that NE-induced antinociception is mediated principally by $\alpha_2$-ARs and involves hyperpolarisation of the superficial dorsal horn where $\alpha_2$-ARs are concentrated.

There are diverse possible mechanisms of $\alpha_2$-AR-mediated noradrenergic antinociception in the spinal cord:

( i ) *Presynaptic inhibition of nociceptive transmission on nociceptive primary afferents.* Descending noradrenergic terminals have been reported to directly inhibit nociceptive primary afferents through the activation of $\alpha_2$-ARs present on central terminals of this type of afferent. Local application of NE to the dorsal horn produced complete inhibition of the noxious mechanical stimuli-evoked release of substance P (SP); and the latter effect was only fully reversed by a selective $\alpha_2$-AR-antagonist (Kuraishi et al., 1985). Inhibition of glutamate release from primary afferents has been also confirmed to accompany the inhibition of SP release (Kamisaki et al., 1993). In addition, release of calcitonin-gene related peptide (CGRP), which is exclusively present on primary afferents (Levine et al., 1993), was

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prevented by the $\alpha_2$-AR-agonist clonidine in an *in vitro* preparation of spinal cord (Solomon et al., 1989).

( ii ) *Postsynaptic inhibition of nociceptive transmission on spinal neurons.* Ionophoretically applied-NE produced a potent selective inhibition of nociceptive responses from multireceptive neurons (i.e. activated by noxious and innocuous cutaneous stimuli) in the dorsal horn. This selective inhibitory effect was only mimicked by $\alpha_2$-AR agonists, not $\alpha_1$ or $\beta$-AR agonists, and for this reason activation of $\alpha_2$-ARs on spinal nociceptive neurons was concluded to be a mechanism for NE-spinal antinociception (Fleetwood-Walker et al., 1985). The $\alpha_2$-ARs mediating this selective inhibitory effect were indicated to be localised to laminae IV-V and to laminae II-III, because ionophoretic administration of NE on either of both areas gave rise to selective nociceptive inhibition of neurons recorded in laminae IV-V (Davies and Quinlan, 1985). Electron microscopic evidence further supports this mechanism. In the monkey, spinothalamic tract neurons in laminae I and IV-V which transmit nociceptive information to supraspinal levels, have been reported to receive axodendritic and axosomatic synapses from D$\beta$H positive axonal boutons; i.e. direct noradrenergic innervation (Westlund et al., 1990). In the cat, dorsal column postsynaptic neurons that project to the dorsal column nuclei have been reported to be a target of noradrenergic terminations since D$\beta$H positive axonal boutons formed synapses onto their somata and proximal dendrites (Doyle and Maxwell, 1993). This type of neuron is found in laminae III-V and is multireceptive; i.e. respond to innocuous and nociceptive -mechanical- stimuli.

Therefore, the conclusion so far is that $\alpha_2$-ARs have an important role in the mediation of NE-induced antinociception and that this action very likely involves inhibition of nociceptive primary afferents and dorsal horn neurons. But what could be the role of *spinal interneurons*? Interestingly noradrenergic terminals concentrate especially in the superficial dorsal horn where many interneurons are known to exist. It has already been noted that lamina II interneurons hyperpolarise after NE application (North and Yoshimura, 1984), but what is the functional significance of this observation? Does it reflect the situation in vivo? Hökfelt and collaborators (1977) proposed the hypothesis that stimulation-produced analgesia is related to activation of spinal interneurons forming axoaxonic synapses with SP-containing primary afferents in the superficial laminae of the

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dorsal horn. Indeed, the role of inhibitory interneurons as inhibitory units of nociceptive primary afferent input has been substantially accepted (reviewed in Rudomin and Schmidt, 1999). In contrast, little evidence supports a role for interneurons in NE-antinociception as possible inhibitory units of nociceptive neurons (Peng et al., 1996; Millar and Williams, 1989) and as commented above this notion is in disagreement with current evidence. Nevertheless, the investigation carried out by Davies and Quinlan (1985) suggests an underlying role for interneurons in NE-mediated antinociception. Application of NE in the superficial laminae II and III inhibited nociceptive neurons in laminae IV and V. NE could cause this effect by acting on postsynaptic $\alpha_2$-ARs present on the distal dendrites of deep nociceptive neurons. Alternatively NE could be acting on presynaptic $\alpha_2$-ARs present on axon terminals of primary afferents and/or interneurons, which contact the distal dendrites of deep nociceptive neurons as they extend into the superficial laminae. If we consider that in laminae II and III interneuronal terminals are predominant and that NE has a major hyperpolarising action on lamina II interneurons (North and Yoshimura, 1984), then the observations of Davies and Quinlan are probably indicative of an interneuronal mechanism of neuronal inhibition, in which excitatory interneurons would be inhibited by NE. Therefore, on the light of current evidence it can be suggested that interneurons are involved in NE-induced antinociception, although the details of the interneuronal circuits and mechanisms of action are yet to be uncovered.

## 4. Functioning of $\alpha_2$-adrenergic receptors

### 4.1. *Effector mechanism*

$\alpha_2$-ARs belong to the superfamily of seven-transmembrane spanning domain G-protein-coupled receptors and share common signal transduction pathways mediated through the inhibitory G-proteins $G_i$ and $G_o$ (Crain et al., 1987; Hoehn et al., 1988). The involvement of inhibitory G-proteins was demonstrated by means of the application of pertussis toxin, a substance that blocks the action of inhibitory G-proteins. Pretreatment with pertussis toxin prevented the antinociceptive effects of intrathecally administered NE and morphine (Hoehn et al., 1988). Studies carried out in guinea pig submucosal neurons demonstrated that NE acting on $\alpha_2$-ARs reduces the $Ca^{2+}$ current, i.e. influx, (Surprenant et al., 1990) by decreasing the opening frequency of $Ca^{2+}$ channels and thus the $Ca^{2+}$ channel activity (Shen and Surprenant, 1991). Additionally, NE acting on $\alpha_2$-ARs has been reported

to increase the $K^+$ current, i.e. efflux, (Tatsumi et al., 1990) by increasing the opening of $K^+$ channels (Surprenant and North, 1988; Shen et al., 1992). Application of pertussis toxin to block the action of inhibitory G-proteins, made $\alpha_2$-AR agonists ineffective by preventing the changes in $Ca^{2+}$ and $K^+$ conductances (Surprenant et al., 1990; Tatsumi et al., 1990). Therefore it can be concluded that the effector mechanism of NE-induced hyperpolarisation through $\alpha_2$-ARs which leads to antinociception involves: 1 activation of pertussis toxin-sensitive inhibitory G-proteins $G_i$ and $G_o$; 2 coupling to $Ca^{2+}$ channels, which tend to close resulting in a decreased influx of $Ca^{2+}$; 3 coupling to $K^+$ channels, which tend to open resulting in an increased efflux of $K^+$.

Additionally, activation of $G_i$ and $G_o$-proteins suppresses the activity of adenylate cyclase by inhibiting a pool of such enzymes, which consequently leads to a reduction in the formation of cAMP. $\alpha_2$-AR agonists were reported to inhibit cAMP formation (Hoehn et al., 1988; Uhlen and Wikberg, 1988). $\alpha_2$-ARs that inhibit cAMP formation have been proposed to be present on cells which are accessible to the stimulation of cAMP by forskolin application. Forskolin, vasoactive intestinal peptide (VIP) and capsaicin stimulated the formation of cAMP, however cAMP formation was only inhibited by $\alpha_2$-AR agonist when forskolin, not VIP or capsaicin, had been previously applied (Uhlen and Wikberg, 1989). This raises the possibility of a dynamic behaviour of the $\alpha_2$-AR dependent on micro-environmental neurochemistry. For instance, could peptides like VIP modulate the functioning of $\alpha_2$-ARs and interfere in the formation of cAMP? If so, how could this happen?

It has been pointed out that the different subtypes of $\alpha_2$-ARs: $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$ (Bylund et al., 1994) exhibit a different receptor-effector coupling leading to the inhibition of cAMP. The $\alpha_{2A}$-AR is coupled with high efficacy but with low sensitivity to the effector, while the $\alpha_{2C}$-AR is coupled with both high efficacy and high sensitivity (Jansson et al., 1994a, 1994b). However not much is known about the functional relevance of such subtype-related specificities.

The general effector mechanisms maybe summarised as follows: activation of $\alpha_2$-ARs by NE triggers the coupling of the receptor to a G-protein, which acts on $Ca^{2+}$ and $K^+$ channels and inhibits adenylate cyclase activity resulting in a decrease of cAMP. Initially it was reported that inhibition of adenylate cyclase was responsible for decreasing neuronal excitation (Limbird, 1988; Uhlen and Wikberg, 1988). However, later investigations

support that inhibition of adenylate cyclase, and thus cAMP, by $\alpha_2$-AR activation is not directly linked to the antinociception mediated through such receptors and that differing $\alpha_2$-AR pathways seem to mediate antinociceptive effects (Uhlen et al., 1990). Indeed, controversy exists about the role of the cAMP signal. Raise in intracellular cAMP has been related to the loss or attenuation of the noradrenergic and opioidergic suppressant effect; i.e. loss of drug-sensitivity and antinociception (Crain et al., 1987). But, paradoxically cAMP is considered to be a signal that induces an increase in $\alpha_2$-AR expression and hence receptor availability (Limbird, 1988), which would favour noradrenergic effectiveness. We can conclude that cAMP acts as a signal or messenger in a variety of pathways leading to diverse events taking place in the cell; and therefore the decrease on cAMP resulting from $\alpha_2$-AR activation cannot be considered as a signal purely involved in the mediation of noradrenergic antinociception.

## 4.2. *Agonist-promoted activation*

Chronic exposure to certain $\alpha_2$-AR agonists (eg. clonidine), or opiate agonists (eg. morphine) leads to the development of tolerance; in other words loss of sensitivity to the behavioural effects of the drug including antinociception. The molecular substrate for such behavioural phenomenon has been investigated and it has been found to lie at receptor level (reviewed in Liggett and Raymond, 1993). It is known that certain exogenously applied compounds require a great amount of available $\alpha_2$-ARs. For instance, clonidine requires more available $\alpha_2$-ARs than dexmedetomidine and the former develops tolerance while not the latter (Hayashi et al., 1995). It is possible then that under long term-agonist exposure the requirement of $\alpha_2$-ARs is not satisfied by the expression of new $\alpha_2$-ARs, leading to the loss of clonidine sensitivity, i.e. effectiveness. The specific receptor behaviour of each subtype ($\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$) in response to agonists is starting to be elucidated.

A series of experiments combining pharmacology with $\alpha_2$-AR site directed mutagenesis, revealed differences in the regulation of the diverse subtypes of $\alpha_2$-AR in response to agonist-activation. Both $\alpha_{2A}$ and $\alpha_{2B}$-ARs, have been shown to undergo short-term desensitisation; while not the $\alpha_{2C}$-AR. Such desensitisation has been reported to require the phosphorylation of more than two serine residues on an EESSSS motif located at the C-terminal of the adrenergic receptor. Because this motif is present in the $\alpha_{2A}$ and $\alpha_{2B}$-ARs and absent in the $\alpha_{2C}$-AR, it is considered to represent a molecular determinant of

desensitisation. In contrast, the $\alpha_{2C}$-AR is thought to be susceptible to long-term downregulation, although there is no evidence of it, while the $\alpha_{2A}$-AR has been reported not to undergo long-term downregulation (reviewed in Liggett and Raymond, 1993). What is the significance of this difference? Is the activation or availability of different $\alpha_2$-ARs time-dependent; are they functionally synchronised? Not much is known about the response pattern of the $\alpha_{2C}$-AR to short and long-term agonist activation. It would be interesting to investigate the $\alpha_{2C}$-AR behaviour, since the expression of this receptor has been reported to be maintained or even increased after different models of peripheral nerve injury (Stone et al., 1999); which indicates the $\alpha_{2C}$-AR as a potential mediator of the reported antinociceptive effects of clonidine or other $\alpha_2$-AR agonists, in neuropathic conditions (Fairbanks et al., 2002).

## 5. Control of noradrenaline release

NE-induced analgesia is known to involve cholinergic activation. $\alpha_2$-AR agonists increase the concentration of ACh in the CSF (Detweiler et al., 1993; De Kock et al., 1997) and in microdialysates (Klimscha et al., 1997). Application of cholinergic antagonists further supports the involvement of cholinergic stimulation because they reduce the antinociceptive effects of $\alpha_2$-AR agonists (Gorth et al., 1989). Additionally, antinociception induced by $\alpha_2$-AR agonists has been reported to be dependent not only on cholinergic activation, but also on nitric oxide (NO) mechanisms. Perfusion of *in vitro* preparations with cholinergic and $\alpha_2$-AR agonists stimulates NO synthesis and the antinociception induced by the latter agonists can be diminished by nitric oxide synthase (NOS) inhibitors (Xu et al., 1996a, 1996b, 1997). Extensive evidence supports that $\alpha_2$-AR agonists produce antinociception under both normal and neuropathic pain conditions (Yaksh, 1985; Ossipov et al., 1997; Fairbanks et al., 2000). Cholinergic activation has been demonstrated to occur in both situations; under normal conditions both muscarinic and nicotinic receptors are similarly activated, while under pathological conditions muscarinic receptors play a predominant role. In both cases activation of cholinergic receptors by ACh leads to the synthesis and release of NO (Xu et al., 2000).

Much controversy exists regarding the role of $\alpha_2$-AR activation on NE release in the spinal cord. The classical view contends that $\alpha_2$-ARs are present on noradrenergic terminals and function as auto-inhibitory receptors to reduce NE release, whilst a more

recent view proposes that the net effect of stimulating $\alpha_2$-ARs in the spinal cord is to increase NE release. These views support the concept of antagonistic feedback loop-like mechanisms: a negative *versus* a positive feedback on NE release.

## 5.1. *Evidence for a positive feedback loop*

$\alpha_2$-AR agonists applied *in vivo* increase NE release as measured by dorsal horn tissue microdialysis (Klimscha et al., 1997). Injection of $\alpha_2$-AR antagonists decreases the release of NE into CSF following stimulation of noxious input (Eisenach et al., 1996) and also decreases systemic opioids (Bouaziz et al., 1996). NE-induced NO synthesis has been related to the control of NE release. Chiari and colleagues (2000) have proposed a *NE-$\alpha_2$-AR cascade mechanism* that exerts a positive feedback on NE release in the spinal cord: The release of NE from descending terminals leads to activation of $\alpha_2$-ARs, which in turn leads to the release of ACh that activates cholinergic receptors. Both muscarinic and nicotinic receptors induce the synthesis of NO which is released. NO combines with NE to form 6-nitro-NE (de la Breteche et al., 1994). There is evidence supporting the involvement of 6-nitro-NE in the regulation of NE release. Intrathecal injection of either 6-nitro-NE or NE, produced antinociception, thus suggesting that 6-nitro-NE leads to NE action and is a potential element in a positive feedback loop for NE release. In addition, because destruction of NE nerve terminals decreased the 6-nitro-NE-induced antinociception mentioned above, it can be concluded that 6-nitro-NE acts on NE descending terminals to lead to the NE cycle of action (Chiari et al., 2000). The same research group, has reported that 6-nitro-NE inhibits NE reuptake by acting directly on noradrenergic fibres and in doing so increases the released NE extracellularly (Li et al., 2000b), which would favour the maintenance of NE action. In addition, 6-nitro-NE has been suggested to be acting as an endogenous amphetamine-like substance; it enters noradrenergic terminals via the NE transporter, where it induces NE release by a $Ca^{2+}$-independent mechanism via reverse transport (Li et al., 2000b).

Aimar and colleagues (1998) proposed a *NMDA-NO cascade mechanism* that leads to spinal hyperexcitability and increased pain perception. Glutamate released from primary afferents acts on NMDA receptors located on interneurons that produce NO; newly synthesised NO is released and in turn triggers the release of peptides like SP from primary afferents (Aimar et al., 1998). If this is the situation *in vivo*, the effect of NO on primary afferents could be neutralised by the presence of NE, as combination of both substances

would lead to the formation of 6-nitro-NE. This could represent one of the mechanisms by which NE leads to suppression of pain transmission. Indeed it begs the question, how does NE action lead to the release/synthesis of ACh and NO?

5.2. *Evidence for a negative feedback loop: autoinhibitory $\alpha_2$-adrenergic receptors*

It has been assumed that $\alpha_{2A}$-ARs are presynaptic autoinhibitory receptors at spinal terminals of noradrenergic descending fibres, because high levels of $\alpha_{2A}$-AR immunoreactivity and mRNA were detected on the cell bodies of noradrenergic supraspinal nuclei (Nicholas et al., 1993; Rosin et al., 1993; Scheinin et al., 1994; Guyenet et al., 1994). NE is thought to induce inhibition of NE release from noradrenergic terminals by acting on these autoinhibitory $\alpha_2$-ARs located on these spinal terminals (Langer et al., 1985; Trendelenburg et al., 1999). However only recent evidence supports the existence of autoinhibitory $\alpha_2$-ARs in the spinal cord. In an *in vitro* preparation of spinal cord tissue devoid of synaptic circuits, application of NE decreased NE release, indicating that NE can produce direct inhibition of NE terminals through $\alpha_2$-ARs and in this way decrease the NE release (Li et al., 2000a). However anatomical investigations have failed to identify autoinhibitory $\alpha_2$-ARs in the spinal cord. Experiments combining radio-ligand binding with treatments for noradrenergic destruction (i.e. intrathecal 6-hydroxydopamine and cervical spinal hemisection) were carried out to investigate the origin of $\alpha_2$-ARs in the cord. The results indicated that $\alpha_2$-ARs are not present either in noradrenergic descending fibres, or in other type of descending fibres because $\alpha_2$-AR binding sites did not decrease following any of the treatments (Howe et al., 1987a). This has been consequently confirmed by immunocytochemical studies. Neither $\alpha_{2A}$ nor $\alpha_{2C}$ subtypes of adrenergic receptors appear to exist on descending noradrenergic terminals since no colocalisation was observed between $\alpha_{2A}$ or $\alpha_{2C}$-AR immunoreactivities and D$\beta$H or TH immunoreactivities, being both enzymes involved in NE biosynthesis (Stone et al., 1998). Therefore, although investigations indicate the presence of autoinhibitory $\alpha_2$-ARs at the cord, there is no anatomical evidence supporting a presynaptic autoinhibitory mechanism controlling NE release, i.e. a negative feedback circuit.

In summary, there is extensive evidence indicating the presence of a positive feedback mechanism involving a spinal circuit on which the described cascade relays [*NE-($\alpha_2$-AR)-ACh-CHOLINERGIC receptors-NO-(6-nitro-NE)-NE RELEASE*]. The negative feedback loop could exist, even though autoinhibitory $\alpha_2$-ARs have not been detected

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anatomically. Indeed, this could be due to the existence of only a small proportion of autoinhibitory $\alpha_2$-ARs, which in turn could explain the predominance of the positive loop to increase NE release that is consistent with multiple studies referred to above.

## 6. Candidate $\alpha_2$-AR subtypes as mediators of spinal noradrenergic antinociception

A number of studies have attempted to investigate the subtypes of $\alpha_2$-AR involved in spinal noradrenergic antinociception. These studies can be broadly classified, according to the techniques applied, into pharmacological, genetic and localisation studies. Recently investigations have become more integrative and frequently apply a combination of techniques from the different approaches.

### 6.1. *Pharmacological studies*

Pharmacological studies can provide information about the involvement of a receptor in specific actions by using receptor specific agonists and antagonists. However the agonist/antagonist approach is limited to resolve the roles of the different $\alpha_2$-AR subtypes because of the unspecificity of exogenous compounds that selectively bind to the different $\alpha_2$-AR subtypes (even though the subtypes have been well characterised). For this reason, the population of spinal $\alpha_2$-ARs that mediates NE-antinociceptive effects, has been determined, pharmacologically, as being of indistinguishable characteristics; i.e. specific affinity properties for each subtype have not been found (Yaksh, 1985). A diversity of strategies has been applied in order to characterise the subtype-selectivity of diverse ligands, but only marginal subtype-selectivity has been found. Competition with radio-labelled ligands is one of the strategies that has been used to characterise the subtype-selectivity of a ligand. For instance, oxymetazoline is an $\alpha_2$-AR agonist that binds with high affinity to $\alpha_{2A}$-ARs and with low affinity to $\alpha_{2B}$ and $\alpha_{2C}$-ARs (Wikberg-Matsson et al., 1995). A drawback of this kind of pharmacological study is that they are performed in membrane preparations, which contain a high concentration of the receptor. This differs from the situation *in vivo* where the amount of receptor varies across tissues and species and diverse receptors coexist in the same cell in variable proportions. Additionally, the agonist or antagonist can have affinity for multiple receptors and bind to other adrenergic receptors ($\beta$ or $\alpha_1$), or even to non-adrenergic receptors (e.g. imidazoline receptors) (Fairbanks and Wilcox, 1999). All these factors make the interpretation of the observations difficult when investigating adrenergic receptors with ligands.

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Although only marginal activity of $\alpha_2$-AR agonists and antagonists has been confirmed, the $\alpha_{2A}$-AR subtype has been suggested as mediator of antinociception in mice, based on the potent specificity of the $\alpha_{2A}$-AR-antagonist, idazoxan, which abolished the antinociceptive action of the $\alpha_2$-AR agonist, UK 14,304 (Millan, 1992; Millan et al., 1994). It has been proposed that the site of action for the antinociceptive effect of $\alpha_2$-AR agonists maybe at either $\alpha_{2A}$-ARs or non-$\alpha_{2A}$-ARs depending on the agonist used which suggests a role for the $\alpha_{2C}$-AR in spinal noradrenergic analgesia (Takano and Yaksh, 1992; Takano et al., 1992; Graham et al., 2000). The $\alpha_{2B}$ or $\alpha_{2C}$ subtypes have been indicated as mediators of analgesia based on the fact that adrenergic receptor agonists produced inhibition of neurotransmitter release from spinal cord preparations by a prazosin-sensitive receptor; i.e. the $\alpha_{2B}$-AR and/or $\alpha_{2C}$-AR, because prazosin is a partially selective antagonist with a binding affinity much greater for them than for the $\alpha_{2A}$-AR (Harrison et al., 1991; Takano and Yaksh, 1992; Marjamäki et al., 1993). In addition, it is known that the $\alpha_{2B}$-AR is predominantly, if not exclusively, located in the periphery (Nicholas et al., 1993), which would indicate the $\alpha_{2C}$-AR as a mediator of the $\alpha_2$-AR agonist-induced analgesia.

Therefore, conventional pharmacological investigations strongly prove that noradrenergic antinociception is mediated by $\alpha_2$-ARs (Yaksh, 1985), but do not provide definitive information about the subtype/s responsible for it.

Investigations that combine pharmacology with genetic manipulation techniques (e.g. gene deletion -knock-out-; gene substitution -functional knock-out-, antisense oligonucleotides -knock-down-) have helped to elucidate the role of the different $\alpha_2$-AR subtypes in noradrenergic analgesia. The $\alpha_{2A}$-AR was first reported to be the primary mediator of $\alpha_2$-AR-mediated spinal analgesia (Hunter et al., 1997; Lakhlani et al., 1997; Stone et al., 1997b). By using a genetically modified mouse line expressing a point mutation in the $\alpha_{2A}$-AR, which is considered to be a functional knock-out of the $\alpha_{2A}$-AR, it was observed that application of an $\alpha_2$-AR agonist results in a dramatic loss of analgesic effect. This is in contrast with the situation in the wild type mice where the agonist induces analgesia. Recent characterisation of a novel $\alpha_2$-AR agonist, moxonidine (Armah and Stenzel, 1981), has helped to demonstrate that the $\alpha_{2C}$-AR plays an important role in $\alpha_2$-AR-mediated antinociception; the role of the receptor has been described as subtle but clear (Fairbanks et al., 2002). Spinal antinociceptive activity of moxonidine appears largely independent from $\alpha_{2A}$-AR activation and for this reason it has been used to test for an $\alpha_{2C}$-

AR role in analgesia (Fairbanks and Wilcox, 1999). Moxonidine-induced antinociception is minimally decreased in functional $\alpha_{2A}$-AR-knock-out mice, while moderately, but significantly decreases in $\alpha_{2C}$-AR-knock-out mice. No change is detected in $\alpha_{2B}$-AR-knock-out mice. In addition, $\alpha_{2C}$-AR-knock-down using antisense oligodeoxinucleotides, not only decreases $\alpha_{2C}$-AR immunoreactivity, but also significantly reduces moxonidene-induced antinociception.

## 6.2 *Genetic studies*

Molecular biology by means of the application of homologous recombination strategies has clarified the classification of $\alpha_2$-ARs in order to find out about the functional contributions of each of the subtypes. Currently it is known that the three subtypes ($\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$) are encoded by three different genes, each one being located on a different chromosome. The $\alpha_{2A}$-AR-gene is on C10; the $\alpha_{2B}$-AR-gene is on C2; and the $\alpha_{2C}$-AR-gene on C4. All three subtypes have been cloned and sequenced in a variety of species (reviewed in Kable et al., 2000). Different functions are likely to be mediated by different subtypes, and much effort is being directed toward understanding the physiological roles of the various subtypes. Mice with altered $\alpha_2$-AR genes have been generated in order to elucidate the subtype-specific functions of the three $\alpha_2$-AR subtypes. These *in vivo* behavioural studies indicate that the $\alpha_{2A}$-AR is the main mediator of spinal antinociception, as well as of most of the other classical functions mediated by $\alpha_2$-ARs: hypotension, sedation, hypothermia, anaesthesia and analgesia. The $\alpha_{2B}$-AR has been suggested as the principal mediator of hypertension. Finally the $\alpha_{2C}$-AR has been related to many CNS processes such as startle reflex, stress response and locomotion, as well it has been reported to be involved in hypothermia and dopamine turnover. Both subtypes, $\alpha_{2A}$ and $\alpha_{2C}$ are considered important in the presynaptic inhibition of NE release, although they seem to have distinct regulatory roles still undefined (reviewed in Kable et al., 2000).

In the literature, the $\alpha_{2C}$-AR is regarded as an enigmatic receptor. The reason for this is that no obvious function was initially elucidated as only subtle differences were found between the $\alpha_{2C}$-AR-knock-out and the wild type mice. This was rather surprising given the $\alpha_{2C}$-AR is extensively conserved throughout evolution and therefore expected to have an important role. It has been argued that the $\alpha_{2C}$-AR function could be compensated by other receptors or mechanisms as a consequence of its constant functional absence. In addition, the finding of cell-specific gene expression indicates a specific $\alpha_{2C}$-AR function.

The $\alpha_{2C}$-AR has been hypothesised to modulate the modulator; i.e. to modulate NE, which in turn modulates the activity of neurotransmitter pathways and circuits. This could imply that the $\alpha_{2C}$-AR alone has a marginal activity (reflected in the subtle differences of $\alpha_{2C}$-AR-knock-outs), but that at the same time is essential for physiological efficiency (MacDonald et al., 1997). It was careful analysis by later investigations of the subtle differences between $\alpha_{2C}$-AR-knock-out, $\alpha_{2C}$-AR-overexpressing and wild type mice, which elucidated the involvement of the $\alpha_{2C}$-AR in a multiplicity of processes (mentioned above). Indeed, Fairbanks and colleagues (2002) have added a new role to the list of $\alpha_{2C}$-AR functions: antinociception.

From the evidence above we can conclude that both the $\alpha_{2A}$ and $\alpha_{2C}$ subtypes of adrenergic receptor are involved in spinal antinociception.

### 6.3. Localisation studies

Many studies concentrate on the localisation of $\alpha_2$-AR and its different subtypes in order to elucidate their functions.

**Radioligand binding studies** have provided extensive information about the distribution of $\alpha_2$-ARs.

In the dorsal horn, $\alpha_2$-ARs have been reported to be concentrated in superficial laminae, because a high concentration of binding sites were shown in lamina II by autoradiography after application of [$^3$H]-clonidine, i.e. a radioactive $\alpha_2$-AR agonist (Bouchenafa and Livingstone, 1987). A proportion of these binding sites must be present on axons and terminals of primary afferents since dorsal root ganglionectomy decreased the concentration of $\alpha_2$-AR binding sites by a 20%, as detected by [$^3$H]-rauwolscine (Howe et al., 1987b). North and Yoshimura (1984) investigation agrees with the first observation, because NE-induced hyperpolarisation of the superficial dorsal horn, in particular lamina II, is only reversed by $\alpha_2$-AR antagonists, which suggests lamina II as a major location of $\alpha_2$-ARs (North & Yoshimura, 1984). The second observation is also in agreement with other experimental evidence which is discussed later in this chapter (Nicholas et al., 1993; Cho et al., 1997; Stone et al., 1998; Shi et al., 2000)

In contrast to the observations in the dorsal horn, radioligand binding studies concluded that $\alpha_2$-ARs are not present on ventral horn motoneurons, because ventral rhizotomy does not alter the density of $\alpha_2$-AR binding sites (Howe et al., 1987b). However this conclusion is not correct if we considered that ventral rhizotomy, unlike dorsal

rhizotomy, does not necessarily imply degeneration of the central component, i.e. motoneuron cell bodies.

Based on binding studies, $\alpha_2$-ARs are not found on descending axons or if so only in very small proportions. The preceding evidence is derived from an experiment in which two different treatments (i.e. intrathecal 6-hydroxydopamine or cervical spinal hemisection) to eliminate descending innervation, effectively decreased the release of NE, while the density of $\alpha_2$-AR binding sites was not altered (Howe et al., 1987a), or even increased at specific areas like the superficial dorsal horn which was consequently described as supersensitive (Roudet et al., 1994).

Radioligand binding techniques have the following limitations: the anatomical resolution is low, and no subtype-specific distribution can be obtained since no subtype-selective ligands are available. The exception is [$^3$H]-rauwolscine, which appears to discriminate between the $\alpha_{2C}$ and $\alpha_{2A}$ subtypes in the rat, by binding primarily $\alpha_{2B}$ and $\alpha_{2C}$-ARs (Boyajian et al., 1987). Radioligand binding studies of $\alpha_2$-AR densities using [$^3$H]-rauwolscine as ligand revealed the presence of $\alpha_2$-ARs throughout the whole gray matter with a preferential location in the superficial dorsal horn; such a pattern is maintained at all rostrocaudal levels of the cord (Sullivan et al., 1987; Roudet et al., 1994). These binding sites very probably represent the distribution of the $\alpha_{2C}$-AR, because $\alpha_{2B}$-ARs are mainly found in the peripheral nervous system (Nicholas et al., 1993).

**Immunocytochemical studies** have provided information about the distribution of the different $\alpha_2$-AR subtypes thanks to the availability of subtype-specific antibodies. Such information can help us to understand the roles of the different subtypes. To a great extent the results from immunocytochemical studies agree with other approaches which generally confirm findings obtained with this method. Throughout the CNS both adrenergic receptors, $\alpha_{2A}$ and $\alpha_{2C}$, are widely distributed, while comparatively the $\alpha_{2B}$-AR appears to be expressed at lower levels and in a more restricted distribution.

- **Immunoreactivity for the $\alpha_{2A}$-AR** has been detected in a subset of dorsal root ganglion (DRG) cells (Gold et al., 1997); on cell bodies of neurons in the spinal cord dorsal horn (laminae I-II and IV-V), central canal (lamina X) and intermediolateral cell column (Rosin et al., 1993; Talley et al., 1996); as well as on axon terminals at all levels of the superficial dorsal horn (laminae I-II) (Stone et al., 1998). $\alpha_{2A}$-ARs have been shown to be present on spinal terminals of SP-containing primary afferents; as

well as on CGRP-containing primary afferents (Stone et al., 1998), which very likely correspond to nociceptors (Levine et al., 1993). $\alpha_{2A}$-AR immunoreactivity did not coexist with enkephalin or preprodynorphin, both endogenous opioid peptides. The fact that enkephalin is not present in primary afferents (Hökfelt et al., 1977, Johansson et al., 1978; Seybold and Elde, 1980) further suggests that the $\alpha_{2A}$-AR is found predominantly on primary afferent terminals.

- **Immunoreactivity for the $\alpha_{2C}$-AR** has been detected on DRG cells (Birder and Perl, 1999), on cell bodies of neurons in the dorsal horn (laminae IV-VI), central canal (lamina X), intermediolateral cell column, and ventral horn. Some of the latter cell bodies displayed typical motoneuronal soma staining (Rosin et al., 1996). In addition $\alpha_{2C}$-AR immunoreactivity, as for the $\alpha_{2A}$ subtype, is present on axon terminals of the superficial layers (laminae I and II) of the dorsal horn; however the $\alpha_{2C}$-AR was more prevalent in deeper layers of the dorsal horn. $\alpha_{2C}$-AR immunoreactivity was also detected in the lateral spinal nucleus (Stone et al., 1998). $\alpha_{2C}$-ARs were found to be present on enkephalin containing terminals (Stone et al., 1998), which, in turn, indicates that the receptor is expressed by spinal enkephalin-containing neurons, since enkephalin is absent from primary afferents (Hökfelt et al., 1977). Colocalisation with somatostatin suggests that $\alpha_{2C}$-ARs could exist on somatostatin-containing primary afferents, which are known to be small diameter unmyelinated primary afferents that lack SP (Hökfelt, 1976; Tuchscherer and Seybold, 1985). However, probably the majority, if not all of the $\alpha_{2C}$-AR terminals colocalising with somatostatin, are located on terminals of a population of spinal somatostatin-containing neurons, because in the rat somatostatin is mainly of spinal origin (Nagy et al., 1981).

Double-labelling of the $\alpha_{2A}$ and $\alpha_{2C}$-AR subtypes showed no colocalisation between these receptor subtypes and it was suggested that the two subtypes are located on different neuronal populations (Stone et al., 1998). This agrees with the situation described in the brain, where the $\alpha_{2A}$ and $\alpha_{2C}$-AR subtypes exhibit a distinctive pattern of distribution (Rosin et al., 1996).

In conclusion the existing evidence indicates that the superficial dorsal horn is a target for noradrenergic influence, with distinct $\alpha_2$-AR sites of action:

- $\alpha_{2A}$-AR-containing primary afferent terminals
- $\alpha_{2A}$-AR somata in the superficial and deep dorsal horn
- $\alpha_{2C}$-AR-containing spinal terminals of interneurons in the superficial dorsal horn
- $\alpha_{2C}$-AR somata in the deep dorsal horn

*In situ* **hybridisation studies** permit the distribution of the mRNAs encoding a particular receptor subtype to be visualised; i.e. they indicate the site of receptor synthesis. Several *in situ* hybridisation studies have been carried out in order to provide information about the sites of synthesis of the different $\alpha_2$-AR subtypes. They involve the use of subtype-specific probes (cRNA or oligonucleotides) to visualise the subtype-specific mRNAs.

- $\alpha_{2A}$-**AR mRNA** has been reported to exist in a subset of DRG neurons (Nicholas et al., 1993; Cho et al., 1997; Shi et al., 2000), which probably correspond to the reported $\alpha_{2A}$-AR-immunoreactive (IR) DRG neurons (Gold et al., 1997) that very likely give rise to the central $\alpha_{2A}$-AR-IR terminals reported by Stone and colleagues (1998). $\alpha_{2A}$-AR mRNA has been detected in spinal cord dorsal and ventral horn neurons (Zeng and Lynch, 1991; Winzer-Serhan et al., 1997a) and the intermediolateral cell column (Nicholas et al, 1993). While most of these studies agree with the immunocytochemical findings, $\alpha_{2A}$-AR mRNA has been detected in ventral horn neurons (Zeng and Lynch, 1991; Winzer-Serhan et al., 1997a), where no $\alpha_{2A}$-AR immunoreactivity has been reported.

- $\alpha_{2C}$-**AR mRNA** in the rat spinal cord has been detected on the majority of both large and small DRG neurons (Nicholas et al., 1993; Cho et al., 1997; Gold et al., 1997; Shi et al., 2000), this is controversial *a priori* because while $\alpha_{2C}$-AR immunoreactivity exists on DRG cell bodies, no immunoreactivity, or very little appears to be present on central primary afferent projections. A reconciling possibility is that the $\alpha_{2C}$-AR is synthesised in the DRG and mainly trafficked to the peripheral terminal. This idea is supported by studies which claim a role for the $\alpha_{2C}$-AR in peripheral antinociception (Khasar et al., 1995). Surprisingly there is very little evidence of $\alpha_{2C}$-AR mRNA in the spinal cord dorsal and ventral horns (Winzer-Serhan et al., 1997b) and indeed many

studies have failed to report $\alpha_{2C}$-AR mRNA in the spinal cord; this leaves unresolved the question of which neurons synthesise the $\alpha_{2C}$-ARs that are present on spinal origin terminals at the superficial dorsal horn.

The current picture provided by *in situ* hybridisation studies is rather incomplete; this could be a consequence of methodological limitations. It is possible that the applied techniques are not sensitive enough to detect small amounts of mRNA; or that the receptor mRNA is in a non-detectable conformation.

Immunocytochemistry provides a special advantage with respect to other techniques. While radioligand binding detects the receptors on the membrane, typically considered as the functional receptors; and *in situ* hybridisation detects the receptor mRNA at the nucleus, considered as the source of the receptor; only immunocytochemistry can pinpoint the distribution of the receptor at subcellular level. Although these observations are not specific to the spinal cord, the $\alpha_{2A}$ and $\alpha_{2C}$ subtypes have been reported to display a distinct pattern of subcellular distribution:

- The $\alpha_{2A}$-AR antibody appears to bind to two different types of substrate. One of these was punctate in nature, intracellular and associated with vesicle-like structures. The other one was diffuse and labelled cell bodies, neurites and glial cells (Talley et al., 1996).

- The $\alpha_{2C}$-AR antibody mainly localised the $\alpha_{2C}$-AR-IR substrate intracellularly (perinuclear), and unlike the $\alpha_{2A}$-AR immunoreactivity, it was not found in the neuropil, i.e. regions of synaptic interaction. However this lack of neuropil labelling could result from a limited sensitivity of the staining (Rosin et al., 1996). Indeed, initially no $\alpha_{2A}$-AR staining was appreciated in distal dendrites and only after methodological modifications distal dendritic labelling was achieved (Talley et al., 1996).

Very limited **immunoelectron microscopic evidence** is available regarding the $\alpha_{2}$-AR subtypes at the ultrastructural level. In the rat LC, $\alpha_{2C}$-ARs were observed to be postsynaptic on catecholaminergic dendrites that receive axodendritic asymmetric synapses (Lee et al., 1998a). $\alpha_{2A}$-ARs were presynaptic on axon terminals and postsynaptic on heterogeneous dendrites (approximately 50% were catecholaminergic) (Lee et al., 1998b). Prior to the present study there was no information available about $\alpha_{2}$-ARs in the spinal cord at the electron microscope level.

# 7. Morphological and neurochemical characteristics of superficial dorsal horn interneurons

The evidence up to date available indicates that the receptor in present in the superficial dorsal horn on the terminals of interneurons; for this reason a brief account is given regarding the morphological and neurochemical characteristics of interneurons in the superficial dorsal horn.

Interneurons in lamina II have been divided into limitrophes or stalked cells and central or islet cells (Ramon y Cajal, 1909; Gobel 1978, 1979). In the rat, Todd and Lewis (1986) found that neurones resembling Gobel's stalked and islet cells made up the majority of lamina II cells. Stalked cells are larger than islet cells and their somata are located in lamina $II_o$. Their dendritic tree has a cone shape, which is directed ventrally and passes through laminae II-IV. The main axonal destination of this type of cell is lamina I. Gobel suggested (1978) that these cells are excitatory. Later studies support the latter suggestion, since lamina II stalked cells did not show GABA-IR (Todd and McKenzie, 1989). Stalked cells may contain somatostatin, enkephalin, dynorphin or tachykinins, which could be coexisting within the same cell (review in Todd and Spike, 1993). Recordings from these cells indicated that they are either nociceptive specific or wide-dynamic-range (Bennett et al., 1980). Islet cells are very numerous in lamina II. Their dendritic trees extend within lamina II and are oriented in the rostrocaudal plane. Similarly, their axons terminate mainly within lamina II (Gobel, 1978, 1979; Shoenen, 1982). Gobel (1978) suggested that islet cells are inhibitory interneurons. Indeed, numerous large islet cells have been reported to contain GABA and in occasions also glycine; however small islet cells have been reported to be non-GABAergic (Todd and McKenzie, 1989; Todd and Sullivan, 1990). Small islet cells may contain neurotensin or somatostatin, whereas large islet cells that were only immunoreactive for GABA (i.e. non-glycinergic) could be containing enkephalin or neuropeptide Y (reviewed in Todd and Spike, 1993). Gobel described three other types of cell: arboreal, II-III Border and spiny (Gobel 1975, 1978). In the rat substantia gelatinosa some of these cell types were found, however often they had mixed structural features corresponding to diverse cell types (Todd and Lewis, 1986). Many interneurons also exist in lamina I and lamina III, but very little information is available that correlates their morphology and neurochemical content. In lamina III neurons with dorsoventral dendritic trees were found to be non-immunoreactive for GABA, indicating that these are very likely excitatory. Differently lamina III islet cells were found to be GABA-IR. Cholinergic

neurons, bearing a characteristic morphology, have been found in laminae III-V. These cells have dendrites which pass dorsally into lamina II and extend in rostral and caudal directions. Their axons terminate mainly in lamina II and III. Lamina I neurons are thought to be mainly excitatory, because 72% of the lamina I neurons were reported to be not containing GABA or glycine (reviewed in Todd and Spike, 1993).

**Figure 1.1. A model of spinal noradrenergic antinociception adapted from Peng et al.,** **1996.** NE released from descending terminals acts on $\alpha_1$-ARs to excite inhibitory interneurons in the superficial dorsal horn; in turn inhibitory interneurons release inhibitory transmitters (GABA and/or glycine) and inhibit projection neurons in deep dorsal horn by acting on GABA and/or glycine receptors (GABA$_R$ and/or Glycine$_R$). Projection neurons are also directly inhibited by NE released from descending terminals, which acts on $\alpha_2$-ARs.

