THE INVOLVEMENT OF ALPHA\textsubscript{1}-ADRENOCEPTORS IN THE INTERMEDIOLATERAL CELL COLUMN OF THE SPINAL CORD IN REGULATING SYMPATHETIC ACTIVITY

BY

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TO JIM AND MY FAMILY WITH MUCH LOVE AND THANKS
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ABSTRACT

Catecholamines are known to influence sympathetic function through regulation of the activity of sympathetic preganglionic neurones at the spinal level. Neuropharmacological, neurophysiological and autoradiographic techniques have been used to study the possible involvement of α₁-adrenoceptors in the intermediolateral cell column of the spinal cord in regulating the activity of sympathetic preganglionic neurones in the intact animal.

The location of α₁-adrenoceptor ligand binding sites in the cat spinal cord was investigated autoradiographically using [³H]prazosin. High levels of [³H]prazosin binding sites were found in the intermediolateral cell column. Non-specific binding was very low, indicating that the binding was to α₁-adrenoceptors.

In anaesthetised rats extracellular recordings were made from antidromically identified sympathetic preganglionic neurones and effects of iontophoretic applications of α₁-adrenoceptor agonists in the vicinity of these neurones studied. Iontophoresis of phenylephrine and methoxamine (both highly selective α₁-adrenoceptor agonists) caused increases in the firing rate of the majority of neurones studied. SPNs were also analyzed for respiratory- and ECG-related activity, and the axonal conduction velocities of the neurones were calculated to determine whether there was relationship between the physiological characteristics of a neurone and its response to α₁-adrenoceptor agonists. The results indicated that the effect of α₁-adrenoceptor agonists on an SPN is not related to its physiological characteristics.

Recordings were made from the inferior cardiac nerve of anaesthetised cats to determine whether sympathetic preganglionic neurones innervating this nerve are excited by α₁-adrenoceptor agonists microinjected into the intermediolateral cell column. Microinjection of 100 nl of phenylephrine caused increases in inferior cardiac nerve activity, an effect which could be antagonised by microinjection of the α₁-adrenoceptor antagonist alfuzosin. This antagonism appeared selective for phenylephrine since alfuzosin did not block the excitatory effects of
microinjections of glutamate and 5-HT.

It is concluded that α₁-adrenoceptors in the intermediolateral cell column may be involved in mediating excitatory input onto sympathetic preganglionic neurones in the intact animal. The possible roles and supraspinal sources of this input are discussed.
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<td>A5</td>
<td>A5 region of the pons</td>
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<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ADP</td>
<td>Afterdepolarisation</td>
</tr>
<tr>
<td>AHP</td>
<td>Afterhyperpolarisation</td>
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<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
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<tr>
<td>C1</td>
<td>C1 region of the rostral ventrolateral medulla</td>
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<tr>
<td>CB-HRP</td>
<td>Cholera toxin-B conjugated to horseradish peroxidase</td>
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<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSN</td>
<td>Cervical sympathetic nerve</td>
</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>[3H]</td>
<td>Tritiated (tritium labelled)</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HRP-WGA</td>
<td>Horseradish peroxidase conjugated with wheatgerm agglutinin</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>IC</td>
<td>Intercalated nucleus</td>
</tr>
<tr>
<td>ICpe</td>
<td>Nucleus intercalatus pars paraependymalis</td>
</tr>
<tr>
<td>ICN</td>
<td>Inferior cardiac nerve</td>
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<tr>
<td>ILf</td>
<td>Nucleus intermediolateralis thoracolumbalis pars funicularis</td>
</tr>
<tr>
<td>IML</td>
<td>Intermediolateral cell column</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>L2</td>
<td>Second lumbar segment of the spinal cord</td>
</tr>
<tr>
<td>Lamina X</td>
<td>Lamina X of the dorsal horn</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>Definition</td>
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<tr>
<td>NTS</td>
<td>Nucleus of the tractus solitarius</td>
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<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PAG</td>
<td>Phosphate activated glutaminase</td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus vulgaris leucoagglutinin</td>
</tr>
<tr>
<td>PNMT</td>
<td>Phenylethanolamine-N-methyl-transferase</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
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<tr>
<td>RSND</td>
<td>Renal sympathetic nerve discharge</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SPN</td>
<td>Sympathetic preganglionic neurone</td>
</tr>
<tr>
<td>T2</td>
<td>Second thoracic segment of the spinal cord</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>&gt;</td>
<td>Greater than</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
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CHAPTER 1
GENERAL INTRODUCTION
Sympathetic preganglionic neurones (SPNs) form the final common pathway from the central nervous system in the regulation of sympathetic function. They are a functionally heterogeneous group of neurones involved in regulating the heart, adrenal medulla, vascular smooth muscle, liver, kidney and many other organs (see Cabot 1990). The activity of these neurones is regulated by inputs of supraspinal and spinal origin which influence the neurones directly and/or via multisynaptic pathways.

SPNs have been studied extensively and substantial information on the morphology and location of these neurones and their axonal projections has been accumulated. This knowledge has been of vital importance for the studies presented in this thesis. This thesis is concerned with the catecholaminergic innervation of SPNs, and especially with the $\alpha_1$-adrenoceptor mediated input onto these neurones. This introduction therefore reviews the literature on the anatomy of SPNs and the CNS regions involved in the control of sympathetic neuronal activity, focusing on the catecholaminergic groups of the brainstem.

1.1 LOCATION AND MORPHOLOGY OF SPNS

Early studies using Golgi staining of SPNs indicated that these neurones were found in a region of the thoracolumbar region of the spinal cord on the lateral border of the grey matter (the intermediolateral cell column; IML; Clarke, 1851). Since then, more comprehensive studies have been carried out on the location and morphology of SPNs in the spinal cord. One of the first studies was that of Petras and Cummings (1972). Following thoracic or abdominal sympathectomy in the rhesus monkey, chromolytic neurones were found in distinct groups in the thoracic and lumbar sections of the spinal cord. Since this study, many investigators have retrogradely labelled SPNs in different species using horseradish peroxidase (HRP) injected into various sympathetic nerves or ganglia. These studies include those carried out in rat (Rando, Bowers and Zigmond, 1981; injection into superior cervical ganglion; Schramm, Adair, Stribling and Gray, 1975; Bacon and Smith, 1988; injection into adrenal medulla); guinea pig (Rubin and Purves, 1980; HRP applied to single white rami from T4-T8) and cat.
Investigators have also used monoclonal antibodies to choline acetyltransferase to label SPNs in the spinal cord of rat (Barber, Phelps, Houser, Crawford, Salvaterra and Vaughn, 1984; Markham and Vaughn, 1990). The morphology of SPNs has also been studied by filling individual antidromically identified neurones with HRP in various regions of the spinal cord (cat, Dembowsky, Czachurski and Seller, 1985a; neonate rat, Forehand, 1990; pigeon, Cabot and Bogan, 1987). All these investigations have observed a similar morphology and distribution of SPNs to that shown in the original degeneration study.

SPNs are found in four topographically distinct groups in the spinal cord. The majority (75% in rat; Rando et al. 1981; 78.2% in cat; Chung et al. 1979) of these neurones are located in the nucleus intermediolateralis thoracolumbalis pars principalis, or the intermediolateral cell column (IML) as it is commonly known. The cell population of the IML consists nearly entirely of SPN somata. A large number of these neurones have multipolar (triangular) or fusiform (spindle-shaped) cell bodies, while a small group of neurones with round soma are also present. The proportion of each type of neurone located within segment is not uniform throughout the spinal cord, in the first and second thoracic segment of the cat cell bodies are predominantly multipolar while cells in more caudal segments are generally fusiform (Oldfield and McLachlan, 1981). The neurones form bilateral columns in the IML, however, at distances along the column, clusters of 20-150 SPNs are found (Petras and Cummings 1972; Chung et al. 1979; Oldfield and McLachlan, 1981). These clusters or nests are located at regular intervals throughout the segments although the inter-nest distance decreases in the lumbar region of the cord. According to the species, these inter-nest distances vary from 100-500 \( \mu \text{m} \) in length. The dendritic trees of these neurones lie mainly in a rostrocaudal direction with little dorsoventral arborization. The dendritic trajectory of SPNs in the adult cat IML ranges from 1.5-2.5 mm which can span
a number of SPN clusters (Dembowsky et al. 1985a). Some SPNs within the clusters (and especially in the first and second thoracic segments; Oldfield and McLachlan, 1981) also give rise to medially projecting dendrites which mingle with the dendrites of SPNs in another region of the cord, the intercalated nucleus (Barber et al. 1984). On occasion, laterally extending dendritic bundles were also observed coursing into the white matter. It may be that SPNs with round cell bodies give rise to dendrites which are orientated in a mediolateral direction while the dendrites of fusiform SPNs extend in a rostrocaudal direction (see Bacon and Smith, 1988).

Another group of SPNs is found in the nucleus intermediolateralis thoracolumbalis pars funicularis (ILf), a region lateral to the IML in the white matter of the cord. In more rostral segments, a high proportion of SPNs are found in this area, scattered through the lateral funiculus (Chung et al. 1979; Strack, Sawyer, Marubio and Loewy, 1988). More caudally, the cells become fewer and merge into the lateral gray matter and IML of the cord. The cells tend to be larger than those in the IML with fusiform or multipolar soma. The dendritic bundles extend in a mediolateral plane to the IML and to the white matter (where they intermingle with laterally projecting dendrites from SPNs in the IML; Petras and Cummings, 1972; Barber et al. 1984).

The intercalated nucleus (IC) consists of transverse bands of SPNs which stretch from the central canal across to the IML. These bands of SPNs occur at regular intervals (300-400 μm in guinea pig; Rubin and Purves, 1980) along the length of the spinal cord but do not share a 1:1 relationship with the clusters of SPNs in the IML. Cells in this region are mostly of a large elongated fusiform variety with their dendrites running in a mediolateral plane (Petras and Cummings, 1972; Oldfield and McLachlan, 1981), intermingling with dendrites from SPNs in the IML.

SPNs are also situated in the nucleus intercalatus pars paraependymalis (ICpe; or central autonomic nucleus). These neurones are fusiform or multipolar with their
dendrites oriented in a transverse plane, projecting medially towards the central canal (and sometimes crossing over to the contralateral hemisection) and laterally towards the IML.

In order to determine whether SPNs which innervate diverse postganglionic nerves arise from different autonomic nuclei, the distribution of SPNs retrogradely labelled from a number of sympathetic nerves or ganglia has been studied (Jänig and McLachlan, 1986; Strack et al. 1988; Anderson, McLachlan and Srb-Christie, 1989). SPNs labelled from more rostral ganglia are located mainly in the IML and ILf, with only a small percentage of neurones in the IC and ICpe. SPNs labelled from the inferior mesenteric ganglia are predominantly located in the ICpe (up to 80%). SPNs retrogradely labelled from the lumbar sympathetic chain are located in the lateral area of the IML and the ILf, while neurones in the hypogastric nerve arise from cell bodies in the medial IML and the intercalated nucleus. Thus, there appears to be some degree of topographic distribution of SPNs within the autonomic nuclei.

The initial segments of sympathetic preganglionic neuronal axons are found either on the soma or the primary or secondary dendrites some distance from the soma (Dembowsky et al. 1985a). For SPNs in the IML and ILf, the axon follows a path along the lateral border of the ventral horn, while axons of SPNs situated around the central canal follow the medial border of the ventral horn, before exiting in the ventral rootlets. Sympathetic preganglionic axons are either unmyelinated or are small diameter myelinated axons, according to the species. The majority of axons in the rat are unmyelinated (Gilbey, Peterson and Coote, 1982b; Gilbey, Numao and Spyer, 1986) while in the cat, both unmyelinated and myelinated axons are found (Coggeshall and Galbraith, 1978; Kuo, Yang, Yamasaki and Krauthamer, 1982).

It has been suggested that axons project through several segments of the cord before exiting in the ventral roots. After sectioning of all white rami except T1 or T2, HRP injections into the stellate ganglion labelled cell bodies in spinal cord
segments from C8-T8 (Chung et al. 1979). Similar results were also seen in the
dog (Faden and Petras, 1978). This widespread labelling of SPNs in the spinal
cord may have been due to leakage of HRP which was subsequently taken up into
the cut ends of nerves. Application of HRP to a single cut white ramus in the
guinea pig, cat and hamster only labelled SPNs in the corresponding section of
the spinal cord with no labelling seen in adjacent segments both rostral and
caudal (Rubin and Purves, 1980). There is some evidence that in the neonatal
rat (Forehand, 1990) and pigeon (Cabot and Bogan, 1987; Bogan and Cabot,
1991) a small number of axons do give rise to an intraspinal axon branch but the
role of these collaterals is as yet unknown.

After exiting the spinal cord sympathetic preganglionic neuronal axons branch and
synapse on many postganglionic neurones (divergence) while each postganglionic
neurone receives input from a number of SPNs (convergence). SPNs innervate
neurones in more than one ganglion (e.g. the superior cervical and the stellate
ganglia; Lichtman, Purves and Yip, 1980). Retrograde labelling of SPNs from the
adrenal medulla and sympathetic cervical chain in rats (Appel and Elde, 1988) or
the intermesenteric and caudal lumbar trunk in golden hamster (Hancock, 1982)
did not result in double labelling of SPNs indicating that, after exiting the spinal
cord, axons travel either rostrally or caudally to innervate postganglionic
neurones, but not in both directions.

Postganglionic neurones are innervated by SPNs arising from a number of
segments in the cord. HRP injections into sympathetic ganglia have demonstrated
that neurones innervating specific ganglia are topographically organised in a
rostrocaudal manner. For example, SPNs innervating the cat stellate ganglion are
found in segments C8-T9 with the highest density originating in the first and
second thoracic segments (Chung et al. 1979; Oldfield and McLachlan, 1981;
Pardini and Wurster, 1984) while SPNs which innervate the more caudal lumbar
splanchnic nerves are located in segments L2 to L5 with the majority in L3 and
L4 (Baron et al. 1985). However, there is a large degree of overlap in the
distribution of SPNs innervating different ganglia. In the rat, SPNs innervating
the superior and middle cervical, and the stellate ganglia are located in segments C8-T5; C8-T7 and C8-T8 respectively (Strack et al. 1988). Thus, within one segment of the cord, SPNs are found which innervate different ganglia and end organs.

1.2 ONGOING ACTIVITY OF SPN

Extracellular recordings from SPNs in anaesthetized preparations have shown that a high proportion of SPNs (e.g. 76%; Gilbey et al. 1982b) do not display ongoing activity (Hongo and Ryall, 1966; Polosa, 1967,1968; Taylor and Gebber, 1973; Mannard and Polosa, 1973; Guyenet and Cabot, 1981; Coote, Macleod, Fleetwood-Walker and Gilbey, 1981; Guyenet and Stornetta, 1982; Gilbey et al. 1982b; Kadzielawa, 1983; Gilbey et al. 1986; Numao and Gilbey, 1987; Gilbey and Stein, 1991). However, intracellular recordings demonstrate that most SPNs receive a background excitatory input, primarily in the form of excitatory postsynaptic potentials (epsp's) that are subthreshold for initiation of action potentials (Dembowsky et al. 1985b).

There are two main components contributing to this excitatory input, a supraspinal component (mentioned in detail later) and a purely spinal component. In a spinal preparation, tonic activity is reduced but still present - severing the dorsal roots reduces the tonic activity of SPNs still further (Mannard and Polosa, 1973). Stimulation of somatic or visceral afferents causes up to 4 waves of reflex excitation in pre- or postganglionic nerves and single SPNs; early, intermediate, late and very late (Coote and Downman, 1966; Coote and Perez-Gonzalez, 1970; Foreman and Wurster, 1975; Dembowsky et al. 1985b). The early and intermediate reflexes persist after spinalisation indicating that they are spinal (Coote and Downman, 1966; Laskey, Schondorf and Polosa, 1979). The early reflex is probably mediated by a polysynaptic pathway onto SPNs since there is no anatomical evidence of a monosynaptic pathway between segmental afferents and SPNs (Petras and Cummings, 1972; Rethelyi, 1972), while the intermediate reflex may involve long propriospinal pathways. The role of propriospinal pathways in the control of SPNs has not been studied to any great degree and
may important in controlling the level of activity of SPNs. Stimulation of the femoral nerve (the afferents of which enter the spinal cord at the sixth and seventh lumbar segments) excited the majority of SPNs tested in the upper thoracic segments in the spinal cat (Laskey et al. 1979). However, much work is needed to elucidate the exact role of intersegmental input onto SPNs. The propriospinal pathways may not be exclusively excitatory since electrical or chemical stimulation of the cervical cord in spinalised rats inhibited renal sympathetic nerve activity (Schramm and Livingstone, 1987). The fact that chemical stimulation caused similar effects as electrical stimulation indicates that this inhibition is generated by cervical cell bodies rather than fibres of passage. The late reflex disappears on cooling the medulla oblongata which implies that the pathway ascends to and descends from the brainstem (Coote and Downman, 1966).

Not all sensory afferent inputs onto SPNs are excitatory. Stimulation of myelinated afferents of sciatic or ulnar nerves (Wyszogrodski and Polosa, 1973) or a number of visceral afferent nerves (eg superior laryngeal nerve; Bachoo and Polosa, 1985; inferior cardiac nerve; Weaver, 1977) can inhibit tonically active SPNs through supraspinal and spinal pathways.

1.2a Rhythmic modulation of ongoing activity

(i) Respiratory-related activity of SPNs

Adrian, Bronk and Phillips, (1932) first demonstrated respiratory-related activity in sympathetic nerves. Since then many investigations have provided evidence of respiratory-related activity patterns in sympathetic nerves in the rat (Numao, Koshiya, Gilbey and Spyer, 1987); cat (Gootman and Cohen, 1974; Barman and Gebber, 1976); dog (Okada and Fox, 1967) and human (recording from peripheral nerve bundles innervating human skeletal muscle; Hagbarth and Vallbo, 1968). Whole nerve recording, however, cannot determine whether SPNs with axons projecting in the nerve all have similar patterns of activity or whether there are groups of SPNs with different firing patterns. Studies by Gilbey and coworkers (Gilbey et al. 1986; Numao et al. 1987) have provided evidence for a number of
different respiratory-related firing patterns in SPNs with axons projecting in the same nerve. They recorded both whole nerve activity in the cervical sympathetic nerve (CSN) and single sympathetic preganglionic neuronal activity from neurones whose axons travel in the CSN. The CSN exhibited maximal discharge in the early expiratory phase and minimal discharge during inspiration. In contrast, SPNs which were antidromically activated by stimulation of the CSN showed 3 distinct respiratory-related firing patterns; maximal discharge during phrenic nerve activity (inspiratory-related); maximal discharge during phrenic silence (expiratory-related) and a firing pattern unrelated to phrenic nerve discharge. The results were obtained from SPNs which either had ongoing activity or were silent (and could be activated by glutamate). The majority of SPNs which had ongoing activity fired maximally during expiration. It is this overall pattern of activity which dictates the expiratory-related firing pattern seen in the CSN. Similar patterns of respiratory-related activity have been observed in SPNs in the cat (Preiss, Kirchner and Polosa, 1975; Preiss and Polosa, 1977; Gerber and Polosa, 1978, 1979), although the majority of SPNs innervating the CSN had inspiratory-related activity. Interestingly, recent recordings from SPNs in the more caudal regions of the cord in the cat (L2-L3; Gilbey and Stein, 1991) and rat (T13-L2; Zhou and Gilbey, 1992) have observed both quantitative and qualitative differences in the respiratory-related activity of these neurones and the activity of those recorded at more rostral levels. It appears that the activity of SPNs is not always modulated by central respiratory drive and those neurones that are modulated display different patterns of respiratory-related activity which may be related to function. Bachoo and Polosa (1985) suggested that a proportion of the neurogenic vasoconstrictor tone in the cat hind limb was attributable to an inspiration-synchronous component of sympathetic discharge, since stimulation of the superior laryngeal (which suppresses the burst firing in inspiration of SPNs; Gerber and Polosa, 1979) caused hind limb vasodilatation. Therefore SPNs which innervate the hind limb would be expected to display some degree of respiratory related firing pattern.
(ii) Baroreceptor modulation of SPN activity

Adrian et al. (1932) first showed that sympathetic nerve activity was grouped at a frequency equal to the heart beat in cats and rabbits. Tying the carotids or sectioning the vagi abolished this rhythmicity indicating that it was due to periodic depression of SPN activity by the baroreceptor reflex. Activation of the baroreceptor reflex caused decreases in the activity of pre- and postganglionic nerves of the cat (Cohen and Gootman, 1970; Taylor and Gebber, 1975; Harada, Ando, Imaizumi, Hirooka, Sunagawa and Takeshita, 1991); dog (Kedzi and Geller, 1968) and human (Hagbarth and Vallbo, 1968). Similar results were also obtained from intracellular and extracellular recordings of single SPNs in the cat (Polosa, 1968; McLachlan and Hirst, 1980) and the rat (Numao and Gilbey, 1987).

R-wave triggered histograms reveal ECG-related patterns of activity in single SPNs which are not always obvious from studying the ongoing activity. A number of studies recording from single SPNs in the cat have shown that some SPNs fire in relation to the cardiac cycle (Mannard and Polosa, 1973; Coote and Westbury, 1974; Jänig and Szulczyk, 1980; Backman and Henry, 1984; Backman, Sequiera-Martinho and Henry, 1990; Gilbey and Stein, 1991; Boczek-Funcke, Habler, Jänig and Michaelis, 1991) as do those in the rat (Gilbey et al. 1982b).

The mechanism involved in the decrease in SPN activity following baroreceptor activation has been studied using intracellular recordings from individual neurones (McLachlan and Hirst, 1980; Dembowski et al. 1986). Baroreceptor activation caused postsynaptic membrane hyperpolarisations which were often accompanied by a decrease in membrane resistance, and which could be reduced by making the membrane potential more negative. This suggests a direct inhibitory effect on the postsynaptic membrane. However, in some neurones hyperpolarisation of the membrane occurred without a change in cell input resistance, indicating that the hyperpolarisation was due to a disfacilitation rather than inhibition. Therefore two mechanisms may be responsible for the baroreceptor modulation of sympathetic preganglionic neuronal activity, a withdrawal of excitatory drive and a postsynaptic inhibition of the neurones. Studies on the firing patterns of
supraspinal descending sympathoexcitatory neurones in the rostral ventrolateral medulla (RVLM); A5 region of the pons; the raphe nuclei and the paraventricular nucleus (PVN) and lateral regions of the hypothalamus have indicated that many of these neurones display cardiac-related firing patterns which are time-locked to sympathetic nerve discharge (Morrison and Gebber, 1982; McAllen, 1986; Barman, 1990; Huangfu, Koshiya and Guyenet, 1991). Thus increasing blood pressure decreases the firing rate of both neurones in the supraspinal regions and SPNs in the spinal cord. Also located in the raphe pallidus are putative sympathoinhibitory neurones which also have cardiac related activity and are excited by increases in blood pressure (Morrison and Gebber, 1982; 1984). Another component of baroreceptor modulation of SPNs may be through activation of these raphe neurones which inhibit the activity of SPNs. It is not known whether these effects are due to direct inhibition of SPNs or whether inhibitory interneurones in the cord are involved.

1.3 SUPRASPINAL CONTROL OF SYMPATHETIC ACTIVITY

1.3a Early studies
For many years it has been known that SPNs are under a high degree of supraspinal control. Bernard (1863) first indicated that the tonic discharge of sympathetic vasoconstrictor nerves is generated primarily by supraspinal neuronal circuits since transection of the spinal cord at the medullospinal border caused a large decrease in blood pressure. Sherrington (1906) showed that after recovery from spinal shock, a second transection below the level of the first had no further effect on blood pressure. He concluded that the initial depressor response was due to a loss of tonically active supraspinal influences on the sympathetic nervous system. Since then it has been demonstrated that spinalisation decreases the number of SPNs which have ongoing activity and the levels of activity seen in those neurones which are still firing which leads to an overall decrease in whole nerve activity (Alexander, 1946; Polosa, 1968; Cohen and Gootman, 1970; Mannard and Polosa, 1973; Meckler and Weaver, 1988 Dembowski et al. 1985b). In addition, the discharge patterns of SPNs or whole nerves are affected by spinalisation - any cardiac or respiratory-related activity is lost (Cohen and
Oswjannikow (1871) carried out serial transections of the cat and rabbit brain and monitored the effect of these lesions on blood pressure. It was observed that blood pressure remained stable until the level of the pons was reached where a cut made 1-2 mm behind the caudal border of the inferior colliculus caused a decrease in blood pressure. Transections carried out caudal to this area progressively decreased blood pressure until the region just rostral to the obex in the medulla was reached. At this level blood pressure was similar to that observed after spinalisation and further cuts caudal to this had no effect on blood pressure. Similar results were observed by Dittmar (1873) in the rabbit using knife cuts in the brainstem. In addition, he located more precisely an area in the ventral medulla which was important in the maintenance of blood pressure. These studies were repeated and extended by Alexander (1946) in the cat, who recorded from the inferior cardiac nerve and showed that activity in this nerve was decreased by sectioning of the medulla at the pontomedullary border. When the medulla was sectioned at the point where the decrease in blood pressure was maximal, inferior cardiac nerve activity disappeared completely. Moreover, a further section at the medullospinal border caused a slight increase in cardiac nerve activity. This indicated for the first time that there were tonically active descending sympathoinhibitory neurones situated more caudal to the pressor region. Wang and Ranson (1939) and Alexander (1946) electrically stimulated the brainstem and mapped pressor and depressor regions. These investigations indicated that there are a number of regions within the brainstem which may be involved in controlling blood pressure.

1.3b The location of supraspinal regions innervating the IML

The above studies demonstrated the importance of the brainstem in maintaining blood pressure and sympathetic activity. However, it was the advent of neuroanatomical tract tracing techniques which determined the precise location of those supraspinal regions which project to the IML and are therefore likely to be intimately involved in sympathetic control.
Amendt, Czarchuski, Dembowsky and Seller (1979) injected HRP into the IML of the cat which labelled neurones in a number of regions in the brain stem, namely the nucleus of the tractus solitarius (NTS); the ventrolateral region of the medulla and a ventral medial region including the caudal raphe. Injections of fluorescent tracers into the grey matter of the rat spinal cord resulted in labelling of neurones in the hypothalamus (PVN and lateral hypothalamus); pons, (A5 and Kolliker-Fuse) and medulla (NTS and the rostral ventrolateral medulla; RVLM; Tucker and Saper, 1985).

These studies give an overall view of the projections from areas in the brain to the IML of the spinal cord in rat and cat. It is this information which prompted investigators to perform further neuroanatomical and electrophysiological studies on these regions, thus gaining insight into the role of the different brainstem regions in controlling sympathetic activity. These studies are reviewed in the following sections.

1.3c The rostral ventrolateral medulla

(i) Neuroanatomical studies

The RVLM consists of a population of neurones projecting to a number of supraspinal and spinal sites, including the IML. It is located caudal to the facial nucleus in a region ventral to the nucleus reticularis parvocellularis and ventrolateral to the nucleus reticularis gigantocellularis.

Projections to the IML from the RVLM have been observed using a number of retrograde and anterograde tracing techniques. Injection of HRP either into the spinal cord (Blessing, Goodchild, Dampney and Chalmers, 1981; Ross, Ruggiero, Joh, Park and Reis, 1984) or more precisely into the IML (Caverson, Ciriello and Calaresu, 1983) retrogradely labelled cells in the RVLM. Similar results were also seen with injection of fluorescent tracers into the spinal cord (Ross, Armstrong, Ruggiero, Pickel, Joh and Reis, 1981; Sawchenko and Bohn, 1989) or IML (Tucker, Saper, Ruggiero and Reis, 1987); injections of fluorogold into the spinal cord (Haselton and Guyenet, 1989) or IML (Jeske and McKenna, 1989)
and injections of colloidal gold conjugated with cholera Toxin B (CB) into the spinal cord (Llewellyn-Smith, Minson, Wright and Hodgson, 1990; Minson, Llewellyn-Smith, Neville, Somogyi and Chalmers, 1990).

These results must be interpreted with caution since some of the injections made were large (eg 2 µl injections into rat spinal cord; Ross et al. 1981; 1984) and may have spread into other areas of the cord especially the lateral funiculus which contains a large proportion of descending fibres from different areas of the brain. Caverson et al. (1983) provided the best control experiments when they showed that injections made in the same way into areas of the spinal cord which did not include the IML led to few labelled cells in the brainstem.

Following injections of anterograde tracers into the RVLM, labelled boutons were observed in the IML of the spinal cord (Loewy, Wallach and McKellar, 1981; Ross et al. 1984). These boutons were seen in close apposition to SPNs which were retrogradely labelled from the adrenal medulla (Ross et al. 1984). Furthermore, Zagon and Smith (1990) demonstrated monosynaptic connections between anterogradely labelled axons and the somata, proximal and distal dendrites of retrogradely labelled SPNs in the rat IML after injections of PHA-L into the RVLM and CB-HRP into the adrenal medulla.