NE

SUPRASPINAL
NORADRENERGIC
NEURON

supraspinal
projection

$\alpha_1$

$\alpha_2$

GABA
Glycine

GABA$_R$
or Glycine$_R$

SUPERFICIAL
DORSAL HORN
NEURON

DEEP
DORSAL HORN
NEURON
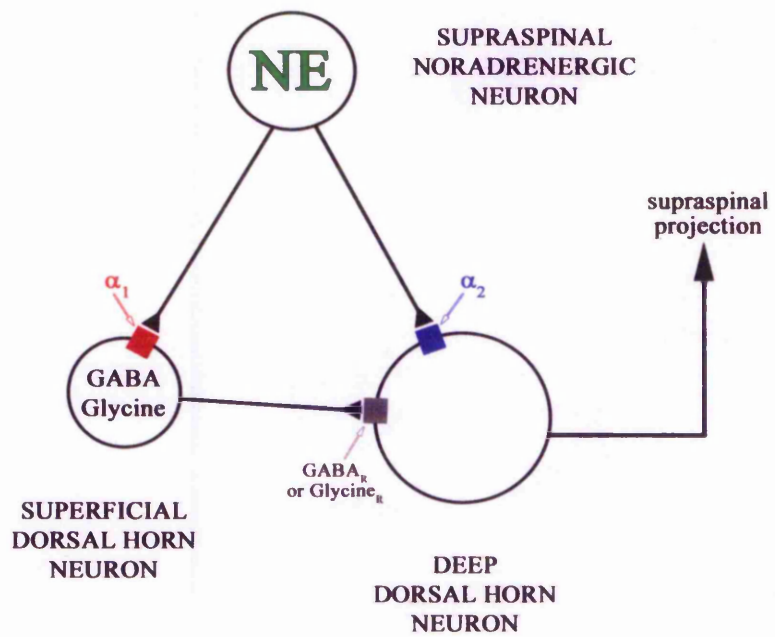
*Chapter 2*

# Aims and General Experimental Procedures

## 1. Aims

The purpose of the first section in this chapter is to give a brief account of the principal aims pursued, and hypothesis tested, in each of the investigations as well as the experimental approaches used. The specific experimental procedures, that were applied in each of the three investigations carried out, are explained in detail in the corresponding results chapter.

Investigations 1 and 2 focus on the organisation of the $\alpha_{2C}$-AR in the dorsal horn of the rat spinal cord, while the topic of investigation 3 is the organisation of the receptor in the lateral spinal nucleus (LSN), a nucleus in the dorsal lateral funiculus.

### *Investigation 1*

Aim: to study the origin and properties of axon terminals that possess $\alpha_{2C}$-ARs in the rat dorsal horn

Hypothesis 1: $\alpha_{2C}$-ARs are located on axon terminals of spinal origin.

Double-labelling immunocytochemistry for confocal microscopy was used to study the colocalisation patterns of $\alpha_{2C}$-ARs with diverse markers for descending systems, primary afferent fibres and excitatory or inhibitory interneurons. Two methods of quantification were applied in order to estimate the extent of colocalisation of $\alpha_{2C}$-ARs immunoreactivity with other markers immunoreactivity. One method measured the area of overlap of immunoreactivities while the other one provided the frequency of double-labelled terminals.

Immunoelectron microscopy was used to investigate at the ultrastructural level the characteristics of $\alpha_{2C}$-AR-IR structures in the neuropil of the superficial dorsal horn. Quantitative analysis of the $\alpha_{2C}$-AR-IR terminals involved classification of the terminals according to the type of synaptic arrangement and type of synapse they formed, as well as the presence or absence of dense-core vesicles (DCVs) within them. A morphometric analysis of the terminals was also produced.

*Investigation 2*

Aim: to study the relationship of axon terminals possessing $\alpha_{2C}$-ARs with projection neurons that express the neurokinin-1 receptor (NK-1) in the dorsal horn

Hypothesis 1: NK-1 projection neurons are innervated by terminals containing $\alpha_{2C}$-ARs.

Retrograde labelling of projection neurons was combined with triple-labelling immunocytochemistry for confocal microscopy to reveal cholera toxin B subunit (CTb; i.e. the retrograde tracer), NK-1 and $\alpha_{2C}$-ARs. NK-1 retrogradely labelled neurons were identified and scanned to be reconstructed three-dimensionally. Contacts formed by $\alpha_{2C}$-AR-IR terminals on the neurons were mapped on the reconstructions, which allowed the study of the distribution of "$\alpha_{2C}$-AR innervation" on NK-1 projection cells.

Hypothesis 2: terminals containing $\alpha_{2C}$-ARs form synaptic contacts on NK-1 projection neurons.

A combined confocal-electron microscopy method permitted the identification of terminals making contact on a neuron with confocal microscopy and consequent visualisation of the ultrastructural detail of the same terminals with electron microscopy. With this method it was possible to test if appositions from $\alpha_{2C}$-AR-IR terminals form synaptic contacts on NK-1 projection cells.

Hypothesis 3: terminals containing $\alpha_{2C}$-ARs which innervate NK-1 projection neurons are excitatory.

A sequential immunocytochemical method was applied in order to determine if the $\alpha_{2C}$-AR appositions on NK-1 projection cells are excitatory or inhibitory.

Hypothesis 4: NK-1 projection neurons that receive innervation from $\alpha_{2C}$-AR-containing terminals are responsive to noxious stimulation.

Expression of the immediate early gene c-Fos was induced by application of peripheral noxious thermal stimulation to investigate if NK-1 projection neurons in the dorsal horn are activated by nociceptive stimulation. Combination of this approach with retrograde labelling of projection cells and multiple-labelling immunocytochemistry was used to determine if c-Fos expressing cells, i.e. responsive to noxious stimulation, are associated with $\alpha_{2C}$-AR-IR terminals.

*Investigation 3*

Aim: to study the organisation of $\alpha_{2C}$-ARs in the LSN

Hypothesis 1: $\alpha_{2C}$-ARs in the LSN are located on axon terminals of spinal origin.

As in *Investigation 1* for the dorsal horn, double-labelling immunocytochemistry for confocal microscopy and immunoelectron microscopy were used to study the colocalisation patterns of $\alpha_{2C}$-ARs and the characteristics of $\alpha_{2C}$-AR-IR structures at the ultrastructural level, respectively.

Hypothesis 2: LSN neurons receive innervation from terminals possessing $\alpha_{2C}$-ARs and are not responsive to noxious stimulation

Expression of c-Fos by application of noxious stimulation was combined with retrograde labelling of projection neurons and multiple-labelling immunocytochemistry to investigate the identity of LSN neurons, their involvement in nociception and their relationship with $\alpha_{2C}$-AR-IR structures.

## 2. General Experimental Procedures

This section gives an account of some relevant aspects regarding immunocytochemistry and confocal microscopy, which were both used consistently throughout the present work. The aim is to provide general information to facilitate the understanding of the specific experimental protocols applied and described in chapters 3,4 and 5.

### 2.1. *Multiple immunolabelling for confocal microscopy*

This technique allows the identification of more than one antigen in the same section of spinal cord. The antigens can be neurotransmitters, enzymes, peptides or receptors. The rationale of the protocol can be summarised to two essential general steps (Fig. 2.1, diagram):

1. Incubation of spinal cord sections with primary antibodies that have been raised in different species. Normally a primary antisera cocktail contains two or three primary antibodies and each one is derived from a different species. Currently is also possible to include four primary antibodies provided they have been raised in a different species.

2. Incubation of sections in a cocktail containing species specific-secondary antibodies, that are coupled each one to a different fluorophore. Generally a

secondary antibody is an immunoglobulin (Ig) raised in donkey, which is directed against an Ig of a particular X species. The resulting secondary antibody will bind to any antigen of the X species and will be readily identified by means of the fluorophore coupled to it.

Following the incubations and rinses, the sections are mounted and finally they are ready to be scanned with the confocal microscope. Immunoreactivity for each of the antigens can be independently visualised as revealed by the corresponding fluorophore-coupled secondary antibody. Images from the same optical section can be merged in order to study the relative spatial distribution of the antigens. For instance it can be assessed if two antigens colocalise (Fig. 2.1, images).

Exceptionally two primary antibodies from the same species can be used provided they are known to identify antigens associated with separate cellular compartments. For instance, immunoreactivity for the NK-1 receptor is localised to the cell membrane while c-Fos immunoreactivity is present essentially in the nucleus of the cell. For this reason both antigens can be labelled with a primary antibody of the same species and the same secondary antibody and still it will be possible to discern both types of immunoreactivity. Figure 5.8 (b) illustrates immunoreactivity for NK-1 and c-Fos that has been obtained using primary antibodies raised in the same species (i.e. rabbit anti-NK-1 and rabbit anti-c-Fos), which were revealed with the same secondary antibody (i.e. donkey anti-rabbit Ig coupled to the fluorophore fluorescein isothiocyanate, FITC).

It is also possible to use two primary antibodies of the same species if the incubation is performed sequentially. This procedure implies additional steps: 1 incubation in the primary and secondary antisera cocktails, 2 scanning of the regions of interest in the section, 3 re-incubation with the additional primary antibody (this will be of the same species as one of the originally used antibodies) followed by incubation with exactly the same secondary antibody that was used to reveal the original primary antibody of the same species, 4 re-location and re-scanning of the previously selected area of interest, 5 comparison of the scans obtained before and after re-incubation allows detection of extra-labelling in the corresponding channel which represents immunoreactivity for the sequentially added antibody (Fig. 2.2).

## 2.2. Confocal microscopes

Two different confocal laser scanning microscopes have been used in the present investigations: a Bio-Rad MRC 1024 and a Radiance 2100 (Bio-Rad, Hemel Hempstead, UK).

The Bio-Rad MRC 1024 microscope is equipped with a Krypton Argon laser, which can emit light of three different wavelengths. Each of the wavelengths can excite specifically the fluorophores coupled to the secondary antibodies (Table 2.1, below). This microscope was used to scan sections of spinal cord, which had been labelled with a maximum of three secondary antibodies each one coupled to a different fluorophore. Scanning with the BioRad MRC 1024 was performed sequentially to avoid bleed-through between channels.

The Radiance 2100 microscope is equipped with four lasers: Argon, Green Helium Neon, Red diode and Blue diode, which allowed the scanning of sections that had been labelled with four secondary antibodies each one being coupled to a different fluorophore (Table 2.1, below). Figure 5.7 illustrates the scanning of tissue labelled with four secondary antibodies coupled each one to a different fluorophore. With the microscope Radiance 2100, scanning could be performed simultaneously.

**Table 2.1.** Excitation-emission wavelengths corresponding to the fluorphores used

| Fluorophore | excitation $\lambda$ | emission $\lambda$ |
|---|---|---|
| Fluorescein isothiocyanate (FITC) | 494 | 518 |
| Rhodamine-red | 570 | 590 |
| Cyanine 5.18 (CY-5) | 650 | 670 |
| *Pacific blue | 416 | 451 |

*\* Only the Radiance 2100 confocal microscope was equipped to scan labelling with this fluorophore.*

**Figure 2.1. Diagram and confocal images to illustrate double-labelling immunofluorescence.** Above, a flow diagram simplifies the two basic steps of a protocol for double-labelling immunocytochemistry of the $\alpha_{2C}$-AR (a2C) and somatostatin (SOM). Below, confocal images exemplify how the distribution of each antigen can be studied independently in the same optical plane; $\alpha_{2C}$-AR (a2C, green) and somatostatin (SOM, red). The relative spatial distribution of the antigens can be assessed by merging the images. In this case the merged image shows an overlap of the immunoreactivities (in yellow) indicating the antigens colocalise.

Double -labelling immunocytochemistry for:

Axon terminals containing **α2c-AR**

Axon terminals containing **somatostatin**

PRIMARY ANTIBODIES

*guinea pig* anti-**α2c-AR**

*rabbit* anti-**somatostatin**

SECONDARY ANTIBODIES  +  FLUORESCENT PARTICLE

donkey anti-*guinea pig*   +   FITC

donkey anti-*rabbit*   +   Rhodamine

**FITC**

**Rhodamine-Red**



a2C

SOM
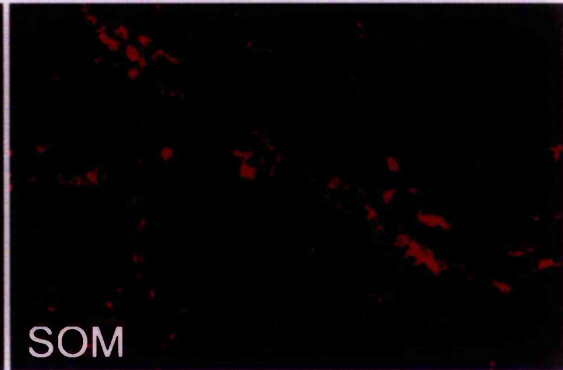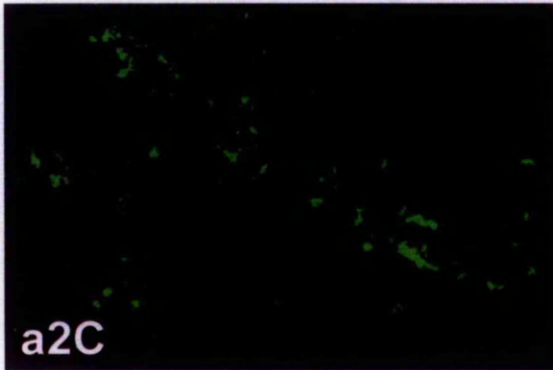
**MERGED IMAGE**
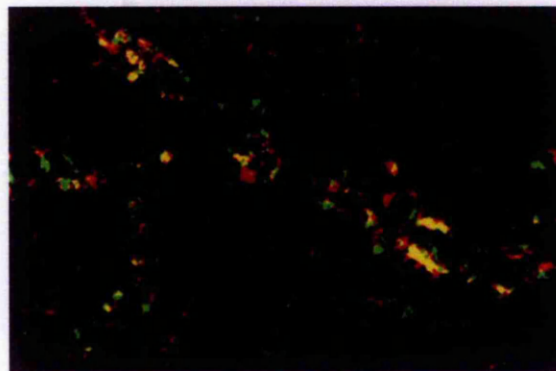
**Figure 2.2. Flow diagram and confocal images to illustrate triple-labelling and sequential immunofluorescence.** Above, a flow diagram indicates the essential steps to perform triple-labelling immunocytochemistry for the $\alpha_{2C}$-AR (A2C), the NK-1 receptor (NK1), and the retrograde tracer: cholera toxin B subunit (CTb). **A,** A series of four confocal images exemplifies how the three types of labelling can be visualised independently (NK1, green; CTb, blue; A2C, red) and how the images can be merged to study the relative spatial distribution of the antigens. In order to perform sequential immunocytochemistry with and additional primary antibody, the section is removed from the slide and re-incubated in the primary and then secondary antisera cocktail. In this example, the sequentially added primary antibody is a rabbit-anti-vesicular glutamate transporter 2 (VGLUT2), which is revealed with a donkey anti-rabbit Ig coupled to the fluorophore FITC; i.e. the same secondary antibody used originally to label NK-1. Once the section is mounted the same area is re-located and re-scanned. **B,** A series of four confocal images shows the resulting labelling. The additional VGLUT2 labelling can be differentiated from the NK-1 labelling by comparing **A** and **B**. The extra-green labelling in **B** represents VGLUT2 immunoreactivity (NK1+VGLUT2); CTb (CTb) and the $\alpha_{2C}$-AR immunoreactivities are not altered (A2C). Merging of the images reveals the localisation of VGLUT2 in relation to the rest of the antigens. (Scale bars=20$\mu$m)

Triple-labelling immunocytochemistry for:

Axon terminals containing **α2c-AR**

Cells expressing **NK-1 receptor**

**CTb**-retrogradely labelled cells

| PRIMARY ANTIBODIES | SECONDARY ANTIBODIES + FLUORESCENT PARTICLE | | |
|---|---|---|---|
| *guinea pig* anti-α2c-AR | donkey anti-*guinea pig* | + | LRSC |
| *rabbit* anti-NK-1 | donkey anti-*rabbit* | + | FITC |
| *goat* anti-CTb | donkey anti-*goat* | + | CY5 |

Scan cells

NK1   CTb   A2C
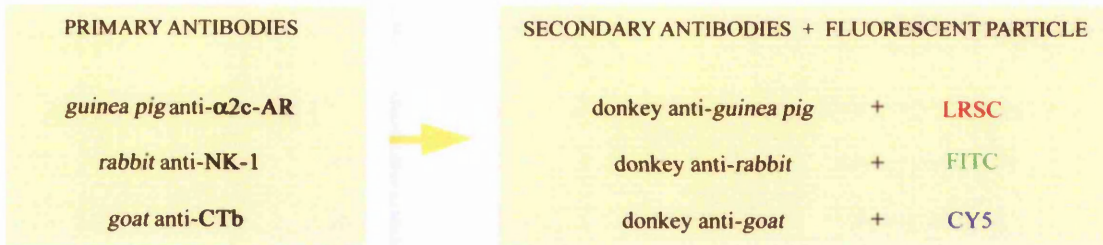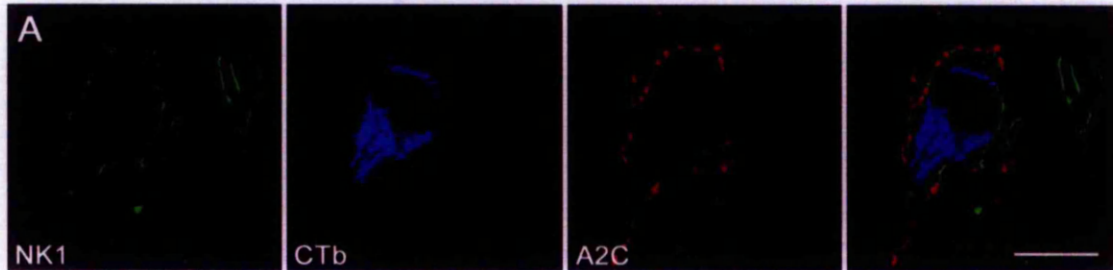
Removal of spinal cord section from slide

Sequential incubation with a fourth antibody against
**Vesicular Glutamate Transporter2 (VGLUT2)**

| *rabbit* anti-VGLUT2 | donkey anti-*rabbit* | + | FITC |
|---|---|---|---|

Re-scan cells

NK1+VGLUT2   CTb   A2C
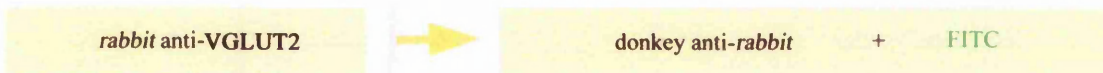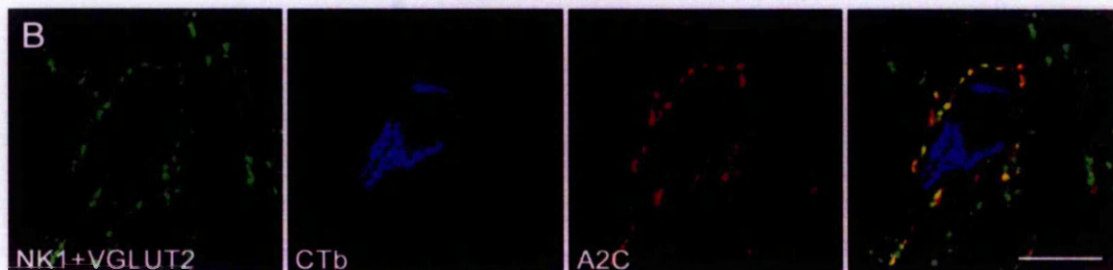
*Chapter 3*

<u>Investigation 1</u>:

**Origin and properties of axon terminals that possess**

**$\alpha_{2C}$-adrenergic receptors in the rat dorsal horn**

# 1. Introduction

Noradrenergic systems in the dorsal horn of the spinal cord are involved in modulation of nociceptive transmission but the mechanisms and circuitry that underlie these actions are poorly understood. Pharmacological evidence indicates that the antinociceptive action of NE is mediated principally by $\alpha_2$-ARs (Howe et al., 1983; Yaksh, 1985; Proudfit, 1988). Neurochemical and immunocytochemical studies show that noradrenergic fibres, which originate from nuclei in the brainstem, are present in all areas of the spinal cord grey matter but are particularly concentrated in the superficial laminae of the dorsal horn (Dahlström and Fuxe, 1965; Schroder and Skagerberg, 1985; Rajaofetra et al., 1992; Doyle and Maxwell, 1991a, 1991b). The superficial dorsal horn also has a high concentration of $\alpha_2$-AR binding sites (Sullivan et al., 1987; Howe et al., 1987). Three subtypes of $\alpha_2$-ARs ($\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$) have been sequenced (Bylund et al., 1994; Kable et al., 2000) and the $\alpha_{2A}$ and the $\alpha_{2C}$ subtypes are widely distributed in the CNS (Rosin et al., 1996; Talley et al., 1996). Unfortunately it is not possible to differentiate between these two subtypes of $\alpha_2$-AR pharmacologically because subtype-selective compounds are not available. "Knock-out" techniques support the idea that $\alpha_{2A}$-ARs mediate NE-induced antinociception, while no function had been established for the $\alpha_{2C}$-AR in the dorsal horn at the time this investigation was carried out (Kable et al., 2000). Recently Fairbanks and co-workers (2002) have reported a role for the $\alpha_{2C}$-AR in noradrenergic antinociception.

The $\alpha_{2A}$-AR is found on axons of the superficial dorsal horn which contain SP and CGRP (Stone et al., 1998) which are likely to be terminals of nociceptive primary afferents (Levine et al., 1993). Immunoreactivity for the $\alpha_{2C}$-AR is also concentrated on axons of superficial layers of the dorsal horn (Stone et al., 1998). The numbers of $\alpha_{2A}$-AR-IR terminals in the superficial dorsal horn decrease dramatically as a consequence of dorsal rhizotomy or neonatal capsaicin treatment whereas $\alpha_{2C}$-AR immunoreactivity is not significantly reduced by either of these treatments (Stone et al., 1998). This evidence indicates that $\alpha_{2A}$-ARs are located principally on small diameter primary afferents whereas $\alpha_{2C}$-ARs do not appear to be associated with primary afferent fibres. Double-labelling experiments, using antibodies specific for the $\alpha_{2A}$ and $\alpha_{2C}$ subtypes, also show that the two subtypes are located on different axonal populations (Stone et al., 1998). Some $\alpha_{2C}$-AR immunoreactivity is associated with axons that contain enkephalin and somatostatin indicating that it is present on terminals of some types of local circuit neuron. The

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immunocytochemical evidence is, however, at variance with findings of *in situ* hybridisation studies where $\alpha_{2C}$-AR mRNA was not detected in intrinsic neurons of the dorsal horn but was detected on the majority of DRG neurons (Nicholas et al., 1993; Cho et al., 1997; Gold et al., 1997; Shi et al., 2000). In one of these studies $\alpha_{2A}$-AR mRNA was also detected on a small proportion of DRG neurons (Nicholas et al., 1993; Cho et al., 1997; Shi et al., 2000).

There is clearly disagreement about the distribution of the $\alpha_{2C}$-AR in the superficial dorsal horn and its function is obscure. In the present investigation, the cellular and sub-cellular organisation of this receptor was characterised in order to gain further insight into its role in the superficial dorsal horn. Quantitative immunocytochemical approaches in conjunction with confocal microscopy were used to determine patterns of distribution of $\alpha_{2C}$-ARs in relation to neurochemical markers that label axons of primary afferents, descending systems and populations of inhibitory or excitatory interneurons. The sub-cellular distribution of $\alpha_{2C}$-AR imunoreactivity was also examined with electron microscopy to investigate the synaptic organisation of axon terminals that possess the receptor.

## 2. Experimental Procedures

### *Confocal Microscopy*

### *Fixation of tissue*

Three male Wistar rats (250g; Harlan, UK) were deeply anaesthetised with sodium pentobarbitone (1ml intraperitoneally, i.p.) and perfused through the left ventricle with saline followed by a fixative containing 4% formaldehyde in phosphate buffer pH 7.6. All animal procedures were conducted according to British Home Office regulations and steps were taken to avoid suffering and minimise the numbers of animals used in the experiments. The L4 lumbar segment was removed from each animal and postfixed in the same fixative solution for 8 hours.

### *Double-labelling immunocytochemistry*

Transverse sections (50μm thick) were cut with a Vibratome. Sections were treated with 50% ethanol (30 minutes) to enhance antibody penetration, which was followed by blocking in 10% normal donkey serum for 1 hour. Double-labelling immunofluorescence was performed with a guinea pig anti-$\alpha_{2C}$-AR antiserum (diluted 1:500; Neuromics,

Minneapolis, U.S.A.; see Stone et al., 1998 for details) which was incubated for 48 hours with each one of the following antisera: rabbit anti-dopamine β-hydroxylase (DβH; 1:500), rabbit anti-serotonin (5HT; 1:200), rabbit anti-calcitonin gene-related peptide (CGRP; 1:500; all from Affiniti, Exeter, U.K.), rat anti-substance P (SP; 1:200; Sera-Lab, Loroughborough, U.K.), rabbit anti-neurotensin (1:1000), rabbit anti-somatostatin (1:1000), rabbit anti-met-enkephalin (1:500), rabbit anti-neuropeptide Y (1:500), rabbit anti-galanin (1:500; all from Peninsula Labs., St. Helens, U.K.), goat anti-choline acetyltransferase (ChAT; 1:100), rabbit anti-nitric oxide synthase (NOS; 1:500; both from Chemicon, Harrow, U.K.), sheep anti-glutamic acid decarboxylase (GAD; 1:400; Oertel et al., 1981) and rabbit anti-glycine transporter 2 (Gly-T2; 1:2500; Zafra et al., 1995), rabbit anti-vesicular glutamate transporter 1 (VGLUT1; 1:5,000) and rabbit anti-vesicular glutamate transporter 2 (VGLUT2; 1:5,000), which are markers for glutamate (Takamori et al., 2000; Varoqui et al., 2002; both obtained from Synaptic Systems, Göttingen, Germany) and goat anti-cholera toxin B subunit (CTb; 1:5,000; List Laboratories, California, USA), which was used to label myelinated primary afferent terminals (see below). The anti-$\alpha_{2C}$-AR antiserum was also incubated with biotinylated isolectin B4 (IB4; Sigma-Aldrich, Dorset, U.K.) for 72 hours (1:200), which labels unmyelinated non-peptidergic primary afferent fibres predominantly (Silverman and Kruger, 1990). Sections were rinsed and incubated for 3 hours in solutions containing species-specific secondary antibodies (all raised in donkey and diluted 1:100) coupled to fluorophores: FITC, to identify $\alpha_{2C}$-AR immunoreactivity, or rhodamine-red to identify the marker (Jackson ImmunoResearch, Luton, U.K.). The presence of the IB4 lectin was identified by incubating sections in avidin-rhodamine for 3 hours (1:1000). Antibodies were diluted in phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 1% normal donkey serum. Following the incubations, sections were mounted in anti-fade medium (Vectashield, Vecta Laboratories, Peterborough, UK) and stored in a freezer at -20°C.

*Labelling of myelinated primary afferent terminals in the lumbar spinal cord*

Three male Wistar rats (250g; Harlan, UK) were deeply anaesthetised with halothane, and 4μl of 1% CTb (List Laboratories, California, USA) were injected into the left sciatic nerve to label terminations of myelinated primary afferents (LaMotte et al., 1991; Rivero-Melian et al., 1992). Three days later, the animals were deeply anaesthetised with sodium pentobarbitone (1ml of 60mg/ml ip) and perfused transcardially. Following postfixation the tissue was processed for double-labelling as described above.

*Quantitative methods of colocalisation: automated and visual-assessment*

Once the sections were mounted, fields from the central region of the superficial dorsal horn (one from lamina I and one from lamina II; except for: CTb, one from lamina I and one from lamina III; and VGLUT1, both from laminae II/III) were systematically scanned with a BioRad MRC 1024 confocal laser scanning microscope. Each field (70x70μm) consisted of six pairs of optical sections gathered sequentially with a X40 oil-immersion lens at 0.5μm intervals in the Z-axis and a zoom factor of 3. Multiple fields were collected for each of the seventeen combinations. Four fields (two from the right horn and two from the left horn) were collected from six transverse sections for each animal. Thus, a total of 24 fields per marker was collected from each of the three animals (i.e.72 fields in total for each marker). CTb was only scanned from the left dorsal horn -ipsilateral to the sciatic injection-.

In the first place we applied an automated quantitative method by using a Kronton KS400 image analysis system (Kontron Elektronik, GmbH), which measures the extent of overlap of $\alpha_{2C}$-AR immunoreactivity with immunoreactivity for neurochemical markers. The analysis programme converted pairs of optical images, corresponding to the same optical plane, into binary images and calculated the total area occupied with $\alpha_{2C}$-AR immunoreactivity and the percentage of this area that was masked by immunoreactivity for the marker (Maxwell et al., 1996). A total of 432 pairs of images was analysed for each marker (i.e. 6 optical sections x 72 fields). The percentage overlap value estimated for each of the markers is expressed as the mean value (±standard deviation, SD) of this percentage for the three animals.

The automated method provides relative (not absolute) values and is suitable for comparing proportions of overlap of various markers. In addition it was decided to apply a visual-assessment method to produce an accurate estimate of numbers of $\alpha_{2C}$-AR-IR puncta that possess immunoreactivity for markers of excitatory *versus* inhibitory terminals of interneurons (i.e. VGLUT2 and GAD, respectively). For this purpose, 100 $\alpha_{2C}$-AR-IR puncta per animal (i.e. 300 in total) were sampled from the scanned fields with the aid of NeuroLucida for Confocal software (MicroBrightField, Inc., Colchester, VT). For each animal, a random sample of 10 boutons per marker was extracted from the third and fourth optical sections of 10 fields collected. Sampled $\alpha_{2C}$-AR-IR boutons were assessed individually to determine if they contained immunoreactivity for the marker. The numbers

of double-labelled $\alpha_{2C}$-AR puncta were then counted for each animal and the final overall mean percentage value ($\pm$SD) was calculated for the three animals.

## Electron microscopy

### Fixation of tissue

Three male Wistar rats (250g; Harlan, UK) were prepared for immunoelectron microscopy. The animals were deeply anaesthetized with sodium pentobarbitone (1ml i.p.) and perfused with saline followed by a fixative containing 1% glutaraldehyde and 1% formaldehyde in phosphate buffer (pH 7.6). L4 segments were removed and placed in the same fixative for 8 hours.

### Processing for immunoelectron microscopy

L4 segments were cut into 50µm transverse sections with a Vibratome. The sections were treated with 50% ethanol for 30 minutes to improve antibody penetration and then with 1% sodium borohydride for 30 minutes to counteract the effects of glutaraldehyde. After blocking in 10% normal donkey serum for 1 hour, sections were incubated for 48 hours in guinea pig anti-$\alpha_{2C}$-AR antiserum (1:2,500; Neuromics, Minneapolis, U.S.A.), rinsed in PBS and then placed for 2 hours in biotinylated donkey anti-guinea pig Ig (1:500; Jackson ImmunoResearch, Luton, U.K.). Primary and secondary antibodies were diluted in PBS supplemented with 1% normal donkey serum. After a further rinse in PBS, sections were incubated in avidin-biotin-HRP complex (Vector Elite, Peterborough, U.K.) for 1 hour and then reacted with 3, 3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide to reveal peroxidase activity, i.e. HRP. The processed sections were treated for 30 minutes with 1% osmium tetroxide in phosphate buffer, dehydrated in a series of acetone solutions, stained *en bloc* with uranyl acetate and flat-embedded between acetate foils in Durcupan resin (Fluka, Switzerland). Selected sections were mounted onto blocks of cured resin and trimmed to include the superficial dorsal horn (laminae I-III). Ultrathin sections were cut with a diamond knife and collected on Formvar-coated single-slot grids. Sections were examined with a Philips CM100 electron microscope.

### Analysis of $\alpha_{2C}$-AR-IR profiles

A sample of 100 $\alpha_{2C}$-AR-IR profiles was randomly collected from each animal for the quantitative analysis. The analysis involved classification of the immunoreactive terminals according to the synaptic arrangements they formed (axodendritic or axoaxonic), the presence or absence of DCVs and whether they formed symmetric or asymmetric

synaptic junctions. The frequency of these categories was then calculated and an image analysis programme was used to estimate the dimensions of $\alpha_{2C}$-AR-IR profiles (Kronton KS400, Kontron Elektronik, GmbH).

## 3. Results

*Neurochemical profile of axons possessing the $\alpha_{2C}$-adrenergic receptor*

Immunoreactivity for the $\alpha_{2C}$-AR was concentrated on axon terminals in laminae I and II of the dorsal horn and in the lateral spinal nucleus (Fig. 3.1). This pattern of labelling was identical to that reported by Stone and co-workers (1998). We used markers to identify axons originating from descending systems, primary afferents, and excitatory or inhibitory interneurons in order to characterise the types of neuron that possess the $\alpha_{2C}$-AR. Typical examples of images for the $\alpha_{2C}$-AR and these markers are shown in Figure 3.2, 3.3 and 3.4. The results derived from both types of quantitative analysis are summarised in Table 3.1 (below) and in two histograms in Figure 3.5.

We could find no evidence that either noradrenergic or serotoninergic axons possess the receptor (Fig. 3.2, a-b), as there was almost no overlap with immunoreactivity for D$\beta$H or 5-HT (0.48% and 0.54% respectively).

Similarly, unmyelinated primary afferents that bind the lectin IB4 and primary afferents containing CGRP also displayed minimal overlap (0.73% and 0.42% respectively; Fig. 3.2, c-d). This latter finding is consistent with the fact that SP-containing axons also do not possess the receptor (1.00% overlap) since the majority of these axons will also contain co-localised CGRP (Fig. 3.3, a). We could find no evidence to show that CTb labelled myelinated primary afferent terminals in lamina I or in lamina III possess the receptor (0.21%; Fig. 3.2, iii, f) even though numerous CTb labelled axons were present in these regions (Fig. 3.2, i,ii). In addition, no overlap was found between the receptor and VGLUT1 (0.37%, Fig. 3.2, e), a marker for glutamate which is associated mainly with large myelinated primary afferent terminals (Todd et al., 2003).

Markers for galanin; Gly-T2, which labels terminals of glycinergic neurons (Zafra et al., 1995; ChAT, which labels cholinergic cells (Barber et al., 1984; Borges and Iversen, 1986; Sheriff and Henderson, 1994) and NOS also showed minimal overlap (2.09%, 0.92%, 0.77% and 0.86% respectively; Fig. 3.3, b-e). The majority of terminals labelled with these markers are likely to originate from inhibitory local circuit neurons although galanin is also present in primary afferent terminals (Todd and Spike, 1993). However

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there was evidence of colocalisation with neuropeptide Y, a marker for axons of inhibitory cells (Rowan et al., 1993), which displayed moderate overlap with the $\alpha_{2C}$-AR (6.09%; Fig. 3.3, f). The receptor was also present on axons containing neurotensin (10.81%) and somatostatin (17.48%; Fig. 3.3, g-h) which probably originate from excitatory interneurons (see Discussion). Finally axons labelled with enkephalin displayed a great amount of overlap with immunoreactivity for the receptor (27.67%; Fig. 3.3, i).

**Table 3.1.** Automated and visual quantification of colocalisation patterns of the $\alpha_{2C}$-AR in the superficial dorsal horn

| | marker | Overlap | ±SD |
|---|---|---|---|
| | DβH | 0.48 | 0.15 |
| | 5-HT | 0.54 | 0.38 |
| | IB4 | 0.73 | 0.5 |
| AUTOMATED | CGRP | 0.42 | 0.34 |
| | VGLUT1 | 0.37 | 0.22 |
| METHOD | CTb-sciatic | 0.21 | 0.17 |
| | SP | 1.00 | 0.05 |
| | Galanin | 2.09 | 0.61 |
| | Gly-T2 | 0.92 | 0.49 |
| | ChAT | 0.77 | 0.49 |
| | NOS | 0.86 | 0.24 |
| | Neuropeptide Y | 6.09 | 0.91 |
| | Neurotensin | 10.81 | 2.38 |
| | Somatostatin | 17.48 | 1.20 |
| | Enkephalin | 27.67 | 2.77 |

| | marker | Frequency | ±SD |
|---|---|---|---|
| VISUAL-ASSESSMENT | GAD | 11.33 | 1.53 |
| METHOD | VGLUT2 | 83.67 | 2.52 |

*Inhibitory versus excitatory $\alpha_{2C}$-AR-IR terminals*

In order to estimate the proportion of $\alpha_{2C}$-AR axons that are likely to have an excitatory or inhibitory action we applied a visual-assessment quantitative method in addition to the automated method. For this purpose we used markers that label terminals of spinal interneurons containing excitatory (i.e. VGLUT2) or inhibitory (i.e. GAD) amino acid transmitters. Numerous $\alpha_{2C}$-AR-IR puncta contained immunoreactivity for VGLUT2 (Fig. 3.4, a-a'') and a small number of $\alpha_{2C}$-AR-IR terminals were associated with GAD

immunoreactivity (Fig 3.4, b-b''). The percentage of overlap of VGLUT2 and GAD with the $\alpha_{2C}$-AR is smaller than the percentage of double labelled $\alpha_{2C}$-AR-IR terminals. This can be explained by the characteristics of the labelling produced by the markers. The intensity of labelling of these markers, especially for VGLUT2, varied considerably in terminals from very intense to weak and all intensities of immunoreactivity were considered positive for the purposes of the quantitative analysis (see arrows in Fig. 3.4). Quantitative analysis confirmed that 83.67±2.52% of $\alpha_{2C}$-AR axons contain VGLUT2 but that only 11.33±1.53% contain GAD (Fig. 3.5, histogram 2).

*Ultrastructure of terminals containing the $\alpha_{2C}$-AR*

A total of 300 $\alpha_{2C}$-AR-IR profiles was collected randomly (100 profiles from each of the three experiments) and classified according to the synaptic arrangements they formed, the presence or absence of DCVs and whether they formed symmetric or asymmetric synaptic junctions. Figure 3.6 illustrates examples of each type of profile. All $\alpha_{2C}$-AR-IR profiles observed were presynaptic to other structures; the vast majority formed axodendritic synaptic arrangements (95.33%; Fig. 3.6, A-D), and a small proportion of $\alpha_{2C}$-AR-IR terminals formed axoaxonic synapses (4.67%; Fig. 3.6, E-F). Approximately one third of terminals in axodendritic arrangements and one half of terminals in axoaxonic arrangements (Table 3.2, below) contained DCVs. The majority of immunoreactive terminals (76.33%) formed asymmetric synapses whereas the remainder formed symmetric synapses (23.67%). However, all immunoreactive terminals at axoaxonic complexes established symmetric synapses (4.67%), whereas terminals forming axodendritic synapses were found to form asymmetric (76.33%) or symmetric (19%) synapses (Table 3.2, below). The area and diameter of its equivalent circle were measured for each of the profiles collected. The average area of profiles is $0.62\mu m^2$ and the average diameter of the equivalent circle is $0.87\mu m$ (SD=±$0.19\mu m^2$; range: 1.38-0.41$\mu m$). On several occasions $\alpha_{2C}$-AR-IR terminals were seen to cluster around large dendritic profiles especially in lamina I. These terminals often contained DCVs and formed synapses with the dendritic profile (Fig. 3.7).

**Table 3.2.** Table summarising the quantification of electron microscopic observations of the $\alpha_{2C}$-AR in the superficial dorsal horn

| | AXODENDRITIC | | AXOAXONIC | | |
|---|---|---|---|---|---|
| average % | 95.33 | | 4.67 | | |
| | DCV | no DCV | DCV | no DCV | |
| average % | 39.67 | 55.67 | 2 | 2.67 | |
| ASYMMETRIC | 28.33 | 48.00 | 0 | 0 | 76.33 |
| SYMMETRIC | 11.33 | 7.67 | 2 | 2.67 | 23.67 |

Data pooled from three experiments, which synthesise the ultrastructural properties of $\alpha_{2C}$-AR-IR terminals. Boutons were classified according to whether they formed axodendritic or axoaxonic synapses, symmetric or asymmetric junctions and possessed DCVs.

## 4. Discussion

*Methodological considerations*

The specificity and staining properties of the $\alpha_{2C}$-AR antibody used in this study have been discussed by Stone and coworkers (1998). Anti-peptide antisera were generated against the C-terminal portion of the rat $\alpha_{2C}$-AR (Lanier et al., 1991). The sequence of the epitope was: HILFRRRRRGFRQ. A series of controls of specificity were carried out by Stone and coworkers. MDCK cells, transfected with the $\alpha_{2C}$-AR or untransfected, were stained with the guinea pig-derived antisera generated against the $\alpha_{2C}$-AR and $\alpha_{2C}$-AR-IR was observed only on cells transfected with the $\alpha_{2C}$-AR. The $\alpha_{2C}$-AR-IR was only blocked by preabsorption of the antiserum with the peptide corresponding to the $\alpha_{2C}$-AR. These results demonstrated that the antibody used recognizes the receptor against which it was generated. In addition, the distribution pattern of $\alpha_{2C}$-AR immunoreactivity obtained in the present investigation is identical to the pattern described by Stone and co-workers (1998); $\alpha_{2C}$-AR-IR is concentrated in the superficial laminae of the dorsal horn (laminae I-II) on axon terminals and is also found in the adjacent lateral spinal nucleus.

The automated computer-based method used to quantify patterns of overlap of $\alpha_{2C}$-AR with other markers has been discussed in Maxwell et al., 1996. This method avoids the subjectivity of a human-assessed analysis. Any errors that occur are likely to be consistent and therefore values of overlap for different markers can be compared. In addition, Maxwell et al., (1996) compared the accuracy of a visual assessment method and the automated image analysis method used in the present study and concluded that there was good agreement between the two approaches. The values provided by the automated method are not absolute; indeed the validity of the results resides on the fact that they are relative values. Finally, application of an automated system enables the quantification of very large samples of images, which would be very laborious and time-consuming if a human-based visual assessment technique was used.

The visual assessment method lacks the advantages provided by the automated method but its use is required in order to estimate numbers of terminals positive or negative for a marker; i.e. a proportion of terminals (frequency), rather than a proportion of overlap (area). In addition, in the case of GAD and VGLUT2 these two parameters (i.e. frequency and area) are especially divergent. This can be explained largely by the variability observed in the intensity of labelling of these markers which ranges from very weak to very strong. The ultimate reason for the use of a visual-assessment method is that the presence of a weakly labelled terminal versus a non-labelled terminal is a major difference of physiological significance; such differences can only be detected currently by means of a visual assessment.

## Interpretation of results

### $\alpha_{2C}$-ARs are of spinal origin

Quantitative analysis of overlap patterns for $\alpha_{2C}$-ARs immunoreactivity with the markers investigated in this study confirms that the receptor is not present on descending terminals of the noradrenergic or serotoninergic systems and also is not found on myelinated, unmyelinated or peptidergic primary afferents labelled with CTb, IB4 and CGRP respectively. In addition, the receptor was not found on terminals labelled with VGLUT1, a marker that is associated mainly with large myelinated primary afferents (Todd et al., 2003). Our results are in agreement with previous reports that showed no significant reduction of $\alpha_{2C}$-AR immunoreactivity following rhizotomy or neonatal capsaicin treatment (Stone et al., 1998). Therefore, it is presumed that the $\alpha_{2C}$-AR-IR axons in the superficial dorsal horn are not terminals of primary afferents. Nevertheless, *in*

*situ* hybridisation studies indicate that both large and small DRG neurons contain $\alpha_{2C}$-AR mRNA (Nicholas et al., 1993; Cho et al., 1997; Gold et al., 1997; Shi et al., 2000) and therefore this receptor would be expected to be present on central terminals of primary afferent fibres. Taken together with the previous findings, our evidence suggests that few, if any, $\alpha_{2C}$-AR-IR axons in the superficial dorsal horn, originate from primary afferents. It is difficult to reconcile this conclusion with the findings of the *in situ* hybridisation studies but it is possible that $\alpha_{2C}$-ARs manufactured in DRG cells are not trafficked to central terminations and, in this respect, may be similar to the neuropeptide Y Y1 receptor (Zhang et al., 1994). Alternatively some $\alpha_{2C}$-ARs may be present on a subgroup of primary afferents that are not labelled by CTb, IB4 or CGRP.

Our results therefore support the suggestion made by Stone et al., (1998) that $\alpha_{2C}$-AR-IR axons originate mainly from spinal interneurons. However, Stone et al., (1998) also reported that $\alpha_{2C}$-AR immunoreactivity was present on some CGRP-IR fibres. We found no evidence for this. Furthermore such an observation would be unexpected since the majority of CGRP-containing primary afferents also contain SP (Todd and Spike 1993), and no evidence was found for the presence of $\alpha_{2C}$-ARs on SP-IR axons in our study or in the study of Stone et al., (1998). The present work extends our knowledge of the neurochemical characteristics of spinal neurons expressing $\alpha_{2C}$-ARs. The results show that $\alpha_{2C}$-ARs are predominantly found on axon terminals of excitatory interneuronal populations but are also present on terminals of inhibitory interneurons and that subpopulations of $\alpha_{2C}$-AR-possessing interneurons can be differentiated on the basis of their peptidergic content.

*Interneurons that possess $\alpha_{2C}$-AR-IR terminals are predominantly excitatory*

The principal finding derived from the quantification based on a visual-assessment method is that in the superficial dorsal horn over 80% of $\alpha_{2C}$-AR-IR terminals also contain VGLUT2. This evidence suggests that $\alpha_{2C}$-ARs are associated with axons of glutamatergic interneurons that predominantly have an excitatory action (Todd et al, 2003). We also confirmed that only a small proportion (approximately 11%) of these axons contain GAD. Most GABAergic neurons in the superficial dorsal horn contain GABA or a mixture of GABA and glycine (Todd and Spike, 1993) and the GAD marker would be predicted to label the entire population of inhibitory axons that release classical inhibitory amino acid transmitters. Therefore it is likely that only a small proportion of $\alpha_{2C}$-AR-IR axons have an

inhibitory action. The conclusion that most $\alpha_{2C}$-AR-IR terminals are excitatory is also supported by the electron microscopic observations, which show that more than 75% of $\alpha_{2C}$-AR-IR terminals form asymmetric axodendritic synapses. Terminals forming this type of synapse are predicted to have an excitatory action (Uchizono, 1965). In addition, the automated quantitative analysis indicated that many $\alpha_{2C}$-AR-IR terminals also contain enkephalin, somatostatin or neurotensin and all of these types of peptidergic terminal are associated with high proportions of VGLUT2 immunoreactivity (Todd et al., 2003). These findings strongly support that the majority of $\alpha_{2C}$-AR-IR terminals contain glutamate and have an excitatory action.

*Subpopulations of interneurons with $\alpha_{2C}$-AR-IR terminals*

In the superficial dorsal horn, GABA and glycine are colocalised in the majority of inhibitory cells although a subpopulation of cells containing GABA only is also present (Todd and Spike, 1993). Immunoreactivity for the $\alpha_{2C}$-AR was not present on axons which express Gly-T2, (a marker for glycinergic axons) and it can be concluded that the population of inhibitory neurons possessing the $\alpha_{2C}$-AR belong to the group of GABAergic cells, which do not contain co-localised glycine. In addition, $\alpha_{2C}$-AR-IR axons were found to possess neuropeptide Y, which has been reported to coexist only in GABAergic interneurons that do not contain glycine (Rowan et al., 1993). Therefore it is likely that a proportion of $\alpha_{2C}$-AR-IR axons, derived from inhibitory interneurons, originates from the subpopulation of GABAergic cells, which also contains neuropeptide Y.

The presence of the $\alpha_{2C}$-AR on axons, which are also immunoreactive for neurotensin and somatostatin, suggests that there are at least two different subpopulations of excitatory interneurons that express the receptor. Neurotensin and somatostatin are present in separate neuronal populations in the dorsal horn (Proudlock et al., 1992). Neurotensin has been shown to be present in axon terminals that do not show GABA immunoreactivity and are enriched with glutamate (Todd et al., 1992; Todd et al., 1994b), and cells which contain somatostatin are not immunoreactive for GABA and are presumed to be excitatory (Proudlock et al., 1992). Somatostatin is also found in a population of CGRP-containing primary afferent fibres (Leah et al., 1985), but it is likely that the somatostatin-containing fibres which possess the $\alpha_{2C}$-AR are derived entirely from spinal interneurons, since CGRP-IR axons do not possess the receptor and this peptide is present only in peptidergic primary afferents (Levine et al., 1993). The highest value of overlap

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with a peptidergic marker was obtained for enkephalin (almost 30% of $\alpha_{2C}$-AR-IR), which is found in a mixed population of excitatory and inhibitory interneurons (Todd and Spike, 1993). However, in view of the relatively low numbers of inhibitory axons that possess the receptor and that the majority of enkephalinergic terminals are glutamatergic (Todd et al., 2003) it is likely that the majority of $\alpha_{2C}$-AR and enkephalin-containing axons originate from excitatory cells. Enkephalin and somatostatin have been reported to colocalise in the dorsal horn (Todd and Spike, 1993) therefore a proportion of $\alpha_{2C}$-AR-IR axons may contain both enkephalin and somatostatin. Stone et al. (1998) also observed colocalisation of the $\alpha_{2C}$-AR with enkephalin and somatostatin but did not quantify the degree of colocalisation.

*Majority of $\alpha_{2C}$-AR-IR terminals are presynaptic to dendrites*

The electron microscope analysis confirmed that $\alpha_{2C}$-AR-IR profiles were axon terminals and showed that the vast majority of them formed axodendritic synapses, although a small minority formed axoaxonic synapses. Therefore most axon terminals possessing the $\alpha_{2C}$-AR are likely to have direct postsynaptic actions on their target cells, while a minority of them may have a presynaptic action. Axoaxonic synapses are found exclusively on terminals of primary afferent fibres (see Alvarez, 1998) and therefore such presynaptic $\alpha_{2C}$-AR-IR axons would be predicted to influence primary afferent transmission. About 76% of the synapses formed by $\alpha_{2C}$-AR-IR terminals made asymmetrical junctions; this type of synapse is generally associated with excitatory transmitters (Uchizono, 1965) and therefore supports our hypothesis that it is predominantly excitatory neurons that possess the $\alpha_{2C}$-AR. A smaller number of $\alpha_{2C}$-AR-IR terminals (23%) formed symmetric junctions, which are thought to be indicative of an inhibitory action (Gray, 1962; Uchizono, 1965). The presence of DCVs within numerous $\alpha_{2C}$-AR-IR terminals is also consistent with the confocal microscope findings, which show that there is extensive colocalisation of the $\alpha_{2C}$-AR with neuropeptides, as neuropeptides are thought to be stored within DCVs (Merighi et al., 1989). However about two thirds of terminals which form asymmetric junctions did not contain DCVs. This indicates that many $\alpha_{2C}$-AR-possessing terminals contain an amino acid transmitter that is not colocalised with a peptide. In the case of terminals forming asymmetric junctions, this transmitter would be expected to be glutamate. Indeed, this is largely in keeping with the

confocal microscopic observations which showed that the vast majority of $\alpha_{2C}$-AR-IR terminals were glutamatergic.

Finally, concentrations of $\alpha_{2C}$-AR-IR terminals (mostly containing DCVs) were found on many occasions to surround large dendritic profiles in lamina I and form synapses with them. This evidence indicates that axons possessing the receptor target specific cells in lamina I. It is likely that $\alpha_{2C}$-AR axons will have a powerful postsynaptic influence upon these cells and that NE will modulate this influence.

*Functional implications*

In this study we have shown that $\alpha_{2C}$-ARs are present on axon terminals of spinal interneurons and that the majority of these form axodendritic synapses, although a small number of axoaxonic synapses were also found. Therefore, NE acting on $\alpha_{2C}$-ARs is likely to modulate transmission in polysynaptic pathways to projection cells or spinal interneurons, as well as influence activity at axoaxonic synapses on primary afferent terminals. The action of NE at such synapses is almost certain to operate through volume transmission, firstly because noradrenergic terminals do not form axoaxonic synapses within the superficial dorsal horn (Doyle and Maxwell, 1991a, 1991b) and secondly because we could find no evidence to support the idea that $\alpha_{2C}$-AR-IR profiles are postsynaptic to other axons. Indeed it is generally accepted that axoaxonic arrangements are found only on primary afferent terminals (e.g. see Alvarez, 1998). NE, acting on $\alpha_{2C}$-ARs, could reduce the efficacy of synaptic transmission at these sites by inducing neuronal membrane hyperpolarisation (Hoehn et al., 1988; Surprenant et al., 1990; Shen et al., 1992). Consequently, it can be hypothesised that if the $\alpha_{2C}$-AR-containing terminal is excitatory the excitability of the postsynaptic element would be inhibited, whereas if it is inhibitory the postsynaptic element would be disinhibited. Since most $\alpha_{2C}$-AR immunoreactivity is associated with terminals of excitatory interneurons which form axodendritic synapses, the predominant effect of NE acting on presynaptic $\alpha_{2C}$-ARs would be predicted to be depressive. Neuropharmacological studies show that the principal effect of NE, and $\alpha_2$-AR agonists, on dorsal horn neurons is inhibitory (North and Yoshimura, 1984; Fleetwood-Walker et al., 1985; Davies and Quinlan, 1985) but, as was discussed in the *Introduction*, it is not possible to differentiate between $\alpha_{2A}$-AR and $\alpha_{2C}$-AR effects with current pharmacological agents. One intriguing possibility is that some of the large dendrites that were observed in lamina I, and received multiple synapses from $\alpha_{2C}$-AR-IR

profiles, belong to projection cells. If this is the case then NE could have a major influence on the activity of cells in this region, which are likely to have an involvement in nociceptive transmission (Naim et al., 1997; Todd et al., 2002). This is the topic of the next chapter.
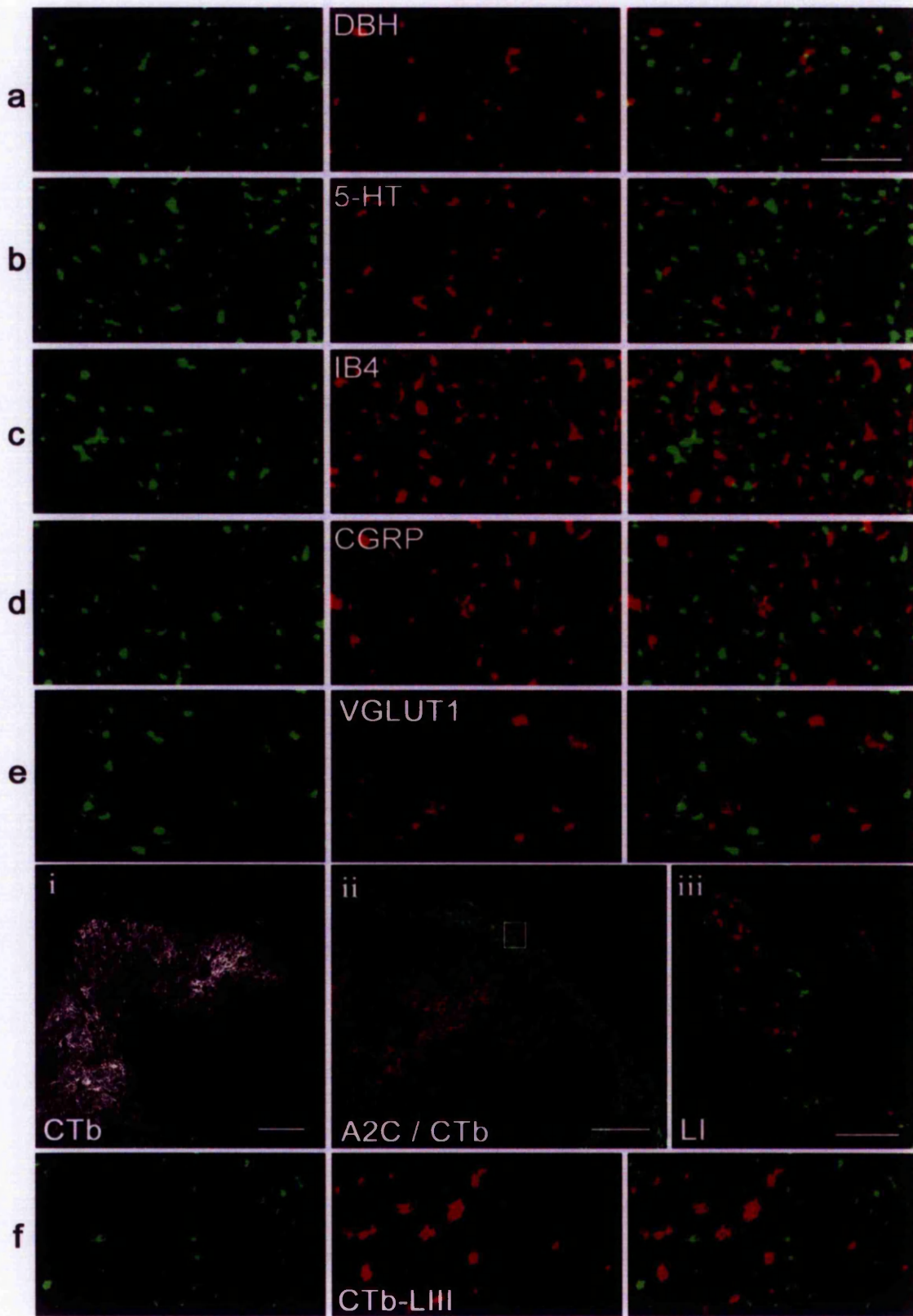
**Figure 3.1. Immunoreactivity for the $\alpha_{2C}$-AR in the dorsal horn.** A low magnification confocal microscope image of a transverse section of the dorsal horn at the L4 segment. Immunoreactivity for the $\alpha_{2C}$-AR (A2C) is concentrated in the superficial dorsal horn and lateral spinal nucleus and is restricted to axon terminals. (Scale bar=100μm)
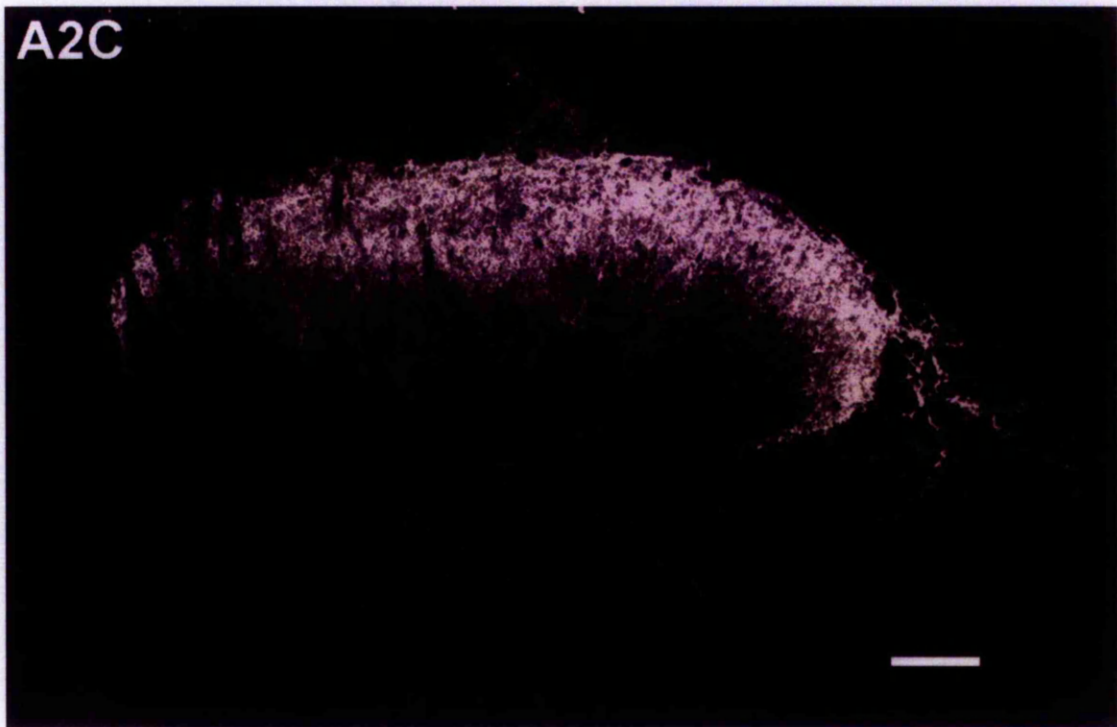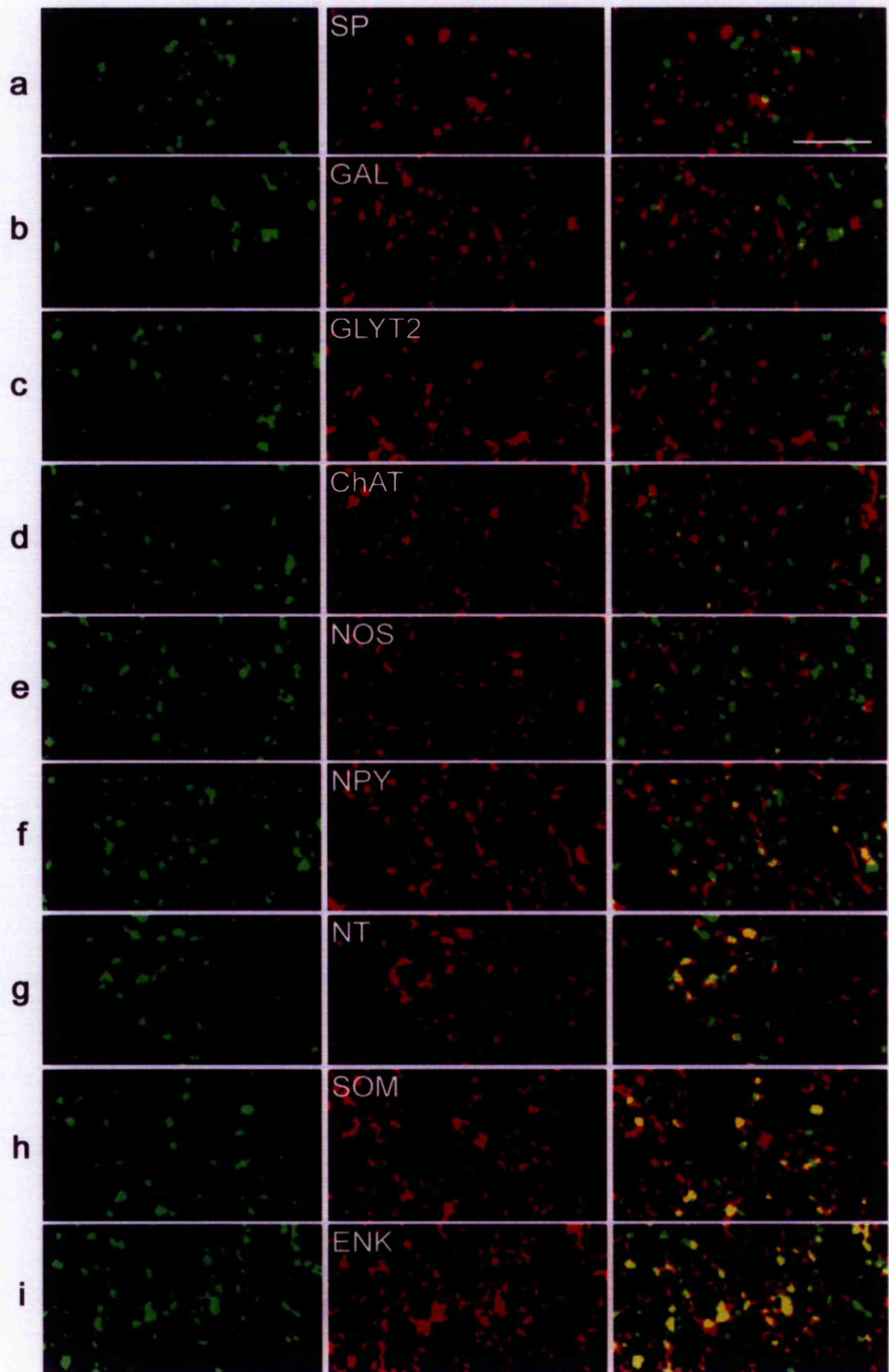
**Figure 3.2. Double-labelling of the $\alpha_{2C}$-AR with D$\beta$H, 5HT, IB4, CGRP, VGLUT1 and CTb.** Six sets of single optical sections are shown (**a-f**). In each set, the first image shows immunoreactivity for the $\alpha_{2C}$-AR (green), the second image shows labelling of a neurochemical marker (red), and the third one is the result of merging both images where overlap could be detected by the presence of yellow colour. Note that there is no overlap between the $\alpha_{2C}$-AR and any of the markers. **f** and **iii** show the absence of overlap with CTb, in lamina III and lamina I respectively. **(i)** A low-power image showing the pattern of labelling in the dorsal horn with CTb following injection into the sciatic nerve. **(ii)** A magnified view of the dorsolateral area of the dorsal horn illustrated in **(i)** showing the relationship between immunoreactivities for the $\alpha_{2C}$-AR (A2C, green) and CTb (red). **(iii)** Details of the boxed area in **(ii)** showing CTb labelling in lamina I (red) and immunoreactivity for the $\alpha_{2C}$-AR (green). Note that there is no overlap of CTb and $\alpha_{2C}$-AR puncta in either lamina I **(iii)** or lamina III **(f)**. (Scale bars: **a**=10μm; **i**=100μm; **ii**=50μm; **iii**=10μm; scale bar in **a** valid for **b-f**)

*(DBH, dopamine β-hydroxylase; 5-HT, serotonin; IB4, isolectin B4; CGRP, calcitonin gene-related peptide; VGLUT1, vesicular glutamate transporter 1; CTb, B subunit of cholera toxin)*

a DBH

b 5-HT

c IB4

d CGRP

e VGLUT1

i CTb ii A2C / CTb iii LI

f CTb-LIII

**Figure 3.3. Double-labelling of the $\alpha_{2C}$-AR with SP, galanin, Gly-T2, ChAT, NOS, neuropeptide Y, neurotensin, somatostatin and enkephalin.** Nine sets of single optical sections are shown (**a-i**). In each set, the first image shows immunoreactivity for the $\alpha_{2C}$-AR (green), the second image shows labelling of a neurochemical marker (red), and the third one is the result of merging both images. Overlap can be detected by the presence of yellow colour in **f-i**. (Scale bar: **a**=10μm; scale bar in **a** valid for **b-i**)

*(SP, substance P; GAL, galanin; GLYT2, glycine transporter T2; ChAT, choline acetyltransferase; NOS, nitric oxide synthase; NPY, neuropeptide Y; NT, neurotensin; SOM, somatostatin; ENK, enkephalin.)*

SP

GAL

GLYT2

ChAT

NOS

NPY

NT

SOM

ENK

**Figure 3.4. Double-labelling of the $\alpha_{2C}$-AR with VGLUT2 and GAD.** Two sets of single optical sections are shown. In each set, the first image shows immunoreactivity for the $\alpha_{2C}$-AR (**a** and **b**), the second image shows labelling for the vesicular glutamate transporter 2 (VGLUT2) and glutamic acid decarboxylase (GAD) (**a'** and **b'**, respectively) and the third image is the result of merging both images (**a''** and **b''**). The presence of yellow profiles in **a''** and **b''** indicates overlap of immunoreactivity. Arrows indicate selected $\alpha_{2C}$-AR-IR puncta that are double-labelled with intense VGLUT2 and arrowheads show terminals that are associated with weak VGLUT2 in series **a**. Arrows indicate selected structures that are double labelled with GAD in series **b**. (Scale bar: **a**= 10μm, valid for all images)
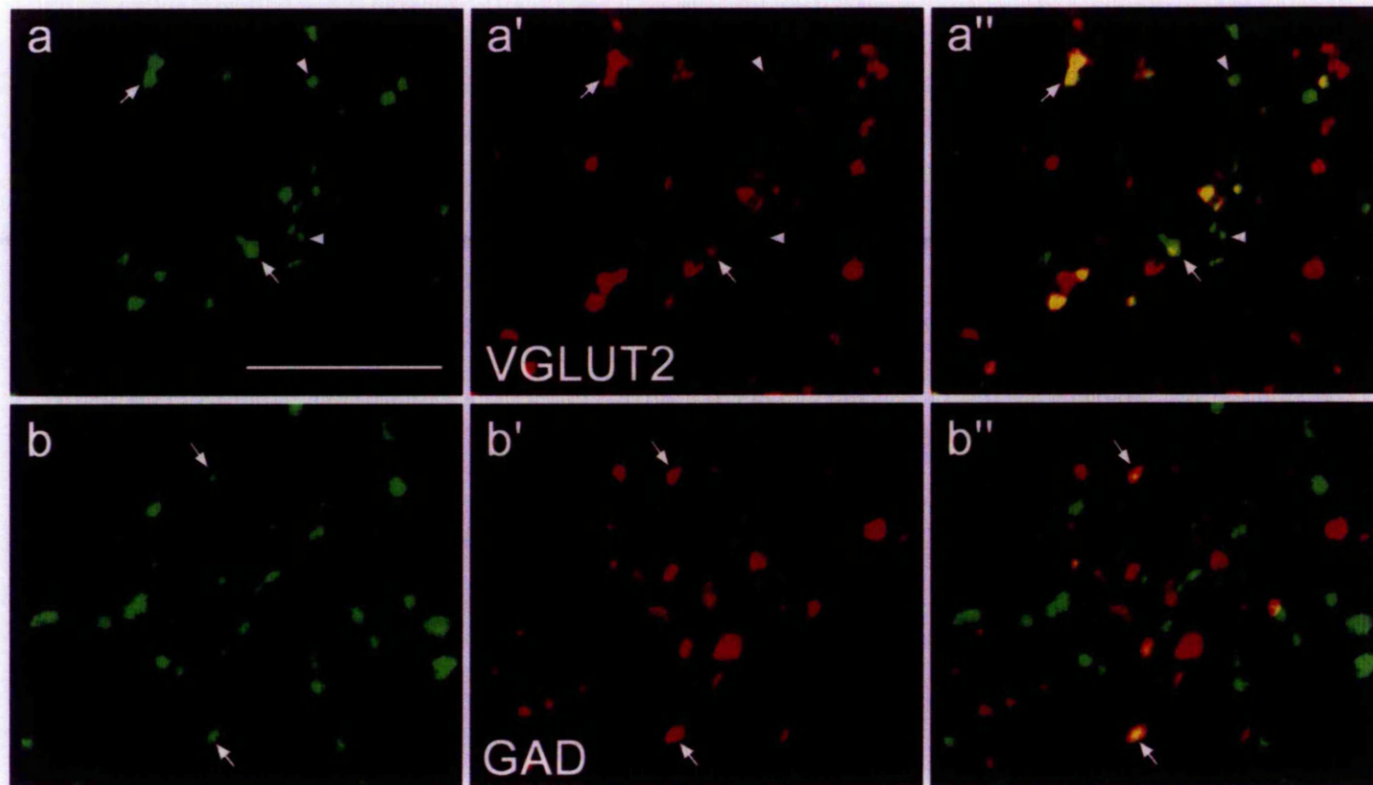
**Figure 3.5. Histograms summarising the quantification of colocalisation patterns of the $\alpha_{2C}$-AR in the superficial dorsal horn.** Histogram 1 shows the average percentages of overlap of the $\alpha_{2C}$-AR with fifteen different markers. Histogram 2 shows the average percentage values of numbers of $\alpha_{2C}$-AR immunoreactive puncta that were double-labelled with the markers VGLUT2 or GAD. A substantial number of $\alpha_{2C}$-AR puncta were immunoreactive for VGLUT2 and a small number were immunoreactive for GAD. (n=3; Error bars=standard deviation)

*(DBH, dopamine β-hydroxylase; 5-HT, serotonin; IB4, isolectin B4; CGRP, calcitonin gene-related peptide; VGLUT1, vesicular glutamate transporter 1; CTb, B subunit of cholera toxin; SP, substance P; GAL, galanin; GLYT2, glycine transporter-T2; ChAT, choline acetyltransferase; NOS, nitric oxide synthase; NPY, neuropeptide Y; NT, neurotensin; SOM, somatostatin; ENK, enkephalin; GAD, glutamic acid decarboxylase; VGLUT2, vesicular glutamate transporter 2)*

Histogram 1.  Overlap of $\alpha_{2C}$-AR immunoreactivity with diverse markers in the dorsal
horn
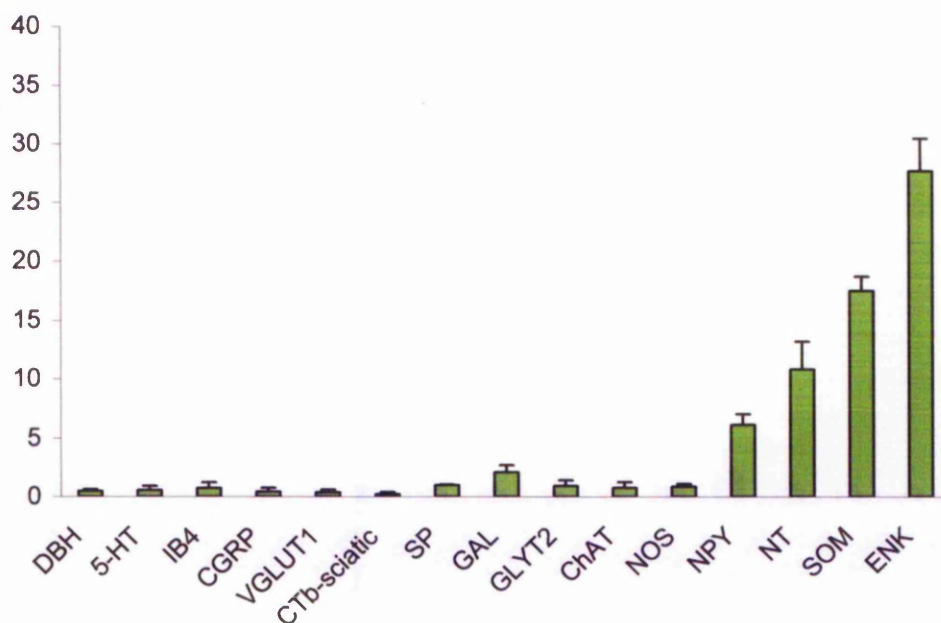


Histogram 2.  Frequency of excitatory and inhibitory $\alpha_{2C}$-AR-IR terminals in the dorsal
horn.
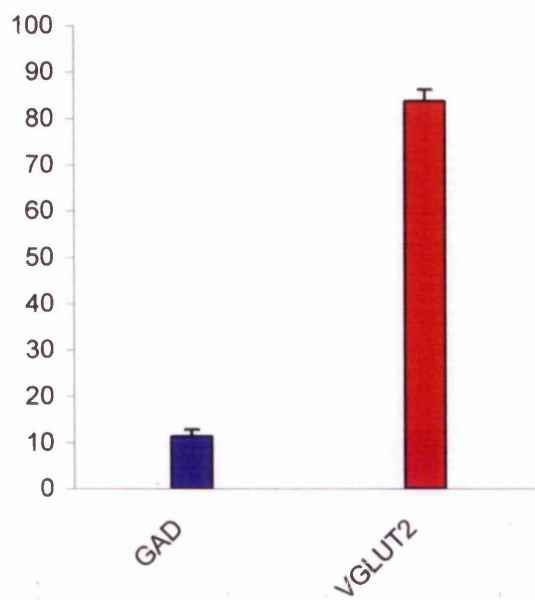
**Figure 3.6. Electron micrographs of $\alpha_{2C}$-AR-IR profiles in the superficial dorsal horn.**
Examples of $\alpha_{2C}$-AR-IR profiles forming axodendritic **(A-D)** and axoaxonic **(E-F)** synapses. The synapse in **F** is associated with a central bouton of a glomerulus. In **A** and **C** immunoreactive terminals form asymmetric synapses, while **B, D, E, F** they make symmetric synapses. Dense-core vesicles (DCVs) are indicated by white arrowheads. (Den=dendrite; Ax=axon; scale bars=0.5μm)
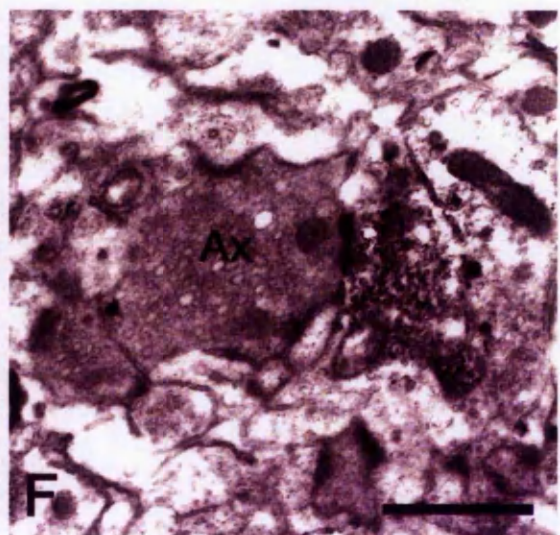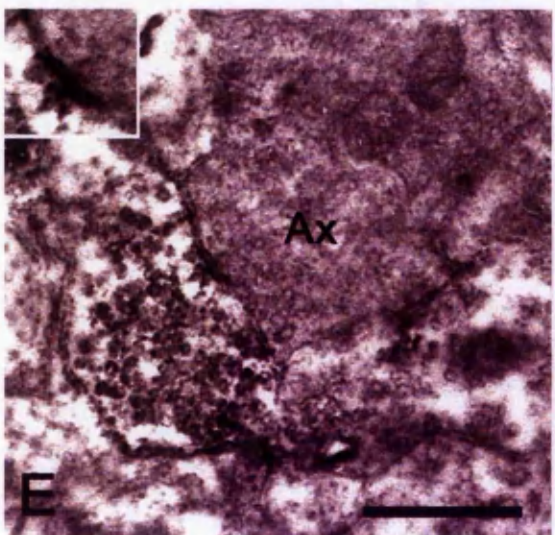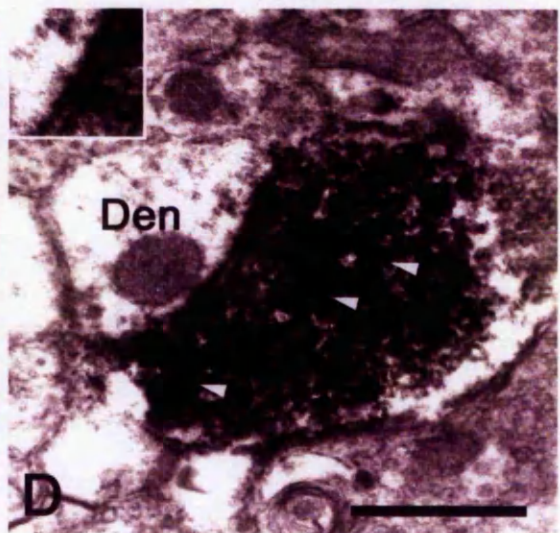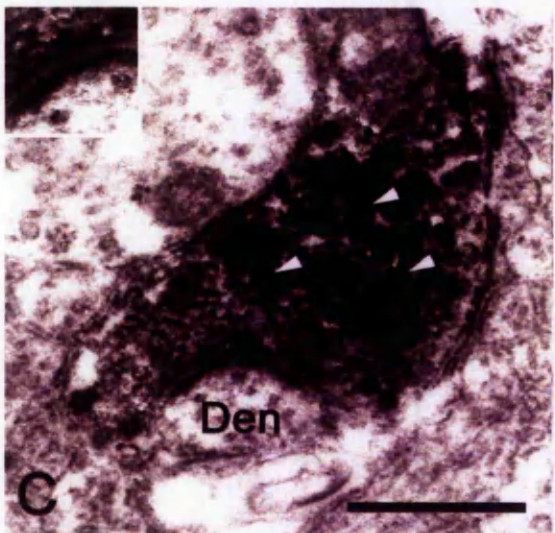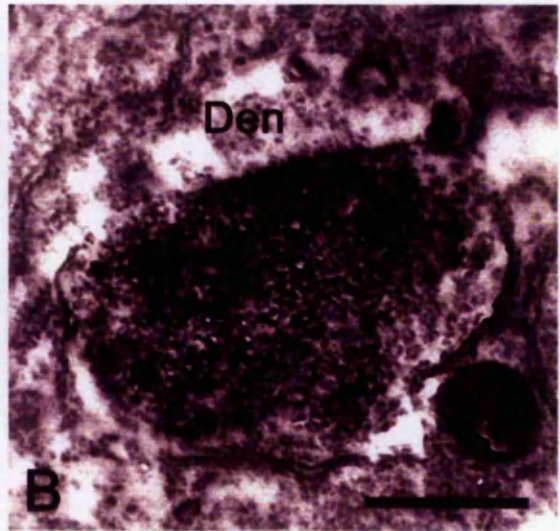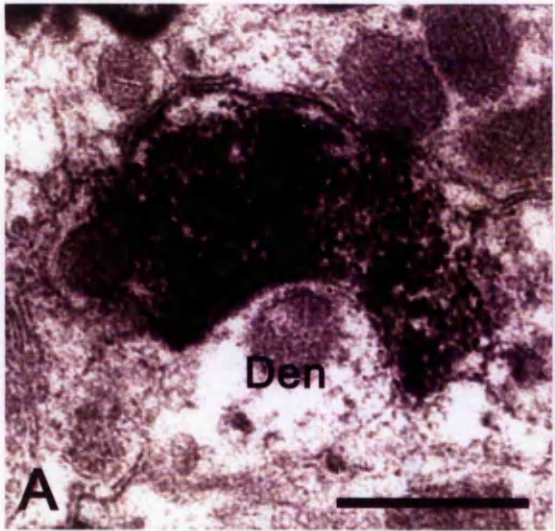
**Figure 3.7. Association of $\alpha_{2C}$-AR-IR terminals with large profiles in lamina I.** A and B, Electron micrographs showing concentrations of $\alpha_{2C}$-AR-IR terminals around large dendritic profiles in lamina I. Insets 1, 2 and 3 show magnified images of the corresponding areas indicated by the numbered arrows in A and B, and confirm that synaptic junctions are formed by the imunoreactive terminals with the dendrites. (Scale bars=1μm)
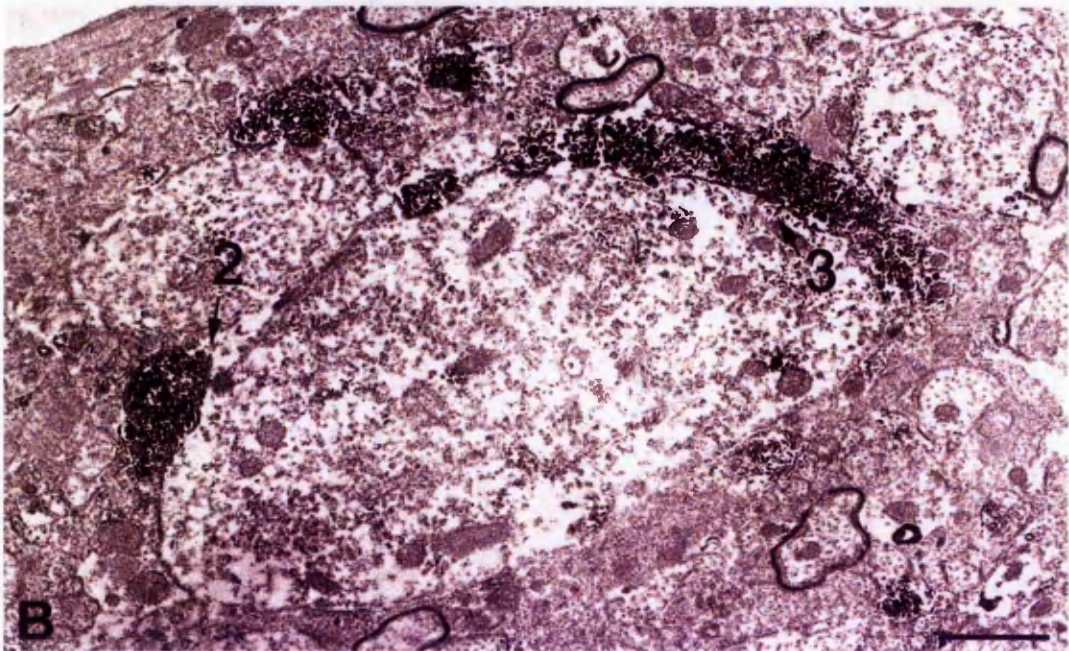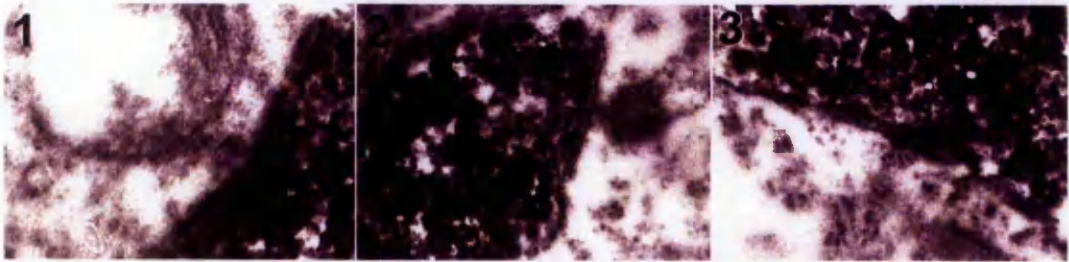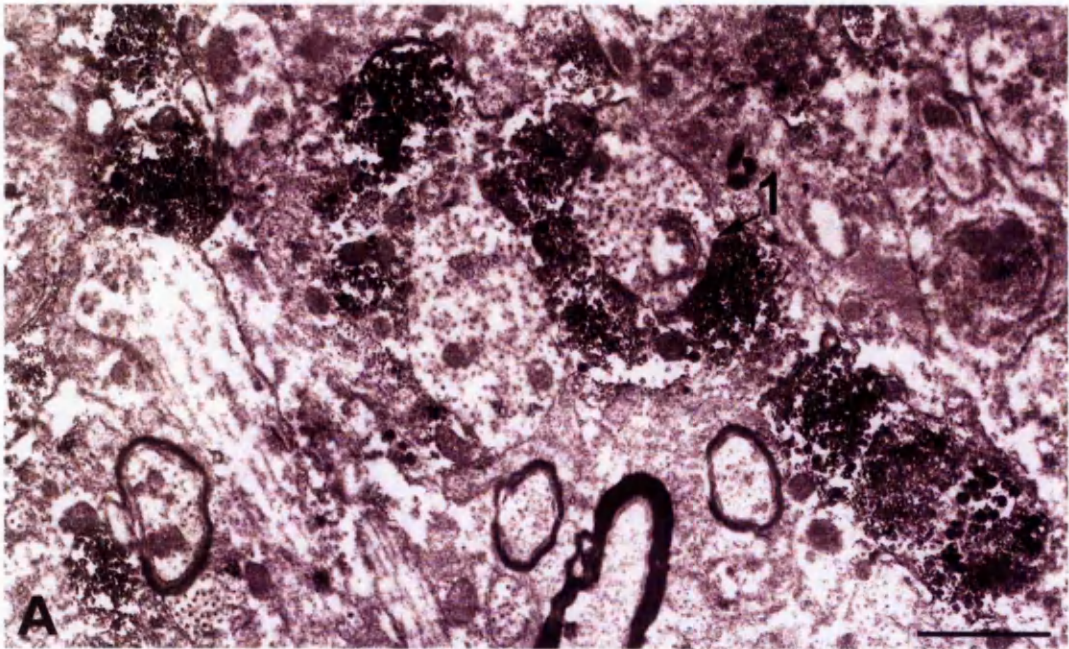
*Chapter 4*

<u>Investigation 2</u>:

**Relationship of axon terminals possessing $\alpha_{2C}$-adrenergic receptors with neurokinin-1 projection neurons in the rat dorsal horn**

# 1. Introduction

Both $\alpha_{2A}$ and $\alpha_{2C}$ subclasses of adrenergic receptor are involved in NE-induced antinociception (Stone et al., 1997; Li and Eisenach, 2001; Fairbanks et al., 2002) and immunoreactivity for both receptor subclasses is concentrated in the superficial dorsal horn. Experiments using antibodies specific for the $\alpha_{2A}$ and $\alpha_{2C}$ subclasses show that they are associated with different axonal populations (Stone et al., 1998). The $\alpha_{2A}$-AR is found in axons that contain SP and CGRP (Stone et al., 1998) which are likely to be terminals of nociceptive primary afferents (Levine et al., 1993) whereas the $\alpha_{2C}$-AR is present in axon terminals of spinal origin (Stone et al., 1998; Olave and Maxwell, 2003). Most (84%) $\alpha_{2C}$-AR terminals are immunoreactive for VGLUT2 (Olave and Maxwell, 2003) and therefore are likely to have an excitatory action; however a small proportion (11%) of terminals contain GAD and therefore are considered inhibitory (Olave and Maxwell, 2003). Ultrastructural observations of $\alpha_{2C}$-AR-IR axon terminals show that they form multiple synapses with large dendritic profiles in lamina I and indicate that they may target certain cells in this region (Olave and Maxwell, 2002). The identity of these target cells is not known but if they prove to be projection neurons that are activated by nociceptive stimuli, then NE could specifically inhibit excitatory polysynaptic input to such cells. This type of arrangement could be particularly important, because the $\alpha_{2C}$-AR is a potentially interesting target for selective analgesics since sedative effects mediated via $\alpha_{2A}$-ARs could be avoided (Guo et al., 1999; Fairbanks et al., 2002).