(ii) The C1 region of the RVLM

The RVLM has been shown to contain many neurones which are immunoreactive for phenylethanolamine-N-methyl-transferase (PNMT: the enzyme involved in catalysing the conversion of noradrenaline to adrenaline), the region containing these neurones has been designated the C1 area (Hökfelt, Fuxe, Goldstein and Johansson, 1974).

Retrograde labelling of RVLM neurones after injections of tracers into the spinal cord combined with immunohistochemical labelling of neurones demonstrated that a high proportion of PNMT-containing neurones project to the spinal cord (Ross et al. 1981; Haselton and Guyenet, 1989; Sawchenko and Bohn, 1989;
Llewellyn-Smith et al. 1990; Minson et al. 1990) and the region of the IML (Tucker et al. 1987; Jeske and McKenna, 1989). Moreover, retrograde transneuronal labelling of neurones in the RVLM was combined with immunofluorescence to see if adrenergic bulbospinal neurones project to SPNs which innervate the adrenal gland (Strack, Sawyer, Platt and Loewy, 1989b). Using this technique, over half of virally infected cells in the RVLM were shown to contain PNMT.

These investigations have shown that a high number of spinally projecting neurones in the RVLM contain PNMT. Equally however, a large number of these neurones were not immunoreactive for PNMT. This may be due to problems involving the immunohistochemical techniques. Some antibodies do not readily penetrate sections of tissue therefore retrogradely labelled neurones containing PNMT which are located deeper in the section may not be seen as immunoreactive for PNMT. However, there is evidence that other neurotransmitters are also localised in bulbospinal neurones in the RVLM. Numerous studies have shown spinally projecting neurones in the RVLM which contain NPY (Blessing, Oliver, Hodgson, Joh and Willoughby, 1987; Strack et al. 1989b); somatostatin (Strack et al. 1989b); enkephalin (Hökfelt, Terenius, Kuypers and Dann, 1979; Menetrey and Basbaum, 1987; Strack et al. 1989b); substance P (Helke, Neil, Massari and Loewy, 1982; Strack et al. 1989b). In addition there is evidence that adrenaline may coexist with other neurotransmitters including NPY (Everitt, Hökfelt, Terenius, Tatemoto, Mutt and Goldstein, 1984; Blessing et al. 1987; Murakami, Okamura, Pelletier and Ibata, 1989); substance P (Lorenz, Saper, Wong, Ciarenello and Loewy, 1985); enkephalin (Murakami et al. 1989; Ceccatelli, Millhorn, Hökfelt and Goldstein, 1989) and glutamate or gamma-aminobutyric acid (GABA; demonstrated using antibodies to phosphate activated glutaminase; PAG, an enzyme involved in synthesis of glutamate and GABA; Kaneko, Akiyama, Nagatsu and Mizuno, 1990).
(iii) Effects of stimulation within the RVLM

(a) Electrical stimulation
Many of the first studies involved electrical stimulation of the RVLM which elicited increases in blood pressure in the cat (Guertzenstein and Silver, 1974; Thomas, Ulrichsen and Calaresu 1977); rat (Ross, Ruggiero, Joh, Park and Reis 1983; Conner and Drew, 1987; Routledge and Marsden, 1987; Routledge, Marshall and Marsden, 1988) and rabbit (Dampney and Moon, 1980). This effect appeared to involve activation of sympathetic nerves since intravenous injections of guanethidine (an adrenergic neurone blocker) markedly reduced the pressor-effect (Conner and Drew, 1987).

Single shock electrical stimulation of the RVLM evoked excitatory potentials in the inferior cardiac nerve (cat; Thomas et al. 1977); the lumbar sympathetic chain (rat; Guyenet and Brown, 1986) and the splanchnic nerve (rat; Morrison, Milner and Reis, 1988). In the sympathetic chain and splanchnic nerve two peaks of excitation were observed indicating that 2 groups of spinally projecting RVLM neurones with different conduction velocities were excited.

(b) Chemical Stimulation
Results from the above studies indicate a sympathoexcitatory role for RVLM neurones, however, electrical stimulation activates nerve endings and fibres of passage as well as cell bodies, therefore many investigators have studied the effect of applying excitatory amino acids (which activate cell bodies only) to RVLM neurones.

Microinjections of glutamate into the area of the RVLM in the rat increased blood pressure; heart rate (Ross et al. 1983; Willette, Krieger, Barcas and Sapru, 1983; Willette, Barcas, Krieger and Sapru, 1984; Conner and Drew, 1987) and the resistance of various vascular beds (Willette, Punnen-Grandy, Krieger and Sapru, 1987). In the conscious rat microinjections of glutamate into the RVLM elicited increases in blood pressure but also caused bradycardia which may have been due to activation of the baroreceptor reflex since the vasopressor effects were much
larger than those observed in anaesthetized rats (Bachelard, Gardiner and Bennett, 1990). Lesions of the RVLM (Granata, Ruggiero, Park, Joh and Reis, 1985) or applications of inhibitory neurotransmitters (Benarroch, Granata, Ruggiero, Park and Reis, 1986; Yardley, Stein and Weaver, 1989) into this region decreased blood pressure, heart rate and sympathetic postganglionic nerve activity indicating that RVLM sympathoexcitatory neurones are tonically active.

(iv) Firing patterns of RVLM neurones and their response to activation of the baroreceptor reflex

Researchers have attempted to determine whether RVLM neurones are modulated by baroreceptor inputs and whether their firing patterns are related to sympathetic activity and the cardiac cycle.

Recordings were made from RVLM neurones which were antidromically activated by stimulation of the spinal cord. Many of these neurones had ongoing activity which was phasically linked to the cardiac cycle. In addition, the discharge of RVLM neurones was synchronised with the cardiac-related bursts in postganglionic sympathetic nerve activity (Brown and Guyenet, 1984; 1985; Barman and Gebber, 1985; Sun and Guyenet, 1986a; McAllen, 1986; Morrison et al. 1988; Haselton and Guyenet, 1989). In baroreceptor denervated rats, activity of both RVLM neurones and sympathetic nerve discharge showed a 2-6 Hz rhythm (Morrison et al. 1988). A high proportion of RVLM neurones had their activity decreased by activation of the baroreceptor reflex, either by intravenous injections of a vasopressor agent or by increasing carotid sinus pressure (Brown and Guyenet, 1984; 1985; Barman and Gebber, 1985; Sun and Guyenet, 1986a; McAllen, 1986; Morrison et al. 1988; Haselton and Guyenet, 1989). This effect seemed to be due to a direct inhibition rather than withdrawal of an excitatory input since, when recording intracellularly from RVLM neurones, carotid baroreceptor stimulation caused short latency hyperpolarisation of these neurones associated with a change in membrane resistance (Dembowsky and McAllen, 1990). This inhibition may be mediated by GABA receptors since injections of bicuculline (a GABA antagonist) into the RVLM also abolished the
sympathoinhibitory and hypotensive effects of vagal nerve stimulation (Sun and Guyenet, 1987). In addition, iontophoretic application of bicuculline onto a single RVLM neurone antagonised the reduction in the unit activity due to increased blood pressure (Sun and Guyenet, 1985). Application of GABA onto the ventral surface of the medulla (Guertzenstein, 1973; Benarroch et al. 1986) caused decreases in blood pressure and heart rate, as did injections of muscimol (a GABA agonist) into the RVLM (Willette et al. 1983; 1987). Iontophoretic application of GABA also inhibited the activity of single RVLM neurones and eliminated their pulse-synchronous discharge (Sun and Guyenet, 1985; Sun and Spyer, 1991). This GABAAergic inhibitory input may be tonically involved in the regulation of blood pressure since application of a GABA antagonist, bicuculline, into the RVLM caused a large increase in blood pressure (Willette et al. 1984) and iontophoretic application of bicuculline onto single RVLM neurones increased their firing rate (Sun and Guyenet, 1985). There is evidence that inhibition of RVLM activity following activation of the baroreceptor reflex is mediated by an indirect pathway from the nucleus of the tractus solitarius (NTS; which is the site of termination for baroreceptor afferents; see Spyer, 1981) via the caudal ventrolateral medulla (CVLM). Injection of the glutamate antagonist kynurenic acid into the CVLM blocked the inhibitory response of RVLM neurones to increases in blood pressure or stimulation of the NTS (Agarwal, Gelsema and Calaresu, 1990).

(v) Importance of the RVLM in maintaining sympathetic tone
Many investigators have argued on the strength of the above observations that the RVLM is the sole area responsible for the maintenance of sympathetic vasomotor tone. In the light of recent experiments, however, this may not be the case. Cochrane and co-workers (Cochrane, Buchholz, Hubbard, Keeton and Nathan, 1988; Cochrane and Nathan, 1989) re-investigated the effect of lesioning the RVLM on blood pressure in anaesthetized and conscious rats. Lesions made in the RVLM of rats anaesthetized with urethane, α-chloralose or sodium pentobarbitone caused immediate decreases in blood pressure and heart rate. Thirty minutes after lesioning, however, the blood pressures of those rats
anaesthetized with α-chloralose or pentobarbitone had returned to near control levels although heart rate remained low. Subsequent spinalisation caused a large decrease in the blood pressure of rats anaesthetized with α-chloralose or pentobarbitone while the blood pressure of rats anaesthetized with urethane was not further decreased. Lesions of the RVLM were also made in rats which were allowed to recover from this surgery. One day after lesioning, the blood pressures and heart rates of these conscious rats were similar to prelesion control levels. Baroreflex-mediated tachycardia to a decrease in blood pressure was attenuated but bradycardia due to increase in blood pressure was not affected by the RVLM lesions.

These results suggest that the RVLM is not the only supraspinal region capable of maintaining vasomotor tone. The many experiments concentrating on this area have neglected other regions in the brain which project to the IML and which may be equally or more important in the regulation of sympathetic outflow.

1.3d The A5 region of the medulla
(i) Neuroanatomical studies
The A5 region of the brainstem is a column of cells lying adjacent to the superior olivary nucleus and facial nucleus in the ventrolateral pontine reticular formation. Early studies by Dahlström and Fuxe (1965) using a histochemical fluorescence method for demonstration of catecholamines combined with transection experiments failed to show that this region projected to the spinal cord. Since then, however, investigations have shown a dense projection of A5 neurones to the IML and other autonomic areas of the spinal cord. Loewy, McKellar and Saper (1979) injected tritiated amino acids into the A5 region of the rat and traced descending fibres in the lateral funiculus of the spinal cord which terminated densely in the IML and intercalated nucleus of the thoracic and upper lumbar cord. Other anterograde tracers such as HRP conjugated with wheat germ agglutinin (HRP-WGA; Byrum and Guyenet, 1987) and PHA-L (Fritschy and Grzanna, 1990) injected into the A5 region of rat also labelled fibres in the IML of the spinal cord. Injections of HRP into the spinal cord of the rat (Loewy
et al. 1979) or rabbit (Blessing et al. 1981) was combined with catecholamine fluorescence labelling in the A5 region to show that the same neurones in A5 that were retrogradely labelled with HRP also contained catecholamines. Pretreatment with 6-hydroxydopamine (6-OHDA), which is a catecholamine neurotoxin, abolished both retrograde and anterograde labelling of neurones in the IML or A5 region of the rat. In addition, experiments involving the retrograde transport of antibody to dopamine-β-hydroxylase (which labels catecholaminergic neurones) labelled neurones in the A5 region of the rat (Westlund, Bowker, Ziegler and Coulter, 1981).

Very little work has been carried out to date on the projections of the A5 region in the cat. Fleetwood-Walker and Coote (1981a) electrolytically lesioned various brain-stem regions and examined the noradrenaline content in the IML. Lesions of the A5 region did not significantly affect the levels of catecholamines in the ventral part of the spinal cord, although A1 lesions caused a decrease in the catecholamine concentration in the cord. They concluded that there was no significant spinal projection from the A5 region in cat, however, an A5 projection to the IML cannot be ruled out entirely as this conclusion was reached from the result obtained in only one cat and the amount of time allowed for depletion of noradrenaline may not have been long enough to allow for a full reduction in noradrenaline levels.

(ii) Neurophysiological studies
Experiments investigating the role of the A5 region of the brainstem in control of the sympathetic nervous system give conflicting results. Electrical stimulation of the region increased blood pressure and reflexly decreased heart rate in the rat (Loewy, Gregorie, McKellar and Baker, 1979a) and rabbit (Woodruff, Baisden and Whittington, 1986; Drye, Baisden, Whittington and Woodruff, 1990), effects which were abolished by pretreatment of the animals with 6-OHDA. However, in the rat, microinjection of glutamate or NMDA decreased blood pressure and heart rate (Neil and Loewy, 1982; Close, Neil and Loewy, 1982); increased blood flow and decreased cardiac output, stroke volume and vascular resistance (Stanek,
Neil, Sawyer and Loewy, 1984). These effects could also be abolished by pretreatment with 6-OHDA. In a later experiment in the rat, however, intraspinal injections of 6-OHDA blocked the decrease in blood pressure by only 60% and the decrease in heart rate by only 30% (Loewy, Marson, Parkinson, Perry and Sawyer, 1986). Results from this study also implicated the NTS in mediating some of the effects of chemical stimulation of the A5 region since injections of 6-OHDA into both the NTS and spinal cord led to a greater attenuation of the heart rate and blood pressure responses to A5 stimulation (up to 80%). Similar decreases in heart rate and blood pressure were observed in rabbits after glutamate injections into the A5 region, effects which were only reduced by lesions of the NTS or injections of 6-OHDA (Drye et al. 1990). These results suggest that the depressor response to chemical stimulation of the A5 region may be only partially mediated by a descending noradrenergic pathway. It must be noted that depletion studies with 6-OHDA will by no means completely deplete the spinal cord of noradrenaline and the levels left in the spinal cord may be sufficient to mediate the depressor response.

The above investigations concluded that the pressor responses due to electrical stimulation of the A5 cell group were probably due to stimulation of fibres passing through the region and not a result of activation of cell bodies. However, recordings made from spinally projecting A5 neurones have shown that some of these neurones are inhibited by increases in blood pressure, an effect which is attenuated by baroreceptor denervation (Guyenet, 1984; Guyenet and Byrum, 1985). This indicates that these neurones are sympathoexcitatory and a recent study by Huangfu et al. (1991) has supported this view. They recorded from neurones in the A5 region which were antidromically activated by stimulation of the spinal cord. Injections of 6-OHDA into the lateral funiculus which would destroy the noradrenergic axons coursing through this region, abolished antidromic activation of these neurones suggesting that the recorded neurones were noradrenergic. A high percentage of these neurones were inhibited by increases in blood pressure or aortic nerve stimulation although very few neurones exhibited pulse-related activity. Analysis of the correlation between splanchnic
sympathetic nerve discharge and the firing of A5 neurones using spike-triggered averaging revealed a clear correlation between the neuronal discharge and sympathetic nerve activity. Thus the A5 region may be involved in both sympathoexcitation and sympathoinhibition.