The aim of the present investigation was to study if axons of excitatory interneurons possessing $\alpha_{2C}$-ARs target nociceptive projection neurons. In the first place retrograde labelling of spinomedullary projection neurons with CTb was combined with triple-immunofluorescence to examine the relationship of projection neurons that possess the SP (NK-1) receptor and axons that possess $\alpha_{2C}$-ARs. Neurons of this type are likely to be involved in the transmission of nociceptive information (Naim et al., 1997; Todd et al., 2002). Furthermore, mice lacking NE display a SP-dependent chronic thermal hyperalgesia (Jasmin et al., 2002), which suggests that NK-1 neurons are components of the circuitry involved in NE antinociception. A combined confocal and electron microscopic method was used to determine if $\alpha_{2C}$-AR terminals make synapses with this type of neuron; and additionally a sequential immunocytochemical method with a VGLUT2 antibody was applied to determine if the $\alpha_{2C}$-AR terminals that contact NK-1 projection cells are likely to have an excitatory action on these cells. Finally, the involvement of NK-1 projection

neurons in nociceptive transmission was studied. For this purpose, the expression of the immediate early gene c-Fos was analysed within the nucleus of the neurons following the peripheral application of a thermal nociceptive stimulus (Hunt el al., 1987). NK-1 projection neurons that expressed c-Fos were assessed for $\alpha_{2C}$-AR terminal innervation.

## 2. Experimental Procedures

### *Confocal microscopy and neuronal reconstruction*

Three male Wistar rats (250g; Harlan, UK)) were deeply anaesthetised (ketamine/xylazine mixture, 7.33 and 0.73mg/100g ip) and an aqueous solution of CTb (200nl of 1% CTb; Sigma, Poole, Dorset, UK) was injected stereotaxically within the left caudal ventrolateral medulla (CVLM; co-ordinates = AP -4.8; DV -0.6; ML +2.1; Paxinos and Watson, 1997). Following three days survival, the animals were anaesthetised with sodium pentobarbitone (1ml ip) and perfused through the left ventricle with saline followed by a fixative containing 4% formaldehyde in phosphate buffer pH 7.6. The L4 lumbar segment was removed from each animal and postfixed in the same solution for 8 hours. Parasagittal sections (70μm thick) were cut with a Vibratome. Sections were treated with 50% ethanol (30 minutes) to enhance antibody penetration, which was followed by blocking in 10% normal donkey serum for 1 hour. Triple-labelling immunofluorescence was performed with a guinea pig anti-$\alpha_{2C}$-AR antiserum (diluted 1:500; Neuromics, Minneapolis, U.S.A.; see Stone et al., 1998 for details), rabbit anti-NK-1 antiserum (diluted 1:10,000; Sigma, Poole, Dorset, UK), and goat anti-CTb antiserum (diluted 1:5,000; List Laboratories, California, USA). After a 48 hour incubation period, sections were rinsed and incubated for 3 hours in solutions containing species-specific secondary antibodies coupled to the following fluorophores (all raised in donkey and diluted 1:100): rhodamine-red to identify $\alpha_{2C}$-AR immunoreactivity; FITC to identify NK-1 receptor immunoreactivity; and CY5 to identify CTb (all obtained from Jackson ImmunoResearch, Luton, U.K.). Antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% normal donkey serum. The sections were mounted in anti-fade medium (Vectashield; Vector Laboratories, Peterborough, UK) and stored at -20°C. Double-labelled cells (i.e. retrogradely labelled cells with NK-1 receptor immunoreactivity) contralateral to the side of the injection were systematically scanned using a BioRad MRC 1024 confocal laser scanning microscope with a X40 oil-immersion lens at 0.5μm intervals in the Z-axis and a zoom factor of 2. Thirty lamina I and 15 lamina III/IV neurons were reconstructed with

Neurolucida for Confocal software (MicroBrightField, Colchester, VT, USA) and appositions formed by $\alpha_{2C}$-AR axon terminals were plotted on the reconstructions. A Sholl analysis was performed to study the pattern of distribution of contacts for the two populations of neurons; numbers of contacts per 100μm unit length of dendrite contained within concentric spheres with radii which increased at 25μm intervals from the centre of the cell body were estimated.

In order to determine if $\alpha_{2C}$-AR contact densities on NK-1 projection cells were greater than would be expected by chance, we compared them with protein kinase C-γ (PKCγ)-IR cells which are also found in laminae I and II within the dense plexus of $\alpha_{2C}$-AR-IR axons. PKCγ cells are predominantly excitatory interneurons (Polgár et al., 1999) and therefore would be expected to be very different functionally from NK-1 projection neurons. Triple-labelling immunofluorescence was performed as described above, except on this occasion the rabbit anti-NK-1 antiserum was replaced by rabbit anti-PKCγ antiserum (diluted 1:1,000; Santa Cruz Biotechnology, California, USA). Contact densities per 100μm$^2$ of dendritic surface area were calculated for lamina I and lamina III/IV NK-1 projection cells and for PKCγ-IR cells by using the Neurolucida programme. The average contact density was calculated for each animal (n=3 on each occasion; 10 cells from each animal for lamina I and 5 cells from each animal for lamina III/IV and PKCγ) and the overall mean for three animals was calculated (±SD). Statistical comparisons were made by using one-way analysis of variance (ANOVA) and a Tukey's *post hoc* pairwise comparison. P values less than 0.05 were considered to be significant.

*Combined confocal and electron microscopy*

A second set of three male Wistar rats (250g; Harlan, UK) was deeply anaesthetised with ketamine and xylazine and received unilateral stereotaxic injections of 200nl of 1% CTb (Sigma, Poole, Dorset, UK) in the left CVLM as described in the section above. The combined confocal and electron microscopic method used, is a modification of that described by Todd (1997). Following a three-day survival period, the animals were deeply anaesthetized with sodium pentobarbitone (1ml ip) and perfused with saline followed by a fixative containing 4% formaldehyde, 0.2% glutaraldehyde, and 0.2% of saturated picric acid in phosphate buffer (pH 7.6). L4 segments were removed, placed in the same fixative for 8 hours and cut into 50μm horizontal sections with a Vibratome. The sections were treated with 50% ethanol for 30 minutes to improve antibody penetration and also with 1% sodium borohydride for 30 minutes to counteract the effects of glutaraldehyde. Sections

were incubated for 3 days in guinea pig anti-$\alpha_{2C}$-AR antiserum (diluted 1:500; Neuromics, Minneapolis, U.S.A.), rabbit anti-NK-1 antiserum (diluted 1:10,000; Sigma, Poole, Dorset, UK), and a goat anti-CTb antiserum (diluted 1:5,000; List Laboratories, California, USA). Sections were then rinsed in PBS and placed for 1 day in a cocktail of donkey secondary antibodies which consisted of rhodamine-red-anti-guinea pig Ig, FITC-anti-rabbit Ig, CY5-anti-goat Ig (all diluted 1:100), and biotinylated-anti-guinea pig Ig (diluted 1:500; all obtained from Jackson ImmunoResearch, Luton, U.K.). Primary and secondary antibodies were diluted in detergent-free PBS. After rinsing in PBS, sections were incubated in avidin-biotin-HRP complex (diluted 1:1,000; Vector Elite, Peterborough, U.K.) for 1 day. Once the sections were mounted, they were scanned with the confocal microscope and lamina I cells were selected for analysis. A total of six NK-1 receptor-IR CTb-labelled neurons (2 from each animal), which received multiple contacts from $\alpha_{2C}$-AR-IR terminals, were examined. Optical sections were gathered sequentially in order to avoid bleed-through. Multiple scans were performed with a X60 oil-immersion lens at 0.5$\mu$m intervals in the Z-axis and a zoom factor of 1.5 in order to produce a montage of each selected neuron. In addition, scans with a X40, X20, X10 and X4 lenses were performed to gather progressively lower power images that would serve as a frame of reference for identification of each cell with the electron microscope.

Sections containing scanned cells were removed from slides and processed for electron microscopy. Following rinsing, they were reacted with DAB in the presence of hydrogen peroxide. They were then placed in a 1% solution of osmium tetroxide for 30 minutes, dehydrated in acetone, stained *en bloc* with uranyl acetate and finally flat-embedded in Durcupan resin (Fluka, Switzerland) between cellulose acetate sheets. Sections were examined with a light microscope to establish the location of each cell; DAB positive $\alpha_{2C}$-AR-IR terminals surrounding cells and landmarks such as blood vessels were used to identify their location within sections. Sections were mounted onto blocks of cured resin, which were trimmed to include the region containing the cell. Ultrathin sections were cut serially with a diamond knife, collected on Formvar-coated grids and viewed with an electron microscope (Philips CM100).

*Sequential immunocytochemistry*

The rationale of this protocol has been explained in *Chapter 2: 2.1. Multiple immunolabelling for confocal microscopy* (pg 27). A third set of three male rats (Wistar, 250 g; Harlan, UK) was used for this part of the study. The first part of the procedure applied was basically identical to that described in the section above (see for details: *Confocal microscopy and neuronal reconstruction*; pg 65), however additional steps were required. Therefore, following stereotaxic injection of CTb in the left CVLM, a three-day survival period, fixation by cardiac perfusion, postfixation for 8 hours, cutting of horizontal sections (50$\mu$m thick) from the L4 lumbar segments and triple-labelling immunocytochemistry for NK-1, CTb and $\alpha_{2C}$-AR, NK-1 retrogradely labelled neurons associated with $\alpha_{2C}$-AR-IR terminals were scanned from lamina I. When scanning of selected double-labelled neurons was completed, sections were retrieved from the slides and re-incubated in a fourth antiserum: rabbit anti-VGLUT2 antiserum (diluted 1:5,000 from Synaptic Systems, Göttingen, Germany) for 48 hours. They were then rinsed and incubated for 3 hours in a solution containing donkey anti-rabbit Ig coupled to FITC (diluted 1:100, Jackson Immunoresearch, Luton, U.K.). Finally, sections were re-mounted and the same neurons that had been scanned previously were identified and scanned again. The same secondary antibody was used to reveal the rabbit anti-NK-1 and rabbit anti-VGLUT2 primary antibodies, but by comparing the FITC-labelling before and after the re-incubation in VGLUT2 antiserum, it was possible to detect the additional FITC labelling, which represents immunoreactivity for VGLUT2. No additional immunoreactivity was observed in control experiments when the VGLUT2 antibody was omitted in the sequential reaction. Equally the pattern of immunostaining was not altered when performing the sequential incubation in a reverse order for the NK-1 and VGLUT2 labelling, i.e. first incubation containing the rabbit anti-VGLUT2 antiserum in the cocktail of primary antisera followed by sequential incubation in rabbit anti-NK-1 antiserum.

*Induction of c-Fos expression*

Three rats were used for this set of experiments (Wistar, 250 g; Harlan, UK). Following stereotaxic injection of CTb in the left CVLM (see for details: *Confocal microscopy and neuronal reconstruction;* pg 65) and a three-day survival period, animals were prepared for c-Fos induction. The rats were deeply anaesthetised (ketamine/xylazine mixture, 7.33 and 0.73mg/100g ip) and then the right hind paw -contralateral to the side of the CTb injection- was immersed in water at a temperature of 52°C for a period of 20

71

seconds. Note that the CTb injection in the CVLM was performed in the left side and the noxious stimulation for c-Fos induction was produced on the right side. The purpose of this was to obtain as many projection cells as possible in the region of maximum c-Fos expression in order to optimise the estimation of the proportion of projection neurons that respond to the peripheral stimulation. The basis for this is that (1) a CTb injection in the left CVLM labels neurons predominantly on the right dorsal horn, because most CVLM neurons are known to project contralaterally (Todd et al., 2000), and (2) peripheral noxious stimulation in the right hind limb induces the expression of c-Fos mainly in neurons in the ipsilateral dorsal horn, i.e. right, especially in the medial portion of the superficial dorsal horn (Hunt et al., 1987; Todd et al., 1994a; Doyle and Hunt, 1999; Todd et al., 2002; see Fig.4.1, a). Animals were maintained under anaesthetic for 2 hours. In order to keep the animals deeply anaesthetised, the corneal reflex was checked regularly and 0.1ml or 0.2ml injections (ketamine/xylazine mixture) were administered as soon as any evidence of the reflex was detected minimally. After the 2 hour period animals were prepared for transcardiac perfusion. Following fixation, L4 lumbar segments were removed and postfixed for 8 hours. Horizontal, parasagittal and transverse sections (50μm thick) were cut with a Vibratome.

Two control experiments were carried out in order to demonstrate that the c-Fos expression is due to the stimulus applied. In the latter experiments animals were treated following exactly the same protocol as described above (*Induction of c-Fos expression*) with the exception that the stimulus was not applied. Immunocytochemical labelling of c-Fos showed almost a complete absence of c-Fos labelling in the spinal cord. The few c-Fos-IR nuclei found were generally located in superficial laminae and around the central canal (Fig.4.1, b).

*Immunofluorescent labelling of four markers*

The rationale of this procedure has been explained previously in *Chapter 2: 2.1 Multiple immunolabelling for confocal microscopy* (pg 27) and it is described in detail below. Sections were treated with 50% ethanol for 30 minutes and blocked in 10% normal donkey serum for 1 hour. Quadruple-labelling immunofluorescence was performed with guinea pig anti-$\alpha_{2C}$-AR antiserum (diluted 1:500; Neuromics, Minneapolis, U.S.A.; see Stone et al., 1998 for details), rabbit anti-NK-1 antiserum (diluted 1:10,000; Sigma, Poole, Dorset, UK), rabbit anti-c-Fos antiserum (diluted 1:5,000; Hunt et al., 1987), and goat anti-CTb antiserum (diluted 1:5,000; List Laboratories, Campbell, CA). After a 48 hour

incubation period, sections were rinsed and incubated for 24 hours in solutions containing three different species-specific secondary antibodies coupled to fluorophores: rhodamine-red-anti-guinea pig Ig to identify $\alpha_{2C}$-AR immunoreactivity; FITC-anti-rabbit Ig to identify NK-1 receptor and c-Fos immunoreactivity; and CY5-anti-goat Ig to identify CTb (all raised in donkey and diluted 1:100; Stratech, Cambridge, U.K). Notice that the NK-1 receptor and c-Fos can be labelled with the same secondary antibody because they are located in clearly different cellular structures, cell membrane and nucleus, respectively, which allows distinction of both types of immunolabelling through the same channel. All antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% normal donkey serum. Once rinsed, the sections were mounted.

*Quantitative analysis of c-Fos expression on dorsal horn NK-1 projection neurons*

In the first place, a general analysis of c-Fos positive cells was carried out. c-Fos-labelled cells present in the ipsi and contra-lateral sides to the stimulation, were counted in order to estimate the relative proportion of c-Fos-IR cells in the ipsilateral *versus* the contralateral dorsal horns (laminae I-IV). The sample for the cell counts consisted of three transverse sections from three different experiments, i.e. nine sections. This analysis agreed with previous investigations using a similar approach (Hunt et al., 1987; Todd et al., 1994a; Todd et al., 2002). The proportion of c-Fos cells in the dorsal horn ipsilateral to the stimulation was almost five times higher than that one in the contralateral side (counts per section: average±SD; ipsilateral 54.89±2.36, contralateral 12.11±1.58). *Student's t-test* confirmed that this difference was highly significant ($P<0.01$).

Secondly, a more specific analysis was produced to study the expression of c-Fos on NK-1 projection neurons. A sample of 30 lamina I and 15 lamina III/IV neurons (10 and 5 respectively from each of the animals), which were double labelled for NK-1 and CTb, were scanned using a Radiance 2100 confocal laser scanning microscope with a X40 oil-immersion lens at 1μm intervals in the Z-axis and a zoom factor of 1. All the neurons sampled were located within the medial portion of the right dorsal horn, which was contralateral to the CVLM injection site and ipsilateral to the c-Fos induction side, because as expected this area contained a high concentration of c-Fos-IR neurons and many retrogradely labelled neurons projecting contralaterally to the CVLM. Lamina I neurons were sampled from horizontal sections of spinal cord, while lamina III/IV neurons were sampled from parasagittal sections. The analysis involved quantification of the proportion of NK-1 retrogradely labelled cells that were c-Fos positive in both samples of lamina I

and lamina III/IV neurons. Finally, lamina I NK-1 retrogradely labelled neurons were assessed for $\alpha_{2C}$-AR-IR terminal innervation. The sample did not include lamina III/IV NK-1 neurons because in the 50μm thick sections examined it was usually not possible to follow their dendrites to the superficial dorsal horn, where they would be expected to receive innervation from $\alpha_{2C}$-AR-IR terminals.

## 3. Results

*NK-1 spinomedullary neurons are innervated by axons that possess the $\alpha_{2C}$-AR*

Spinomedullary neurons were labelled by injection of the retrograde tracer CTb in the left CVLM. Figure 4.2 shows the injection site for one of the experiments, and an example of the extent of the tracer spread in another experiment. As predicted, numerous retrogradely labelled neurons were found in lamina I and lamina III/IV of the spinal dorsal horn, especially contralateral to the injection site (Todd et al., 2000). A large proportion of CTb-labelled neurons was present in lamina I in the right dorsal horn along with immunoreactivity for the NK-1 receptor and axon terminals possessing the $\alpha_{2C}$-AR (Fig. 4.3, a; and Fig. 4.4, a). At high magnification it was possible to identify NK-1 projection cells by the presence of CTb within them and to study their relationship with $\alpha_{2C}$-AR-IR profiles. Cell bodies and proximal dendrites of lamina I cells frequently received large numbers of contacts from $\alpha_{2C}$-AR-IR structures (Fig. 4.3, b-d; and Fig. 4.4, c) as did distal dendrites of labelled cells in lamina III/IV which extended dorsally into lamina I (Fig. 4.4, b, d-f). More than half of the NK-1 projection cells were found to receive contacts from $\alpha_{2C}$-AR terminals (average±SD=57.3±5.26%).

*Distribution and density of $\alpha_{2C}$-AR contacts on NK-1 projection neurons*

The distribution of $\alpha_{2C}$-AR contacts was studied in the two populations of NK-1 projection neurons. The neurons were sampled from parasagittal sections which were contralateral to the injection site. A sample of 30 lamina I neurons was scanned (10 from each of the three experiments), while the sample of lamina III/IV neurons was reduced to 15 (5 from each experiment) as they were found less frequently. The intensity of immunostaining for the NK-1 receptor varied from cell to cell and while some neurons were very strongly labelled others were weakly labelled. Neurons showing any evidence of NK-1 receptor immunoreactivity were included in the analysis. On average 83% of the retrogradely labelled neurons were NK-1 positive (SD=16.7%). Once the neurons were

scanned they were reconstructed and the $\alpha_{2C}$-AR contacts were mapped (see examples in Fig. 4.5). Sholl analysis of the distribution of $\alpha_{2C}$-AR contacts revealed that NK-1 projection neurons with somata in lamina I receive numerous $\alpha_{2C}$-AR contacts on cell bodies and proximal dendrites, while NK-1 projection neurons with somata in lamina III/IV receive most $\alpha_{2C}$-AR contacts on distal dendrites which extend into laminae I-II. The average number of contacts per 100μm of dendritic length was higher for lamina I neurons than for lamina III/IV neurons (average±SD=109.3±38.1 and 69.3±20.7 respectively; see histograms in Fig. 4.5). We also calculated average densities of $\alpha_{2C}$-AR contacts per unit area (100 $\mu m^2$) of dendritic surface for lamina I and lamina III/IV NK-1 projection cells in order to compare them with a population of PKCγ-IR interneurons (Fig. 4.6). Dendrites of lamina I cells had approximately seven times the density of contacts associated with PKCγ cells (average±SD contacts per 100 $\mu m^2$=1.09±0.07 and 0.14±0.01 respectively) and lamina III/IV cells were associated with 3 times the density (0.51±0.09 contacts per 100 $\mu m^2$). Statistical comparisons confirm that these differences are significant (P<0.001, ANOVA; individual differences between all three groups were significant at P<0.05, Tukey's *post hoc* pairwise comparison).

*Axon terminals containing the $\alpha_{2C}$-AR form synapses with NK-1 projection neurons*

By means of the combined confocal and electron microscopic method applied it was possible to firstly visualise $\alpha_{2C}$-AR-IR terminals forming appositions on NK-1 spinomedullary neurons, and secondly, following processing of the tissue for electron microscopy, to determine if such terminals formed synapses with the neuron (Fig. 4.7). In total, six neurons were examined (two from each animal) which received 45 appositions from $\alpha_{2C}$-AR terminals. Electron microscopic analysis confirmed that 42 of these formed synapses with the neurons. Most of these synapses could be classified as asymmetric but occasionally it was difficult to define the type of synapse. The remaining three appositions were not observed to form synapses. Synaptic boutons contained circular agranular vesicles and often granular vesicles also (Fig. 4.7).

*$\alpha_{2C}$-AR-IR axon terminals that innervate NK-1 projection neurons are excitatory*

Sequential immunocytochemistry with a fourth antibody against VGLUT2 was used to determine if $\alpha_{2C}$-AR-IR profiles forming appositions with NK-1 projection cells were immunoreactive for VGLUT2 (Takamori et al., 2000; Varoqui et al., 2002). A sample of thirty NK-1 retrogradely labelled lamina I neurons was scanned (10 from each of the three

animals) from the side contralateral to the injection. An average of 43 $\alpha_{2C}$-AR appositions per neuron was recorded (SD=12). Of these appositions, 100% were found to be VGLUT2 positive when the selected neurons were re-scanned following the sequential incubation in VGLUT2 antiserum (example illustrated in Fig. 4.8).

*NK-1 projection neurons respond to thermal noxious stimulation and are innervated by $\alpha_{2C}$-AR-IR terminals*

The analysis of c-Fos expression included NK-1 projection neurons with cell bodies in lamina I and lamina III/IV that were contralateral to the side of the CTb injection and ipsilateral to the side of peripheral thermal nociceptive stimulation. The majority of NK-1 spinomedullary neurons in medial portion of lamina I were found to express c-Fos in their nuclei (70±10%), while a minimal proportion of NK-1 spinomedullary neurons in lamina III/IV were found to be positive for c-Fos (6.67±5.77%). A high proportion of the analysed lamina I NK-1 projection neurons received innervation from $\alpha_{2C}$-AR-IR terminals (42±5.77%) which included both c-Fos positive (23±5.77%); example illustrated in Figure 4.9) and c-Fos negative (20±0%) neurons.

## 4. Discussion

The major finding of this investigation is that axon terminals containing the $\alpha_{2C}$-AR densely innervate NK-1 spinomedullary neurons that project to the CVLM. Both lamina I and lamina III/IV projection neurons were associated with $\alpha_{2C}$-AR contact densities that were significantly greater than contact densities on interneurons possessing PKC$\gamma$ immunoreactivity and it can be concluded that this represents a specific type of arrangement that has not arisen merely by chance. Terminals were concentrated around cell bodies and proximal dendrites of lamina I neurons, while in lamina III/IV neurons the terminals were apposed predominantly to distal dendrites which extended dorsally into laminae I and II. We were able to demonstrate that axon terminals possessing the $\alpha_{2C}$-AR form synapses with NK-1 projection neurons in lamina I, and that they are likely to have an excitatory action because they also contained VGLUT2 which is a marker for glutamatergic axon terminals (Takamori et al., 2000; Varoqui et al., 2002; Todd et al., 2003). In addition, a considerable proportion of lamina I NK-1 projection neurons were found to be responsive to thermal noxious stimulation as well as receive innervation from axon terminals containing $\alpha_{2C}$-ARs. On this basis, it can be concluded that our hypothesis

is correct and that axons of excitatory interneurons possessing $\alpha_{2C}$-ARs do indeed target nociceptive projection neurons.

*Identification of NK-1 projection neurons*

Our quantitative analysis was confined to neurons contralateral to the CVLM injection site that were NK-1 positive since neurons of this type are very likely to be involved in the transmission of nociceptive information (Naim et al., 1997; Todd et al., 2002). Stereotaxic injections were made into the CVLM because this region of the brainstem is known to receive substantial input from lamina I (Lima et al., 1991; Craig, 1995) and also because lamina I neurons labelled from the CVLM are more numerous than those labelled from other projection targets (i.e. dorsal reticular nucleus, periaqueductal gray, or thalamus; Marshall et al., 1996; Todd et al., 2000). Most spinomedullary neurons labelled from the CVLM project contralaterally and only a minority project ipsilaterally. Neurons retrogradely labelled from the CVLM also include a population of lamina III/IV cells, which have dorsally directed dendrites that terminate in lamina I. Double-labelling studies indicate that most CVLM neurons also have collateral projections to the lateral parabrachial area (Todd, 2002) and therefore many of the cells analysed in this study are likely to project to this area in addition to the CVLM. Both these regions of the brainstem are known to be targets of nociceptive neurons and are intimately involved in nociceptive processing (Gauriau and Bernard, 2002; Lima et al., 2002).

*Functional significance of $\alpha_{2C}$-AR innervation of NK-1 projection neurons*

Our findings indicate that NE can influence NK-1 projection neurons through a presynaptic action on axon terminals that possess $\alpha_{2C}$-ARs. Both lamina I and lamina III/IV NK-1 projection cells receive dense innervation from small diameter primary afferent fibres that contain a combination of SP and glutamate (De Biasi and Rustioni, 1988; Naim et al., 1997; Todd et al., 2002). Glutamate is undoubtedly involved in acute pain signalling mechanisms but the role of SP in nociceptive transmission is subtle. Ablation of lamina I NK-1 cells attenuates the development of thermal and mechanical hyperalgesia (Mantyh et al., 1997; Khasabov et al., 2002) and mice that lack the NK-1 receptor do not display the characteristic amplification and intensity coding of nociceptive reflexes (De Felipe et al., 1998). Such 'knock-out' animals also have reduced descending inhibition evoked by peripheral noxious stimuli (Bester et al., 2001) thus indicating that NK-1 neurons are components of an ascending-descending antinociceptive loop. The NK-1 receptor is directly implicated in the mediation of NE antinociception because genetically

engineered mice that lack NE show a SP-dependent chronic hyperalgesia (Jasmin et al., 2002). These lines of evidence suggest that NK-1 neurons are likely to be components of the circuitry that underlies NE antinociception. NE descending inhibitory systems could be recruited by NK-1 spinomedullary neurons, as these cells terminate in a region close to the lateral reticular nucleus that contains DβH-IR neurons, which in turn, project to the spinal dorsal horn (Lee et al., 2001).

It is well established that the antinociceptive action of NE is mediated by $\alpha_2$-ARs (Howe et al., 1983; Yaksh, 1985; Proudfit, 1988) but it has been shown only recently that both $\alpha_{2A}$-AR and $\alpha_{2C}$-AR subtypes are involved in this process (Stone et al., 1997; Li and Eisenach, 2001; Fairbanks et al., 2002). Budai et al. (1998) reported that periaqueductal gray neurons inhibit nociceptive dorsal horn cells by a presynaptic action on $\alpha_2$-ARs. Our findings also support this proposal, as we have shown that $\alpha_{2C}$-ARs are located on axon terminals that are presynaptic to nociceptive cells. In addition, virtually all of these terminals are likely to be glutamatergic as they are immunoreactive for VGLUT2 and therefore would be expected to have an excitatory action on their target cells. This finding is also in agreement with studies reporting that activation of $\alpha_2$-ARs can reduce the release of glutamate. Pan et al. (2001) showed that the $\alpha_2$-AR agonist clonidine inhibits glutamatergic synaptic input to spinal neurons in outer lamina II by a presynaptic action on $\alpha_2$-ARs. The $\alpha_{2A}$ and $\alpha_{2C}$-ARs not only differ in their cellular location in the dorsal horn, but also differ in their affinity for NE and deactivation kinetics. The $\alpha_{2C}$-AR has a greater affinity for NE than the $\alpha_{2A}$-AR and the $\alpha_{2C}$-AR shows slower deactivation following NE stimulation (Bunemann et al., 2001). These differences suggest that each subclass has distinct physiological functions, even if both types of receptor are implicated in NE antinociception. The results indicate that part of the antinociceptive action of NE could be mediated via $\alpha_{2C}$-ARs present on terminals of excitatory spinal interneurons that are presynaptic to nociceptive neurons in lamina I and distal dendrites of presumed nociceptive neurons in lamina III/IV.

**Figure 4.1. c-Fos labelling with and without peripheral thermal noxious stimulation. a** shows c-Fos labelling in ipsilateral (ipsi) and contralateral (contra) dorsal horns following application of peripheral thermal noxious stimulation. A high concentration of c-Fos-labelled neurons is found in the medial portion of the superficial dorsal horn (green arrowhead). Some c-Fos-IR cells can be seen in the lateral spinal nucleus (blue arrowhead). **b** illustrates the sparse c-Fos labelling obtained in the control experiment when no peripheral thermal noxious stimulation was applied. Few c-Fos cells are found (red arrowheads).

(Scale bar: **b**=100μm, valid for both images)

**a** contra — ipsi — stimulation

**b** contra — ipsi — no stimulation
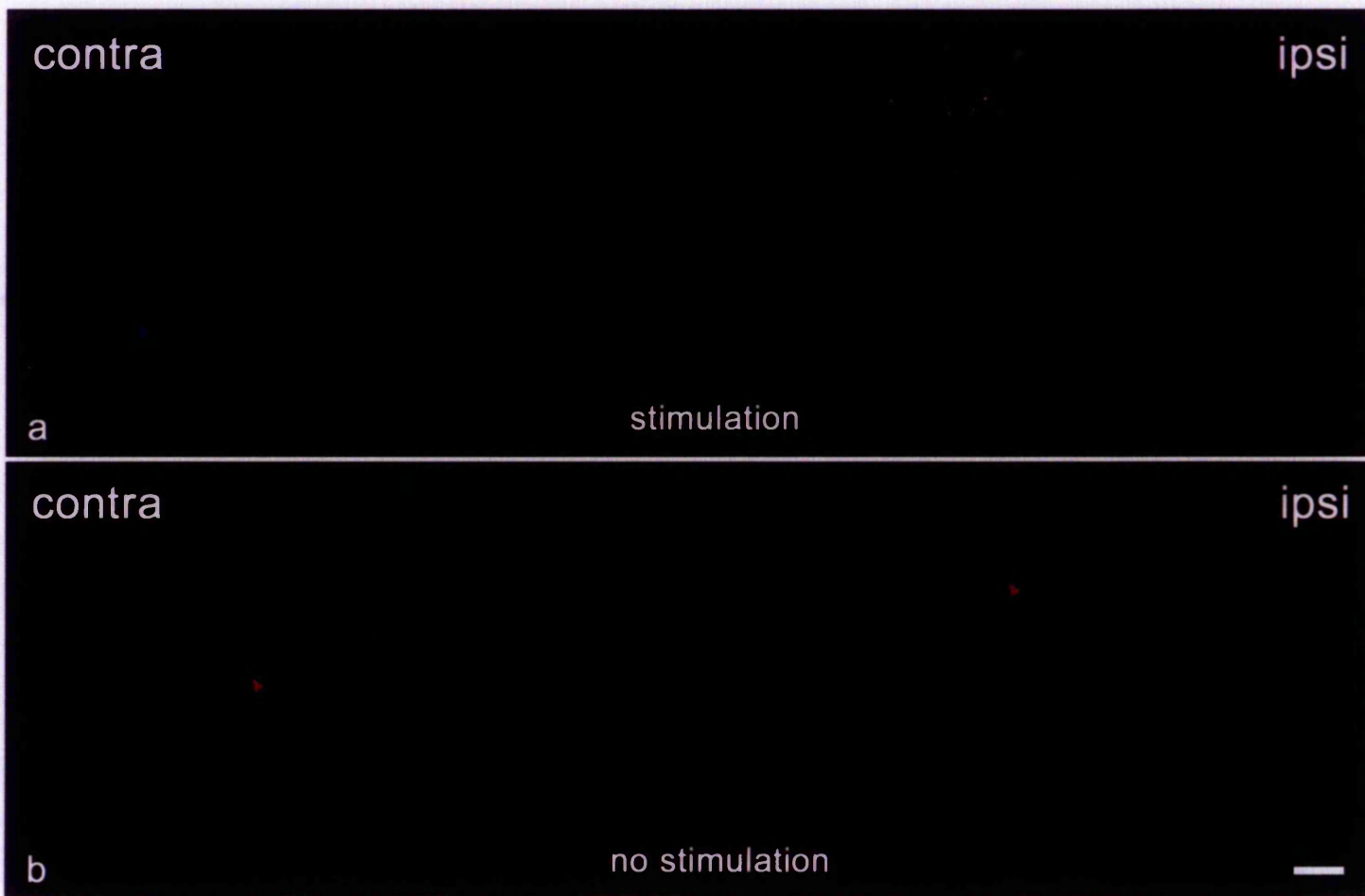
**Figure 4.2. Injection of retrograde tracer in the CVLM. a** Photo micrograph illustrating the CTb-injection site in the left CVLM. 4v, 4$^{th}$ ventricle; cu, cuneate nucleus; cc, central canal; XII, hypoglossal nucleus; spV, spinal trigeminal nucleus; ml, medial lemniscus; LRt, lateral reticular nucleus; vsc, ventral spinocerebellar tract; py, pyramidal tract. **b** Reconstruction of an injection site indicating the interaural co-ordinate (Paxinos and Watson, 1997). The spread of the tracer is represented by the dark grey area.

a

b

-3.8    -4.3    -4.8    -5.3    -5.6
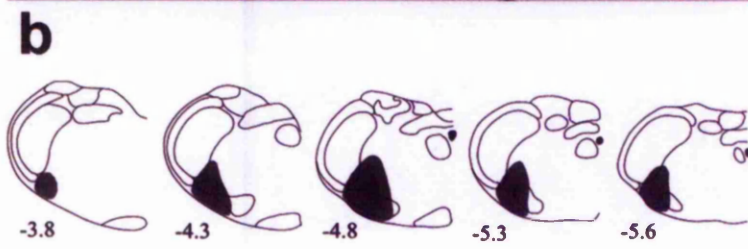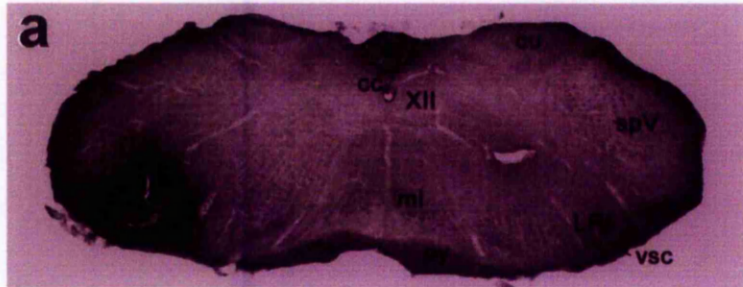
**Figure 4.3. Immunoreactivity for CTb, the NK-1 receptor and $\alpha_{2C}$-AR in a horizontal section of lamina I. a** A merged image of a horizontal section of lamina I (blue, CTb; green, NK-1 receptor; red, $\alpha_{2C}$-AR) illustrating the general pattern of triple-labelling at a low magnification (made from 20 projected confocal images gathered at $1\mu m$ steps with a X20 lens). **b-d**, show projected images of three retrogradely labelled neurons at high magnification (built from 15, 10 and 5 single optical sections for **b, c, d** respectively. Optical sections were gathered at $0.5\mu m$ steps with a X40 lens). Cells shown in **b** and **c** are multipolar, whereas the cell shown in **d** is fusiform. As in **a,** CTb is blue, NK-1 receptor labelling is green and immunoreactivity for the $\alpha_{2C}$-AR is red. All three neurons receive multiple contacts from $\alpha_{2C}$-AR-IR terminals. **b'-d'**, show the corresponding $\alpha_{2C}$-AR immunoreactivity. Note that $\alpha_{2C}$-AR terminals delineate the cell bodies and dendrites of these neurons. (Scale bars: **a**=$100\mu m$; **b**=$10\mu m$; scale in **b** valid for **c-d** and **b'-d'**)
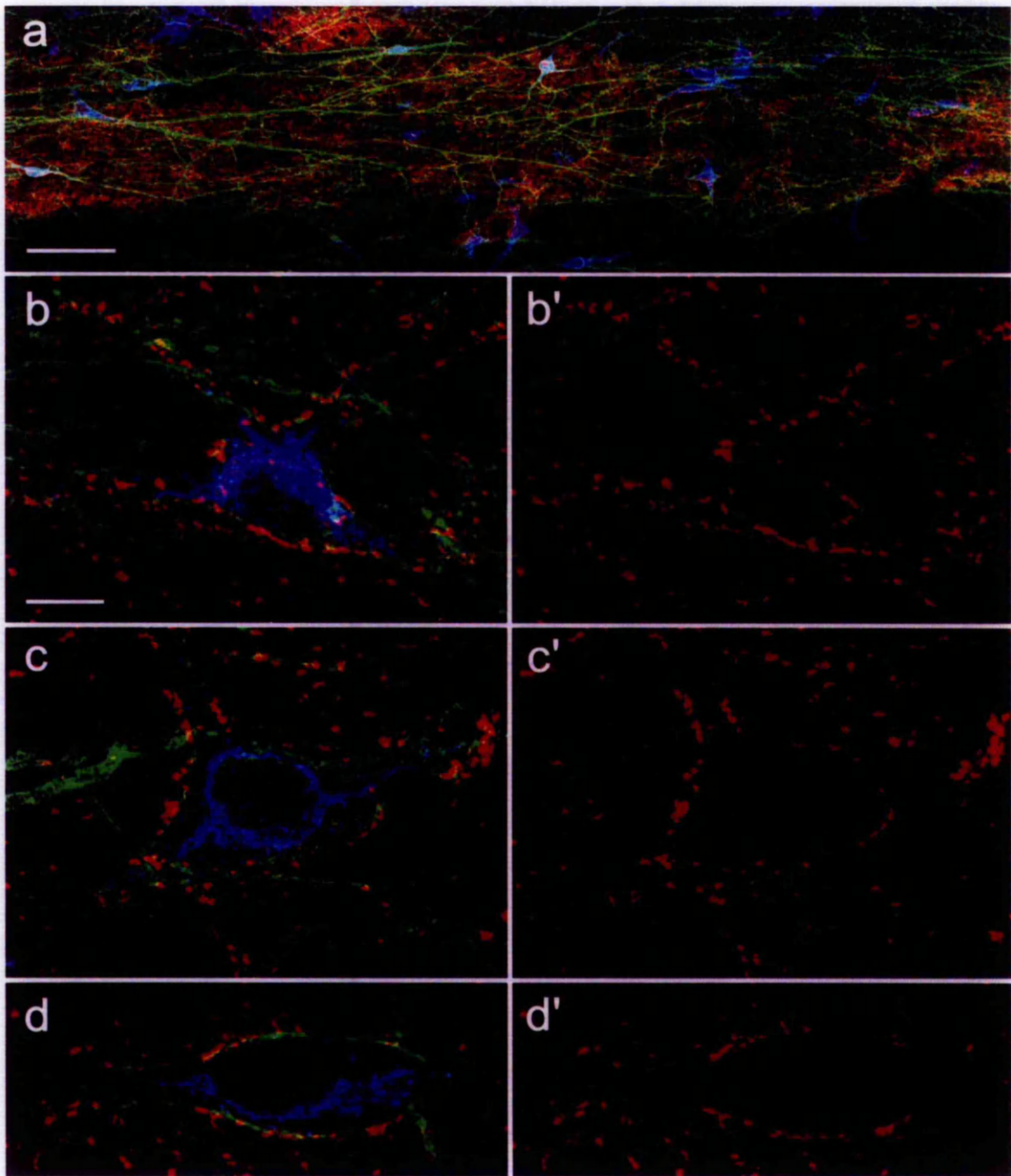
**Figure 4.4. Triple-labelling of CTb, the NK-1 receptor and $\alpha_{2C}$-AR in a parasagittal section. a** A projected image of a parasagittal section built from 30 confocal images which were gathered at $1\mu m$ steps with a X20 lens (blue, CTb; green, NK-1 receptor; red, $\alpha_{2C}$-AR). The locations of laminae I to IV are indicated on the right side of the image. A cell that is labelled with CTb and the NK-1 receptor can be observed in lamina III. This cell has three dorsally orientated dendrites that extend into lamina I. **b** A projected image at high magnification showing the boxed area in **a** which includes one of the dorsal dendrites from the lamina III cell (made from 20 optical sections, gathered at $0.5\mu m$ steps with a X60 lens). **c** A single optical section showing that $\alpha_{2C}$-AR terminals form contacts with the lamina 1 cell seen in **b** which contains CTb and is labelled for the NK-1 receptor. **d** A projected image of the boxed area in **b** (built from 7 optical sections, gathered at $0.5\mu m$ steps with a X60 lens); seven boutons, indicated by the numbered arrows, form appositions with the NK-1 positive dendrite which belongs to the lamina III cell seen in **a**. Appositions are shown in single optical sections: 1 and 3 in **e**, and 2, 4-7 in **f**. (Scale bars: **a**=$100\mu m$, **b**-**c**=$20\mu m$, **d**=$10\mu m$; scale in **d** valid for **e**-**f**)
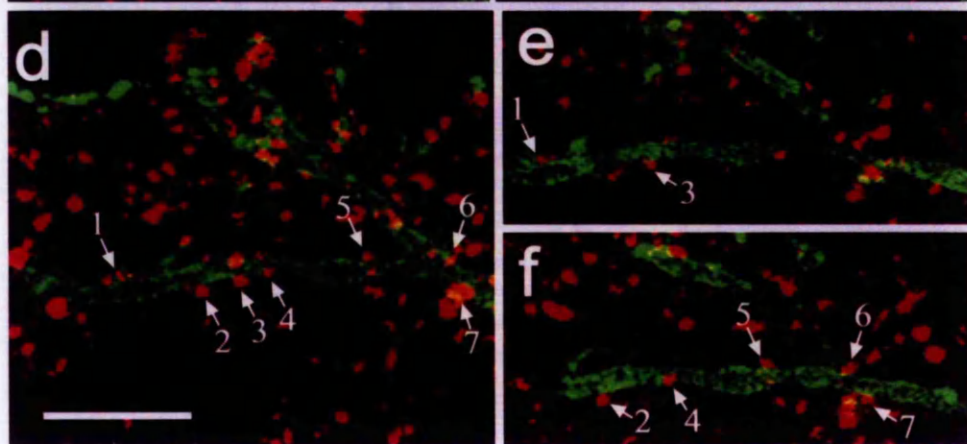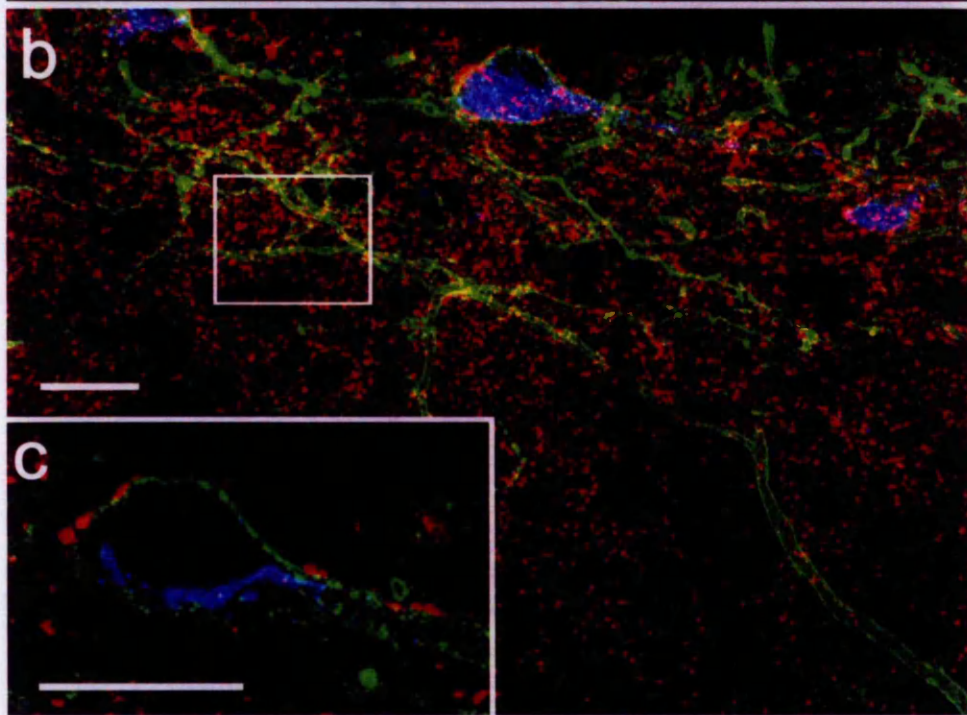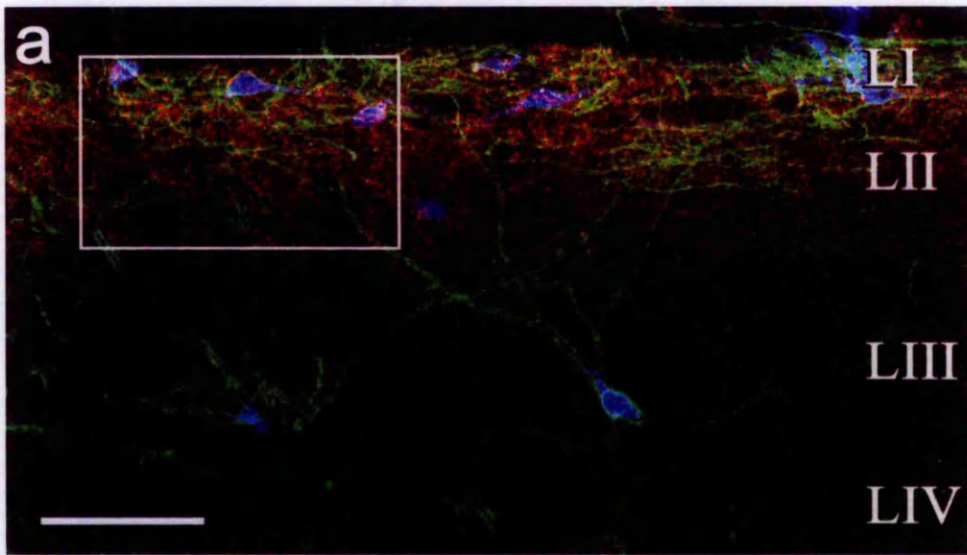
**Figure 4.5. Distribution of $\alpha_{2C}$-AR contacts on NK-1 projection cells.** Left, two examples of NK-1 projection cells reconstructed with Neurolucida. Contacts (black circles) from $\alpha_{2C}$-AR-IR puncta were mapped on the reconstructions. A lamina I cell is shown above and a lamina III/IV cell bellow. (Scale bars=100μm). Right, histograms summarising the Sholl analysis of the distribution of $\alpha_{2C}$-AR contacts on both populations of cells (lamina I, n=30; lamina III/IV, n=15). The number of contacts per 100μm of dendritic length is plotted against the distance from the soma, where 0μm represents the cell body and the consecutive numbers represent the radii of the concentric shells, which increase at 25μm intervals.

LAMINA I NK-1 CELL



LAMINA III/IV NK-1 CELL



Distribution of $\alpha_{2C}$-AR contacts on NK-1 projection cells in Lamina I
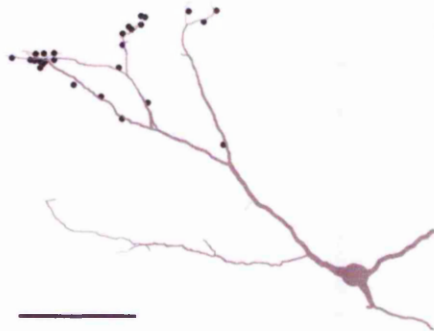


Distribution of $\alpha_{2C}$-AR contacts on NK-1 projection cells in Lamina III/IV
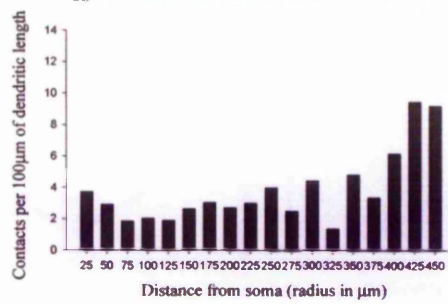
**Figure 4.6. Double-labelling of PKCγ and the α2C-AR in a parasagittal section. a** A projected image of a parasagittal section showing immunoreactivity for PKCγ and the α2C-AR (green and red respectively) at low magnification (made from 20 single optical sections, gathered at 0.5μm steps with a X20 lens). The location of laminae I-III is indicated on the right side of the image. A PKCγ-IR cell can be seen in lamina I, dorsal to the main PKCγ plexus in lamina II. Note that α2C-AR immunoreactivity is dense in the superficial dorsal horn where dorsal PKCγ cells are found. **b** A projected image of the dorsal PKCγ cell at high magnification (made from 15 optical sections, gathered at 0.5μm steps with a X40 lens). **b'** shows the corresponding α2C-AR immunoreactivity; note that α2C-AR terminals do not delineate the outline of the cell. **c** A reconstruction of the dorsal cell illustrated in **a** and **b**; only one α2C-AR terminal was found to form a contact (red circle) with the cell. **d** and **e**, are single optical sections extracted from the z-series in **b** illustrating the paucity of contacts formed by α2C-AR terminals with the cell body and dendrites of the PKCγ cell. (Scale bars: **a**=40μm; **b-c**=20μm; scale in **b** valid for **b'**, **d-e**)
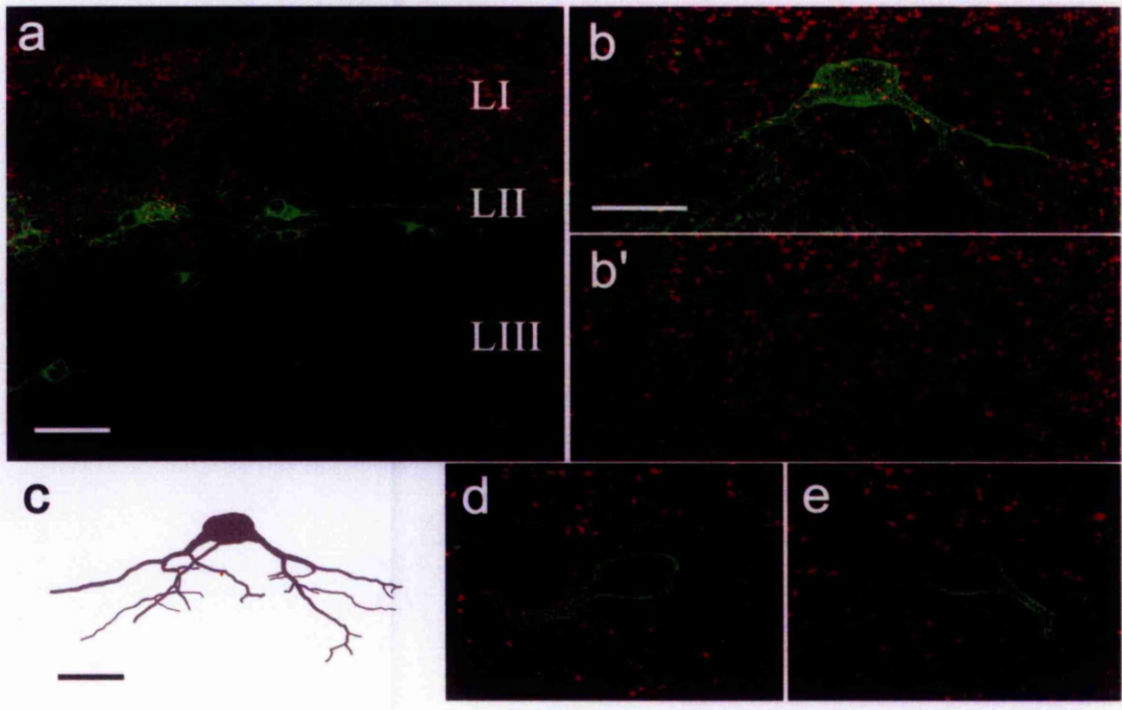
**Figure 4.7. Combined confocal and electron microscopy of $\alpha_{2C}$-AR contacts. a** A single optical section of a NK-1 projection cell in lamina I which is apposed by three $\alpha_{2C}$-AR-IR terminals (arrows numbered 1-3; blue, CTb; green, NK-1 receptor; red, $\alpha_{2C}$-AR) **b** An electron micrograph of the same cell. The $\alpha_{2C}$-AR-IR terminals can be recognised by the dark DAB-reaction product. The three terminals indicated by the arrows (numbered 1-3) correspond to those indicated in **a**. The areas delineated by the purple and blue boxes in **b** are shown at higher magnification in **c** and **f** respectively. **c-e**, show progressively magnified images of the axon terminal indicated by arrow 1. This forms an asymmetric synapse with a proximal dendrite of the cell. **f-h**, are progressively magnified images of the terminal indicated by arrow 3. This bouton forms a long asymmetric synapse. Boutons contained circular agranular vesicles and often also granular vesicles (red arrowheads in **g**). (Scale bars: **a-b**=10µm; **c, f**=1µm; **d, g**=0.5µm; **e, h**=0.25µm)
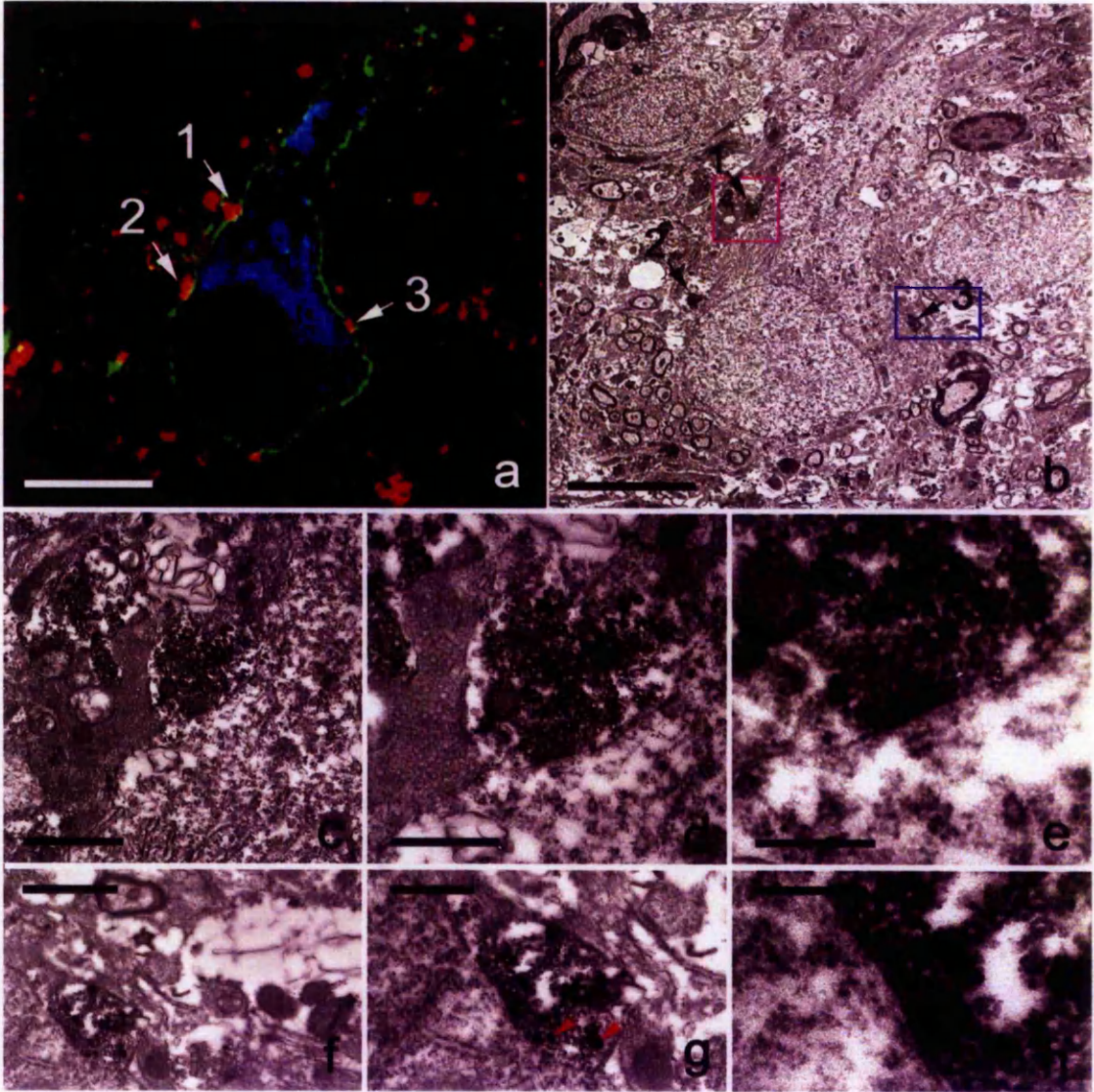
**Figure 4.8. Sequential immunocytochemistry for VGLUT2. a** A single optical section of a NK-1 projection cell from lamina I that receives numerous contacts from $\alpha_{2C}$-AR terminals. Immunoreactivity for the NK-1 receptor (NK1), CTb (CTb), and $\alpha_{2C}$-AR ($\alpha$2C) are shown independently. A merged image formed from the previous three is shown on the right. **b** A single optical section of the same cell which has been re-scanned following sequential incubation with a $4^{th}$ antibody against VGLUT2. The extra-green labelling present in **b**, that was absent in **a**, corresponds to the additional VGLUT2 immunostaining (see NK1+VGLUT2). Notice that all $\alpha_{2C}$-AR-IR terminals, that form appositions on the NK-1 projection cell, are immunoreactive for VGLUT2 (yellow profiles in **b,** merged image on the right) and hence can be considered to be excitatory glutamatergic terminals. (Scale bars: **a** and **b**=20$\mu$m)

a | NK1 | CTb | α2C
b | NK1+VGLUT2 | CTb | α2C
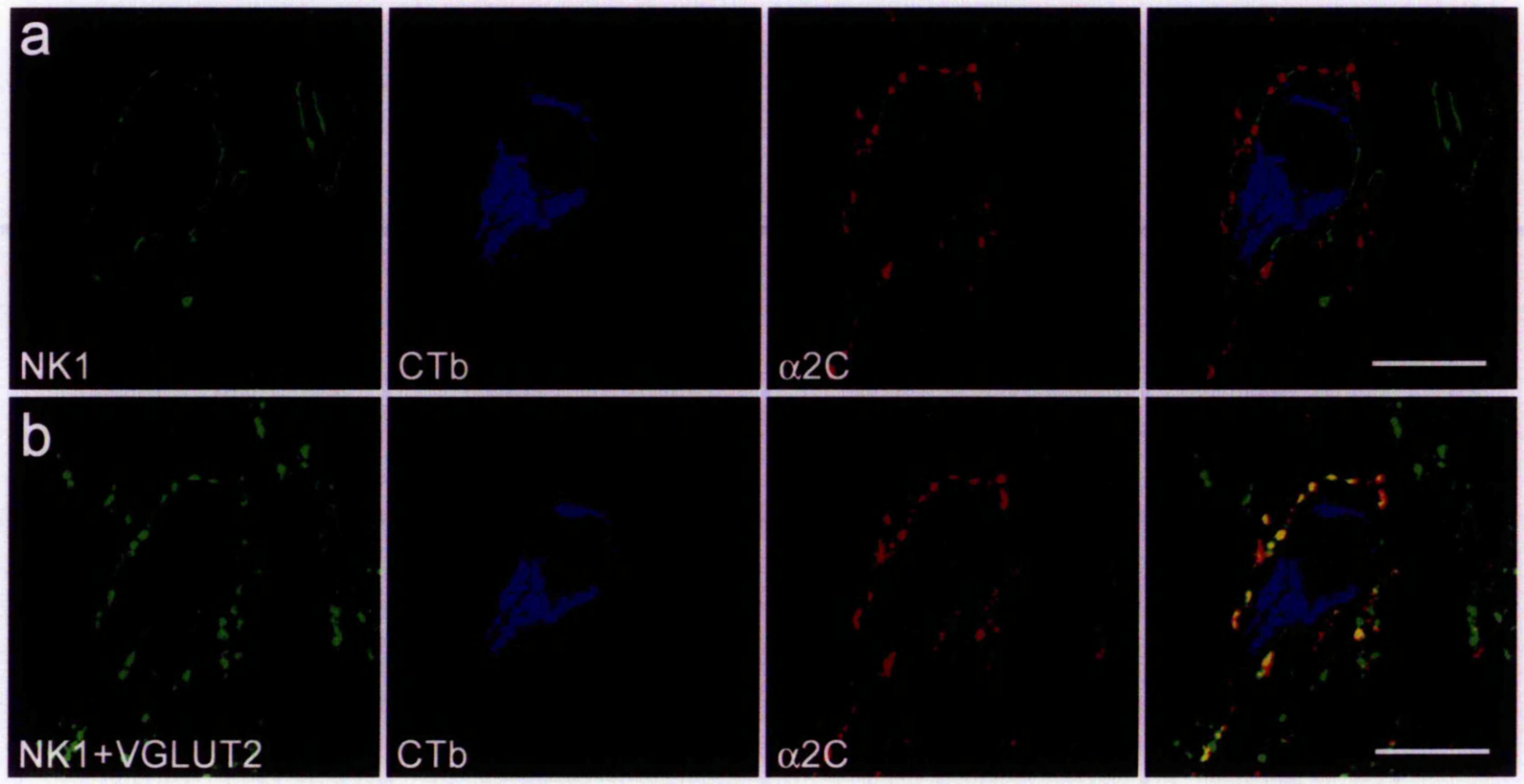
**Figure 4.9. Quadruple-labelling of CTb, the NK-1 receptor, $\alpha_{2C}$-AR and c-Fos in lamina I. d** A projected image of a retrogradely labelled neuron at high magnification (built from 10 single optical sections that were gathered at 0.5µm steps with a X40 lens). The CTb-labelled neuron is positive for NK-1 and c-Fos and receives contacts from $\alpha_{2C}$-AR-IR terminals. **a** shows the distribution of the $\alpha_{2C}$-**AR** ($\alpha$2C); **b** shows the immunoreactivity for c-Fos and NK-1 (c-Fos+NK1); and **c** displays the CTb labelling (CTb). (Scale bar: **a**=20µm, valid for all images)

a  α2C

b  c-Fos+NK-1

c  CTb

d
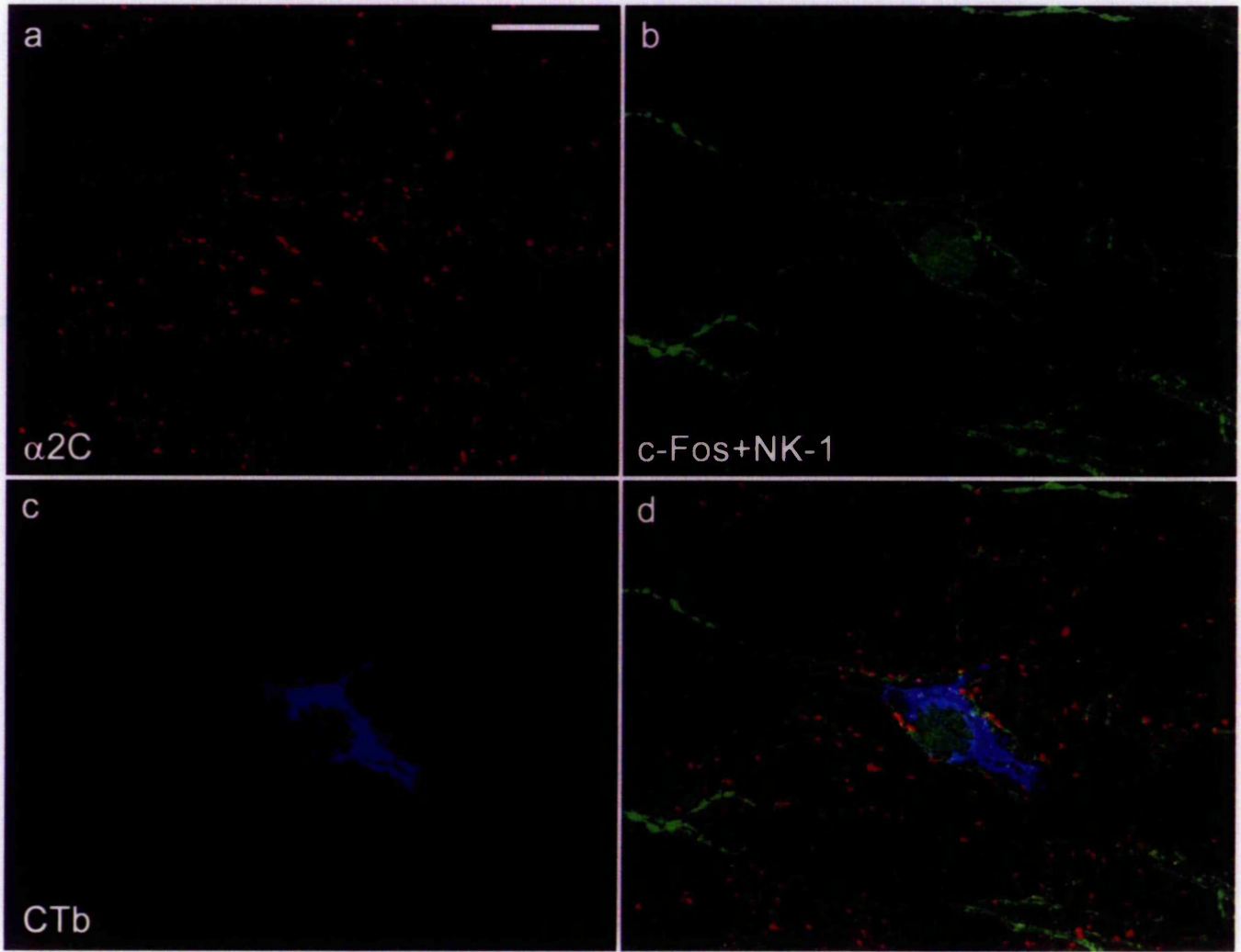
*Chapter 5*

<u>Investigation 3</u>:

**$\alpha_{2C}$-adrenergic receptors in the lateral spinal nucleus**

# 1. Introduction

The lateral spinal nucleus (LSN) is found within the dorsal lateral funiculus at all four levels of the rat spinal cord. However, the LSN is not present in all species, indeed it is found mainly in rodents such as the rat or guinea pig, and is not present in rabbit, cat or human (Gwyn and Waldron, 1969). This nucleus differs from the superficial dorsal horn in the nature of its neuropil. The LSN area contains rostrocaudal myelinated axons, unmyelinated axons and cell bodies encrusted with boutons. On occasions dendrites extend laterally and almost appear to appose the pial surface (Bresnahan et al., 1984). LSN neurons have multipolar and fusiform cell bodies with non-oriented dendrites (Giesler et al., 1979a). Electrophysiological studies indicated that LSN neurons have no obvious cutaneous sensitivity (Giesler et al., 1979b; Menétrey et al., 1980), but that some can be responsive to innocuous movement of joints and deep tissue (Menétrey et al., 1980). Additionally, neurons in the LSN have no spontaneous activity and possess axons with slow conduction velocities (unmyelinated range) that project contralaterally (46%) and bilaterally (40%) (Menétrey and Besson, 1981). Neurochemically, LSN cells were first reported to be positive for ACh (Gwyn and Waldron, 1968), based on the presence of the enzyme acetylcholinesterase; however this is unlikely because later evidence showed that the enzyme is a non specific marker for cholinergic cells (Albanese and Butcher, 1980; Greenfield, 1991). LSN neurons contain SP (Hökfelt et al., 1977; Giesler and Elde, 1985). Leah and coworkers (1988) investigated ascending tract cells in the rat lumbosacral cord containing neuropeptides. Interestingly, 90% of these peptidergic projection cells congregated in two main areas: the LSN, especially, and the region surrounding the central canal. LSN peptidergic neurons contained VIP, bombesin, SP, and dynorphin and were reported to project mainly contralaterally. Retrograde labelling studies showed that LSN neurons project through diverse tracts: the spinoreticular, spinomesencephalic (Menetrey et al., 1982, Pechura and Liu, 1986), spinosolitary (Menétrey and Basbaum, 1987; Leah et al., 1988) and spinohypothalamic tracts (Burstein et al., 1990). An additional characteristic of the LSN is its great content of peptidergic varicosities, which underlies an extensive peptidergic input to LSN neurons. The plexi of SP, enkephalin, dynorphin, somatostatin and FMRF (neuropeptide Y-like substance) present in the LSN were not affected following any experimental lesion that interrupted primary afferent or descending pathways, indicating that the peptidergic afferent input to the LSN arises from the same or nearby segmental levels of the spinal cord (Jessel et al., 1978; Larabi et al., 1983; Seybold and

Elde, 1980; Giesler and Elde, 1985; Cliffer et al., 1988). Not much is known about the function of the LSN, however previous work indicated that $\alpha_{2C}$-ARs are present on axon terminals in the LSN (Stone et al., 1998; Olave and Maxwell, 2002), which suggests that NE is likely to modulate input to LSN neurons by acting at these terminals.

In order to further elucidate the function of the $\alpha_{2C}$-AR in the LSN we investigated the neurochemical profile and morphological properties of $\alpha_{2C}$-AR-IR terminals by using confocal microscopy and electron microscopy, respectively. To study the neurochemical profile of $\alpha_{2C}$-AR-IR terminals we used double-labelling immunocytochemistry and quantitatively studied the patterns of colocalisation of the receptor with diverse markers that label axons of primary afferent, descending systems and populations of inhibitory and excitatory interneurons. In additon, we investigated the involvement of LSN neurons, including CTb retrogradely labelled neurons from the CVLM, in nociceptive transmission by studying the expression of c-Fos following application of a thermal nociceptive stimulus peripherally (Hunt et al., 1987). Finally quintuple immunocytochemistry for NeuN -a neuronal marker-, c-Fos, NK-1, CTb and $\alpha_{2C}$-AR was used to investigate diverse populations of LSN neurons and their relationship with axons that possess $\alpha_{2C}$-ARs.

## 2. Experimental Procedures

### *Double-labelling immunocytochemistry for confocal microscopy*

In this study the experimental procedures used for the analysis of colocalisation with confocal microscopy are basically identical to those described in *Investigation 1 (Chapter 3, pg 36) Confocal Microscopy*, i.e. fixation of tissue, double-labelling immunocytochemistry, labelling of myelinated primary afferent terminals in the lumbar spinal cord, and the quantitative methods of colocalisation including both automated and visual-assessment methods. Double-labelling immunocytochemistry involved combined labelling of the $\alpha_{2C}$-AR with each of the following seventeen markers: DβH, 5-HT, CGRP, SP, neurotensin, somatostatin, enkephalin, neuropeptide Y, galanin, ChAT, NOS, GAD, Gly-T2, VGLUT1, VGLUT2, CTb (injected in the sciatic nerve) and IB4 (for details see; *Chapter 3: Investigation 1*, pg 37). There are a few differences between both investigations regarding the collection of fields with the confocal microscope. Obviously, in the present investigation fields were systematically scanned from the central part of the LSN, rather than the superficial dorsal horn. Two fields, one from the right LSN and one from the left LSN, (with exception for CTb which was only scanned from the left LSN -ipsilateral to the

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sciatic injection-) were collected from six transverse sections (i.e. 12 fields per marker from each of the three animals, making a total of 36 fields per marker). Since each field consists of 6 pairs of optical sections, a total of 216 pairs of images was analysed with the automated method for each marker (i.e. 6 optical sections x 36 fields). An additional specificity of the present study is that several of the markers were absent from the LSN and for this reason they were not scanned for quantification (i.e.VGLUT1, CTb, NOS, IB4, CGRP; Fig. 5.1).

## *Electron microscopy*

The experimental procedures applied in this investigation for electron microscopy have been already described in *Investigation 1* (*Chapter 3*, pg 39) *Electron Microscopy*, i.e. fixation of tissue and processing for immunoelectron microscopy. In this case the area trimmed corresponded to the LSN, instead of the superficial dorsal horn, and the analysis involved qualitative observation of the neuropil and a quasi-quantitative approach.

## *Identification of LSN neurons*

### *Retrograde labelling of spinomedullary neurons*

As described in *Investigation 2* (*Chapter 4*, pg 65) CTb was injected in the left CVLM. Following a three day survival period animals were prepared for induction of the immediate early gene c-Fos.

### *Induction of c-Fos expression*

A set of three rats (Wistar, 250g; Harlan, UK) was deeply anaesthetised (ketamine/xylazine mixture, 7.33 and 0.73mg/100g ip) and then the right hind paw -contralateral to the CTb injection side- was immersed in water at a temperature of 52°C for a period of 20 seconds. This procedure has been explained in detail in *Investigation 2* (*Chapter 4*, pg 68). Animals were maintained under anaesthetic for 2 hours. After this period the animals, which were under deep anaesthesia (ketamine/xylazine mixture), were fixed by perfusion with 4% formaldehyde as previously described (*Chapter 3*, pg 36). For the present investigation transverse sections (50µm thick) were cut with a Vibratome. The c-Fos control experiments described in *Investigation 2* (*Chapter 4*, pg 69) are also valid for the present investigation. Figure 4.1 illustrates the scarcity of c-Fos-labelled neurons when the noxious peripheral stimulation was not applied.

*Immunofluorescent labelling of five markers*

The rationale of multiple-immunolabelling has been explained previously in *Chapter 2* (pg 27); the specific procedure used is described in detail below. Quintuple-labelling immunofluorescence was performed with a guinea pig anti-$\alpha_{2C}$-AR antiserum (diluted 1:500; Neuromics, Minneapolis, U.S.A.; see Stone et al., 1998 for details), rabbit anti-NK-1 antiserum (diluted 1:10,000; Sigma, Poole, Dorset, UK), rabbit anti-c-Fos antiserum (diluted 1:5,000; Hunt et al., 1987), goat anti-CTb antiserum (diluted 1:5,000; List Laboratories, Campbell, CA), and mouse anti-NeuN antiserum (diluted 1:1,000, Chemicon, Harrow, U.K.). After a 48 hour incubation period, sections were rinsed and incubated for 24 hours in solutions containing four different species-specific secondary antibodies raised in donkey. Three of them were coupled to fluorophores: rhodamine-red-anti-guinea pig Ig to identify $\alpha_{2C}$-AR immunoreactivity; FITC-anti-rabbit Ig to identify NK-1 receptor and c-Fos immunoreactivity; and CY5-anti-goat Ig to identify CTb (the three diluted 1:100). The fourth secondary antibody was a biotinylated-anti-mouse Ig which binds to NeuN (diluted 1:500, all obtained from Stratech, Cambridge, U.K.). Notice that the NK-1 receptor and c-Fos can be labelled with the same secondary antibody because they are located in clearly different cellular structures, cell membrane and nucleus, respectively, which allows distinction of both types of immunolabelling through the same channel (discussed in *Chapter 2*, pg 28). Following rinsing, the sections were incubated for 24 hours in solutions containing avidin coupled to the fluorophore pacific blue to identify the biotinylated antibody coupled to NeuN (diluted 1:1,000; Molecular Probes, Leiden, The Netherlands). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% normal donkey serum. Once rinsed, the sections were mounted in anti-fade medium (Vectashield; Vector Laboratories, Peterborough, UK) and stored in a freezer at -20°C.

*Quantitative analysis*

The right and left LSNs were systematically scanned using a Radiance 2100 confocal laser scanning microscope, equipped with Argon/Green Helium Neon/Red diode/Blue diode lasers with a X40 oil-immersion lens at 1μm intervals in the Z-axis and a zoom factor of 1. Each field included the total extent of the LSN (1024x1024pixels=301x301μm) in the transverse plane and consisted of 20 optical sections that represent a field depth of 20μm in the rostro-caudal axis. From each of the three animals, two fields (one from the left LSN and one from the right LSN) were scanned from six transverse sections (i.e. a total of 36 fields, 18 from the left LSN -ipsilateral to the injection site- and 18 from the

right LSN -contralateral to the injection site and ipsilateral to the c-Fos induction side-). The quantitative analysis involved three steps. First, the number of LSN neurons was calculated by counting the neuronal cell bodies, which were revealed by NeuN. Second, the neurons were classified into one of the following eight categories according to their pattern of immunoreactivity:

1.    Only immunoreactive for c-fos
2.    Only immunoreactive for CTb
3.    Only immunoreactive for NK-1
4.    Double-labelled for c-Fos and CTb
5.    Double-labelled for c-Fos and NK-1
6.    Double-labelled for CTb and NK-1
7.    Triple-labelled for c-Fos, CTb and NK-1
8.    Unlabelled by any of the markers but NeuN

Third, LSN neurons were assessed for $\alpha_{2C}$-AR innervation by examining their relationship with $\alpha_{2C}$-AR-IR axon terminals; only LSN neurons labelled with CTb and/or NK-1 (i.e. those corresponding to category 2 to 7) were studied since these were the only markers revealing the outline of the cell, which is essential to assess the relationship between the neuron and the terminals.

Finally, the following calculations were produced to summarise the data; 1 total count of each category of LSN neuron per experiment, 2 average and standard deviation of each category from the three experiments, 3 percentage of each category of LSN neuron to calculate the proportion of each category of neuron in the LSN. Equivalent calculations were done for LSN neurons receiving innervation from $\alpha_{2C}$-AR-IR terminals (i.e. CTb and/or NK-1 neurons, categories 2 to 7). Statistical comparisons between the values obtained from the left and right LSNs were made by using *Student's t-test*. P values less than 0.05 were considered to be significant.

## 3. Results

### *Neurochemical profile of $\alpha_{2C}$-AR-IR terminals in the LSN*

The markers used in this investigation are the same as those used in *Investigation 1* for the superficial dorsal horn. In this case, of the seventeen markers applied only twelve were found in the LSN and therefore were consequently quantified. Images a-e in Figure 5.1, show the lack of labelling in the LSN for CTb (injected in the sciatic nerve), IB4,

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CGRP, VGLUT1 and NOS. Examples of LSN fields for the $\alpha_{2C}$-AR and the rest of the markers are exhibited in Figure 5.2 and 5.3. The results obtained from both types of quantitative analysis (i.e. automated or visual) are summarised in Table 5.1 (below) and in the histograms in Figure 5.4.

No overlap of immunoreactivity was found between the $\alpha_{2C}$-AR and D$\beta$H or 5-HT (0.08% and 0.06% respectively) indicating that neither noradrenergic nor serotoninergic axons possess the receptor (Fig. 5.2, a-b). Similarly, markers for ChAT, which labels cholinergic cells and terminals (Barber et al., 1984; Borges and Iversen, 1986; Sheriff and Henderson, 1994); Gly-T2, which labels glycinergic terminals (Zafra et al., 1995); and galanin exhibited minimal overlap (0.04%, 0.26%, 0.79% respectively; Fig. 5.2, c-e). The majority of terminals labelled with these markers probably originate from inhibitory local circuit neurons (Todd and Spike, 1993). Nevertheless, there was evidence of moderate overlap of the receptor with neuropeptide Y (3.22%; Fig. 5.3, a), a marker for axons of inhibitory cells (Rowan et al., 1993) and neurotensin (4.91%, Fig. 5.3, b), which originates from excitatory interneurons (Tood et al., 1992; Todd et al., 1994b). The $\alpha_{2C}$-AR was also found on axons containing SP, somatostatin and enkephalin with which the receptor displayed a substantial overlap (16.41%, 26.62% and 30.10% respectively; Fig. 5.3, c-e).

*Inhibitory versus excitatory $\alpha_{2C}$-AR-IR terminals in the LSN*

The visual-assessment quantitative method was applied to estimate the proportion of $\alpha_{2C}$-AR-IR terminals that are likely to be excitatory or inhibitory. The markers VGLUT2 and GAD were used to label terminals of spinal interneurons containing excitatory or inhibitory amino acid transmitters respectively. The great majority of $\alpha_{2C}$-AR-IR boutons contained immunoreactivity for VGLUT2 while a minority of the $\alpha_{2C}$-AR-IR axon terminals was associated with GAD immunoreactivity (Fig. 5.3, f-g). The quantitative analysis derived from the visual assessment revealed that 82.33±3.51% of $\alpha_{2C}$-AR-IR boutons contain VGLUT2 and 10.67±4.16% contain GAD. (see Table 5.1 below and Fig. 5.4, histogram 2).

**Table 5.1.** Automated and visual quantification of colocalisation patterns of the $\alpha_{2C}$-AR in the LSN

| | marker | Overlap | ± SD |
|---|---|---|---|
| | DβH | 0.08 | 0.12 |
| | 5-HT | 0.06 | 0.10 |
| | ChAT | 0.04 | 0.05 |
| AUTOMATED | Gly-T2 | 0.26 | 0.26 |
| | Galanin | 0.79 | 0.13 |
| METHOD | Neuropeptide Y | 3.22 | 0.31 |
| | Neurotensin | 4.91 | 2.84 |
| | SP | 16.41 | 0.89 |
| | Somatostatin | 26.62 | 2.24 |
| | Enkephalin | 30.10 | 3.82 |

| | marker | Frequency | ± SD |
|---|---|---|---|
| VISUAL-ASSESSMENT | | | |
| | VGLUT2 | 82.33 | 3.51 |
| METHOD | GAD | 10.67 | 4.16 |

*Ultrastructure of $\alpha_{2C}$-AR-IR terminals in the LSN*

Observation with the electron microscope showed the high content of myelinated and unmyelinated axons in the LSN and the presence of neurons surrounded by axon terminals. Many of these terminals were immunoreactive for the $\alpha_{2C}$-AR and formed synaptic contacts on the cell body and dendrites of LSN neurons. Often many of these synapses could be clearly identified as asymmetric, but occasionally it was difficult to determine if they were symmetrical or asymmetrical. See Figure 5.5. Additionally, no axoaxonic arrangements were found.