1.3e The caudal raphe nuclei

(i) Neuroanatomical studies

The caudal raphe nuclei are positioned on or near the mid-line of the medulla. Early neuroanatomical studies indicated a projection from the brainstem raphe nuclei to the IML in a number of different species. Dahlström and Fuxe (1965) identified a bulbospinal 5-hydroxytryptamine- (5-HT) containing pathway in rats using a histochemical fluorescent technique (which labels 5-HT neurones and axons yellow) combined with lesions made in the spinal cord. The bulbospinal neurones originated in the the nucleus raphe pallidus, obscurus and magnus (B1, B2 and B3 cell groups, respectively; see Dahlström and Fuxe, 1964) and projected to the IML of the spinal cord via the dorsolateral funiculus. These results confirmed, and extended, observations made from lesioning studies in the kitten that neurones in the raphe nuclei had descending projections to the cord (Brodal, Taber and Walberg, 1960). After injections of HRP into the spinal cord, labelled neurones were observed in the raphe nuclei of the cat (Kuypers and Maisky, 1975) and rabbit (Haselton, Winters, Liskowsky, Haselton, McCabe and Schneiderman, 1988). Basbaum, Clanton and Fields (1978) injected [3H]leucine into the nucleus raphe magnus which led to labelling in the IML of cat spinal cord. There is also a raphe-spinal pathway in the pigeon which innervates the sympathetic preganglionic column. This was shown using a combination of retrograde (HRP into the spinal cord) and anterograde ([3H]proline into the raphe nuclei) tracers and anterograde degeneration techniques (Cabot, Wild and Cohen, 1979). A recent study in which injections of anterograde tracer (PHA-L) into the raphe pallidus and raphe magnus were combined with retrograde labelling of adrenal SPNs using cholera-B conjugated to HRP provided evidence of monosynaptic connections between the raphe spinal neurones and SPNs in the IML of rat spinal cord (Bacon, Zagon and Smith, 1990).
So far only 5-HT-containing raphe-spinal neurones have been considered. Evidence suggests that other neurotransmitters are also present in raphe-spinal neurones and that co-existence of these transmitters may occur. Raphe neurones were retrogradely labelled by injections of rhodamine bead-labelled microspheres into the IML and antibodies to substance P, 5-HT and thyrotropin-releasing hormone were used to study the neurotransmitter content of these neurones. Neurones projecting to the IML contained any one of these neurotransmitters or a combination of any two or all three of the transmitters (Sasek, Wessendorf and Helke, 1990). These observations are supported by immunofluorescence studies which demonstrated colocalisation of these three neurotransmitters in the raphe nuclei (Johansson, Hokfelt, Pernow, Jeffcoate, White, Steinbusch, Verhofstad, Emson and Spindel, 1981). In addition, there is evidence of co-existence of 5-HT and neuropeptide Y (Blessing, Howe, Joh, Oliver and Willoughby, 1986); 5-HT and enkephalin (Leger, Charnay, Dubois and Jouvet, 1986); 5-HT and GABA (Belin, Nanapoulos, Didier, Aguera, Steinbusch, Verhofstad, Maitre and Pujol, 1983) and 5-HT and PAG (Kaneko et al. 1990) in neurones located in all 3 raphe nuclei.

(ii) Neurophysiological studies
Investigations involving the raphe nuclei have indicated that this region may have a dual role in the control of sympathetic activity.

Electrical stimulation in the nucleus raphe pallidus (Coote and Macleod, 1974a; 1975) and nucleus raphe obscurus (Neumayr, Hare and Franz, 1974) of the cat caused depressor responses and inhibition of spontaneous and reflexly evoked sympathetic discharge. Lesions of the dorsolateral funiculus of the spinal cord abolished this sympathoinhibition. Henry and Calaresu (1974) recorded field potentials in the raphe nuclei evoked from electrical stimulation of the IML in cat and showed that stimulation of those regions in the raphe caused decreases in heart rate and blood pressure. Extracellular recordings from SPNs in the spinal cord of the pigeon showed that raphe stimulation strongly inhibited SPN discharge in addition to causing significant depressor responses (Cabot et al. 1979).
Lesioning the raphe nuclei caused increases in sympathetic nerve discharge but had no effect on the inhibition of nerve discharge by activation of the baroreceptor reflex, suggesting that at least a proportion of the sympathoinhibition is due to a tonic nonbaroreceptor-mediated inhibition from the raphe nuclei (Barman and Gebber, 1978; McCall and Harris, 1987).

This sympathoinhibition caused by raphe stimulation may be due to activation of 5-HT-containing raphe-spinal pathways. Electrical stimulation of the raphe obscurus or raphe pallidus decreased sympathetic discharge, an effect which was selectively antagonised by intravenous or topical application to the spinal cord of the 5-HT antagonists LSD or methysergide (Gilbey, Coote, Macleod and Peterson, 1981). However, McCall and Humphrey (1985) observed that intravenous injections of the GABA antagonists bicuculline and picrotoxin blocked the decreases in cardiac nerve discharge due to raphe stimulation. This may not necessarily be due to a direct effect of the antagonists on the raphe spinal neurones themselves but may be due to antagonism of inhibitory GABAergic interneurones onto sympathoexcitatory neurones in the raphe or other regions of the brainstem. This may cause an increase in sympathetic activity which overrides the sympathoinhibition due to stimulation of the raphe.

Adair, Hamilton, Scappaticci, Helke and Gillis (1977) electrically stimulated the raphe area of the cat and evoked pressor as well as depressor responses. These results were confirmed in the cat by McCall (1984) who found that raphe pressor and depressor sites were intermingled in the raphe complex with pressor sites more likely to be found in the nucleus raphe magnus. Midcollicular transection failed to alter the sympathoexcitatory effects of raphe stimulation suggesting that a direct raphe spinal pathway was mediating this effect. In the rat, glutamate injections into the raphe obscurus also caused increases in blood pressure (Dreteler, Wouters, Saxena and Ramage, 1991). Intravenous injections of the 5-HT antagonist methysergide attenuated the sympathoexcitatory effects of raphe stimulation in the cat but had no effect on the sympathoinhibitory effect of raphe stimulation (McCall, 1984). This 5-HT sympathoexcitatory pathway may be
tonically active since methysergide inhibits spontaneous sympathetic discharge but not in 5-HT-depleted or spinalised cats (McCall and Humprey, 1982).

Studies of single raphe neurones have shown two groups of neurones both of which have activity patterns which are locked to the 2-6 Hz rhythm in sympathetic nerve discharge (Morrison and Gebber, 1982, 1984). The first group of neurones were excited by activation of the baroreceptor reflex suggesting that these neurones are sympathoinhibitory. Some of these neurones were shown to project to the IML. The second group of neurones were inhibited by increases in blood pressure suggesting a sympathoexcitatory role. A high proportion of these neurones were driven by stimulation of the dorsolateral funiculus suggesting they were spinally projecting, the fact that none could be driven by stimulation of the IML may be due to their terminals being located further medially. These investigations have suggested two roles for raphe spinal neurones in the control of sympathetic activity. It may be that activation of the two groups of neurones leads to simultaneous excitation and inhibition of different groups of SPNs with different functional characteristics. This view is supported by the fact that electrical stimulation of the raphe complex caused a decrease in renal sympathetic nerve discharge and an increase in skeletal muscle vasoconstrictor nerve activity in the cat (Futuro-Neto and Coote, 1982) and decerebrate rat (Yusof and Coote, 1988).

It remains to be elucidated which neurotransmitters are involved in both the sympathoinhibition and sympathoexcitation. Early reports suggest that 5-HT is involved in mediating both responses to raphe stimulation. However, a recent report suggests that 5-HT-containing raphe-spinal neurones were distinct from sympathoexcitatory or sympathoinhibitory raphe-spinal neurones (McCall and Clement, 1989). Furthermore, it must not be forgotten that a large number of raphe neurones projecting to the IML contain more than one neurotransmitter and the importance of this co-existence must be analyzed in order to elucidate the role of the raphe spinal connections.
1.3f The hypothalamic nuclei

(i) Neuroanatomical studies

The hypothalamus consists of a number of different nuclei involved in a variety of functions such as control of gastrointestinal motility and secretion, urinary output, body weight and temperature, in addition to its role in the control of the cardiovascular system. Neuroanatomical techniques have identified the important regions in control of sympathetic activity.

After injections of HRP into the spinal cord of cat (Kuypers and Maisky, 1975; Saper, Loewy, Swanson and Cowan, 1976; Holstege, 1987); rat (Saper et al. 1976; Hosoya 1980,) monkey (Saper et al. 1976) or baboon (Smith, DeVito and Astley, 1990), labelled neurones were located in most areas of the posterior hypothalamus with the highest proportion in the paraventricular nucleus (PVN; Saper et al. 1976) or the lateral hypothalamus (Hosoya, 1980). Injections of HRP or fluorescent dyes into rat spinal cord retrogradely labelled neurones mainly in the parvocellular region of the PVN (Hosoya and Matsushita, 1979; Swanson and Kuypers, 1980; Swanson, Sawchenko, Wiegand and Price, 1980). Within the parvocellular region there is also a degree of segregation between neurones projecting to the median eminence (that produce pituitary releasing factors) and those that project to the spinal cord (Swanson et al. 1980).

Following injections of tritiated amino acids into the hypothalamus (Saper et al. 1976; Holstege, 1987) or PHA-L into the PVN (Gerfen and Sawchenko, 1985; Liuten, Ter Horst, Karst and Steffens, 1985), labelled axons were found in the IML and lamina X of the spinal cord. Furthermore, injections of PHA-L into the PVN labelled axons in the IML which were in close apposition to retrogradely labelled SPNs from the superior cervical ganglion (Hosoya, Sugiura, Okado, Loewy and Kohno, 1991). Although no direct connections were shown in this study it seems likely, on the basis of electrophysiological data, that there is a monosynaptic pathway from the hypothalamus onto SPNs in the spinal cord (Caverson, Ciriello and Calaresu, 1984). Furthermore, Strack, Sawyer, Hughes, Platt and Loewy (1989a) labelled neurones in the PVN after injections of PRV
into various sympathetic ganglia with no evidence of interneurones being labelled in the spinal cord.

The chemical nature of the spinal projections from the hypothalamus has also been studied. Retrograde labelling of neurones in the hypothalamus following injections of fluorescent tracers into the spinal cord has been combined with immunohistochemical labelling of these neurones (Sofroniew, 1980; Sawchenko and Swanson, 1982; Gerfen and Sawchenko, 1985; Cechetto and Saper, 1988). These studies have shown that a large number of retrogradely labelled neurones were immunoreactive for oxytocin and vasopressin (oxytocin immunoreactive cells being more numerous) while few neurones contained somatostatin and leu- and met-enkephalin. Injections of PRV into the adrenal gland were combined with immunofluorescence to deduce the neurochemical nature of labelled cells in the hypothalamus (Strack et al. 1989b). Dopamine was the prevalent neurotransmitter found in these cells, with substance P also present in a number of neurones. Oxytocin and vasopressin were not localised in many virally infected neurones in the hypothalamus. This may be an artifact of the technique involved but is more likely to be due to differential neurochemical input onto subsets of SPNs: Holets and Elde (1982) retrogradely labelled SPNs from the adrenal gland and found neither oxytocin or vasopressin containing terminals even though there was convergence of labelled terminals onto unlabelled neurones in the IML.

(ii) Neurophysiological studies

For many years the lateral hypothalamus has been believed to be important in integrating the defense reaction, which is the characteristic behaviour of an animal to a noxious or threatening stimulus (see Jordan, 1990). The cardiovascular response accompanying the defense reaction involves an increase in heart rate, arterial blood pressure, skeletal muscle vasodilatation and cutaneous, intestinal and renal vasoconstriction (see Hilton and Redfern, 1987). This pattern of responses is mimicked by electrical stimulation of the lateral hypothalamus-perifornical region in addition to other supraspinal regions. Excitatory amino acid injections into this area failed to cause similar patterns of
response in the cat (Hilton and Redfern, 1986), however, in the baboon electrical stimulation of the perifornical region caused the cardiovascular component of the defense reaction, an effect which was substantially reduced by injections of ibotenic acid (which destroys only cell bodies; Smith et al. 1990). It seems likely, therefore, that this region of the hypothalamus is involved in eliciting the cardiovascular component of the defense reaction.

Recordings have been made from neurones in the lateral hypothalamus which have sympathetic related activity (Barman, 1990). Most of these neurones were sympathoexcitatory, however, none of the neurones could be activated by stimulation of the spinal cord, although many were antidromically activated from the other brainstem regions (including the RVLM). This indicated that a polysynaptic pathway is involved in mediating sympathoexcitatory input from this area of the hypothalamus to SPNs. It has also been observed that electrical or chemical stimulation of the lateral hypothalamus increased blood pressure, lumbar sympathetic nerve discharge and excited RVLM neurones, while microinjection of the glutamate antagonist kynurenic acid into the RVLM attenuated the pressor and sympathoexcitatory effects of hypothalamic stimulation (Sun and Guyenet, 1986b). Although these studies indicate that the lateral hypothalamus is important in sympathoexcitation, the evidence suggests that the neurones involved in this control do not directly project to the spinal cord. Anatomical studies demonstrate the presence of a spinally projecting pathway from this region, and the role of this pathway remains to be elucidated.

Early studies involving electrical stimulation of the PVN and supraoptic nucleus caused increases in blood pressure and/or heart rate in cat (Ciriello and Calaresu, 1980). However, later studies suggested a sympathoinhibitory role for these neurones. Electrical or chemical stimulation of the PVN in rats decreased blood pressure and sympathetic outflow (Yamashita, Kannan, Kasai and Osaka, 1987; Kannan, Niijima and Yamashita, 1988). Electrical and chemical stimulation of the PVN in awake rats (Kannan, Hiyashida and Yamashita, 1989) increased blood pressure and renal sympathetic nerve activity. This may be due to spread of
glutamate or electric current to activate neural circuits involved in behavioral and motor response since larger electrical currents and glutamate both elicited alerting and orienting movements of the head and neck. Electrical stimulation of the PVN also inhibited the firing rate of single SPNs in the upper thoracic spinal cord of the rat (Gilbey, Coote, Fleetwood-Walker and Peterson, 1982a). In addition, these investigators observed that iontophoresed oxytocin and vasopressin onto SPNs inhibited the majority of these neurones.