*Relationship of LSN neurons with $\alpha_{2C}$-AR-IR terminals*

The average number of sampled neurons, as detected by the neuronal marker NeuN, was very consistent between experiments and also between the right and left sides of the spinal cord (right LSN, average±SD=54±5.57; left LSN, 53±2.79). Following counting for each of the defined neuronal categories, averages (±SD) and percentages were calculated. Examples of LSN neurons corresponding to each of the eight established categories are shown in Figure 5.6, 5.7 and 5.8. Table 5.2 (below) exhibits the percentages obtained for each of the eight categories of LSN neuron (see first row 'COUNT'), as well as the percentage of neurons in each category that received innervation from $\alpha_{2C}$-AR-IR terminals (see second row 'A2C INPUT'). Statistical comparisons of data corresponding to

the right and the left sides of the lumbar cord revealed that there is no significant difference in the proportion of each neuronal population between the right and the left side (*t-test*, P>0.05).

The average percentage of all c-Fos positive cells (i.e. sum of categories 1, 4, 5 and 7) in the right LSN is similar to that one in the left LSN (23.70±2.65% and 20.73±3.81% respectively). Again, there is very little difference between the right (7.51±1%) and left (6.71±1.06%) LSN regarding the proportion of c-Fos projection cells (i.e. those labelled with c-Fos and CTb corresponding to categories 4 and 7). Indeed, none of these differences were found to be significant statistically (*t-test*, P>0.05). The total proportion of CVLM projection neurons (i.e. categories 2, 4, 6 and 7) was slightly higher in the right LSN (42.77±3.61%), which is contralateral to the injection site in the brainstem, than in the left LSN (35.37±3.81%). Statistical comparison demonstrated that this difference is significant (*t-test*, P=0.035). Note that the P value is close to 0.05, which suggests that the difference is significant but not highly significant. Indeed, this difference is considerably more obvious in the dorsal horn (Todd et al., 2000).

A high proportion of LSN neurons received innervation from $\alpha_{2C}$-AR-IR terminals; 90.10±9.07% and 85.06±3.98% of the neurons in the right and left LSN, respectively, were apposed by terminals positive for the $\alpha_{2C}$-AR. Interestingly, almost all of the NK-1 retrogradely labelled neurons were innervated by $\alpha_{2C}$-AR-IR terminals (right LSN, 100±0%; left LSN, 96.15±16.65%). Neurons that were only positive for c-Fos or NeuN, were not assessed for $\alpha_{2C}$-AR innervation, because the outline of the cell was not consistently defined by any marker.

**Table 5.2.** Table summarising the percentages obtained for each of the eight categories of LSN neuron

| | AVERAGE PERCENTAGE OF EACH CATEGORY | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| **RIGHT** | c-fos | cvlm | nk1 | c-fos+cvlm | c-fos+nk1 | cvlm+nk1 | c-fos+cvlm+nk1 | NeuN only |
| COUNT | 13.87 | 2.89 | 13.29 | 0.58 | 2.31 | 32.37 | 6.94 | 27.75 |
| A2C-INPUT | * | 2.31 | 8.09 | 0.58 | 2.31 | 32.37 | 6.94 | * |
| **LEFT** | c-fos | cvlm | nk1 | c-fos+cvlm | c-fos+nk1 | cvlm+nk1 | c-fos+cvlm+nk1 | NeuN only |
| COUNT | 10.37 | 2.44 | 14.02 | 1.22 | 3.66 | 26.22 | 5.49 | 36.59 |
| A2C-INPUT | * | 1.83 | 8.54 | 1.22 | 3.05 | 25.00 | 5.49 | * |

## 4. Discussion

*$\alpha_{2C}$-ARs in the LSN are of spinal origin*

The results confirm that $\alpha_{2C}$-ARs in the LSN are not present on descending noradrenergic or serotoninergic terminals, or on myelinated, unmyelinated or peptidergic primary afferents labelled with CTb, IB4 and CGRP. Indeed, the absence of CTb, IB4, CGRP and also VGLUT1 (marker associated with large myelinated primary afferents; Todd et al., 2003) from the LSN not only indicates that the receptor is not present on these types of terminal, but also implies that LSN neurons do not receive input from these classes of primary afferent. These results are in agreement with previous studies. Stone and coworkers (1998) reported that the $\alpha_{2C}$-AR is of spinal origin, because immunoreactivity for the receptor is not reduced after rhizotomy or neonatal capsaicin treatment, either in the superficial dorsal horn, or in the LSN. In addition, electrophysiological studies showed that LSN neurons do not have obvious cutaneous sensitivity (Giesler et al., 1979b; Menétrey et al., 1980), which agrees with the observed lack of primary afferent markers in the LSN. This body of evidence indicates that, as in the superficial dorsal horn, few if any $\alpha_{2C}$-AR-IR terminals originate from primary afferents in the LSN. However it is also possible that $\alpha_{2C}$-ARs exist on a subgroup of primary afferents which are not labelled by CTb, IB4 or CGRP.

*$\alpha_{2C}$-ARs in the LSN are predominantly excitatory*

The visual-assessment method of quantification showed that in the LSN over 80% of the $\alpha_{2C}$-AR-IR terminals also contain VGLUT2, which suggests that $\alpha_{2C}$-ARs are present on axons of glutamatergic interneurons and therefore are presumed to have an excitatory action (Todd et al., 2003). A small proportion of $\alpha_{2C}$-AR-IR terminals (~10%) was found to contain GAD, which indicates that these terminals are GABAergic and therefore are expected to have an inhibitory action (Todd and Spike, 1993). Observations derived from the electron microscopic analysis are in agreement with the latter results, because the great majority of $\alpha_{2C}$-AR-IR terminals form asymmetric axodendritic synapses, which are predicted to have an excitatory action (Uchizono, 1965). Results derived from the automated method further support the findings obtained with the visual-assessment method. The former method indicated that $\alpha_{2C}$-AR immunoreactivity overlaps considerably with neuropeptides (neurotensin, SP, somatostatin and enkephalin), which are associated with high proportions of VGLUT2 immunoreactivity (Todd et al., 2003). All

these findings closely resemble the situation in the superficial dorsal horn, however two main differences were found: 1 the proportion of asymmetric synapses is higher in the LSN, which could be explained partly by the lack of axoaxonic synapses; 2 $\alpha_{2C}$-AR immunoreactivity overlaps with SP immunoreactivity in the LSN, which must be of spinal origin, because there is no CGRP labelling. This body of evidence strongly indicates that although differences exist between both spinal regions, the majority of $\alpha_{2C}$-AR-IR terminals contain glutamate and have an excitatory action either in the superficial dorsal horn or in the LSN.

*$\alpha_{2C}$-ARs in the LSN are derived from diverse subpopulations of spinal interneurons*

The results indicate that $\alpha_{2C}$-ARs in the LSN are derived from an inhibitory and an excitatory population of interneurons. Based on the colocalisation of the receptor with the inhibitory markers GAD and neuropeptide Y, and the lack of colocalisation with Gly-T2 -a marker for glycine (Zafra et al., 1995)-, we can conclude that, as in the superficial dorsal horn, such inhibitory population corresponds to the GABA only subpopulation of interneurons, which do not contain glycine, and can also contain neuropeptide Y (Todd and Spike, 1993; Rowan et al., 1993). The excitatory population includes several peptidergic subpopulations of interneurons, which can contain neurotensin, SP, somatostatin and enkephalin. The neurotensin and somatostatin populations have been shown to be present in distinct exclusive populations of interneurons in the dorsal horn (Proudlock et al., 1992) and therefore they can be presumed to represent distinct excitatory subpopulations in the LSN, since peptidergic terminals in this nucleus have been suggested to arise from the same or nearby segmental levels of the spinal cord (Jessel et al., 1978; Larabi et al., 1983; Seybold and Elde, 1980; Giesler and Elde, 1985; Cliffer et al., 1988). Conversely, SP, somatostatin and enkephalin colocalise in the dorsal horn (Todd and Spike, 1993). Indeed preliminary observations indicated that SP and enkephalin colocalise considerably in the LSN (Fig. 5.9) and enkephalin is known to colocalise greatly with somatostatin (Todd and Spike, 1993), which suggests that a considerable proportion of the $\alpha_{2C}$-ARs in the LSN is likely to be derived from an interneuronal subpopulation that expresses the three peptides. The results obtained from *Investigation 1* (*Chapter 3*, pg 40) regarding the neurochemical profile of $\alpha_{2C}$-AR-IR terminals in the superficial dorsal horn are very similar to the present results in the LSN; however an obvious difference can be noticed. Overlap of immunoreactivity between the $\alpha_{2C}$-AR and SP is found in the LSN, while it is absent in the

superficial dorsal horn. This divergence indicates that a specific population of interneurons containing SP gives rise to axons in the LSN which possess $\alpha_{2C}$-ARs on their terminals.

*Axon terminals possessing $\alpha_{2C}$-ARs are presynaptic to LSN neurons*

Observation at the ultrastructural level showed that $\alpha_{2C}$-AR-IR terminals are presynaptic to cell bodies and dendrites of LSN neurons with which they predominantly establish asymmetric synapses. Our results are in agreement with previous electron microscopic studies of the LSN. Bresnahan and coworkers (1984) reported that SP and enkephalin-IR boutons in the LSN form asymmetric synapses on LSN neurons.

*LSN neurons are activated by noxious stimulation*

A great number of LSN neurons was labelled by other marker/s in addition to NeuN, however, a proportion of LSN neurons was only labelled by NeuN (right LSN, 27.75%; left LSN, 36.59%). These NeuN only labelled neurons could be projecting to supraspinal regions different to the CVLM, or alternatively could be local interneurons. Based on our classification the most common type of cell was the NK-1 neuron projecting to the CVLM which occasionally showed c-Fos expression.

The presence of immunoreactivity for the immediate early gene c-Fos in nuclei of the LSN indicates that thermal noxious stimulation activates a proportion of LSN neurons. Although NK-1 projection neurons which expressed c-Fos were not very numerous, the presence of such neurons is bound to be meaningful because omission of the thermal stimulus in the control experiment resulted in lack of c-Fos expression, which demonstrates that the c-Fos detected is most probably due to the noxious stimulation applied.

In addition, the mechanism of cellular activation, as detected by c-Fos, would be expected to be different from that occurring in the dorsal horn for diverse reasons. First, no cutaneous primary afferent input has been detected in LSN neurons. Second, LSN cells were activated bilaterally and no significant difference was found in the proportions of c-Fos-IR neurons between the right and the left LSNs, i.e. ipsi and contra-lateral to peripheral stimulation, unlike the situation in the superficial dorsal horn where neurons must be activated mainly unilaterally since the highest concentration of c-Fos-IR neurons is located on the dorsal horn ipsilateral to the peripheral stimulation (Hunt et al., 1987; Todd et al., 1994a; Todd et al., 2002; and also results from *Investigation 2*, pg 70).

*Possible mechanisms of activation*

LSN neurons could be activated by descending fibres that originate from supraspinal nuclei, which in turn are activated by ascending fibres of spinal projection neurons located in the superficial dorsal horn. There is evidence that superficial dorsal horn neurons expressing NK-1 activate descending pathways, which control spinal excitability (Suzuki et al., 2002). The activation of LSN neurons by descending fibres projecting bilaterally could explain the bilateral appearance of c-Fos.

LSN neurons could be also activated by spinal neurons. Grudt and Perl (2002) reported that some lamina I neurons possess occasional axon collaterals that enter the dorsal lateral funiculus. Interestingly these neurons have characteristic thick axons, which course ventrally and cross contralaterally to project in the ventral lateral funiculus. Therefore, it is possible that this type of lamina I projection neuron is activated by primary afferent input and in turn activates LSN neuron/s through an axon collateral. Interneurons would be expected to influence the LSN, since the dense peptidergic input to the LSN is of spinal origin (Jessel et al., 1978; Larabi et al., 1983; Seybold and Elde, 1980; Giesler and Elde, 1985; Cliffer et al., 1988) and possibly activate neurons because as indicated by the present investigation most of this input is supposed to be excitatory. Also commissural interneurons could be involved in the neuronal circuit responsible for bilateral activation of LSN neurons. Petkó and Antal (2000), using retrograde and anterograde labelling techniques, showed that neurons in the lateral part of the dorsal horn possess commissural axons that project to the lateral aspect of the dorsal horn in the contralateral side. Careful observation of their diagrams provides evidence for labelling of neurons and axons in the LSN, after injections of retrograde and anterograde tracers, respectively, in the lateral dorsal horn. This observation suggests that neurons in the LSN could be activated by ipsi and/or contra-lateral projections from neurons in the lateral dorsal horn. Furthermore, it is possible that the tracer injections partially invaded the LSN (as appears in the photographs), in which case it could be suggested that LSN neurons themselves project contralaterally and can be reciprocally activated. Of course both mechanisms, involving descending and spinal neurons, could be combined to mediate bilateral activation of LSN neurons.

Additionally or alternatively, LSN neurons may be activated by primary afferents not detected. Indeed, unmyelinated primary afferent terminals from muscle have been identified in the dorsal lateral white matter of the guinea pig spinal cord recently (Ling et al., 2003). It is also possible that the nucleus receives visceral primary afferent input;

Sugiura and coworkers (1989) reported the dorsal lateral funiculus as a termination area of visceral unmyelinated fibres.

LSN neurons frequently display dendrites that extend toward the pial surface of the cord where they branch; transmitters circulating in the CSF could have an effect on the outermost distal dendrites of LSN neurons. This is likely since neurons in the dorsal medial funiculus, which possessed similar kind of surface dendrites, have been reported to respond to neurotransmitters and drugs injected in the CSF (Abbadie et al., 1999). Of course drugs in the CSF are expected to influence the whole of the spinal cord and indeed this is the basis of intrathecal administration of pharmacological compounds through canulae. However, in physiological conditions the concentration of neuroactive substances in the CSF is likely to be lower and the superficially located dendrites of LSN neurons could be influenced by neurotransmitters in a more sensitive manner than most of the spinal grey.

*LSN neurons are innervated by axon terminals possessing $\alpha_{2C}$-ARs*

Many of the LSN neurons assessed (i.e. NK-1 and/or CVLM neurons) were found to receive innervation from $\alpha_{2C}$-AR-IR terminals. Indeed, almost all NK-1 projection neurons in the LSN received this type of innervation, which is approximately twice the value obtained in *Investigation 2* for lamina I NK-1 projection neurons (*Chapter 4*, pg 71). This suggests that NE may have a very powerful influence in the modulation of LSN neurons and in particular NK-1 neurons projecting to the CVLM.

*Functional significance*

The LSN may function as an integrative nucleus where neurons could receive inputs from unmyelinated muscle and visceral primary afferents, neurons and interneurons in the spinal grey and as well could be influenced by neurotransmitters circulating in the CSF. Unlike the dorsal horn, the LSN does not receive cutaneous primary afferent input; and interestingly the vast majority of their neurons are densely innervated by spinal terminals containing $\alpha_{2C}$-ARs.

Another interesting specific property of the LSN is its great content of peptidergic projection neurons (Leah et al., 1988). Based on the latter observation, Leah and coworkers (1988) suggested that activation of this type of neuron could lead to release of the peptide/s at supraspinal levels and therefore modulate the activity of diverse nuclei to which LSN neurons project.

LSN neurons and dorsal horn neurons have common supraspinal projections, however two main differences have been reported regarding their ascending projections.

Retrograde labelling studies indicate that LSN neurons, unlike dorsal horn neurons, do not project to the thalamus (Giesler et al., 1979a; Kevetter and Willis, 1983). In contrast, many LSN neurons were retrogradely labelled from the hypothalamus, while only a minority of dorsal horn neurons was labelled (Burstein et al., 1990). This is a very interesting observation; noxious stimulation, mechanical stimulation of nipples during lactation, changes in temperature of the skin, stimulation of pelvic and abdominal viscera are known to trigger the firing of hypothalamic neurons, that in turn regulate autonomic and neuroendocrine functions as heart rate, blood pressure, vasodilation, and feeding, drinking and satiety behaviours. LSN neurons may be an essential element of the circuitry underlying such autonomic and homeostatic functions.

**Figure 5.1. Lack of CTb, IB4, CGRP, VGLUT1 and NOS in the LSN. a** An image of the dorsolateral area of the spinal cord obtained by combining transmitted light with immunofluorescence for the $\alpha_{2C}$-AR (green). The spinal grey matter can be observed to contain intense green ($\alpha_{2C}$-AR) labelling. The adjacent LSN is labelled in the image and also contains $\alpha_{2C}$-AR immunoreactivity. **b-f** show double-labelling immunocytochemistry for the $\alpha_{2C}$-AR (green) and NOS, IB4, CGRP, CTb (injected in the sciatic nerve) and VGLUT1 (all in red), respectively. Note that no immunoreactivity (red) is found in the LSN for any of the markers. (Scale bar: **b**=100μm, valid for all images)
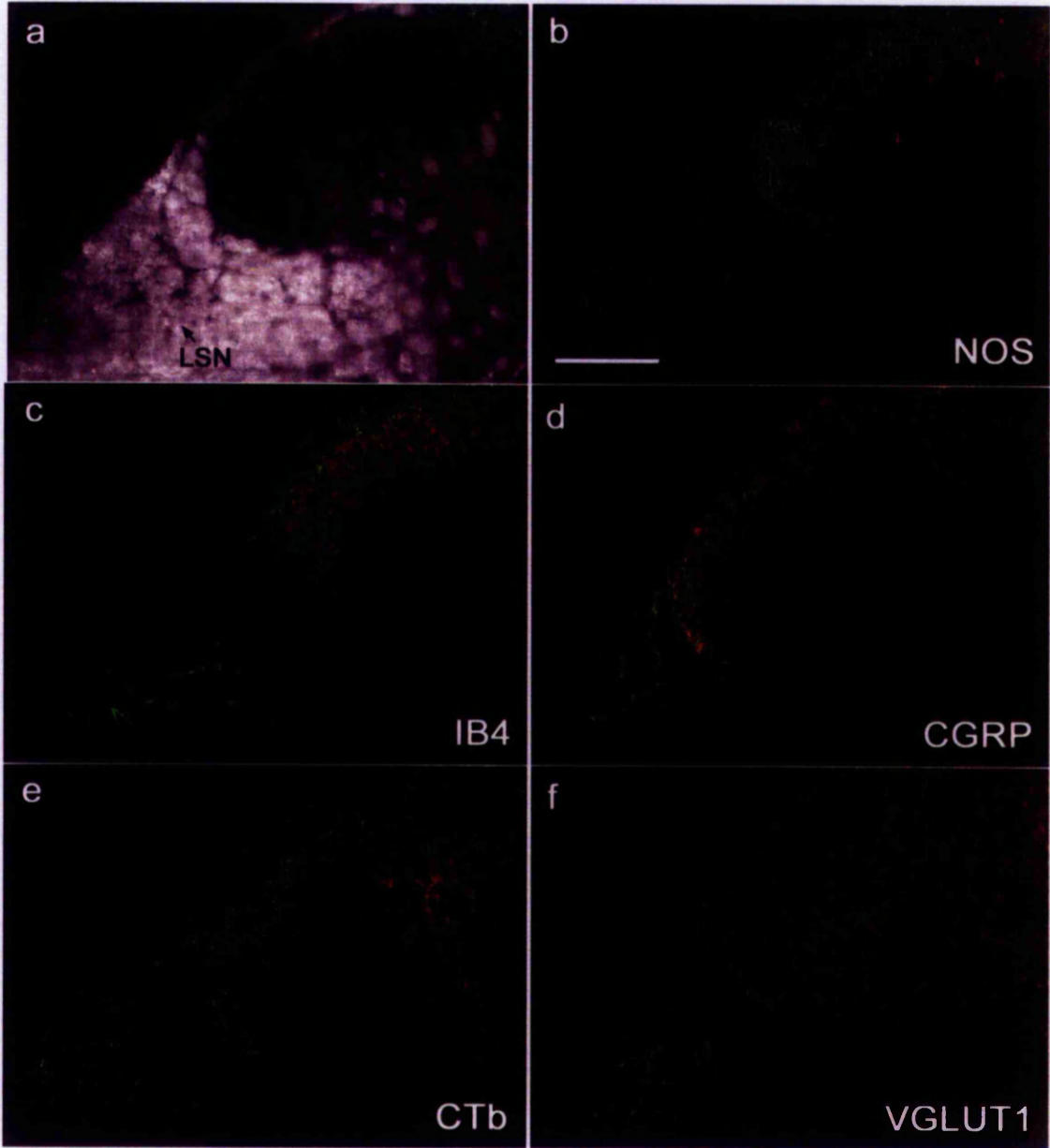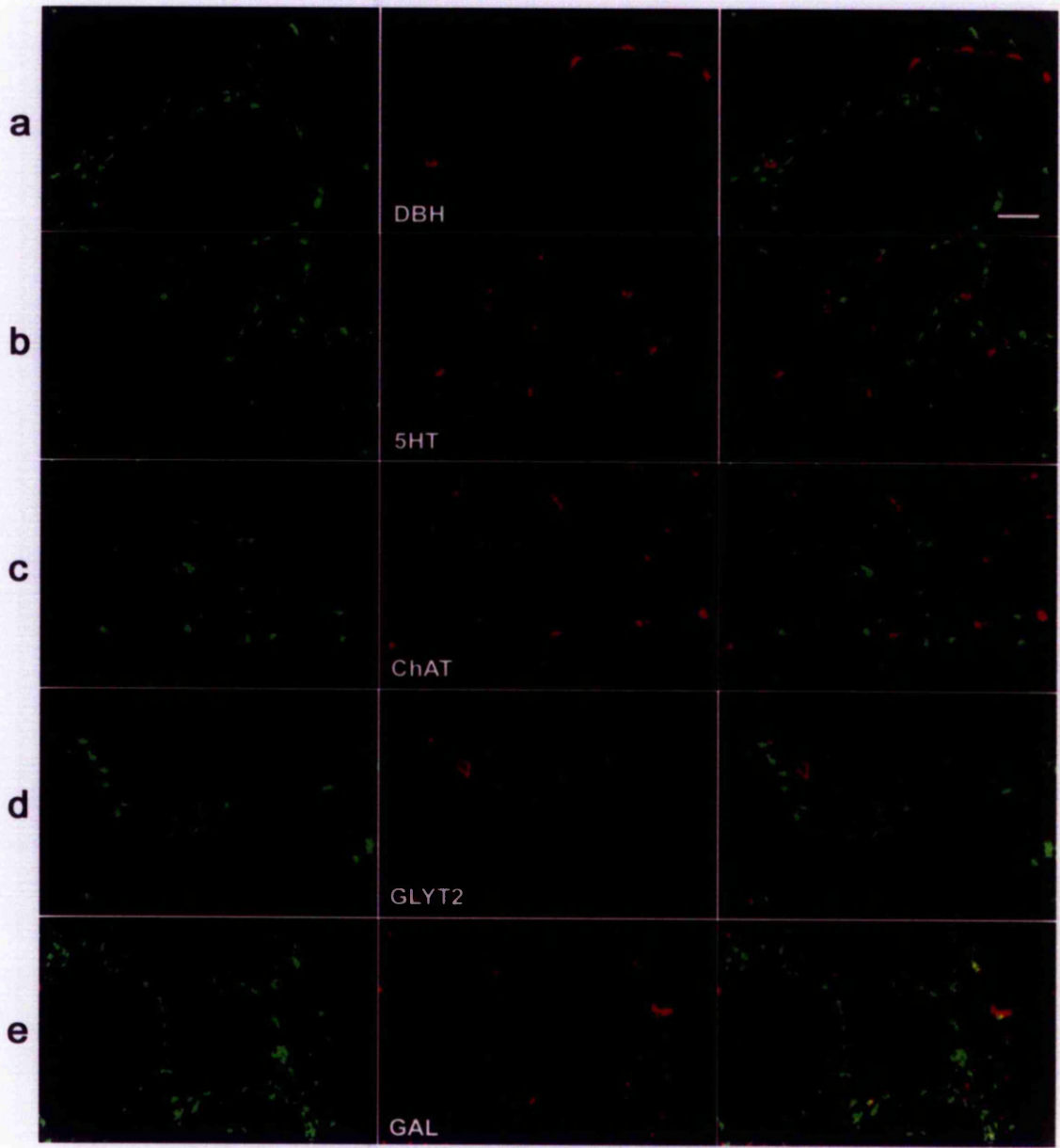
**Figure 5.2. Double-labelling of the $\alpha_{2C}$-AR with D$\beta$H, 5HT, ChAT, Gly-T2 and galanin.** Five sets of three images obtained from the same optical plane (**a-e**). In each set, the first image shows labelling for the $\alpha_{2C}$-AR (green), the second image shows labelling of the corresponding neurochemical marker (red), and the third one is the result of merging both images where no overlap can be detected (note lack of yellow colour).
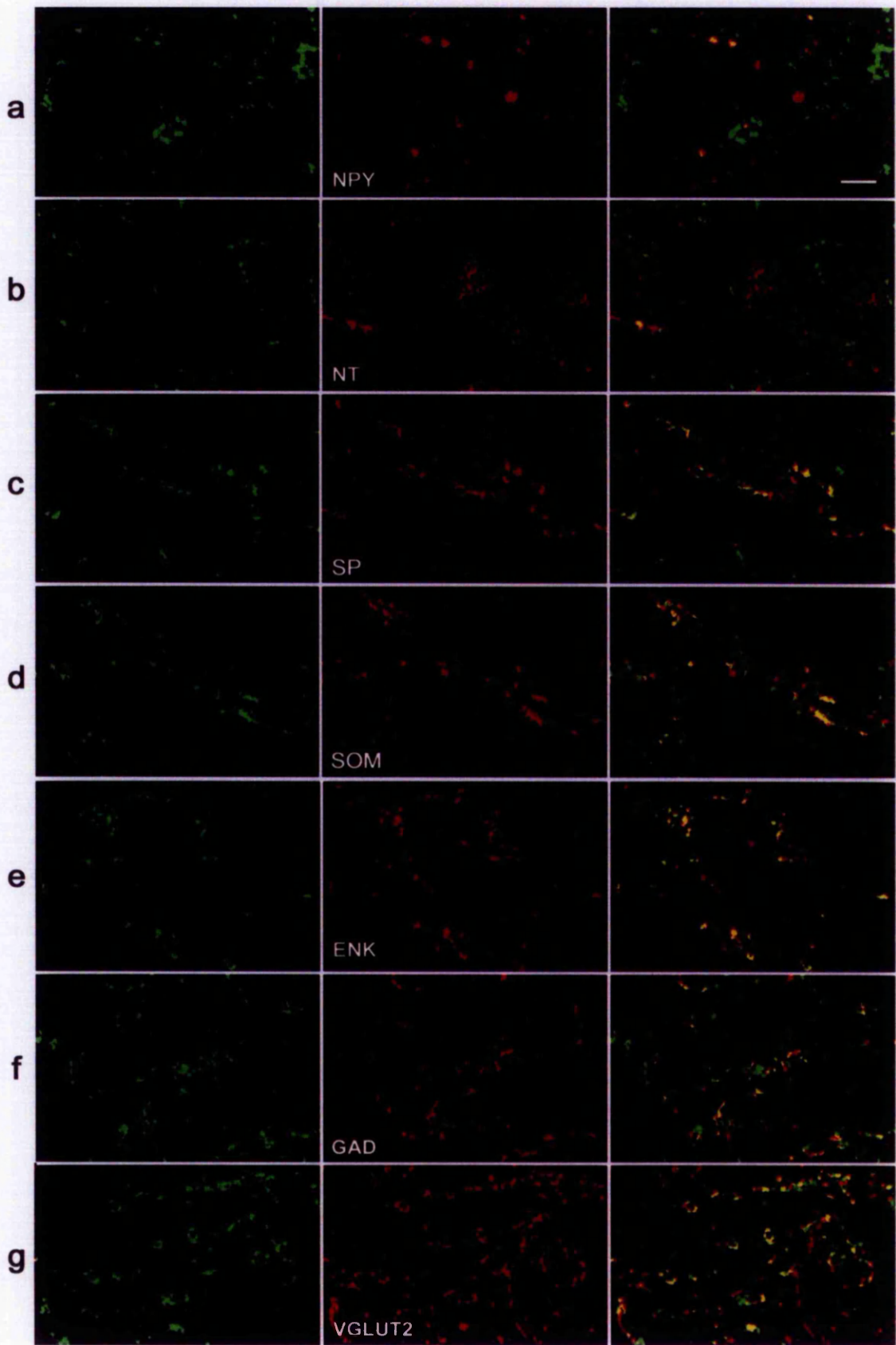(Scale bar: **a**=20μm, valid for all images)

*(DBH, dopamine β-hydroxylase; 5-HT, serotonin; ChAT, choline acetyltransferase, GLYT2, glycine transporter 2; GAL, galanin)*

a    DBH

b    5HT

c    ChAT

d    GLYT2

e    GAL

**Figure 5.3. Double-labelling of the $\alpha_{2C}$-AR with neuropeptide Y, neurotensin, SP, somatostatin, enkephalin, GAD and VGLUT2.** Seven sets of three images obtained from the same optical plane (**a-g**). In each set, the first image shows labelling for the $\alpha_{2C}$-AR (green), the second image shows labelling of the corresponding neurochemical marker (red), and the third one is the result of merging both images where overlap can be detected (note presence of yellow colour).

(Scale bar: **a**=20μm, valid for all images)

*(NPY, neuropeptide Y; NT, neurotensin; SP, substance P; SOM, somatostatin; ENK, enkephalin; GAD, glutamic acid decarboxylase; VGLUT2, vesicular glutamate transporter 2)*

NPY

NT

SP

SOM

ENK

GAD

VGLUT2

**Figure 5.4. Histograms summarising the quantification of colocalisation patterns of the $\alpha_{2C}$-AR in the LSN.** Histogram 1 shows the average percentages of overlap of the $\alpha_{2C}$-AR with each of the ten markers. Histogram 2 shows the average frequency of $\alpha_{2C}$-AR-IR terminals which colocalise with GAD or VGLUT2. The majority of $\alpha_{2C}$-AR-IR puncta were immunoreactive for VGLUT2 and a small number were immunoreactive for GAD. (n=3; Error bars=standard deviation)

*(DBH, dopamine β-hydroxylase; 5-HT, serotonin; ChAT, choline acetyltransferase; GLYT2, glycine transporter 2; GAL, galanin; NPY, neuropeptide Y; NT, neurotensin; SP, substance P; SOM, somatostatin; ENK, enkephalin; GAD, glutamic acid decarboxylase; VGLUT2, vesicular glutamate transporter 2)*
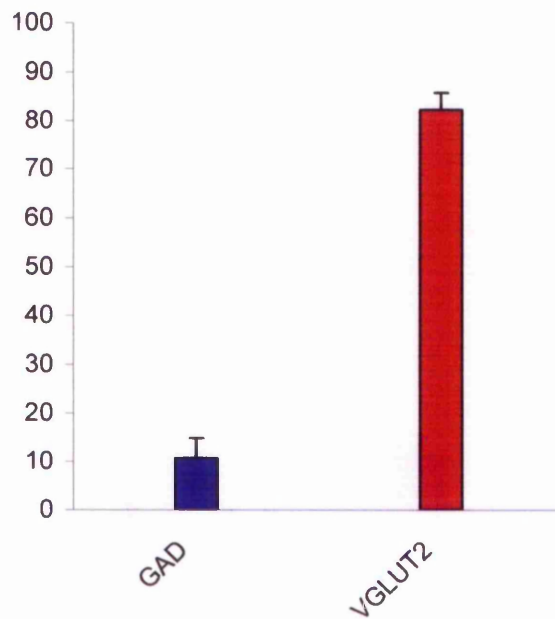
Histogram 1. Overlap of $\alpha_{2C}$-AR immunoreactivity with diverse markers in the LSN
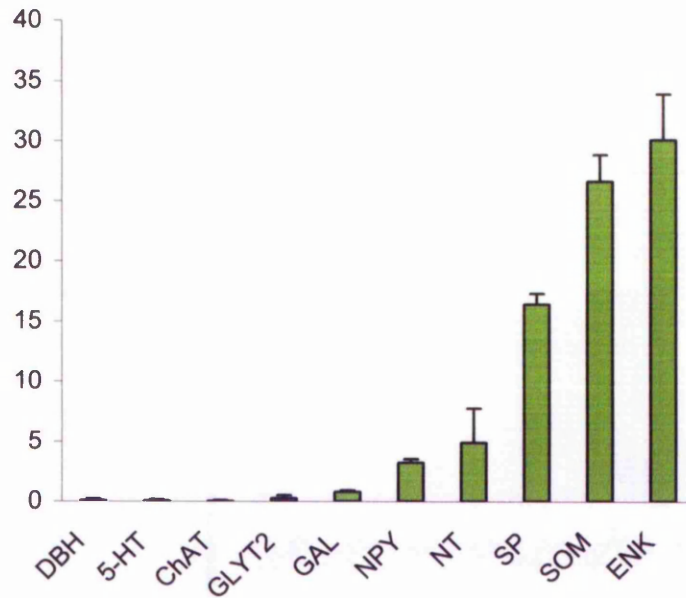


Histogram 2. Frequency of excitatory and inhibitory $\alpha_{2C}$-AR-IR terminals in the LSN

**Figure 5.5. Electron micrographs of $\alpha_{2C}$-AR-IR profiles in the LSN.** All images were obtained from ultrathin sections cut from transverse 50μm thick sections of spinal cord. **a** A low power micrograph of a cell in the LSN; note the numerous myelinated axonal fibres surrounding the cell. **b** A large $\alpha_{2C}$-AR-IR terminal makes contact with a dendrite that extends in a mediolateral direction. **c** Several $\alpha_{2C}$-AR-IR terminals form synapses with dendrites oriented in the rostrocaudal direction (**D1, D2**). The area delineated by the blue (left) and magenta (right) boxes is shown in a magnified view in **d** and **e**, respectively. Both synapses are asymmetric. (Scale bars: **a**=5μm; **b**=2μm, valid for **c**; **d**=0.2μm, valid for **e**).
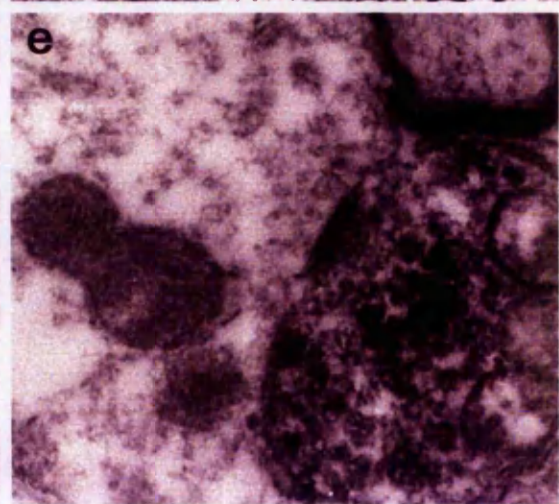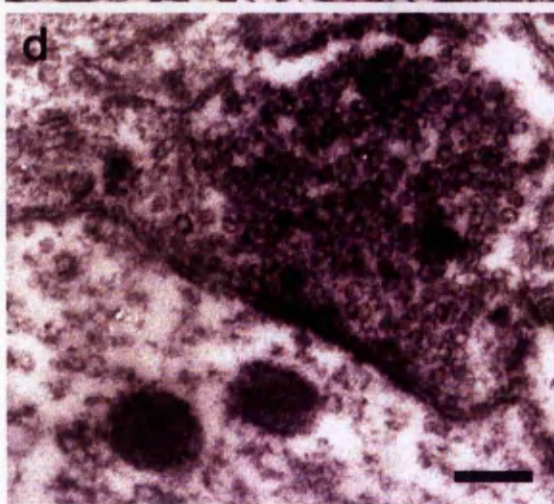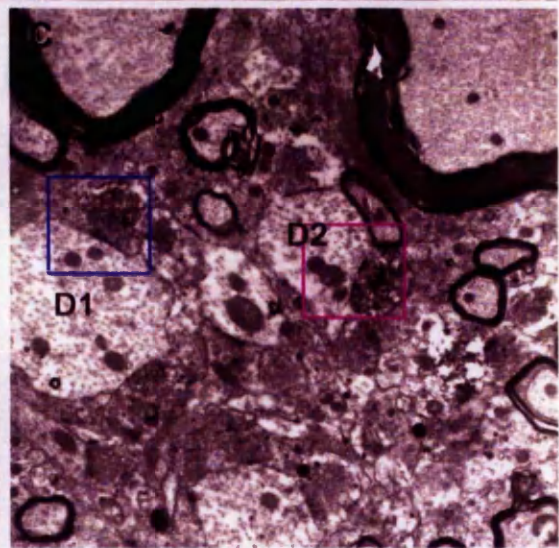
**Figure 5.6. Quintuple-labelling of $\alpha_{2C}$-AR, NK-1, c-Fos, CTb and NeuN in the LSN. a,**
A merged image of a transverse section of the LSN built from 9 projected confocal images
gathered at 1µm steps with a 40X lens (red, $\alpha_{2C}$-AR ($\alpha$2C); green, NK-1 and c-Fos
(NK1+c-Fos); blue, CTb (CTb)). **b,** shows independently the corresponding NeuN
labelling, which allows to identify the number of neurons present in the scanned area.
These images illustrate diverse categories of LSN neuron, which are indicated by the
numbers: **1** only c-Fos-IR; **3** only NK-1-IR; **6** double-labelled for CTb and NK-1; **7** triple-
labelled for c-Fos, CTb and NK-1 and **8** only NeuN-IR. (Scale bar: **a**=20µm, valid for both
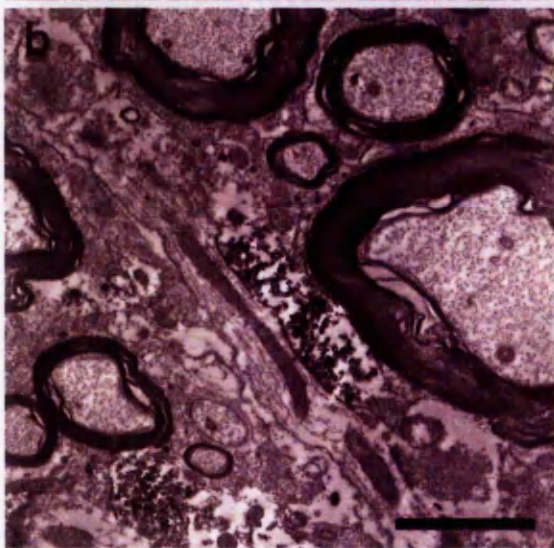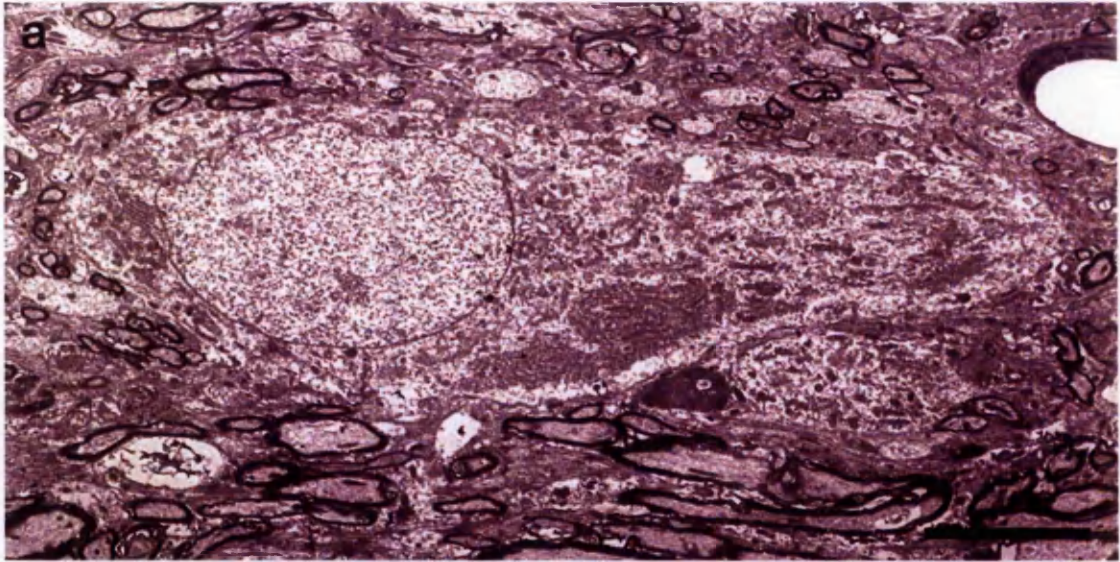images)

Histogram 1. Overlap of $\alpha_{2C}$-AR immunoreactivity with diverse markers in the LSN