It may be that the differences in responses seen to stimulation of the hypothalamus and especially the PVN, are due to species differences. Stimulation of the PVN in cats increased activity in the T2 or T3 white rami and increased blood pressure (Yamashita, Inenaga and Koizumi, 1984). These sympathoexcitatory effects may be mediated via a polysynaptic pathway since Barman (1990) recorded from hypothalamic neurones in the cat which had activity synchronized to inferior cardiac nerve discharge. Some of the neurones were suggested to be sympathoexcitatory (since their activity decreased with increased blood pressure) were positioned in the PVN, however, none of these neurones could be antidromically activated by stimulation of the spinal cord.

It has been noted that chemical stimulation of the PVN evoked an increase in adrenal nerve activity and a decrease in renal nerve activity and blood pressure (Katafuchi, Oonura and Kurosawa, 1988). This differential effect of stimulation of the PVN may be due to the receptors which are mediating these effects. Sympathoadrenal neurones do not seem to be innervated by oxytocin- or vasopressin-containing boutons (which have been shown to inhibit SPNs) while other neurones in the IML are innervated by these boutons. It seems important in the light of these observations to study further the chemical nature of the hypothalamic inputs onto SPNs and the effect on sympathetic activity of stimulating these various pathways.
FIGURE 1.1.

Diagram showing the brainstem and hypothalamic inputs to the IML of the spinal cord.

Abbreviations
PVN  Paraventricular nucleus
A5   A5 cell group of the pons
RVLM Rostral ventrolateral medulla
CRN  Caudal raphe nuclei
IML  Intermediolateral cell column

Adapted from Strack et al. 1989
1.3g Other regions which may be important in sympathetic control

The previous sections have dealt with a number of supraspinal regions known to project directly to the IML of the spinal cord and may be important in controlling sympathetic activity (see figure 1.1). Recent research has indicated that two other regions in the brain stem project to the IML which may also contribute to the regulation of sympathetic outflow. Following injections of fast blue into the spinal cord of rats, a small number of retrogradely labelled cells were located along the medial edge of the nucleus of the tractus solitarius (NTS; Lorenz et al. 1985). Using immunohistochemistry, these cells were identified as PNMT-containing and correspond to the C2 region of the medulla described by Hökfelt et al. (1974). Nicholas and Hancock (1988) injected HRP into rat spinal cord and combined this with immunohistochemical labelling of PNMT-containing cells to show retrogradely labelled cells in the C2 region and a region in the dorsal medulla which is known as the C3 region. Similar results were seen after injections of the transneuronal tracer herpes simplex virus type 1 into the adrenal medulla (Wesselingh, Li and Blessing, 1989). More recently, quantitative analysis of spinally projecting adrenaline-synthesising neurones in the medulla of the rat (Sawchenko and Bohn, 1989; Minson et al. 1990) have shown that around a third of spinally projecting PNMT-containing neurones derive from the C2 and C3 medullary cell groups. The importance of these regions has yet to be determined but it is likely that they contribute to the regulation of sympathetic outflow and may be as important as the C1 region of the RVLM in the catecholaminergic control of SPNs. Other areas in the brainstem also project to the IML of the spinal cord such as the Kolliker Fuse nucleus and ventromedial nucleus (Tucker and Saper, 1985; Strack et al. 1989b), however, less work has been carried out on these areas and their role in sympathetic control is not clear.

In addition to those regions that project directly to the IML, there are a number of supraspinal regions which have an indirect effect on sympathetic activity. One of these regions is the caudal ventrolateral medulla (CVLM) which contains the A1 cell group. This area was thought to be a principal source of catecholamines by Dahlström and Fuxe (1965), a view which was shared by Fleetwood-Walker and Coote (1981a), based on lesioning studies. However, more recent neuroanatomical
studies have indicated that very few axons of the CVLM and particularly the A1 cell
group project to the IML (Blessing et al. 1981; Westlund et al. 1981; McKellar and
Loewy, 1982). Therefore the effects of stimulation of the CVLM on sympathetic
activity are likely to be indirect. Stimulation of the CVLM using injections of
excitatory amino acids caused decreases in blood pressure and heart rate (Blessing
and Reis, 1982; Willette et al. 1983; 1984; 1987; Li and Blessing, 1990; Smith and
Barron, 1990; Masuda, Terui, Koshiya and Kumada, 1991), an effect which may be
due to inhibition of RVLM neurones since injections of the neurotoxin kainic acid
into the RVLM of rats attenuated the decreases in renal sympathetic nerve activity
and blood pressure induced by injections of glutamate into the CVLM (Agarwal et

Other areas which do not project directly to the IML may be important in regulating
sympathetic outflow. These areas may include cortical regions which receive and
integrate afferent input and activate autonomic responses. The cerebellar cortex and
the insular; somatic sensory and the motor cortices receive input from areas involved
in autonomic control and project (often via other areas such as the parabrachial
nucleus) to other supraspinal regions in the central autonomic control system.
However this complex control will not be reviewed in detail in this introduction.

1.3h Complexity of the supraspinal control of sympathetic activity
So far the supraspinal regions that may be involved in control of sympathetic
neuronal activity have been considered as separate entities, each exerting their own
effect on SPNs in the IML. This, however, is far from the case and it seems that
there may be complex interactions between the areas. The NTS receives afferent
input from the baroreceptors and is a vital relay station in the control of sympathetic
(and parasympathetic) activity (see Spyer, 1981). Neurones in the NTS project to
most of the regions which have projections to the IML of the spinal cord or to other
areas in the brain stem. Anatomical studies have demonstrated projections from the
NTS to the RVLM; CVLM; hypothalamus; the C2 region of the NTS; the raphe
(light labelling) and the A5 region of the pons (Loewy and Burton, 1978; Ross,
Ruggiero and Reis, 1985; Byrum and Guyenet, 1987; Hancock, 1988; Agarwal and
Calaresu, 1990; Riche, de Pommery and Menetrey, 1990). In addition, these regions also project to the NTS thus providing reciprocal connections (Basbaum et al. 1978; Holstege, 1987; Byrum and Guyenet, 1987; Dampney, Czachurski, Dembowsky, Goodchild and Seller, 1987; Thor and Helke, 1988; Agarwal and Calaresu, 1990). These studies and others (Sawchenko, Swanson, Grzanna, Howe, Bloom and Polak, 1985; Nicholas and Hancock, 1990; 1991; Agarwal and Calaresu, 1991) have provided anatomical evidence of interconnections between most of the areas described above that are important in sympathetic control. Therefore it seems that each region may cause effects both at the supraspinal and spinal level to regulate sympathetic outflow.

1.4 CATECHOLAMINE-CONTAINING INPUTS ONTO SPNS

1.4a Anatomical Studies

Many investigators have studied the distribution of noradrenergic and adrenergic terminals in the spinal cord of different species using a variety of techniques to label these neurotransmitters. Studies have involved the use of antibodies to PNMT to detect adrenaline-containing neurones in the spinal cord. However, assays for adrenaline have shown low levels in the spinal cord under normal circumstances (Fleetwood-Walker and Coote, 1981b; Sved, 1990). In the presence of monoamine oxidase inhibitors (which prevent degradation of adrenaline) adrenaline levels are much higher (Sved, 1990). Therefore, it may be that adrenaline is not stored in terminals in the spinal cord but is synthesised and released on demand. Thus the use of antibodies to PNMT detects adrenaline-containing terminals which may not be observed with antibodies to adrenaline.

Using immunohistochemical and fluorescence techniques, a similar distribution of labelled fibres and terminals was observed for all species studied, including the rat (Dählstrom and Fuxe, 1965; Chiba and Murata, 1981; Westlund, Bowker, Ziegler and Coulter, 1983; Chiba and Masuko, 1986; Kohno, Shinoda, Kawai, Okuchi, Ono and Shiotani, 1988; Bernstein-Goral and Bohn, 1989; Anderson et al. 1989; Fuxe, Tinner, Bjelke, Agnati, Verhofstad, Steinbusch, Goldstein and Kalia 1990 a and b); cat (Taylor and Brody, 1976; McLachlan and Oldfield, 1981); guinea-pig (Chiba and Masuko, 1987) and monkey (Westlund, Bowker, Ziegler and Coulter, 1984; Chung,
Lee and Westlund, 1989).

Catecholaminergic fibres descend in two distinct groups located in the dorso-lateral and ventro-lateral funiculi. At the midpoint between the two groups the fibres turn medially towards the IML (Chung et al. 1989) and form a discrete accumulation of terminals within and between the autonomic nuclei. There are dense plexa of both noradrenergic and adrenergic terminals in the IML and the central autonomic nuclei which surround the cell bodies in these areas. In addition, there are rostrocaudally and mediolaterally (along the intercalated nucleus) oriented strands of fibres which interconnect these plexa and the terminals in the intercalated nucleus. Studies involving retrograde labelling of SPNs (Anderson et al. 1989; Bernstein-Goral and Bohn, 1989; Fuxe et al. 1990b) or immunohistochemical labelling of choline acetyltransferase containing neurones (Kohno et al. 1988) have shown that this ladder-like distribution of terminals closely follows the distribution of SPNs in the autonomic nuclei.

To determine whether there are catecholaminergic boutons terminating directly on SPNs, it is necessary to take material to the electron microscopic level which allows identification of membrane specialisations associated with the formation of synapses on labelled neurones. In the guinea pig and rat, SPNs were retrogradely labelled following application of HRP onto the cervical sympathetic trunk. Catecholaminergic terminals were labelled using 5-hydroxydopamine which is selectively taken up into the terminals and noradrenergic terminals were identified using immunohistochemistry (Chiba and Masuko, 1986; 1987). Both catecholamine- and noradrenaline-containing terminals were found to synapse onto SPNs, forming both axosomatic and axodendritic synaptic contacts. Catecholaminergic synapses were either symmetric or asymmetric, while the majority of noradrenergic synapses were observed to be symmetric. Asymmetric synapses have been proposed to mediate excitatory responses since they have been found in abundance in areas of the brain containing high proportions of excitatory inputs onto neurones (Gray, 1973). Similar data suggest an inhibitory role for symmetric synapses.
Bernstein-Goral and Bohn (1989) examined the adrenergic innervation of SPNs which project to the adrenal medulla and the ultrastructure of these PNMT-immunoreactive inputs during development. In the young rat (7-30 days) the synapses observed were exclusively symmetric, forming both axosomatic and axodendritic synaptic specialisations. In the adult rat, there appeared to be a substantial degree of synaptic reorganisation with both symmetric and asymmetric synapses observed. However, PNMT-immunoreactive axosomatic synaptic contacts were not observed in the adult rat IML. Those axodendritic synapses that occurred were formed on small diameter dendrites which could not be labelled following HRP injections into the adrenal medulla, therefore, it was not possible to elucidate whether PNMT-immunoreactive terminals directly innervated SPNs in the adult rat.

Retrograde labelling of SPNs from the cervical sympathetic chain combined with immunoautoradiographic labelling of PNMT-containing fibres resulted in the identification of labelled synapses associated with both the somata and dendrites of these neurones (Milner, Morrison, Abate and Reis, 1988). It was noted that axosomatic synapses were exclusively symmetric, while the majority of axodendritic synapses formed were of the asymmetric type. Therefore SPNs which project to the adrenal gland did not appear to have adrenergic axosomatic inputs, while those SPNs which send their axons in the cervical sympathetic nerve were observed to receive synaptic input onto their somata. This discrepancy between the two studies may be due to the differential distribution of adrenergic inputs onto subsets of SPNs.

A recent study has tried to quantify the numbers of tyrosine hydroxylase- (TH) and PNMT-containing terminals in the IML of rat spinal cord (Llewelyn-Smith, Minson, Pilowsky and Chalmers, 1991). Only a small proportion of identified synapses were shown to contain either TH (5%) or PNMT (1-2%). However, immunocytochemical techniques are unlikely to identify all terminals containing a certain neurotransmitter due to problems with penetration of the antibody into the section and morphological preservation of synaptic specialisations.
1.4b Autoradiographic studies on the location of adrenoceptors in the spinal cord

The effect of catecholamines on sympathetic nerve activity is dependant on the types of adrenoceptors which are involved in mediating the response. Therefore autoradiographic techniques have been employed to study whether there are catecholamine receptors in the IML and other autonomic nuclei in the spinal cord. Homogenate studies were carried out using membrane fragments from different areas of the thoracic spinal cord. Significant levels of $\alpha_1$-adrenoceptor binding sites were found in the IML using $[^3H]$WB4101 while dopamine and $\beta$-adrenoceptor levels were negligible (Coote, Fleetwood-Walker and Mitchell, 1979). However, in vitro autoradiographic binding studies on the distribution of adrenoceptors in the cat spinal cord failed to detect any $\alpha_1$-adrenoceptors in the IML even though $\alpha_2$-adrenoceptors were abundant in this area (Dashwood, Gilbey and Spyer, 1985). A recent study on the localisation of $\beta$-adrenoceptors in the chicken spinal cord using a histochemical fluorescence technique demonstrated binding in the autonomic nuclei of this species (Bondok, Botros and El-Mohandes, 1988).

1.4c Origin of catecholaminergic inputs onto SPNs

Most of the supraspinal catecholaminergic inputs onto SPNs have been discussed in earlier sections. These inputs include the C1 area of the RVLM; the C2 region in the NTS; and the C3 region in addition to the A5 region in the pons and the A11 group in the hypothalamus (which is probably dopaminergic).

1.4d Functional studies on the catecholaminergic input onto SPNs

Early pharmacological studies on the role of catecholaminergic inputs on to SPNs involved intravenous or intrathecal injections of drugs. Intravenous injections of L-dopa, (which is presumed to be taken up into nerve terminals to cause release of endogenous monoaminergic neurotransmitter) or clonidine in the cat or dog decreased both pre- and post-ganglionic sympathetic activity, an effect which could be reversed by administration of yohimbine (Schmitt, Schmitt and Fenard, 1973; Sinha, Atkinson and Schmitt, 1973; Coote and Macleod, 1974). These effects were assumed to be acting at a spinal level since, in spinalised animals, clonidine still reduced splanchnic nerve discharge (Sinha et al. 1973). Furthermore, intrathecal
injections of noradrenaline decreased activity in the lumbar sympathetic chain (LoPachin and Rudy, 1983). Intravenous injections of yohimbine enhanced the spinal component of the somatosympathetic reflex in T3 white ramus (Dembowsky, Czachurski, Amendt and Seller, 1980) and caused increases in preganglionic sympathetic nerve activity (Ramage and Tomlinson, 1985) suggesting that the "inhibitory" catecholaminergic input onto SPNs is tonically active.

Taylor and Brody (1976) stimulated the lateral funiculus (which is known to contain descending catecholaminergic fibres) in spinal cats and caused increases in blood pressure and vasoconstriction of the hind limb. Intrathecal injections of the α-adrenoceptor antagonist phentolamine attenuated these responses. In the spinal rat, superfusion of dopamine elicited a dose dependant increase in renal sympathetic nerve activity (Simon and Schramm, 1983). Furthermore, intravenous injections or infusions of α₁-adrenoceptor antagonists such as prazosin, indoramin, doxazosin and alfuzosin caused decreases in preganglionic and postganglionic nerve activity (McCall and Humprey, 1981; Ramage, 1986a and b), in addition to a decrease in heart rate and blood pressure which suggested a central action of these drugs. These results also infer that the sympathoexcitatory input is tonically active.

Intrathecal injections of noradrenaline at the T10 level of rat spinal cord caused both increases and decreases in renal sympathetic nerve discharge (RSND), depending on the dose given, while adrenaline caused mainly sympathoinhibition (Shi, Lewis and Coote, 1988). The sympathoexcitatory effects seemed to be due to activation of α₁-adrenoceptors since methoxamine caused similar increases in RSND and this and the effect of noradrenaline could be blocked by intrathecal prazosin. The α₂-adrenoceptor agonist guanabenz caused decreases in RSND and this effect and the inhibitions seen with adrenaline and noradrenaline could be antagonised by yohimbine. Therefore pharmacological experiments suggest a dual role for catecholamines in the control of sympathetic activity, which is dependant on the type of receptor activated. However these experiments do not allow precise localisation of the receptors involved.
One of the many techniques used for studying the functions of SPNs in the spinal cord is that of extracellular recording from these neurones using glass multibarrelled micropipettes. This enables the combination of extracellular recording from an SPN with iontophoretic application of drugs in the vicinity of that neurone to study the inputs onto individual SPNs.

To date, there have been a large number of iontophoretic studies on the effects of catecholamines on the firing rate of SPNs in both the cat and pigeon. Hongo and Ryall (1966) were unable to show an effect of noradrenaline on SPNs in unanaesthetised decorticated cats, however, a later study by DeGroat and Ryall (1967) in anaesthetised cats showed that noradrenaline depressed the firing rate of 4 out of 10 SPNs and had no effect on the remainder. These observations were confirmed by Coote et al. (1981b) since iontophoresed adrenaline or dopamine onto SPNs inhibited a small proportion of the neurones tested. Iontophoresis of these drugs onto SPNs was never associated with an increase in neuronal firing rate. Similar results were also obtained by Kadzielawa (1983) using α-methylnoradrenaline and endogenous catecholamines, the inhibitions in this study could be consistently blocked by iontophoresis of the α2-adrenoceptor antagonist piperoxan. Guyenet and coworkers (Guyenet and Cabot, 1981; Guyenet and Stornetta, 1982) iontophoresed a number of catecholamines in the vicinity of SPNs with ongoing activity in the pigeon and observed inhibitions in the firing rate of all neurones tested. Iontophoresis of the α2-adrenoceptor agonist, clonidine also inhibited all SPNs tested. This effect of clonidine and the inhibitions seen with catecholamines were antagonised by iontophoresis of yohimbine and piperoxan suggesting that the inhibitions were mediated by an α2-adrenoceptor.

These iontophoretic studies have suggested an exclusively inhibitory role for catecholamines in the control of the firing rate of SPNs. However recent in vitro studies demonstrated that noradrenaline and adrenaline can have both excitatory and inhibitory effects on SPNs. Superfusion of noradrenaline in the adult cat spinal cord slice elicited both long-lasting depolarisations and hyperpolarisations in SPNs (Yoshimura, Polosa and Nishi 1987 a, b, c and d). The noradrenaline-induced
Depolarisations were antagonised by applications of the selective α₁-adrenoceptor antagonist prazosin while yohimbine blocked the hyperpolarisations. Similar results were obtained upon pressure ejection or superfusion of adrenaline onto SPNs in the neonatal rat spinal cord slice (Miyazaki, Coote and Dun, 1989). Superfusion of noradrenaline onto SPNs in the neonatal rat slice caused slow depolarisations in 23/38 identified SPNs (Ma and Dun, 1985). These effects were assumed to be α₁-adrenoceptor-mediated since they were blocked by prazosin but not yohimbine or propanolol.

Thus iontophoretic data suggested that catecholamines induce exclusively α₂-adrenoceptor-mediated inhibitions of SPNs, whereas in vitro studies (and the pharmacological studies) observed both α₁-adrenoceptor-mediated excitations in addition to the α₂-adrenoceptor-mediated inhibitions.

Iontophoretic and in vitro studies were carried out in the same animal (cat) which excludes the possibility of a species difference in the types of α-adrenoceptor found on SPNs. Discrepancies using the two techniques may possibly be explained by differences in the access of the drugs to the adrenoceptors on the SPN. Extracellular recording from single SPNs in vivo requires positioning of the microelectrode close to the cell soma to obtain a good neuronal signal over the noise level. Therefore, when drugs are iontophoresed from this microelectrode in the vicinity of SPNs, they come into contact initially with the cell body before diffusing away into the surrounding tissue. In contrast, bath superfusion or pressure ejection of drugs onto SPNs in the spinal cord slice preparation allows access of the drug to the SPN, including the dendrites, almost simultaneously. Recently, immunoautoradiographic labelling identified asymmetric and symmetric PNMT-containing (and therefore presumed adrenergic) synapses on retrogradely labelled SPNs (Milner et al. 1988). It was noted that axosomatic synapses were exclusively symmetric (inhibitory), while the majority of axodendritic synapses formed were of the asymmetric type (excitatory). The two categories of synapses may correspond to the two types of α-adrenoceptor and the positioning of these receptors could explain the discrepancies in the results obtained from the in vitro and iontophoretic studies.
In the adult cat spinal cord slice other effects of noradrenaline on SPNs were observed in addition to the slow depolarisations and hyperpolarisations. Action potentials in SPNs are made up of two components, a fast component which is due to an influx of sodium and a slow element due to influx of calcium which causes a shoulder on the repolarising phase of the spike (Yoshimura et al. 1986). Superfusion of noradrenaline can abolish the shoulder of the repolarising phase by decreasing the inward calcium (Ca\(^{2+}\)) current so that the spike becomes narrower and repolarisation occurs almost at a constant rate. The spike was followed by a long-lasting afterhyperpolarisation (AHP) which had a fast phase (caused by an increase in potassium (K\(^{+}\)) conductance) and a slow phase which is due to an increase in calcium-dependant K\(^{+}\)-conductance. Noradrenaline depresses the slow AHP either by decreasing the Ca\(^{2+}\) influx during the spike or by affecting one of the intermediate steps coupling Ca\(^{2+}\) influx to activation of K\(^{+}\) conductance. This suppression of the slow AHP results in increased responsiveness of the SPN to excitatory input. In addition noradrenaline produced a marked afterdepolarisation (ADP) which generated spikes when the depolarisation reached threshold. It has been suggested that a calcium-activated sodium conductance is the ionic mechanism underlying the production of ADP. Noradrenaline was also seen to induce rhythmic bursting which were associated with the spontaneous appearance of oscillations of the membrane potential. These oscillations were abolished at potentials below -65 mV and were attenuated by low calcium.

1.5 THE PRESENT INVESTIGATIONS

To date, although \textit{in vitro} studies have proposed that \(\alpha\)-adrenoceptors in the IML of the spinal cord may mediate excitatory input onto SPNs, this has not been corroborated by observations from \textit{in vivo} experiments. Moreover autoradiographic experiments have failed to show the presence of \(\alpha\)-adrenoceptor binding sites in the IML of the cat. This investigation therefore endeavoured to re-examine the localisation of \(\alpha\)-adrenoceptors in the cat spinal cord using \textit{in vitro} autoradiography. In addition, selective \(\alpha\)-adrenoceptor agonists were applied in the vicinity of SPNs by iontophoresis or by microinjection techniques to determine the effect of these drugs on sympathetic activity in the intact animal. This is important since, in the
spinal cord slice preparation, the firing rates of SPNs are no longer influenced by supraspinal or segmental inputs onto the neurones and therefore it is possible to study the effect of activating one input alone. In the intact animal the situation is much different and the activity of one SPN may be controlled by a large of number of supraspinal or segmental inputs. In this situation, application of $\alpha_1$-adrenoceptor agonists may have little or no effect on the firing rate of SPNs. This may be due to the fact that tonically active catecholaminergic inputs are firing at a rate such that most of the $\alpha_1$-adrenoceptors on the SPN are occupied and the agonists cannot activate enough free receptors to cause an effect of the firing rate. Alternatively, it may be that the $\alpha_1$-adrenoceptor mediated input onto SPNs is almost redundant in the intact animal. This may be due to the fact that the ion channels normally affected by activation of $\alpha_1$-adrenoceptors are already in an active state due to the action of another neurotransmitter. This concept of two neurotransmitters affecting the same current has been described by Pape and McCormick (1989) in the thalamus where noradrenaline and 5-HT both modulate burst firing by enhancing a cation channel. If either of these situations exists in the intact animal then application of $\alpha_1$-adrenoceptor agonists will not affect the firing rate of SPNs. Spinalisation will remove any tonically active supraspinal descending inputs onto SPNs and may then unmask an effect of these agonists. In order to investigate whether $\alpha_1$-adrenoceptors are involved in the control of SPNs in the intact animal, applications of selective $\alpha_1$-adrenoceptor agonists in the vicinity of SPNs were made in the in vivo preparation, using both spinalised animals and those with an intact neuraxis.
CHAPTER 2
AUTORADIOGRAPHIC LOCALISATION OF $\alpha_1$-ADRENOCEPTORS
IN THE SPINAL CORD OF THE CAT
INTRODUCTION

2.1a Homogenate Studies

Radioactive ligand binding techniques have been used for many years to study the distribution of different receptors within the periphery and the central nervous system. Early studies examined the binding of radiolabelled ligands (radioligands) to tissue homogenates in the periphery - one of the first such studies analyzed $[^{125}\text{I}]{\text{ACTH}}$ binding to adrenal cortex homogenates (Lefkowitz, Roth and Pastan (1970). This technique was adapted for use in the central nervous system and binding of $[^{3}\text{H}]{\text{catecholamines}}$ (noradrenaline and adrenaline) was demonstrated to sites in a number of calf brain regions, including the cerebellum and cerebral cortex (Greenberg, U’Prichard and Snyder, 1976; U’Prichard and Snyder, 1977a and b). Two radioligands were used to determine whether $\alpha_1$-adrenoceptors were present in brain homogenates, $[^{3}\text{H}]$WB4101 (U’Prichard, Greenberg and Snyder, 1977) and $[^{3}\text{H}]$prazosin (Greengrass and Bremner, 1979). Although these studies reported saturable binding to sites in homogenated brain membranes, indicating the presence of $\alpha_1$-adrenoceptors, they provided very little information regarding the distribution of these receptors within each area studied.

2.1b In vitro autoradiography.

Young and Kuhar (1979) developed a technique of labelling receptors in lightly fixed tissue sections mounted onto microscope slides. These sections were incubated in radioligand in a similar way to that used for the homogenate studies and were then apposed to emulsion coated coverslips for production of autoradiographs. In this way it was possible to visualise the distribution of high and low levels of ligand binding in sections of tissue. The first such study examined the distribution of opiate receptor binding sites in the rat frontal brain and demonstrated similar overall levels of binding to that observed in homogenates. In addition, there was good accessibility of the ligands to sites within the sections, as shown by the similarity of the $B_{\text{max}}$ values obtained for ligands from both the homogenate and the in vitro techniques (Young and Kuhar, 1979). This technique has been used extensively to study the regional distribution of many receptors in the brain and spinal cord as well as the periphery. Again,
both \(^{3}H\)prazosin (Rainbow and Biegon, 1983) and \(^{3}H\)WB4101 (Young and Kuhar, 1979, 1980) were used to study the location of \(\alpha_{1}\)-adrenoceptors in the central nervous system. Using this technique, differences in the distribution of the two radioligands within the brain were noted. Unnerstall, Fernandez and Orensanz (1985) observed that the distributions of binding of the two radioligands were vastly different in the cortex, thalamus and hippocampus. Moreover, there were inconsistencies in the degree of displacement from the radioligand binding sites seen with unlabelled ligands. Any increased \(^{3}H\)WB4101 binding in certain areas may have been because there is now evidence that WB4101 is also a 5-HT\(_{1A}\) antagonist (Norman, Battaglia and Creese, 1985; Newberry and Gilbert, 1989), therefore some of the \(^{3}H\)WB4101 binding may be to 5-HT\(_{1A}\) binding sites in the brain. Furthermore, the fact that WB4101 is selective for a subgroup of \(\alpha_{1}\)-adrenoceptor, the \(\alpha_{1a}\)-adrenoceptor (Morrow and Creese, 1986), might account for the lower amounts of binding of \(^{3}H\)WB4101 observed in some brain areas.

### 2.1c Adrenoceptors in the IML

To date the only in vitro autoradiographic investigation into the distribution of \(\alpha_{1}\)-adrenoceptors in the spinal cord has been carried out by Dashwood, Gilbey and Spyer (1985) using \(^{3}H\)prazosin as the \(\alpha_{1}\)-adrenoceptor ligand. They also examined the binding of \(^{3}H\)rauwolscine and \(^{3}H\)yohimbine (both of which are \(\alpha_{2}\)-adrenoceptor antagonists). This study failed to show \(\alpha_{1}\)-adrenoceptor ligand binding in the IML of the cat spinal cord, although high concentrations of \(\alpha_{2}\)-adrenoceptor ligand binding sites were observed. On the strength of this study and the observation that iontophoresis of catecholaminergic agonists caused only \(\alpha_{2}\)-adrenoceptor mediated inhibitions of SPNs (see general introduction), they suggested that \(\alpha_{2}\)-adrenoceptors are the important adrenoceptors at the spinal level involved in mediating the effects of catecholamines on SPNs. Recently, however, studies in cat spinal cord slices have indicated a direct \(\alpha_{1}\)-adrenoceptor-mediated increase in the excitability of SPNs (Yoshimura et al,. 1987a, see general introduction). Furthermore, an autoradiographic study on the distribution of 5-HT receptor subtypes within the cat spinal cord indicated the presence of \(\alpha_{1}\)-adrenoceptors in the IML (Dashwood and Gilbey unpublished observations).
This study involved the use of \[^{3}\text{H}\] ketanserin, an antagonist at 5-HT\(_2\) receptors, which is also known to have some affinity for \(\alpha_1\)-adrenoceptors (McCall and Schuette, 1984). To control for the possibility that some of the binding of \[^{3}\text{H}\] ketanserin was to \(\alpha_1\)-adrenoceptors, total binding of the radioligand was displaced by excess amounts of prazosin. Displacement by prazosin resulted in a significant decrease in the amount of binding of \[^{3}\text{H}\] ketanserin compared to that seen in sections incubated in \[^{3}\text{H}\] ketanserin alone, indicating that some of the \[^{3}\text{H}\] ketanserin binding was to \(\alpha_1\)-adrenoceptors.