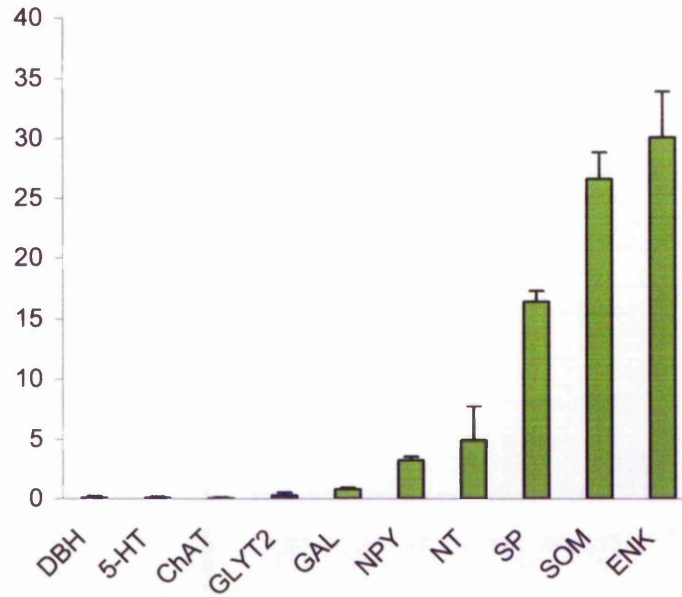Histogram 2. Frequency of excitatory and inhibitory $\alpha_{2C}$-AR-IR terminals in the LSN

a  α2C/c-Fos+NK1/CTb

b  NeuN
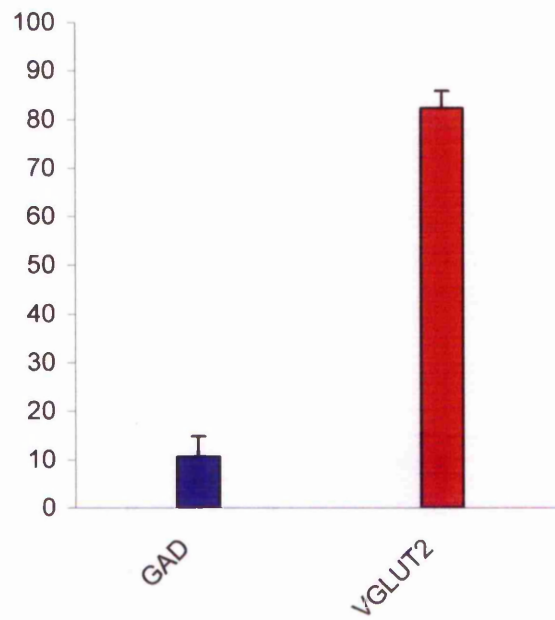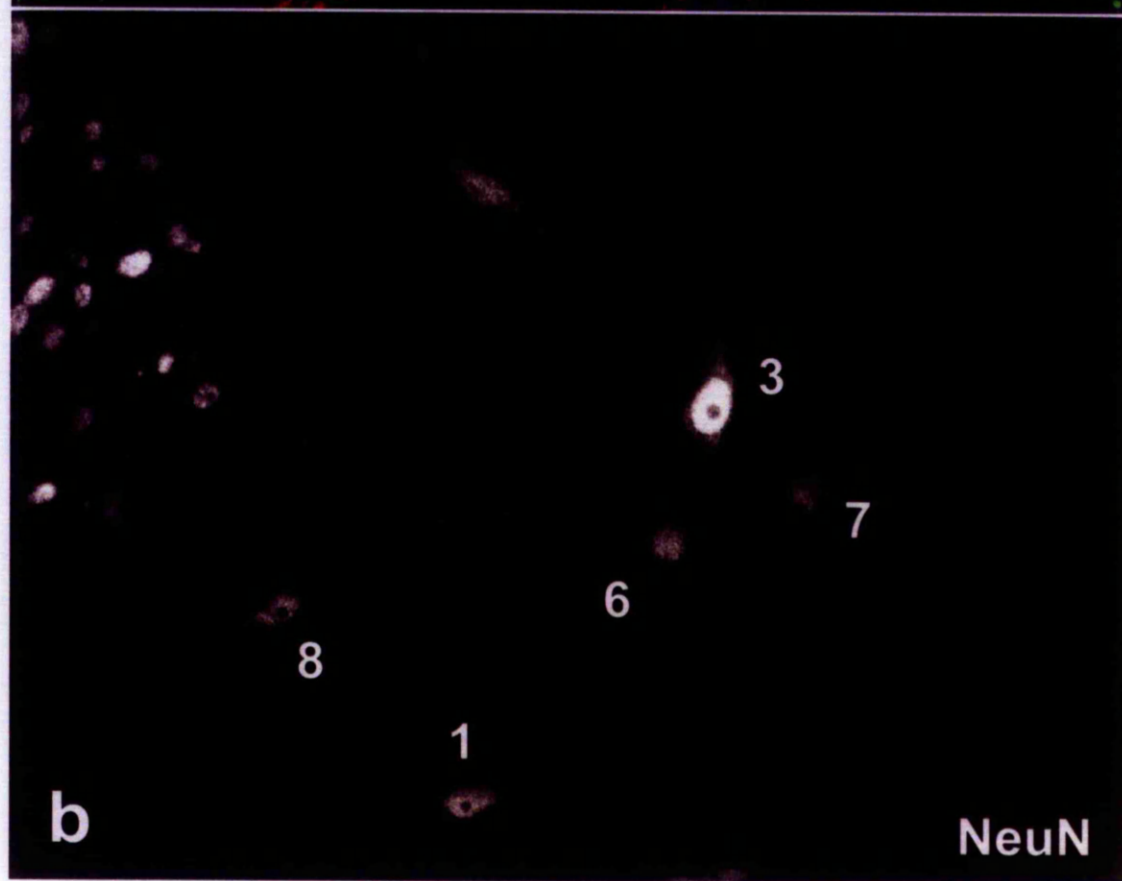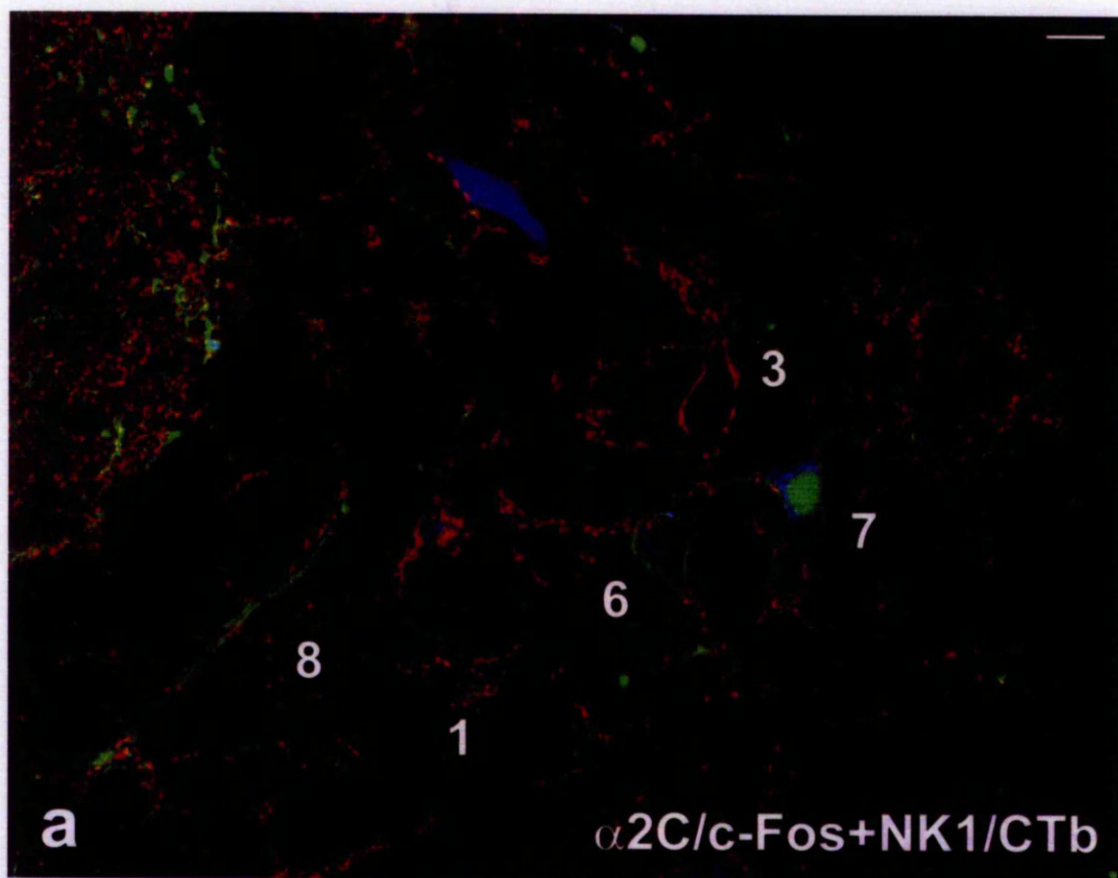
**Figure 5.7. Independent visualisation of four fluorophores with confocal microscopy. a, b, c** and **d** show, respectively, immunoreactivities for $\alpha_{2C}$-AR ($\alpha$2C); NK-1 and c-Fos (NK1+c-Fos); CTb (CTb); and NeuN (NeuN) corresponding to image shown in Figure 5.6. Independent visualisation is essential for optimal assessment of the pattern of immunoreactivity of the cells and their consequent classification. Numbers indicate the same LSN neurons as in Figure 5.6. (Scale bar: **a**=20$\mu$m, valid for all images)

a    α2C

b    c-Fos+NK1

c    CTb

d    NeuN
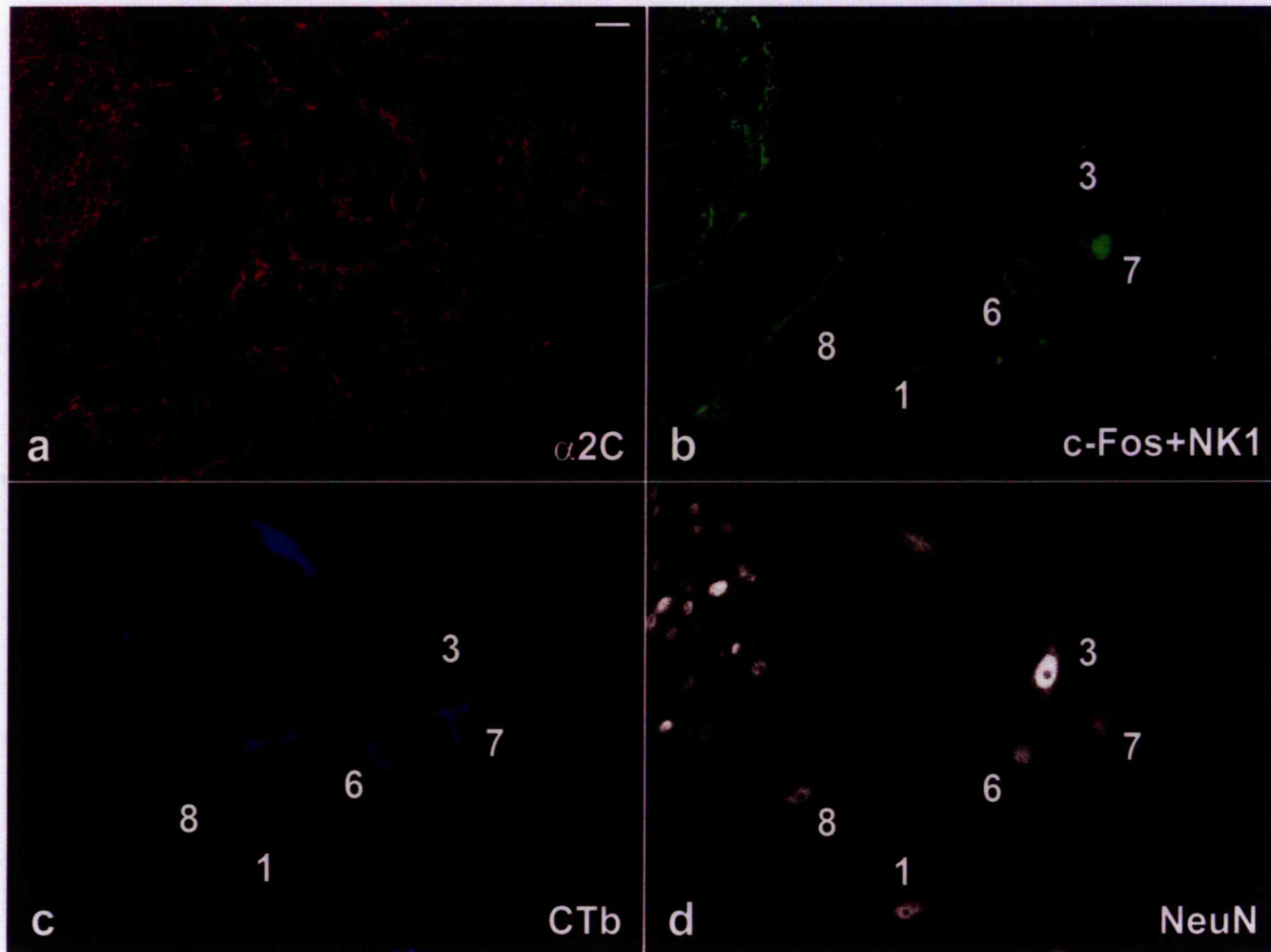
**Figure 5.8. Examples of LSN neurons.** Merged images of transverse sections of the LSN are shown in **a** and **b**, both built from 9 projected confocal images gathered at 1 μm steps with a 40X lens (red, $\alpha_{2C}$-AR; green, NK-1 and c-Fos; blue, CTb). Numbers in **a** indicate LSN neurons corresponding to categories **2** only CTb-IR, **3** only NK-1 and **5** double-labelled for c-Fos and NK-1. Number **4** in **b** highlights a LSN neuron from category **4** doubled-labelled for c-Fos and CTb. (Scale bar: **a**=20 μm, valid for both images)
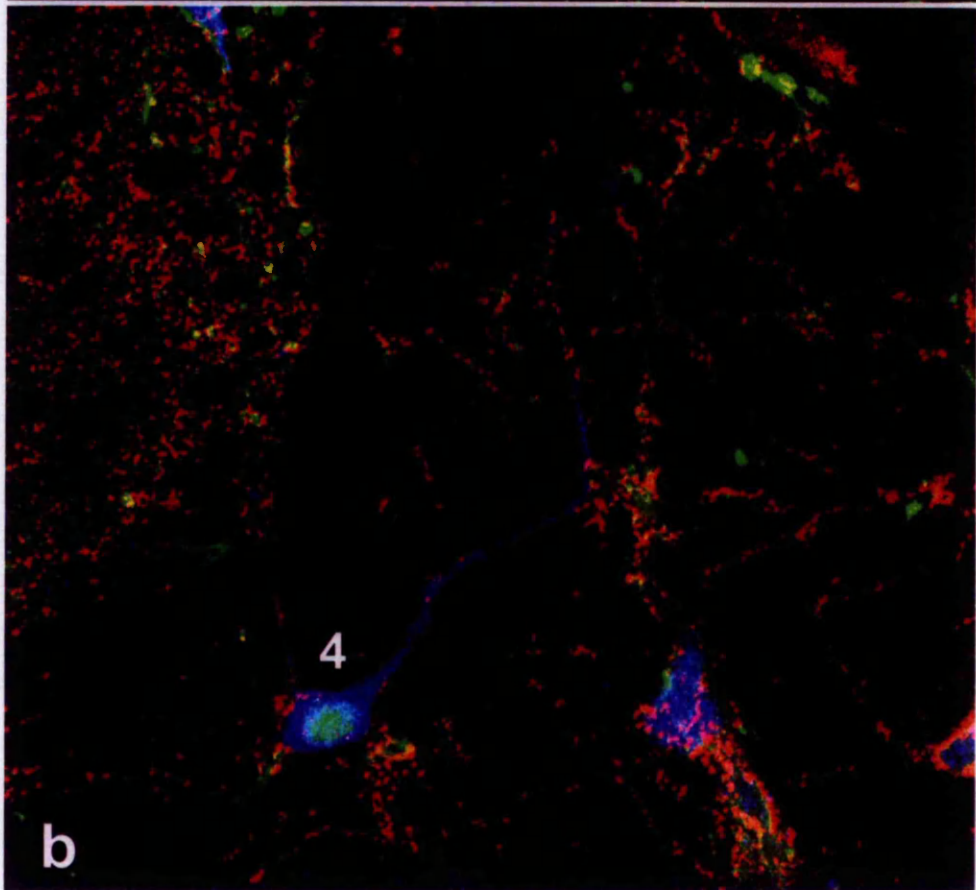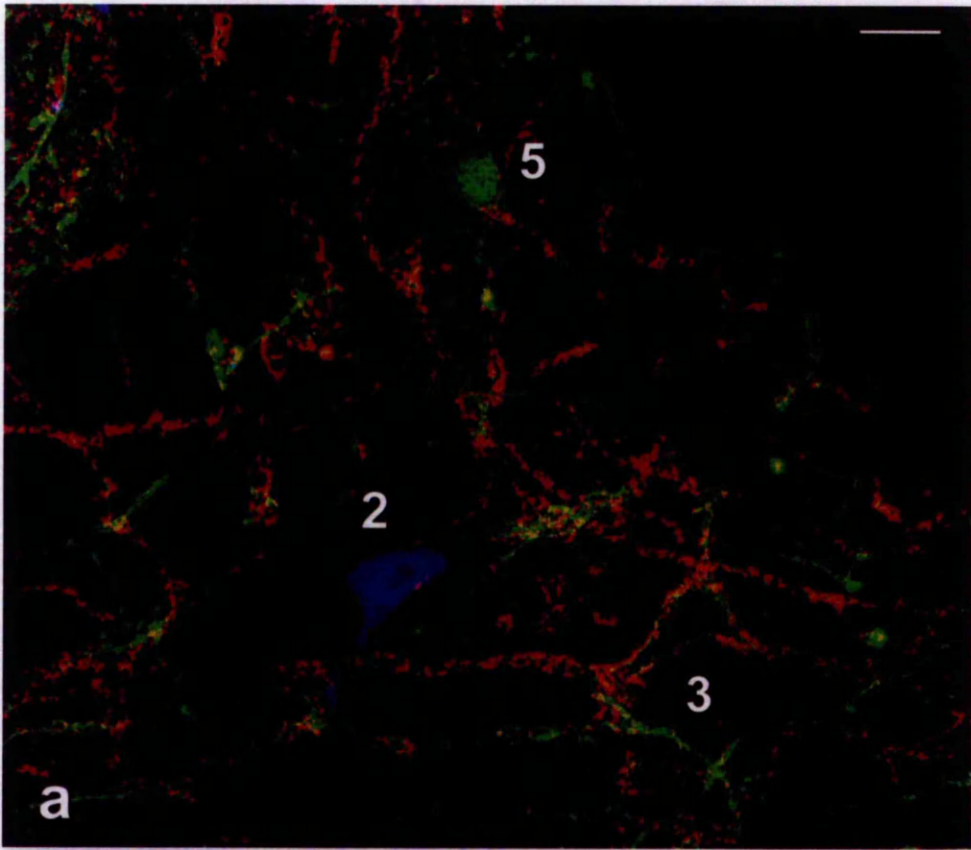
**Figure 5.9. Triple-labelling of $\alpha_{2C}$-AR, enkephalin and SP in the LSN.** $\alpha_{2C}$-AR (green), enkephalin (ENK, red) and SP (SP; blue). The three sets of images correspond to the same optical section. Notice the extensive colocalisation between enkephalin and SP (ENK/SP) and the numerous $\alpha_{2C}$-AR-IR terminals which contain enkephalin and SP as observed in the merged images on the right side of the figure (triple-labelled boutons appear white). (Scale bar=20μm, valid for all images)

ENK

SP

ENK / SP
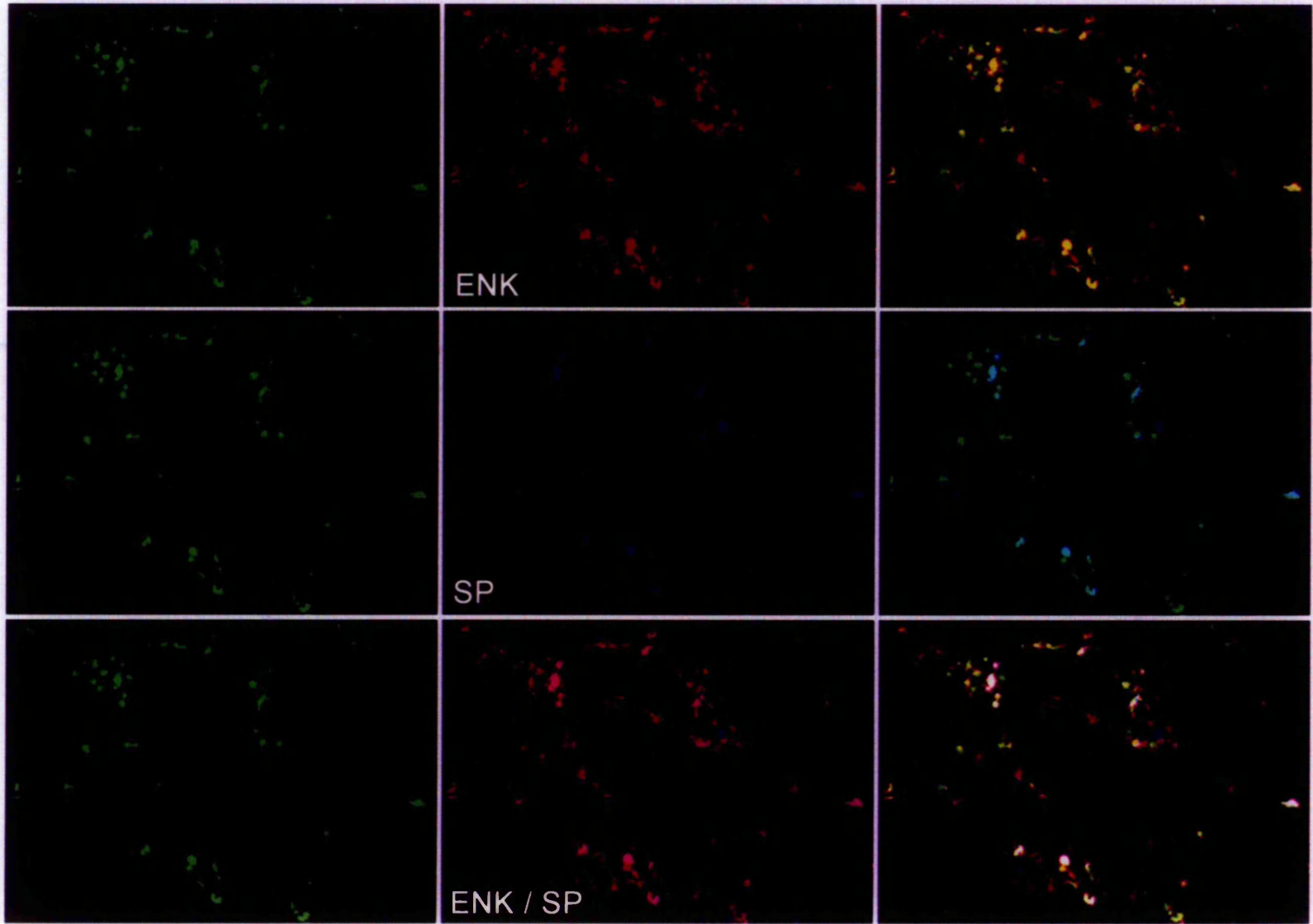
*Chapter 6*

# General Discussion

The aim of this chapter is to discuss further the functional significance of the results obtained from the different investigations that were carried out. For this purpose the main conclusions of each investigation are summarised briefly followed by hypothetical functional models.

## 1. Conclusions and models

*Investigation 1*: Axon terminals possessing the $\alpha_{2C}$-AR in the rat dorsal horn are of spinal origin. The majority of these terminals is excitatory and presynaptic to dendrites, however a minority is inhibitory and can be presynaptic to axons.

*Action of NE through $\alpha_{2C}$-ARs on excitatory versus inhibitory terminals*

In the superficial dorsal horn, there is a dense plexus of NE fibres (Rajaofetra et al., 1992) which largely overlaps with areas of intense $\alpha_{2C}$-AR imunoreactivity. Noradrenergic axons do not form axoaxonic synapses in this region (Doyle and Maxwell, 1991a, 1991b), indeed axoaxonic synapses have been found only on primary afferent terminals and are not formed with terminals of interneurons (Alvarez, 1998). NE therefore cannot act upon interneuron axons through a classical synaptic mechanism and any interaction with terminals possessing adrenergic receptors must occur via volume transmission. The results indicate that NE could modulate transmission at the superficial dorsal horn by acting through $\alpha_{2C}$-ARs which are present on the terminals of interneurons. Figure 6.1 illustrates possible actions of NE on terminals containing the $\alpha_{2C}$-AR and in turn the consequent effect on the postsynaptic element. The action of NE on $\alpha_{2C}$-ARs is expected to induce hyperpolarisation (Surprenant et al., 1990; Shen et al., 1992) of the membrane and therefore lead to a reduction in the efficacy of synaptic transmission (Fig. 6.1, A and B). If the terminal contains an excitatory transmitter, the action of NE on $\alpha_{2C}$-ARs would be predicted to induce suppression of excitatory transmission (Fig. 6.1, A). If the terminal is of an inhibitory nature the action of NE on $\alpha_{2C}$-ARs would be predicted to induce suppression of inhibitory transmission or disinhibition (Fig. 6.1, B). This implies that NE could lead to opposite effects in the postsynaptic element by acting at inhibitory or excitatory terminals containing the $\alpha_{2C}$-AR. Alternatively, the action of NE on inhibitory terminals could be different to the one on excitatory terminals, i.e. excitatory, so that the resulting effect on the postsynaptic element would be facilitation of the inhibitory transmission (Fig. 6.2, C). Indeed it has been reported that activation of presynaptic $\alpha_2$-

ARs increases GABAergic synaptic transmission in the substantia nigra (Cathala et al., 2002). If this is also the case in the spinal cord, then it is possible that the action of NE on presynaptic terminals that contain the $\alpha_{2C}$-AR could result in a net inhibitory effect of the postsynaptic targets by suppressing excitatory transmission and facilitating inhibitory transmission.

Theoretically, the action of NE on excitatory terminals which contain the $\alpha_{2C}$-AR could be hypothesised to result in facilitation of excitatory transmission. however this appears unlikely and for this reason this possibility has not been included in the diagram in Figure 6.1. NE or $\alpha_2$-AR agonists have been reported to inhibit the release of glutamate and SP from primary afferents terminals (Kuraishi et al., 1985; Kamisaki et al., 1993) suggesting that NE inhibits excitatory transmission from excitatory terminals by acting on $\alpha_2$-ARs. Although these receptors would be expected to be of the $\alpha_{2A}$ subclass because they are the predominant type on primary afferents (Stone et al., 1998), it is likely that the effect of NE on $\alpha_{2C}$-ARs located on interneuron terminals resembles the effect occurring on $\alpha_{2A}$-ARs of primary afferent terminals. Furthermore, the above studies could be including both types of receptors since the experiments were not carried out with $\alpha_2$-subtype-specific compounds, which are still currently unavailable. In addition, the $\alpha_1$-AR has been recently reported to prime excitatory synapses increasing the excitatory drive in the hypothalamus (Gordon and Bains, 2003), which suggests that although it is possible, it would be unexpected, that $\alpha_2$-ARs mediate a similar effect.

It should be kept in mind that we do not know much about the identity of the postsynaptic elements. They can be projection neurons or interneurons of excitatory or inhibitory nature, as well as primary afferent fibres. In the case of postsynaptic neurons these are more likely to be excitatory, because the majority of neurons in the dorsal horn is excitatory (Todd and Spike, 1993). Uncovering the identity of the postsynaptic targets of $\alpha_{2C}$-AR terminals can help us to elucidate the circuitry involved in NE modulation of nociception and by extension increase our understanding of the functional organisation of the dorsal horn. *Investigation 2* reveals the identity of a postsynaptic target.

*Investigation 2*: NK-1 projection neurons in the rat dorsal horn receive synaptic contacts from excitatory axons that possess $\alpha_{2C}$-ARs and can be responsive to thermal noxious stimulation.

*An $\alpha_{2C}$-AR-mediated mechanism of noradrenergic antinociception*

A model of a possible mechanism of antinociception mediated through $\alpha_{2C}$-ARs is presented in Figure 6.2. The action of NE on an excitatory terminal containing $\alpha_{2C}$-ARs would be predicted to induce membrane hyperpolarisation (Surprenant et al., 1990; Shen et al., 1992), which would lead to a reduction in the efficacy of synaptic transmission between $\alpha_{2C}$-AR-containing terminals of excitatory interneurons and projection neurons. This would selectively suppress excitatory polysynaptic input to these neurons, which, in turn, would attenuate transmission of nociceptive information to supraspinal structures. In addition, it is possible that NK-1 neurons projecting to the CVLM are responsible for the spinal release of NE. Activation of such projection neurons could lead to the recruitment of NE descending inhibitory systems as these neurons terminate close to the lateral reticular nucleus, which contains noradrenergic neurons that in turn project to the spinal dorsal horn (Lee et al., 2001).

*Model of noradrenergic modulation of nociception through $\alpha_2$-ARs*

Both presynaptic actions on primary afferent terminals (Kuraishi et al., 1985; Kamisaki et al., 1993) and postsynaptic actions on dorsal horn cells (Fleetwood-Walker et al., 1985; Davies and Quinlan, 1985) have been proposed as mechanisms for NE modulation of nociceptive transmission through $\alpha_2$-ARs. The investigations carried out suggest that a third mechanism can also operate: i.e. that NE modulates transmission at terminals of interneurons. These three mechanisms could operate in a complementary manner. A model is shown in Figure 6.3 which is an attempt to integrate the diverse mechanisms of $\alpha_2$-AR-mediated antinociception within the circuitry of the dorsal horn. It is likely that both $\alpha_{2A}$-ARs, located on primary afferent terminals and dorsal horn neurons, as well as $\alpha_{2C}$-ARs on interneuron terminals and dorsal horn cells (Rosin et al., 1993; Rosin et al., 1996; Talley et al., 1996; Stone et al., 1998) are involved in these modulatory processes.

*Noradrenergic system versus serotoninergic system*

The NE descending system, along with the serotoninergic system, performs a major role in the regulation of nociceptive transmission in the dorsal horn. However the

mechanisms of action of these two monoamines on NK-1 neurons are likely to be different. Many projection cells that possess the NK-1 receptor in lamina I and laminae III-IV are heavily targeted by 5HT-IR axons which form numerous contacts with their cell bodies and proximal dendrites (Stewart and Maxwell, 2000; Polgár et al., 2002) but NE contacts on these cells are very sparse indeed (Stewart and Maxwell, 2000; Stewart, 2001). This evidence suggests that 5HT operates directly upon NK-1 cells via a postsynaptic action whereas the effect of NE is more likely to be diffuse and operate mainly via volume transmission.

*Investigation 3*: Axon terminals possessing $\alpha_{2C}$-ARs in the LSN are of spinal origin, predominantly excitatory, and exclusively presynaptic to dendrites and cell bodies. LSN neurons are densely innervated by axons that possess $\alpha_{2C}$-ARs, and a small proportion can be activated by thermal noxious stimulation.

*Noradrenergic modulation of the LSN through $\alpha_{2C}$-ARs*

Although the LSN and the dorsal horn share common characteristics, they both possess unique properties (*Chapter 5: Investigation 3, Discussion*, pg 105), which suggests that these closely related areas subserve divergent functions that probably are complementary. While the superficial dorsal horn is involved extensively in the processing and transmission of nociception, the specific functions of the LSN are currently unclear and interestingly it has been suggested to have a role in autonomic and homeostatic functions. The results show that the LSN is involved, to a certain extent, in nociceptive processing, and that NE could have an important modulatory action on this nucleus through $\alpha_{2C}$-ARs. Notice the LSN is also represented in the model in Figure 6.3. LSN neurons that were responsive to noxious stimulation, potentially could have been activated by excitatory spinal interneurons as suggested by the presence of excitatory terminals in the LSN. Many of these terminals also possess $\alpha_{2C}$-ARs on which NE can act to induce hyperpolarisation of the excitatory terminal and reduce the efficacy of synaptic transmission. Therefore, NE could inhibit the excitatory interneuronal input to the LSN and in turn attenuate excitatory transmission to projection neurons, which would suppress the transmission of nociceptive or other types of information that they transmit supraspinally.

In the previous chapter a body of evidence was presented supporting that the LSN displays a set of unique characteristics and has a specific function/s (*Chapter 5: Investigation 3*, pg 107); this evidence also favours the idea that species without LSN, are likely to possess a functional equivalent. It has been hypothesised that neurons subserving

an equivalent function and with similar characteristics to the LSN are integrated within the superficial dorsal horn (Leah et al., 1988). If this is the case, the LSN, present in rat, mice (preliminary observations) and other rodents, could be a very convenient model to investigate the role of "LSN-like" neurons.

**Figure 6.1. Model of possible actions of NE on $\alpha_{2C}$-ARs located on excitatory or inhibitory axon terminals. A** represent the effect of NE on the terminal of an excitatory interneuron, while **B** and **C** represent two possible effects of NE on the terminal of an inhibitory interneuron. In all three cases, NE acts by volume transmission on $\alpha_{2C}$-ARs. In **A** and **B** NE has an inhibitory effect on the presynaptic terminal, i.e. either on excitatory or inhibitory terminals, which results in opposite net postsynaptic effects depending on the nature of the terminal (see labels on the right side of the diagram). In **C**, NE has an excitatory effect on the inhibitory presynaptic terminal which results in facilitation of the inhibitory transmission, i.e. an inhibitory net postsynaptic effect as in **A**. Blue boxes denote inhibitory net effect; red box denotes excitatory net effect. The postsynaptic element is generic and could be a projection neuron, an interneuron or a primary afferent axon.
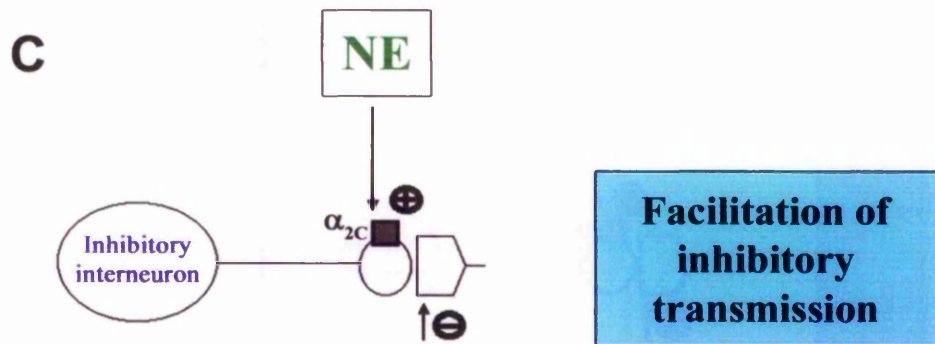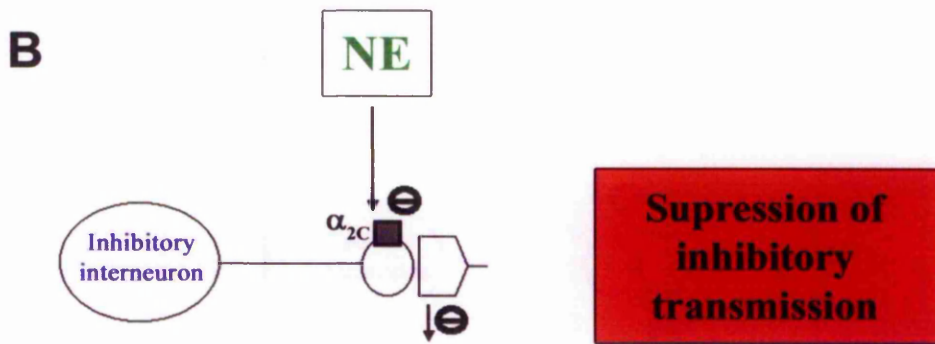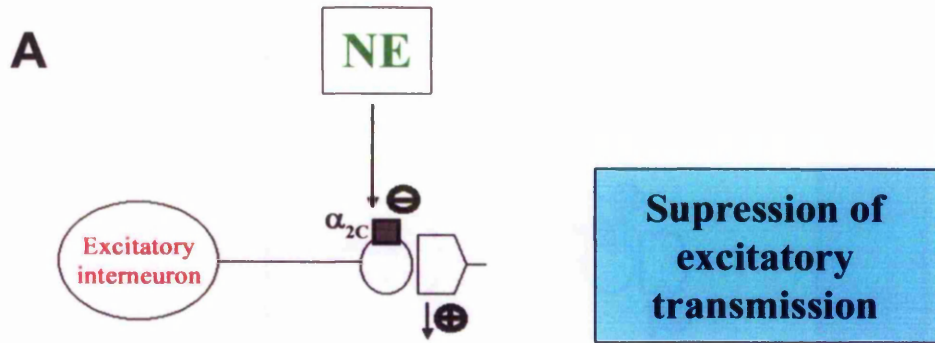
**Figure 6.2. A model of a possible mechanism of noradrenergic antinociception mediated by $\alpha_{2C}$-ARs.** Under nociceptive stimulation NK-1 neurons projecting to the CVLM are activated and could induce the release of NE. **1** NE acts by volume transmission on $\alpha_{2C}$-ARs. **2** NE induces hyperpolarisation of the excitatory interneuron axon terminal by acting on $\alpha_{2C}$-ARs. **3** Reduction in the efficacy of synaptic transmission results in **4**, inhibition of supraspinal transmission of nociceptive information.
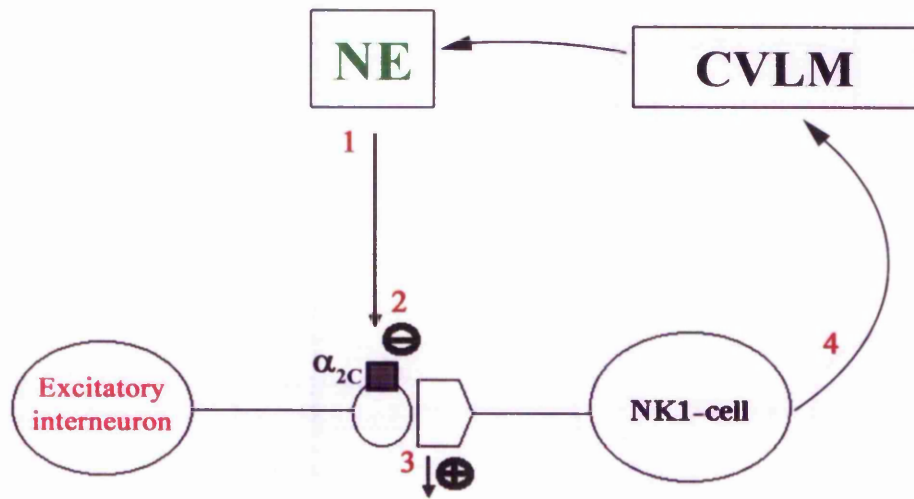
**Figure 6.3. Presynaptic and postsynaptic mechanisms of noradrenergic modulation through $\alpha_2$-ARs.** Presynaptic mechanisms involve terminals of small diameter primary afferents containing $\alpha_{2A}$-ARs (green terminals) and terminals of interneurons containing $\alpha_{2C}$-ARs (red terminals). Although cell bodies of interneurons that synthesise $\alpha_{2C}$-ARs have not been detected in the superficial dorsal horn, these must exist and are drawn in the diagram with a red circle within them that represents the synthesis of the $\alpha_{2C}$-AR in the soma. NE acts by volume transmission at $\alpha_{2A}$ and $\alpha_{2C}$-ARs located on presynatic terminals and attenuates the transmission to postsynaptic nociceptive projection neurons (blue cells 1 and 2). Postsynaptic mechanisms involve cell bodies expressing $\alpha_2$-ARs; potentially NE could act on superficial and deep dorsal horn neurons that possess $\alpha_{2A}$-ARs on the cell bodies (green cells), as well as on deep dorsal horn neurons that possess $\alpha_{2C}$-ARs on the cell bodies (red cells). Noradrenergic terminals could act directly (synaptically) on projection neurons; based on the immunocytochemical distribution of $\alpha_2$-AR on cell bodies it is possible that superficial projection cells possess $\alpha_{2A}$-ARs (blue cell, 1), while deep projection cells could contain $\alpha_{2C}$-ARs or $\alpha_{2A}$-ARs (blue cell, 2). The purple arrows represent supraspinal axonal projections. In the dorsal horn: Cell 1 is nociceptive and Cell 2 is nociceptive multireceptive; in the LSN: Cell 3 is nociceptive and/or visceral. LSN neurons can also be modulated by NE through a presynaptic mechanism that involves terminals of interneurons containing $\alpha_{2C}$-ARs (red terminals in LSN).

peptidergic
nociceptive
primary afferent

LI
LII
LIII
LIV

(LI-II)

(LIV-VI)

(LIV-V)

LSN

nociceptive

nociceptive
(multireceptive)

nociceptive
visceral
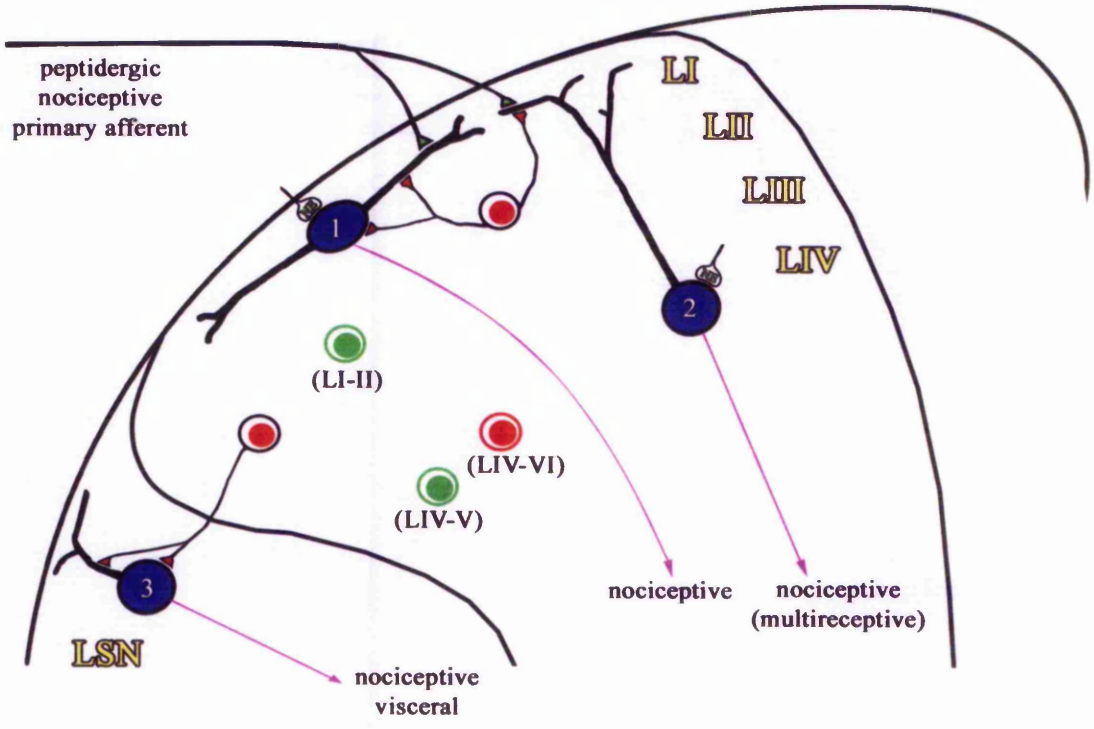
# References

Abbadie C, Skinner K, Mitrovic I, Basbaum AI (1999). Neurons in the dorsal column white matter of the spinal cord: Complex neuropil in an unexpected location. Proc. Natl. Acad. Sci. (USA) 96:260-265

Abhold RH and Bowker RM (1990). Descending modulation of dorsal horn biogenic amines as determined by in vivo dialysis. Neurosci. Lett. 108:231-6

Aimar P, Pasti L, Carmignoto G, Merighi A (1998). Nitric oxide-producing islet cells modulate the release of sensory neuropeptides in the rat substantia gelatinosa. J. Neurosci. 18:10375-10388

Alvarez FJ, Kavookjian AM, Light AR (1993). Ultrastructural morphology, synaptic relations and CGRP immunoreactivity of physiologically identified C-fibre terminals in monkey spinal cord. J. Comp. Neurol. 329:427-490

Alvarez FJ (1998). Anatomical basis for presynaptic inhibition of primary sensory fibers. In: Rudomin P., Romo R., Mendell L. (eds) Presynaptic inhibition and neural control. Oxford University Press, New York, pp 13-49

Albanese A and Butcher LL (1980). Acetylcholinesterase and catecholamine distribution in the locus ceruleus of the rat. Brain Res. Bull. 5:127-134

Armah IB and Stenzel W (1981). BE-5895, a new clonidine-type antihypertensive aminopyrimidine derivative. Naunyn-Schmeidelberg's Arch. Pharmakol. 316 (Suppl R):42

Arnsten AF, Steere JC, Jentsch DJ, Li BM (1998). Noradrenergic influences on prefrontal cortical cognitive function: opposing actions at postjunctional alpha 1 versus alpha 2-adrenergic receptors. Adv. Pharmacol. 42:764-7

Barber RP, Phelps PE, Houser CR, Crawford GD, Salvaterra PM, Vaughn JE (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. J. Comp. Neurol. 229:329-346

Belcher G, Ryall RW, Schaffner R (1978). The differential effects of 5-hydroxytryptamine, noradrenaline and raphe stimulation on nociceptive and non-nociceptive dorsal horn interneurones in the cat. Brain Res. 151:307-21
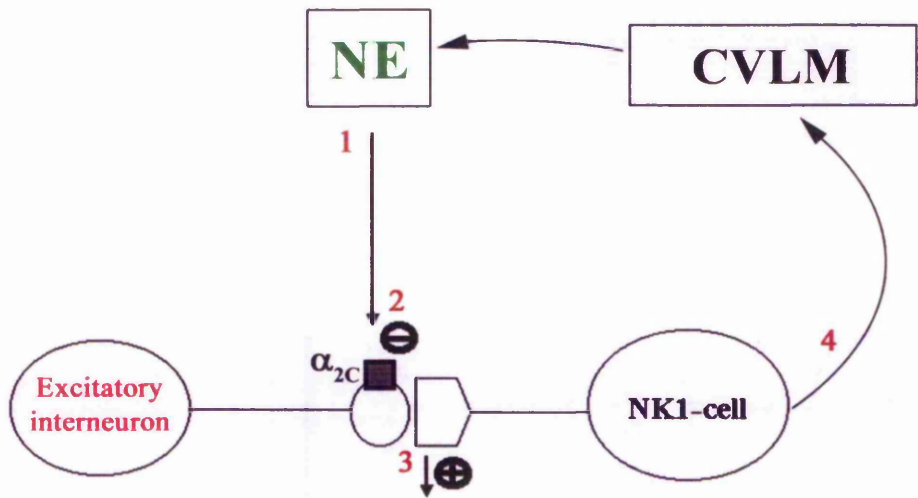
Bunemann M, Bucheler MM, Philipp M, Lohse MJ, Hein L (2001). Activation and deactivation kinetics of alpha 2A- and alpha 2C-adrenergic receptor-activated G protein-activated inwardly rectifying K+ channel currents. J. Biol. Chem. 276:47512-47517

Burstein R, Cliffer KD, Giesler GJ (1990). Cells of origin of the spinohypothalamic tract in the rat. J. Comp. Neurol. 291:329-344

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr, Tredelenburg U (1994). IVth International Union of Pharmacology nomenclature of adrenoceptors . Pharmac. Rev. 46:121-136

Cathala L, Guyon A, Eugene D, Paupardin-Tritsch D (2002). Alpha 2-adrenoceptor activation increases a cationic conductance and spontaneous gabaergic synaptic activity in dopaminergic neurons of the rat substantia nigra. Neuroscience 115:1059-1065

Chiari A, Li XH, Xu Z, Pan HL, Eisenach JC (2000). Formation of 6-nitro-norepinephrine from nitric oxide and norepinephrine in the spinal cord and its role in spinal analgesia. Neuroscience 101:189-196

Cho H-J, Kim D-S, Lee N-H, Kim J-K, Lee K-M, Han K-S, Kang Y-N, Kim K-J (1997). Changes in the a2-adrenergic receptor subtypes gene expression in rat dorsal root ganglion in an experimental model of neuropathic pain. NeuroReport 8:3119-3122

Christensen BN and Perl ER (1970). Spinal neurons specifically excited by noxious or thermal stimuli: marginal zone of the dorsal horn. J. Neurophysiol. 33:293-307

Clark FM and Proudfit HK (1991a). The projection of noradrenergic neurons in the A7 catecholamine cell group to the spinal cord in the rat demostrated by anterograde tracing combined with immunocytochemistry. Brain Res. 547:279-288

Clark FM and Proudfit HK (1991b). The projection of locus coeruleus neurons to the spinal cord in the rat determined by anterograde tracing combined with immunocytchemistry. Brain Res. 538: 231-245

Clark FM and Proudfit HK (1993). The projections of noradrenergic neurons in the A5 catecholamine cell group to the spinal cord in the rat: Anatomical evidence that A5 neurons modulate nociception. Brain Res. 616: 200-210

Cliffer KD, Urca G, Elde RP, Giesler GJ (1988). Studies of peptidergic input to the lateral spinal nucleus. Brain Res. 460:356-60

Craig AD (1995). Distribution of brainstem projections from spinal lamina I neurons in the cat and monkey. J. Comp. Neurol. 361:225-248

Crain SM, Crain B, Makman MH (1987). Pertussis toxin blocks depressant effects of opioid, monoaminergic and muscarinic agonists on dorsal-horn network responses in spinal cord-ganglion cultures. Brain Res. 400:185-190

Dahlström A (1971). Regional distribution of brain catecholamines and serotonin. Neurosci. Res. Program Bull. 9:197-205

Dahlström A and Fuxe K (1965). Evidence for the existence of an outflow of noradrenaline nerve fibres in the ventral roots of the rat spinal cord. Experientia 21:409-410

Davies J and Quinlan JE (1985). Selective inhibition of responses of feline dorsal horn neurones to noxious cutaneous stimuli by tizanidine (DS103-282) and noradrenaline: involvement of alpha 2-adrenoceptors. Neuroscience 16:673-682

De Biasi S and Rustioni A (1988). Glutamate and substance P co-exist in primary afferent terminals in the superficial laminae of spinal cord. Proc. Natl. Acad. Sci. 85: 7820-7824.