2.1d The present study

In the light of the above observations, the distribution of \(\alpha_1\)-adrenoceptor binding sites within the IML of the cat spinal cord was re-examined. Due to its high degree of selectivity, \[^{3}\text{H}\] prazosin was chosen as the radioligand in these studies. One of the most comprehensive studies on the binding characteristics of \[^{3}\text{H}\] prazosin was carried out by Greengrass and Bremner (1979). By displacing \[^{3}\text{H}\] prazosin binding with a large number of unlabelled compounds, they demonstrated that the binding of \[^{3}\text{H}\] prazosin to homogenated brain membranes was saturable and selective for \(\alpha_1\)-adrenoceptor binding sites. The \(\alpha\)-adrenoceptor agonists and antagonists were potent competitors for binding sites in the brain while those compounds such as 5-HT, histamine and acetylcholine as well as dopamine and \(\beta\)-adrenoceptor agonists had very little effect on the levels of \[^{3}\text{H}\] prazosin binding. In this study the time period for exposure of the \[^{3}\text{H}\] prazosin-incubated slides to the Hyperfilm was increased to twelve weeks from the five week period used in the experiments by Dashwood et al. (1985). This should be long enough to produce a higher density image and thus visualise any binding of the radioligand to sites in the spinal cord that has taken place.
METHODS

2.2a Removal of tissue and preparation of sections

Three cats were used in total for this study; in each experiment, tissue was used for both low resolution and high resolution autoradiography. However, for the first cat, tissue used for the high resolution study was fixed and dipped in emulsion, while for the remaining two cats, the coverslip method was used. Cats were anaesthetised with either an intraperitoneal injection of sodium pentobarbitone (60 mg/kg) or an intravenous injection of α-chloralose (80 mg/kg) and sodium pentobarbitone (12mg).

At a level of deep surgical anaesthesia (determined by the absence of the palpebral and flexor reflexes), the chest cavity was opened and a hypodermic needle pushed through the wall of the left ventricle into the heart. The needle was attached via a polyethylene tubing to either a 50 ml syringe or an infusion pump for delivery of the buffers and fixative. The jugular veins were sectioned to allow outflow from the cardiovascular system. The animals were perfused initially with 30% sucrose (which acts as cryoprotectant) in 1000 ml of ice-cold 100 mM phosphate buffer (pH 7.4) until no more blood was seen in the outflow. They were subsequently fixed with 1000 ml of 0.1% formaldehyde in 100mM phosphate buffer. This caused light fixation of the tissue suitable for autoradiography since it has little effect on receptor binding (Young and Kuhar, 1979).

The first thoracic to the third lumbar vertebrae were removed using bone cutting forceps. The spinal cord was removed following sectioning of the dorsal and ventral rootlets. The dura mater was removed using scissors and the cord was cut either into individual thoracic or lumbar segments (one experiment) or longer pieces (e.g. upper thoracic, middle thoracic etc).

The segments were mounted in Ames OCT compound and frozen onto the chuck of a Bright cryostat (Cambridge rocking microtome, model FS/CS) for transverse...
sectioning. 20 μm serial sections were cut from each segment and thaw mounted onto acid washed, subbed (dipped into a solution of 1.25g gelatin and 124 mg chrome alum in 250 ml of distilled water) microscope slides. Slides that were to be used for high resolution autoradiography had sections thaw mounted onto the lower half of the slide only. After each segment of spinal cord had been cut, the slides were allowed to dry and were stored well wrapped in a -20 °C freezer for short periods before incubation or, if the tissue was not be used immediately, in a -70 °C freezer.

2.2b Incubation of sections
Slides were removed from the freezer and allowed to reach room temperature. All incubations were carried out at 4 °C to minimise dissociation of the radioligand from the binding sites in the cord. Slides were first preincubated in 170 mM Tris HCl (pH 7.4) in order to reduce the level of endogenous neurotransmitter in the tissue sections. All sections were then incubated in 5 nM \(^{3}\text{H}\text{]prazosin (22Ci/mol, Amersham UK) in 170 mM Tris HCl for 60 minutes as described by Dashwood (1986). Adjacent slides were incubated in the presence of 5 nM \(^{3}\text{H}\text{]prazosin in the presence of excess unlabelled phentolamine (a ligand specific for α-adrenoceptors) to control for nonspecific binding to low affinity sites in the cord. These incubations were repeated for high resolution autoradiographs.}

All slides were then washed twice (2 x 10 minutes) in Tris HCl to decrease low affinity non-specific binding and dried for one hour in a stream of cold air.

2.2c Exposure of the slides and subsequent development of autoradiographs
Slides were exposed to produce either high or low resolution autoradiographs.

(i) Low resolution Autoradiographs
Slides were fixed using double sided tape to the mounting board of Ilford X-ray cassettes (24 x 30 cm) so that the tissue was uppermost. Sections were apposed to \(^{3}\text{H}\text{]Hyperfilm (Amersham UK) in a dark room under an Ilford F904 brown safety light. Cassettes were then sealed in a black plastic bag and stored at 4 °C
for twelve weeks.

At the end of this period the cassettes were allowed to reach room temperature. The cassettes were opened under a brown safety light and the hyperfilms removed. The films were developed in Kodak D19 for 5 minutes at 20 °C, briefly placed in stop bath, fixed in Ilford IF25 (diluted 1 in 5) for 5 minutes and then allowed to dry. Once the images from incubated sections had been examined and marked, the slides were removed from the cassettes and stained to show the histology of the underlying cord using 0.1% thionine heated to 37 °C. The sections were dipped for one minute, rinsed in distilled water and then taken through 95% and absolute alcohol for dehydration. The slides were cleared in three solutions of histoclear for at least 30 minutes in total then mounted and coverslipped.

The Hyperfilm was placed on a light box and viewed with a binocular microscope. Areas of binding of the radioactive ligand were evident as grains on the tritium-sensitive film, the amount of radioactivity representing the total binding to the tissue (specific and non-specific). Images on the hyperfilm were photographed using a Nikon Macro system.

A flow diagram of the procedures involved in producing low resolution autoradiographs is illustrated in figure 2.1.

(ii) High resolution Autoradiography
(a) Dipping technique
Slides were first fixed by placing them in a dessicator with paraformaldehyde powder to stop dissociation of the ligand from its binding site. The dessicator was partially evacuated and heated in an oven (80 °C) for 2 hours to produce a paraformaldehyde vapour for fixation. The slides were removed and left overnight in a fume cupboard. Slides were immersed in histoclear for 5 minutes to defat the tissue and ensure close adhesion of the emulsion with the sections and rehydrated using clean alcohols (100, 95, 70 and 50 %).
FIGURE 2.1.

Diagram of the experimental protocol for the autoradiographical experiments.
PREINCUBATE

INCUBATE (Experimental)

INCUBATE (Control + Displacer)

Examine Detail

DEVELOP

X-Ray Cassette

DRY

EXPOSE

RINSE
The tritium sensitive emulsion was prepared using equal quantities of Ilford K2 emulsion (15 ml) and 2% glycerol. The emulsion was placed first in a glass container and melted at 43 °C then the glycerol was carefully added down the side of the container. The solution was mixed by stirring very slowly with a glass rod to minimise the production of air bubbles. The consistency of the emulsion was tested by dipping a blank microscope slide slowly into the emulsion and looking for any streakiness or air bubbles. Once a uniform consistency was obtained, incubated slides were dipped in pairs for five seconds, removed slowly and the backs of the slides wiped. They were then placed onto a cooled metal tray for 15 minutes until the emulsion started to gel (this slowed down the drying process so that the emulsion did not crack). Slides were then stored vertically overnight to complete drying after which they were packed with silica gel in black light-tight boxes. The boxes were sealed, covered with black plastic bags and stored for 12 weeks at 4 °C.

At the end of this period, the boxes were removed from the refrigerator and left for an hour until they reached room temperature. The slides were developed under an Ilford F904 safety light in Kodak D19 for 5 minutes, agitating the slides every 30 seconds. At the end of this period slides were transferred to 2% acetic acid (which stops the developing process) for 1 minute and fixed using Hypam (diluted 1 + 4) for 2 minutes before washing (3 x 10 minutes) in distilled water.

(b) The Coverslip Method
This involved exposing the sections to emulsion coated coverslips rather than dipping the tissue in the emulsion. Fixing the tissue before exposure to the emulsion-coated coverslips was unnecessary using this technique.

The emulsion was prepared as described above and alcohol washed coverslips were dipped in the same way as the microscope slides and allowed to dry in a vertical position. Once dried a coverslip was attached to each slide by dropping a small amount of cyanoacrylate adhesive on the top of the coverslip which was then placed (emulsion side down) on top of the slide. The coverslip was kept in
close apposition to the slide during exposure by placing a small piece of cork over
the coverslip secured by an elastic band. The slides were once more placed in
black light-tight boxes as above and the coverslips were exposed for 12 weeks at
4 °C. For developing, the coverslip was very gently prised away from the slide
and held with a piece of polyethylene tubing so that complete development of the
emulsion was possible. Development was carried out as for the dipped slides.

2.2d Viewing the high resolution autoradiographs
All sections were stained using 0.1% thionine at 37 °C for 1 minute and rinsed in
distilled water. The slides were dehydrated using 95% (1 minute) and 100%
alcohol (2 x 1 minute) and finally left in histoclear for at least 1 hour before
mounting the slides. Those with coverslips were mounted by dropping a small
amount of Xam mountant between the two surfaces, while others were mounted
using alcohol washed coverslips.
Areas of radioligand binding to sites in the tissue were viewed as silver grain
deposits on the tritium-sensitive emulsion under darkfield illumination using an
Olympus Vanox-T system. The distribution of silver grains and the tissue
underlying these areas were photographed with the Vanox-T system.
RESULTS

2.3a Low resolution autoradiographs of [³H]Prazosin binding to cat spinal cord.

(i) Total and non-specific binding of [³H]prazosin

[³H]Prazosin binding sites were identified by incubation of spinal cord sections taken from the first thoracic to the third lumbar segments in 5 nM [³H]prazosin. The binding observed in these sections gave an indication of the total binding of this ligand to sites within the cord. Examples of total binding are shown in figure 2.2.B and 2.3 (the segments from which these were taken are indicated in the figures). Generalised binding of [³H]prazosin, indicated by the density of silver grains in the autoradiograph, was observed throughout the spinal cord, especially in the grey matter. It can be observed that there was a much greater degree of binding in the IML (and also around the central canal and dorsal horn) compared with the rest of the grey matter at all levels of the cord (fig 2.3). In this figure it can be seen that the overall binding in the white and grey matter varies considerably in each picture. This may be due to the positioning of the hyperfilm in the cassettes (some hyperfilms may have been in closer contact with the slides than others so that the level of radioactivity reaching the films may differ, causing some images to look darker than others), or may be due to different numbers of neurones in each section of spinal cord (fewer cells with the same amount of binding sites will lead to lower amounts of [³H]prazosin binding in these sections). This variation in overall binding is very prominent when looking at the illustration as a whole and may distract from the fact that binding is increased in the IML. However, if each section is studied individually, it may be observed that, in each case, the amount of binding in the IML is higher than that in the grey matter as a whole.

Non-specific binding of the tritiated ligand was established by incubation of adjacent sections of spinal cord in [³H]prazosin and excess unlabelled phentolamine. The high concentration of phentolamine displaced [³H]prazosin from the α₁-adrenoceptor binding sites so that the [³H]prazosin remained bound only to non-specific sites. An example of the degree of non-specific binding of
FIGURE 2.2

 Autoradiographic localization of $[^3H]$prazosin binding in the third thoracic segment of cat spinal cord.

(A) The underlying tissue of the cord stained with thionine. The intermediolateral cell column (IML) is marked with an arrow.

(B) Total $[^3H]$prazosin binding in the spinal cord. The grey matter has a high level of binding indicated by the density of grains. The IML has the highest level of binding shown by the higher grain densities.

(C) The degree of non-specific $[^3H]$prazosin binding in a section adjacent to (B) incubated in the presence of excess unlabelled phentolamine. Scale bar = 2 mm
FIGURE 2.3

Total binding of $[^3H]$prazosin to spinal cord sections from segments T3; T4; T8; T13; L1; L2 in the cord.

The density of binding is indicated by the grain density of the autoradiograph. The greater grain densities in the IML indicate the high levels of $[^3H]$prazosin binding.

Scale bars = 1mm
[\textsuperscript{3}H]prazosin is shown in figure 2.2.C and was very low in all segments of the cord.

(ii) Densitometric image analysis

In an attempt to demonstrate specific binding of [\textsuperscript{3}H]prazosin to sites within the spinal cord, computer-assisted densitometric analysis was undertaken. This method measures the optical densities in the film autoradiograph, providing colour coded images according to the density in any one area. Thus areas with high binding have a high optical density and are colour coded red (in the system used in this study), while areas with low binding have low optical densities and are colour coded blue. Using this technique, it was possible to obtain an image of the amount of specific binding of [\textsuperscript{3}H]prazosin to the spinal cord by scanning images of total and non-specific binding, storing these images on the computer and subtracting the amounts of non-specific binding from the total binding.

The amount of total, non-specific and specific binding in a section of spinal cord at the third thoracic level is shown in figure 2.4. The sections are the same as those seen in figure 2.2. and the underlying tissue is shown in figure 2.2.C. The highest optical density corresponding to the greatest amount of binding was found in the IML of the spinal cord. Other areas with high degrees of binding are the superficial layers of the dorsal horn and those around the central canal.