De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJ, Laird JM, Belmonte C, Cervero F, Hunt SP (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature 392:394-397

De Kock M; Eisenach J, Tong C, Schmitz AL, Scholtes JL (1997). Analgesic doses of intrathecal but not intravenous clonidine incrase acetylcholine in cerebrospinal fluid in humans. Anesth. Analg. 84:800-803

de la Breteche M-L, Servy C, Lenfant M and Ducrocq C (1994). Nitration of catecholamines with nitrogen oxides in mild conditions: A hypothesis for the reactivity of NO in physiologic systems. Tetrahedron Lett. 35:7231-7232

DeBernardis JF, Kyncl JJ, Basha FZ, Arendsen DL, Martin YC, Winn M, Kerkman DJ (1986). Conformationally defined adrenergic agents. 2. Catechol imidazoline derivatives: biological effects at alpha 1 and alpha 2 adrenergic receptors. J. Med. Chem. 29:463-467

Detweiler DJ, Eisenach JC, Tong C, Jackson C (1993). A cholinergic interaction in $\alpha_2$ adrenoceptor-mediated antinociception in sheep. Pharmacol. Exp. Ther. 265:536-542

Doyle CA and Hunt SP (1999). Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. Neuroscience 89:17-28

Doyle CA and Maxwell DJ (1991a). Ultrastructural analysis of noradrenergic nerve terminals in the cat lumbosacral spinal dorsal horn: a dopamine-beta-hydroxylase immunocytochemical study. Brain Res. 563:329-333

Doyle CA and Maxwell DJ (1991b). Catecholaminergic innervation of the spinal dorsal horn: a correlated light and electron microscopic analysis of tyrosine hydroxylase-immunoreactive fibres in the cat. Neuroscience 45:161-176

Doyle CA and Maxwell DJ (1993). Direct catecholaminergic innervation of spinal dorsal horn neurons with axons ascending the dorsal columns in cat. J. Comp. Neurol. 331:434-444

Eisenach JC, Detweiler DJ, Tong CY, D'Angelo R, Hood DD (1996). Cerebrospinal fluid norepinephrine and acetylcholine concentrations during acute pain. Anesth. Analg. 82:621-626

Fairbanks CA and Wilcox GL (1999). Moxonidine, a selective alpha2-adrenergic and imidazoline receptor agonist, produces spinal antinociception in mice. J. Pharmacol. exp. Ther. 290:403-412

Fairbanks CA, Nguyen HO, Grocholski BM, Wilcox GL (2000). Moxonidine, a selective imidazoline-alpha2-adrenergic receptor agonist, produces spinal synergistic antihyperalgesia with morphine in nerve-injured mice. Anesthesiology 93:765-773

Fairbanks CA, Stone LS, Kitto KF, Nguyen HO, Posthumus IJ, Wilcox GL (2002). $\alpha_{2C}$-adrenergic receptors mediate spinal analgesia and adrenergic-opioid synergy. Pharmacol. Exp. Ther. 300:282-290

Fleetwood-Walker SM, Mitchell R, Hope PJ, Molony V, Iggo A (1985). An alpha2 receptor mediates the selective inhibition by noradrenaline of nociceptive responses of identified dorsal horn neurons. Brain Res. 334:243-254

Fritschy JM and Grzanna R (1990). Demonstration of two separate descending noradrenergic pathways to the rat spinal cord: evidence for an intragriseal trajectory of locus coeruleus axons in the superficial layers of the dorsal horn. J. Comp. Neurol. 291:553-582

Gauriau C and Bernard JF (2002). Pain pathways and parabrachial circuits in the rat. Exp. Physiol. 87: 251-258

Giesler GJ and Elde RP (1985). Immunocytochemical studies of the peptidergic of fibers and terminals within the lateral spinal and lateral cervical nuclei. J. Neurosci. 5: 1833-1841

Giesler GJ, Menétrey D, Basbaum AI (1979a). Differential origins of spinothalamic tract projections to medial and lateral thalamus in the rat. J. Comp. Neurol. 184:107-126

Giesler GJ, Urca G, Cannon JT, Liebeskind JC (1979b). Response properties of neurons of the lateral cervical nucleus in the rat. J. Comp. Neurol. 186: 65-78

Gobel S (1975). Gogi studies of the substantia gelatinosa neurons in the spinal trigeminal nucleus of the adult cat. Brain Res. 83:333-338

Gobel S (1978). Gogi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp.Neurol. 180:395-414

Gobel S (1979). Neural circuitry in the substantia gelatinosa of Rolando: anatomical insights. Adv. Pain Res. Ther. 3: 175-195

Gold MS, Dastmalchi S, Levine JD (1997). Alpha 2 adrenergic receptor subtypes in rat dorsal root and superior cervical ganglion neurons. Pain 69:179-190

Gordon GRJ and Bains JS (2003). Priming of excitatory synapses by alpha 1 adrenoceptor-mediated inhibition of group III metabotropic glutamate receptors. J. Neuronsci. 23:6223-6231

Gorth T Jr, Jansson I, Hartvig P, Gilberg PG, Post G (1989). Interactions between noradrenergic and cholinergic mechanisms involved in spinal nociceptive processing. Acta anaesth. scand. 33:39-47

Graham BA, Hammond DL, Proudfit HK (2000). Synergistic interactions between two alpha(2)-adrenoceptor agonists, dexmedetomidine and ST-91, in two substrains of Sprague-Dawley rats. Pain 85:135-143

Gray EG (1962). A morphological basis for presynaptic inhibition. Nature 193:82-83

Greenfield SA (1991). A noncholinergic action of acetylcholinesterase (AChE) in the brain: from neuronal secretion to the generation of movement. Cell. Mol. Neurobiol. 11:55-77

Grudt TJ and Perl ER (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J. Physiol. 540:189-207

Guo TZ, Davies MF, Kingery WS, Patterson AJ, Limbird LE, Maze M (1999). Nitrous oxide produces antinociceptive response via alpha 2B and/or alpha2C adrenoceptor subtypes in mice. Anesthesiology 90:470-476

Guyenet PG, Stornetta RL, Riley T, Norton FR, Rosin DL, Lynch KR (1994). Alpha 2A-adrenergic receptors are present in lower brainstem catecholaminergic and serotonergic neurons innervating spinal cord. Brain Res. 646:356-357

Gwyn DG and Waldron HA (1968). A nucleus in the dorsal lateral funiculus of the spinal cord of the rat. Brain Res. 10:342-351

Gwyn DG and Waldron HA (1969). Observations on the morphology of a nucleus in the dorsolateral funiculus of the spinal cord of the guinea-pig, rabbit, ferret and cat. J. Comp. Neurol. 136:233-236

Hammond DL, Tyce GM, Yaksh TL (1985). Efflux of 5-hydroxytryptamine and noradrenaline into spinal cord superfusates during stimulation of the rat medulla. J. Physiol. 359:151-162

Harrison JK, D'Angelo DD, Zeng D, Lynch KR (1991). Pharmacological characterization of rat a2-adrenergic receptors. Mol. Pharmacol. 40:407-412

Hayashi Y, Guo TZ, Maze M (1995). Desensitization to the behavioral effects of alpha 2-adrenergic agonists in rats. Anesthesiology 82:954-962

Hayes RL, Price DD, Ruda M, Dubner R (1979). Suppression of nociceptive responses in the primate by electrical stimulation of the brain or morphine administration: behavioral and electrophysiological comparisons. Brain Res. 167:417-421

Headley PM, Duggan AW, Griersmith BT (1978). Selective reduction by noradrenaline and 5-hydroxytryptamine of nociceptive responses of cat dorsal horn neurones. Brain Res. 145:185-189

Hillarp NA, Fuxe K, Dahlström A (1966). Demonstration and mapping of central neurons containing dopamine, noradrenaline, and 5-hydroxytryptamine and their reactions to psychopharmaca. Pharmacol. Rev. 18:727-741

Hoehn K, Reid A, Sawynok J (1988). Pertussis toxin inhibits antinociception produced by intrathecal injection of morphine, noradrenaline and baclofen. Eur. J. Pharmacol. 146:65-72

Hökfelt T, Ljungdahl A, Terenius L, Elde R, Nilsson G (1977). Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: Enkephalin and substance P. Proc. Natl. Acad. Sci. USA 74:3081-3085

Hökfelt T, Elde R, Johansson O, Luft R, Nilsson G, Arimura A (1976). Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. Neuroscience 1:131-136

Howe JR, Wang JY, Yaksh TL (1983). Selective antagonism of the antinociceptive effect of intrathecally applied alpha adrenergic agonists by intrathecal prazosin and intrathecal yohimbine. J. Pharmacol. Exp. Ther. 224:552-558

Howe JR, Yaksh TL, Go VLW (1987b). The effect of unilateral dorsal root ganglionectomies or ventral rhizotomies on 2-adrenoceptor binding to, and the substance P, enkephalin, and neurotensin content of, the cat lumbar spinal cord. Neuroscience 21:385-394

Howe JR, Yaksh TL, Tyce GM (1987a). Intrathecal 6-hydroxydopamine or cervical spinal hemisection reduces norepinephrine content, but not the density of alpha 2-adrenoceptors, in the cat lumbar spinal enlargement. Neuroscience 21:377-384

Howe JR and Zieglgänsberger W (1987). Responses of rat dorsal horn neurons to natural stimulation and to iontophoretically applied norepinephrine. J. Comp. Neurol. 255:1-17

Hunt SP, Pini A, Evan G (1987). Induction of c-Fos-like protein in spinal cord neurons following sensory stimulation. Nature 328:632-634

Hunter JC, Fontana DJ, Hedley LR, Jasper JR, Lewis R, Link RE, Secchi R, Sutton J, Eglen RM (1997). Assessment of the role of alpha2-adrenoceptor subtypes in the antinociceptive, sedative and hypothermic action of dexmedetomidine in transgenic mice. Br. J. Pharmacol. 122:1339-1344

Jansson CC, Marjamäki A, Luomala K, Savola JM, Scheinin M, Akerman KE (1994a). Coupling of human alpha 2-adrenoceptor subtypes to regulation of cAMP production in transfected S115 cells. Eur. J. Pharmacol. 266:165-174

Jansson CC, Savola JM, Akerman KE (1994b). Different sensitivity of alpha 2A-C10 and alpha 2C-C4 receptor subtypes in coupling to inhibition of cAMP accumulation. Biochem. Biophys. Res. Commun. 199:869-875

Jasmin L, Tien D, Weinshenker D, Palmiter RD, Green PG, Janni G, Ohara PT (2002). The NK1 receptor mediates both the hyperalgesia and the resistance to morphine in mice lacking noradrenaline. Proc. Natl. Acad. Sci. U S A. 99:1029-34

Jessel T, Iversen L, Cuello A (1978). Capsaicin induced depletion of SP from primary sensory neurons. Brain Res. 152:183-188

Johansson O, Hökfelt T, Elde RP, Schultzberg M, Terenius L (1978). Immunohistochemical distribution of enkephalin neurons. Adv. Biochem. Psychopharmacol. 18:51-70

Kable JW, Murrin LC, Bylund DB (2000). In vivo gene modification elucidates subtype-specific functions of alpha(2)-adrenergic receptors. J. Pharmacol. Exp. Ther. 293:1-7

Kamisaki Y, Hamada T, Maeda K, Ishimura M, Itoh T (1993). Presynaptic alpha 2 adrenoceptors inhibit glutamate release from rat spinal cord synaptosomes. J. Neurochem. 60:522-526

Karoum F, Commissiong JW, Neff NH, Wyatt RJ (1980). Biochemical evidence for uncrossed and crossed locus coeruleus projections to the spinal cord. Brain Res. 196:237-241

Kevetter GA and Willis WD (1983). Collaterals of spinothalamic cells in the rat. J. Comp. Neurol. 215:453-464

Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA (2002). Spinal neurons that possess the substance P receptor are required for the development of central sensitization. J. Neurosci. 22: 9086-9098

Khasar SG, Green PG, Chou B, Levine JD (1995). Peripheral nociceptive effects of alpha2-adrenergic receptor agonists in the rat. Neuroscience 66:427-432

Klimscha W, Tong C, Eisenach JC (1997). Intrathecal alpha(2)-adrenergic agonists stimulate acetylcholine and norepinephrine release from the spinal cord dorsal horn in sheep: an in vivo microdialysis study. Anesthesiology 87:110-116

Kuraishi Y, Hirota N, Sato Y, Kaneko S, Satoh M, Takagi H (1985). Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. Brain Res. 359:177-182

Lakhlani PP, Macmillan LB, Guo TZ, McCool BA, Lovinger DM, Maze M, Limbird LE (1997). Substitution of a mutant alpha(2a)-adrenergic receptor via hit and run gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. Proc. Natl. Acad. Sci. USA 94:9950-9955

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

Langer SZ, Duval N, Massingham R (1985). Pharmacologic and therapeutic significance of alpha-adrenoceptor subtypes. J. Cardiovasc. Pharmacol. Suppl 8:1-8

Lanier SM, Downing S, Duzic E, Homcy CJ (1991). Isolation of rat genomic clones encoding subtypes of the alpha 2-adrenergic receptor. Identification of a unique receptor subtype. J. Biol. Chem. 266:10470-10478

Larabi Y, Berger B, Besson J-M, Lombard MC, Gay M (1983). Substance P in the dorsal horns of partially deafferented rats. In Bonica J, Lindblom U, Iggo A (eds): Advances in Pain Research and Therapy, Vol 5. New York: Raven Press, pp 277-282

Leah JD, Cameron AA, Kelly WD, Snow PJ (1985). Coexistence of peptide immunoreactivity in sensory neurons of the cat. Neuroscience 16: 683-690

Leah J, Menétrey D, de Pommery J (1988). Neuropeptides in long ascending spinal tract cells in the rat: evidence for parallel processing of ascending information. Neuroscience 24:195-207

Lee A, Rosin DL, Van Bockstaele EJ (1998a). Ultrastructural evidence for prominent postsynaptic localization of alpha2C-adrenergic receptors in catecholaminergic dendrites in the rat nucleus locus coeruleus. J. Comp. Neurol. 394:218-229

Lee A, Rosin DL, Van Bockstaele EJ (1998b). Alpha2A-adrenergic receptors in the rat nucleus locus coeruleus: subcellular localization in catecholaminergic dendrites, astrocytes, and presynaptic axon terminals. Brain Res. 795:157-169

Lee HS, Waterhouse BD, Mihailoff GA (2001). Evidence that dopamine-beta-hydroxylase immunoreactive neurons in the lateral reticular nucleus project to the spinal cord in the rat. Anat. Rec. 263:268-279

Levine JD, Fields HL, Basbaum AI (1993). Peptides and the primary afferent nociceptor. J. Neurosci. 13:2273-2286

Li X and Eisenach JC (2001). $\alpha2A$-adrenoceptor stimulation reduces capsaicin-induced glutamate release from spinal cord synaptosomes. J. Pharmacol. Exp. Ther. 299:939-944

Li, X., Rose, G., Chiari, A., Pan, H.-L., Tobin, J. R., Eisenach, J. C. (2000b). 6-NO2-Norepinephrine Increases Norepinephrine Release and Inhibits Norepinephrine Uptake in Rat Spinal Synaptosomes. J. Pharmacol. Exp. Ther. 292: 895-899

Li X, Zhao Z, Pan HL, Eisenach JC, Paqueron X (2000a). Norepinephrine release from spinal synaptosomes: auto-alpha2 -adrenergic receptor modulation. Anesthesiology 93:164-172

Liggett SB and Raymond JR (1993). Pharmacology and molecular biology of adrenergic receptors. In: Clinical Endocrinology and Metabolism, by Bouloux G. Ed. Bailliere Tindall, London, pp. 279-306

Light AR and Perl ER (1979b). Spinal terminations of functionally identified primary afferent neurons with slowly conducting myelinatd fibres. J. Comp. Neurol. 186:133-150

Lima D, Mendes-Ribeiro JA, Coimbra A (1991). The spino-latero-reticular system of the rat: projections from the superficial dorsal horn and structural characterization of marginal neurons involved. Neuroscience 45:137-152

Lima D, Albino-Teixeira A, Tavares I (2002). The caudal medullary ventrolateral reticular formation in nociceptive-cardiovascular integration. An experimental study in the rat. Exp Physiol 87: 267-274

Limbird LE (1988). Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. FASEB J. 2:2686-2695

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

MacDonald E, Kobilka BK, Scheinin M (1997). Gene targeting-homing in on alpha 2-adrenoceptor-subtype function. Trends Pharmacol. Sci. 18:211-219

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997). Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 278: 275-279

Marjamäki A, Luomala K, Ala-Uotila S, Scheinin M (1993). Use of recombinant human alpha2-adrenoceptors to characterize subtype selectivity of antagonist binding. Eur. J. Pharmacol. 246:219-226

Marshall GE, Shehab SAS, Spike RC, Todd AJ (1996). Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. Neuroscience 72:255-263

Maxwell L, Maxwell DJ, Neilson M, Kerr R (1996). A confocal microscope survey of serotoninergic axons in the lumbar spinal cord of the rat: co-localisation with glutamate decarboxilase and neuropeptides. Neuroscience 75, 471-80

Mayer DJ and Price DD (1976). Central nervous system mechanisms of analgesia. Pain 2:379-404

Menétrey D, Giesler GF, Besson J-M (1977). An analysis of response properties of spinal cord dorsal horn neurons to non-noxious and noxious stimuli in the spinal rat. Exp. Brain Res. 27:15-33

Menétrey D and Basbaum AI (1987). Spinal and trigeminal projections to the nucleus of the solitary tract: a possible substrate for somatovisceral and viscerovisceral reflex activation. J. Comp. Neurol. 255:439-50

Menétrey D and Besson J-M (1981). Elecrophysiology and location of dorsal horn neurons in the rat, including cells at the origin of the spinoreticular and spinothatlamic attracts. In A Brown and M Rethelyi (eds): Spinal Cord Sensation. Edinburgh: Scottish Academic Press, pp179-188

Menétrey D, Chaouch A, Besson J-M (1980). Location and properties of dorsal horn neurons at origin of spinoreticular tract in lumbar enlargement of the rat. J. Neurophysiol. 44:862-877

Menétrey D, Chaouch A, Binder D, Besson J-M (1982). The origin of the spinopmesencephalic tract in the rat: An anatomical study using the retrograde transport of horseradish peroxidase. J. Comp. Neurol. 206:193-207

Merighi A, Polack JM, Fumagalli G, Theodosis DT (1989). Ultrastructural localization of neuropeptides and GABA in rat dorsal horn: a comparison of different immunogold labeling techniques. J. Histochem. Cytochem. 37, 529-540

Millan MJ (1992). Evidence that an alpha 2A-adrenoceptor subtype mediates antinociception in mice. Eur. J. Pharmacol. 215:355-356

Millan MJ, Bervoets K, Rivet JM, Widdowson P, Renouard A, Le M, Girardon S, Gobert A (1994). Multiple alpha-2 adrenergic receptor subtypes. II. Evidence for a role of rat R alpha-2A adrenergic receptors in the control of nociception: motor behavior and hippocampal synthesis of noradrenaline. J. Pharmacol. Exp. Ther. 270:958-972

Millar J and Williams GV (1989). Effects of iontophoresis of noradrenaline and stimulation of the periaqueductal gray on single-unit activity in the rat superficial dorsal horn. J. Comp. Neurol. 287:119-133

Mouchet P, Manier M, Feuerstein C (1992). Immunohistochemical study of the catecholaminergic innervation of the spinal cord of the rat using specific antibodies against dopamine and noradrenaline. J. Chem. Neuroanat. 5:427-440

Nagy JI, Hunt SP, Iversen LL, Emson PC (1981). Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. Neuroscience 6:1923-1934

Naim M, Spike RC, Watt C, Shebab SA, Todd AJ (1997). Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input form tachykinin-containing primary afferents. J. Neurosci. 17:5536-5548.

Nicholas AP, Pieribone V, Hökfelt T (1993). Distributions of mRNAs for alpha-2 adrenergic receptor subtypes in rat brain: an in situ hybridization study. J. Comp. Neurol. 328:575-594

North RA and Yoshimura M (1984). The actions of noradrenaline on neurones of the rat substantia gelatinosa in vitro. J. Physiol. 349:43-55

Oertel WH, Schmechel DE, Mugnaini E, Tappaz ML, Kopin IJ (1981). Immunocytochemical localization of glutamate decarboxylase in rat cerebellum with a new antiserum. Neuroscience.6:2715-2735

Olave MJ and Maxwell DJ (2002). An investigation of neurons that possess the $\alpha_{2c}$-adrenergic receptor in the rat dorsal horn. Neuroscience 115:31-40

Olave MJ and Maxwell DJ (2003). Axon terminals possessing the $\alpha_{2c}$-adrenergic receptor in the rat dorsal horn are predominantly excitatory. Brain Res. 965:269-273

Ossipov MH, López Y, Bian D, Nichols ML, Porreca F (1997). Synergistic antinociceptive interactions of morphine and clonidine in rats with nerve-ligation injury. Anesthesiology 86:196-204

Pan YZ, Li DP, Pan HL (2001). Inhibition of glutamatergic synaptic input to spinal lamina $II_o$ neurons by presynaptic $\alpha_2$-adrenergic receptors. J. Neurophysiol. 87:1938-1947

Patel R, Kerr R, Maxwell DJ (1997). Absence of co-localized glutamic acid decarboxilase and neuropeptides in noradrenergic axons of the rat spinal cord. Brain Res. 749:164-169

Paxinos G and Watson C. The rat brain in stereotaxic coordinates. 3rd. (1997). New York, Academic Press.

Pechura CM and Liu RP (1986). Spinal neurons which project to the periaqueductal gray and the medullary reticular formation via axon collaterals: a double-label fluorescence study in the rat. Brain Res. 374:357-361

Peng YB, Lin Q, Willis WD (1996). Involvement of alpha-2 adrenoceptors in the periaqueductal gray-induced inhibition of dorsal horn cell activity in rats. J. Pharmacol. Exp. Ther. 278:125-135

Petkó M and Antal M (2000). Propriospinal afferent and efferent connections of the lateral and medial areas of the dorsal horn (laminae I-IV) in the rat lumbar spinal cord. J. Comp. Nerol. 422:312-325

Polgár E, Fowler JH, McGill MM, Todd AJ (1999). The types of neuron which contain protein kinase C gamma in rat spinal cord. Brain Res 833:71-80

Polgár E, Puskár Z, Watt C, Matesz C, Todd AJ (2002). Selective innervation of lamina I projection neurones that possess the neurokinin 1 receptor by serotonin-containing axons in the rat spinal cord. Neuroscience 109:799-809

Proudfit HK (1988). Pharmacologic evidence for the modulation of nociception by noradrenergic neurons. Prog. Brain Res. 77:357-370

Proudfit HK and Clark FM (1991). The projections of locus coeruleus neurons to the spinal cord. In: CD Barnes & O Pompiano (Eds.) The Neurobiology of the Locus Coeruleus. Progress in Brain Res. Vol. 88, Elsevier, Amsterdam, pp 123-141

Proudfit HK, Yeomas DC, Clark FM (1990). Differences in coeruleospinal projections in rat substrains, Soc. Neurosci. Abst. 16:96

Proudlock F, Spike RC, Todd AJ (1992). Immunocytochemical study of somatostatin, neurotensin, GABA, and glycine in rat spinal dorsal horn. J. Comp. Neurol. 327:289-297

Ramón y Cajal S (1909). Histologie du systeme nerveux de l'homme et des vertebres. Vol I. Pp. 908-911. Inst. Cajal, Madred, reprinted in 1952.

Rajaofetra N, Ridet JL, Poulat P, Marlier L, Sandillon F, Geffard M, Privat A (1992). Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. J. Neurocytol. 21:481-494

Rivero-Melian C, Rosario C, Grant G (1992). Demonstration of transganglionically transported choleragenoid in rat spinal cord by immunofluorescence cytochemistry. Neurosci. Lett. 145:114-117

Rosin DL, Talley EM, Lee A, Stornetta RL, Gaylinn BD, Guyenet PG, Lynch KR (1996). Distribution of alpha 2C-adrenergic receptor-like immunoreactivity in the rat central nervous system. J. Comp. Neurol. 372:135-165

Rosin D, Zeng D, Stornetta R, Norton F, RIley T, Okusa M, Guyenet P, Lynch K (1993). Immunohistochemical localization of alpha-2-A adrenergic receptors in catecholaminergic and other brainstem neurons in the rat. Neuroscience 56:139-155

Roudet C, Mouchet P, Feuerstein C, Savasta M (1994). Normal distribution of alpha 2-adrenoceptors in the rat spinal cord and its modification after noradrenergic denervation: a quantitative autoradiographic study. J. Neurosci. Res. 39:319-329

Rowan S, Todd AJ, Spike RC (1993). Evidence that neuropeptide Y is present in GABAergic neurons in the superficial dorsal horn of the rat spinal cord. Neuroscience 53:537-545

Rudomin P and Schmidt RF (1999). Presynaptic inhibition in the vertebrate spinal cord revisited. Exp. Brain Res. 129:1-37

Scheinin M, Lomasney JW, Hayden HD, Schambra UB, Caron MG, Lefkowitz RJ, Fremeau RJ (1994). Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain. Brain Res. Mol. Brain Res. 21:133-149

Schroder HD and Skagerberg G (1985). Catecholamine innervation of the caudal spinal cord in the rat. J. Comp. Neurol. 242:358-368

Seybold V and Elde R (1980). Immunohistochemical studies of peptidergic neurons in the dorsal horn of the spinal cord. J. Histochem. Cytochem. 28:367-370

Shen K-Z, North RA, Surprenant A (1992). Potassium channels opened by noradrenaline and other transmitters in excised membrane patches of guinea-pig submucosal neurons. J. Physiol. (Lond) 445:581-599

Shen K-Z and Surprenant A (1991). Noradrenaline, somatostatin and opioids inhibit activity of single HVA/N-type calcium channels in excised neuronal. Pflugers Arch. 418:614-616

Sheriff FE and Henderson Z (1994). A cholinerfgic propriospinal innervation of the rat spinal cord. Brain Res. 634:150-154

Shi TJS, Winzer-Serhan U, Leslie F, Hökfelt T (2000). Distribution and regulation of alpha(2)-adrenoceptors in rat dorsal root ganglia. Pain 84:319-330

Shoenen J (1982). The dendritic organization of the human spinal cord: the dorsal horn. Neuroscience 7: 2057-2088

Silverman JD and Kruger L (1990). Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and exzyme markers. J. Neurocytol. 19:789-801

Solomon RE, Aimone LD, Yaksh TL, Gebhart GF (1989). Telease of calcitonin gene-related peptide (CGRP) from rat spinal cord in vitro. Neurosci. Abstr. 15:549

Steinbusch HW (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience 6:557-618

Stewart W (2001). The organization of the monoaminergic and cholinergic systems in the spinal cord. Ph.D. Thesis, University of Glasgow.

Stewart W and Maxwell DJ (2000). Morphological evidence for selective modulation by serotonin of a subpopulation of dorsal horn cells which possess the neurokinin-1 receptor. Eur. J. Neurosci. 12:4583-4588

Stone LS, Broberger C, Vulchanova L, Wilcox GL, Hökfelt T, Riedl MS, Elde R (1998). Differential distribution of alpha2A and alpha2C adrenergic receptor immunoreactivity in the rat spinal cord. J. Neurosci. 18:5928-5937

Stone LS, Macmillan L, Kitto KF, Limbird L, Wilcox GL (1997b). The a2a-adrenergic receptor subtype mediates spinal analgesia evoked by a2-agonists and is necessary for spinal adrenergic/opioid synergy. J. Neurosci. 17:7157-7165

Stone LS, Vulchanova L, Riedl MS, Wang J, Williams FG, Wilcox GL, Elde R (1999). Effects of peripheral nerve injury on alpha-2A and alpha-2C adrenergic receptor immunoreactivity in the rat spinal cord. Neuroscience 93:1399-1407

Sugiura Y, Lee CL, Perl ER (1986). Central projections of identified unmyelinated (C) afferent fibres innervating mammalian skin. Science 234:358-361

Sugiura Y, Terui N, Hosoya Y (1989). Difference in distribution of central terminals between visceral and somatic unmyelinated (C) primary afferent fibers. J. Neurophysiol. 62:834-840

Sullivan AF, Dashwood MR, Dickenson AH (1987). Alpha 2-adrenoceptor modulation of nociception in rat spinal cord: location, effects and interactions with morphine. Eur. J. Pharmacol. 138:169-177

Surprenant A and North RA (1988). Mechanisms of synaptic inhibition by noradrenline acting at alpha-2 receptors. Proc. R. Soc. Lond [Biol.] 234:84-114

Surprenant A, Shen KZ, North RA, Tatsumi H (1990). Inhibition of calcium currents by noradrenaline, somatostatin and opioids in guinea-pig submucosal neurones. J. Physiol. (Lond) 431:585-608

Suzuki R, Morcuende S, Webber M, Hunt SP, Dickenson AH (2002). Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nature 5:1319-1326

Takamori S, Rhee JS, Rosenmund C and Jahn R (2000). Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407:189-194

Takano Y, Takano M, Yaksh TL (1992). The effect of intrathecally administered imiloxan and WB4101: possible role of a2-adrenoceptor subtypes in the spinal cord. Eur. J. Pharmacol. 219:465-468

Takano Y and Yaksh TL (1992). Characterization of the pharmacology of intrathecally administered alpha-2 agonists and antagonists in rats. J. Pharmacol. Exp. Ther. 261:764-772

Talley EM, Rosin DL, Lee A, Guyenet PG, Lynch KR (1996). Distribution of alpha 2A-adrenergic receptor-like immunoreactivity in the rat central nervous system. J. Comp. Neurol. 372:111-134

Tatsumi H, Costa M, Schimerlik M, North RA (1990). Potassium conductance increased by noradrenline, opioids, somatostatin, and G-proteins: whole-cell recording from guinea pig submucous neurons. J. Neurosci. 10:1675-1682

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

Todd AJ (2002). Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. Exp. Physiol. 87: 245-249

Todd AJ, Hughes DI, Polgár E, Mackie M, Ottersen PO, Maxwell DJ (2003). The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur. J. Neurosci. 17:13-27

Todd AJ and Lewis SG (1986). The morphology of Golgi-stained cells in lamina II of the rat spinal dosal horn. Journal of Anatomy 149:113-119

Todd AJ, McGill MM, Shehab SAS (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. Eur. J. Neurosci. 12:680-700

Todd AJ and McKenzie J (1989). GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord. Neuroscience 31:799-806

Todd AJ and Millar J (1983). Receptive fields and responses to ionophoretically applied noradrenaline and 5-hydroxytryptamine of units recorded in laminae I-III of cat dorsal horn. Brain Res. 288:159-167

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

Todd AJ, Russell G, Spike RC (1992). Immunocytochemical evidence that GABA and neurotensin exist in different neurons in laminae II and III of rat spinal dorsal horn. Neuroscience 47:685-691

Todd AJ and Spike RC (1993). The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog. Neurobiol. 41:609-645

Todd AJ, Spike RC, Brodbelt AR, Price RF, Shehab SA (1994a). Some inhibitory neurons in the spinal cord develop c-fos-immunoreactivity after noxious stimulation. Neuroscience 63:805-816

Todd AJ, Spike RC, Price RF, Neilson M (1994b). Immunocytochemical evidence that neurotensin is present in glutamatergic neurons in the superficial dorsal horn of the rat. J. Neurosci. 14:774-784

Todd AJ and Sullivan AC (1990). Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J. Comp. Neurol. 296:496-505

Trendelenburg AU, Hein L, Gaiser EG, Starke K (1999). Occurrence, pharmacology and function of presynaptic 2-autoreceptors in at 2A/D-adrenoceptor-deficient mice. Naunyn-Schmiedeberg's Arch. Pharmacol. 360: 540-551

Tuchscherer MM and Seybold VS (1985). Immunohistochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. Neuroscience 14:593-605

Tyce GM and Yaksh TL (1981). Monoamine release from cat spinal cord by somatic stimuli: an intrinsic modulatory system. J. Physiol. 314:513-329

Uchizono K (1965). Characteristics of excitatory and inhibitory synapses in the central nervous system of the cat. Nature 207:642-643

Uhlen S, Persson ML, Alari L, Post C, Axelsson KL, Wikberg JE (1990). Antinociceptive actions of alpha 2-adrenoceptor agonists in the rat spinal cord: evidence for antinociceptive alpha 2-adrenoceptor subtypes and dissociation of antinociceptive alpha 2-adrenoceptors from cyclic AMP. J. Neurochem. 55:1905-1914

Uhlen S and Wikberg JE (1988). Inhibtion of cyclic AMP production by alpha2-adrenoceptor stimulation in the guinea pig spinal cord slices. Pharmacol. Toxicol. 63:178-182

Uhlen and Wikberg JE (1989). Alpha 2-adrenoceptors mediate inhibition of cyclic AMP production in the spinal cord after stimulation of cyclic AMP with forskolin but not after stimulation with capsaicin or vasoactive intestinal peptide. J. Neurochem. 52:761-767

Varoqui H, Schafer MK, Zhu H, Weihe E, Erickson JD (2002). Identification of the differentiation-associated Na+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses, J. Neurosci. 22:142-155

Westlund KN, Bowker RM, Ziegler MG, Coulter JD (1981). Origins of spinal noradrenergic pathways demonstrated by retrograde transport of antibody to dopamine-beta-hydroxylase. Neurosci. Lett. 25:243-249

Westlund KN, Bowker RM, Ziegler MG, Coulter JD (1982). Descending noradrenergic projections and their spinal terminations. Prog. Brain Res. 57:219-238

Westlund KN, Bowker RM, Ziegler MG, Coulter JD (1983). Noradrenergic projections to the spinal cord of the rat. Brain Res. 263:15-31

Westlund KN, Bowker RM, Ziegler MG, Coulter JD (1984). Origins and terminations of descending noradrenergic projections to the spinal cord of monkey. Brain Res. 292:1-16

Westlund KN, Carlton SM, Zhang D, Willis WD (1990). Direct catecholaminergic innervation of primate spinothalamic tract neurons. J. Comp. Neurol. 299:178-86

Wikberg-Matsson A, Wikberg JE, Uhlen S (1995). Identification of drugs subtype-selective for alpha 2A-, alpha 2B-, and alpha 2C-adrenoceptors in the pig cerebellum and kidney cortex. Eur. J. Pharmacol. 284:271-279

Willis WDJ and Coggeshall RE (1991). Functional organisation of dorsal horn interneurons. In Sensory Mechnisms of the Spinal Cord, ed. by W.D. Willis & R.E. Coggeshall, pp. 153-216, Plenum Press, New York.

Willis WD, Haber LH, Martin RF (1977). Inhibition of spinothalamic tract cells and interneurons by brain stem stimulation in the monkey. J. Neurophysiol. 40:968-981

Winzer-Serhan UH, Raymon HK, Broide RS, Chen Y, Leslie FM (1997a). Expression of alpha2-adrenoceptors during rat brain development – I. alpha2A messenger RNA expression. Neuroscience 76:241-260

Winzer-Serhan UH, Raymon HK, Broide RS, Chen Y, Leslie FM (1997b). Expression of alpha2-adrenoceptors during rat brain development – II. alpha2C messenger RNA expression and [$^{3}$H]rauwolscine binding. Neuroscience 76:261-272

Xu Z, Chen SR, C Eisenach J, Pan HL (2000). Role of spinal muscarinic and nicotinic receptors in clonidine-induced nitric oxide release in a rat model of neuropathic pain. Brain Res. 861:390-398

Xu Z, Li P, Tong C, Figueroa J, Tobin JR, Eisenach JC (1996a). Location and characteristics of nitric oxide synthase in sheep spinal cord and its interaction with alpha(2)-adrenergic and cholinergic antinociception. Anesthesiology 84:890-899

Xu Z, Tong C, Eisenach JC (1996b). Acetylcholine stimulates the release of nitric oxide from rat spinal cord. Anesthesiology 85:107-111

Xu Z, Tong C, Pan HL, Cerda SE, Eisenach JC (1997). Intravenous morphine increases release of nitric oxide from spinal cord by an alpha-adrenergic and cholinergic mechanism. J. Neurophysiol. 78:2072-2078

Yaksh TL (1985). Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. Pharmacol. Biochem. Behav. 22:845-858

Zeng D and Lynch KR (1991). Distribution of alpha2-adrenergic receptor mRNA in the rat CNS. Mol. Brain Res. 10:219-225

Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J (1995). Glycine transporters are differentially expressed among CNS cells. J. Neurosci. 15: 3952-3969

Zhang X, Bao L, Xu Z-Q, Kopp J, Arvidsson U, Elde T, Hökfelt T (1994). Localization of neuropeptide Y Y1 receptors in the rat nervous system with special reference to somatic receptors on small dorsal root ganglion neurons. Proc. Natl. Acad. Sci 91:11738-11742

**PUBLICATIONS**