2.3b High resolution autoradiographs of [\textsuperscript{3}H]prazosin binding to cat spinal cord

(i) Dipping sections in tritium-sensitive emulsion

After exposure of the sections for twelve weeks and processing, they were viewed under darkfield illumination to visualise the silver grains. The density of silver grains was uniformly very low throughout the section of spinal cord at all levels suggesting that there was very little specific binding of [\textsuperscript{3}H]prazosin to the sections after dipping the slides in the emulsion. Any binding was assumed to be to non-specific sites in the cord since the levels and distribution of silver grains in the total and non-specific sections were very similar.
Densitometric analysis of \[^3\text{H}\]\text{prazosin} binding within a section of cat spinal cord at the third thoracic level.

(A) Total binding of \[^3\text{H}\]prazosin.
The degree of binding is shown as a measure of the optical density of the section. The optical densities are colour coded so that the highest degree of binding corresponds to areas which are red. The IML is shown to have the greatest degree of binding with other areas such as the superficial dorsal horn and around the central canal also showing high degrees of binding.

(B) Non-specific binding of \[^3\text{H}\]prazosin.
The areas which have abnormally high optical densities correspond to artifacts on the autoradiograph since they occurred randomly throughout the sections, while areas of specific binding occurred consistently in the same areas of the cord. These artifacts are one of the problems associated with long exposure times of autoradiographs and use of these sections to calculate specific binding using image analysis result in misleadingly lower levels of specific binding in the spinal cord.

(C) Specific binding of \[^3\text{H}\]prazosin.
This image was acquired by superimposing the total and non-specific binding so that non-specific binding could be subtracted by the computer system. The IML once more showed a high optical density, indicating an intense degree of specific binding in this area.
(ii) The coverslip method for high resolution autoradiographs

Having failed to show binding by dipping the sections in emulsion, a technique was used which involved apposing sections to tritium-sensitive emulsion-coated coverslips. Binding was visualised as silver grains under darkfield illumination, however, it was necessary to focus through the image on the coverslip in order to show the neuropil of the tissue underlying any areas of high binding. An example of a high resolution autoradiograph showing total binding of \(^3\text{H}\)prazosin to sites in the spinal cord at the third thoracic level is shown in figure 2.5.B. The histology of the underlying tissue is shown in figure 2.5.A. Using this technique, it was difficult to visualise binding to sites in the spinal cord. This was because the tissue was illuminated from below and the light flared through areas of lower density such as the white matter (which is next to the IML). Thus the autoradiograph was obscured by the flare of the underlying tissue. In addition, areas of myelin in the grey matter which were less dense also caused flare around the silver grains making it difficult to discern and count single grains. Taking this problem into account there still did not seem to be any increase in the amount of binding to the IML of the spinal cord above that seen in other areas of the grey matter, although there was an increase in binding in the grey matter above non-specific binding levels.
FIGURE 2.5

High resolution autoradiograph of [³H]prazosin binding to cat spinal cord.

(A) Histology of the underlying tissue of the cord stained with thionine. The IML is marked by an arrow.

(B) Total binding of [³H]prazosin binding to the spinal cord. The boundary between grey and white matter is marked by a dotted line. Binding is represented by silver grains when viewed under darkfield illumination and areas of high binding are seen as accumulations of grains. The tissue is illuminated from below the section and the less dense white matter causes flare of the light so that it is difficult to determine whether areas have a higher degree of binding.

Scale bar = 0.25mm
DISCUSSION

2.4a \([^{3}H]prazosin\) binding to sites in the cat spinal cord

This study has utilised the \textit{in vitro} autoradiographic binding technique to investigate the location of \([^{3}H]prazosin\) binding sites in the cat spinal cord. A relatively high level of \([^{3}H]prazosin\) binding sites was demonstrated in the IML of cat spinal cord from the first thoracic to the third lumbar segments. Non-specific binding of this radioligand was observed to be low throughout the cord, therefore it is likely that the binding in the IML is to specific sites, most likely \(\alpha_{1}\)-adrenoceptors.

These results are in contrast to those obtained in a previous investigation into the distribution of various receptors in the IML of the cat spinal cord (Dashwood et al. 1985). The study reported high concentrations of \([^{3}H]yohimbine\) and \([^{3}H]rauwolscine\) (\(\alpha_{2}\)-adrenoceptor ligands) binding sites within the IML. However, there was a relative paucity of \([^{3}H]prazosin\) binding sites throughout the spinal cord. Their study used a much shorter exposure time (5 weeks compared with the twelve weeks used in this study) and even though the specific activity of \([^{3}H]prazosin\) in that study was slightly higher, (33 Ci/mmol compared with 20 Ci/mmol in this study), it was still not possible to visualise the binding sites in the spinal cord. This study has therefore highlighted the necessity of establishing the right conditions for visualising radioligand binding, thus minimising the risk of obtaining false negative results.

2.4b Is \([^{3}H]prazosin\) binding to \(\alpha_{1}\)-adrenoceptors in the IML?

Although this study provides evidence that \([^{3}H]prazosin\) binding sites are present in the IML of cat spinal cord, recent results have suggested that prazosin may also bind to a subtype of \(\alpha_{2}\)-adrenoceptor. There is a great difference in the affinity of prazosin for the two \(\alpha_{2}\)-adrenoceptor subtypes. Prazosin inhibited the binding of \([^{3}H]yohimbine\) in rat CNS in a biphasic manner, with a high affinity for the \(\alpha_{2b}\)-adrenoceptor site compared with a 20-fold less affinity for the \(\alpha_{2a}\)-adrenoceptor.
site (Brown, MacKinnon, McGrath, Spedding and Kilpatrick, 1990). However, prazosin has a much higher affinity for the $\alpha_1$-adrenoceptor binding site than the $\alpha_{2b}$-adrenoceptor subtype ($K_i$ value of 50 nM for the $\alpha_{2b}$-adrenoceptor site compared with a value of 0.1 nM for the $\alpha_1$-adrenoceptor site; Greengrass and Bremner, 1979; Brown et al. 1990), therefore it is unlikely that the binding in these studies is to $\alpha_{2b}$-adrenoceptor sites.

2.4c High resolution autoradiography

This study also involved the use of high resolution autoradiography in an attempt to localise the binding of $[^3H]prazosin$ to $\alpha_1$-adrenoceptors in the IML. Using this technique it is possible to stain the underlying tissue so that the exact positioning of silver grains can be shown in relation to the neurones underneath. Two techniques were used for high resolution autoradiography. The first technique involves fixation of the tissue after incubation in radioligand. This technique has been successfully used with opioids where irreversible covalent bonds are formed between the radioligand and the binding site so that, during dipping, diffusion of the radioligand is minimised (see Wamsley, 1983). However, fixation of tissue in this study did not prevent the loss of the majority of binding of $[^3H]prazosin$ to sites in the spinal cord suggesting that any bonds made between $[^3H]prazosin$ and sites in the spinal cord were not strong enough to withstand dipping the slides in the emulsion. The second technique involved apposing slides to emulsion-coated coverslips for exposure to visualize $[^3H]prazosin$ binding. Surprisingly, with this technique binding was not observed to be much increased in the IML compared with the rest of the grey matter. It is not clear why this technique failed to reveal a increased level of binding in the IML similar to that observed in the low resolution autoradiographs. The tissue was incubated and dried in exactly the same way as the tissue used for low resolution autoradiographs. There may have been problems with the batch of emulsion used for this study. Alternatively, it may be necessary to expose the slides for a longer period of time in order to distinguish an increase in the specific binding of $[^3H]prazosin$ to the IML. Once more, the importance of establishing the right conditions for visualising binding in these studies must be emphasised.
2.4d Densitometric Analysis

Densitometric analysis was undertaken to elucidate the specific binding of \[^{3}H\]prazosin to sites in the spinal cord. This involved subtraction, by computer, of non-specific binding from the total binding. Using this technique it was possible to show that the specific binding of \[^{3}H\]prazosin was high in the IML. Another advantage of this technique has been to quantify the amount of radioligand binding in various regions of tissue using calibrated radioactive standards placed in the cassettes. This has proved to be a useful technique, enabling direct comparison of binding of a radioligand to different areas of tissue. This technique, however, is not suitable for use in this study due to the anatomical arrangement of SPNs within the IML of the spinal cord (see general introduction). The majority of SPNs in the IML are grouped into clusters which are 150-500 \(\mu m\) apart. A 20 \(\mu m\) transverse section of cat spinal cord may contain many or relatively few SPNs depending on whether the section was taken through a cluster of SPNs. If the binding observed was to SPNs in the IML and a section contains relatively few SPNs the amount of \[^{3}H\]prazosin binding would be relatively lower than the binding seen in a section with many SPNs.

The same problem also exists when the exact amounts of displacement occurring with unlabelled ligands are studied. The section incubated in radioligand alone may have been taken through a cluster which contains many SPNs, therefore binding may be high. An adjacent section incubated in unlabelled ligand may still be through the same cluster therefore these two sections may reasonably be compared quantitatively for the amount of displacement that has occurred. However, the next section taken may contain only a few SPNs and even if the ligand did not displace binding, the amount of radioligand binding would be less and displacement may be assumed to have taken place. Therefore, great care is needed in the interpretation of results in this study and a large number of sections must be used to gain an overall picture of what is occurring.
Location of binding sites in the IML

This study has demonstrated using low resolution autoradiographical techniques that \(^{3}\text{H}\)prazosin binds to specific sites (which are likely to be \(\alpha_{1}\)-adrenoceptors) in the IML of the cat spinal cord. What it fails to show conclusively is whether these binding sites are located on SPNs. The fact that the IML consists almost exclusively of SPNs (Oldfield and McLachlan, 1981) suggests that the sites are most likely to be located on these neurones, either on the cell soma or on the dendrites (a number of which are oriented along the plane of the IML). However, this does not rule out the possibility that the receptors are found on axon terminals which synapse in the IML. In a previous study on the location of various receptors in the IML of rat spinal cord, retrograde labelling of sympathoadrenal SPNs using fast blue injections into the adrenal medulla was combined with receptor autoradiography to determine whether binding sites were associated with these neurones (Seybold and Elde, 1984). The results demonstrated that \(\alpha_{2}\)-adrenoceptors and serotonergic receptors were more highly concentrated over retrogradely labelled neurones than other areas in the IML and grey matter. This supported the idea that these receptors are located on SPNs in the IML. It was not possible to observe whether the receptors were actually found on the membranes on these neurones due to the low resolution of the autoradiographic technique. This is because autoradiographic localisation of binding sites is usually carried out on unfixed or lightly fixed tissue. The degree of fixation does not allow preservation of the ultrastructure of the material to determine the position of the binding sites on neurones. It may be possible to carry out a sympathectomy before removing tissue for autoradiography so that the SPNs have degenerated and any binding sites on the neurones will be lost. In this case, if \(^{3}\text{H}\)prazosin binding was much decreased, it would indicate that the majority of the binding sites were located on the SPN itself.

In conclusion, this study has demonstrated the autoradiographic localization of a relatively high level of \(^{3}\text{H}\)prazosin binding sites in the IML of the cat spinal cord. This indicates that, in contrast to previous observations (Dashwood et al. 1985), \(\alpha_{1}\)-adrenoceptors are present in the cat IML and may be important in
sympathetic control. Thus it has been possible for the first time to provide anatomical evidence that $\alpha_1$-adrenoceptors may be important in mediating input onto SPNs within the IML of the spinal cord.
CHAPTER 3
GENERAL METHODS FOR NEUROPHYSIOLOGICAL
AND NEUROPHARMACOLOGICAL STUDIES
3.1 Animals and anaesthesia

Experiments were carried out on a total of 27 female cats (microinjection studies; weight 1.7-2.9 kg) and 96 male Sprague-Dawley rats (iontophoretic studies; weight 205-375 g). Cats were anaesthetized with either an intravenous injection of α-chloralose (Sigma U.K. Ltd; 80 mg/kg) and sodium pentobarbitone (Sagatal, May and Baker Ltd; 12 mg) given via the cephalic vein, or an intraperitoneal injection of sodium pentobarbitone (40 mg/kg). Anaesthesia was induced in rats by an injection, via a tail vein, of α-chloralose (100 mg/kg) and sodium pentobarbitone (2 mg), or through an intraperitoneal injection of sodium pentobarbitone (60 mg/kg).

In both species anaesthesia was maintained throughout the experiments with bolus doses of α-chloralose (rats; 20-40 mg/kg, cats; 10-20 mg/kg). The adequacy of anaesthesia was judged from recordings of blood pressure, heart rate and phrenic nerve activity (where recorded), and from pupil size and the animal’s palpebral and paw pinch reflexes. The depth of anaesthesia was maintained at a level at which both corneal and flexor reflexes were sluggish. In all experiments during data collection a neuromuscular blocker was administered either by a bolus dose every hour or by constant infusion. During neuromuscular blockade, a miotic pupil, little or no response of pupillary diameter to noxious stimuli applied to the skin and stable blood pressure and heart rate were used to confirm the adequacy of anaesthesia. In addition, all animals were allowed to recover every hour from neuromuscular blockade and the reflexes checked.

3.2 Immobilisation of the preparation

All animals were immobilised to prevent any muscular contractions in response to stimulation of nerves or the spinal cord and to provide stable recording conditions. Rats were paralysed by a bolus intravenous injection of 16 mg/kg gallamine triethiodide (Flaxedil, May and Baker). The initial dose was given just prior to spinalisation or before removal of the vertebra for exposure of the spinal cord and was repeated every hour throughout the recording period. Cats were
paralysed with an infusion of vecuronium bromide (Norcoron; Organon Teknika Ltd.) at dose of 400 μg/kg/hr given in 6 ml of saline.

3.3 Surgical procedures and maintainence of the animal.
3.3a Initial Dissection
All initial dissection was carried out on an operating table. The remaining surgery and recordings were carried out on a pneumatic vibration free table (Ealing Electro-optics Ltd.).

A femoral artery and vein were cannulated with polyethylene tubing filled with heparinised saline and saline, respectively. The venous line was used for administration of supplementary anaesthetic and drugs. The arterial line was connected to a pressure transducer to monitor blood pressure which was displayed on an oscilloscope and pen recorder. Steel needles were placed in the left forepaw and the right hindpaw, and the ECG signal was displayed on an oscilloscope and a pen recorder.

The trachea was exposed, intubated and the animals were artificially respired on oxygen-enriched air. End-tidal CO₂ was monitored continuously using a fast response CO₂-analyser (ADC Ltd). Arterial blood samples were taken periodically using a Corning Blood Gas Analyser to check pH and blood gas tensions which were kept in the following ranges: pH 7.35-7.45; P<sub>aCO₂</sub> 30-40 mmHg; P<sub>aO₂</sub> > 80 mmHg. The values were maintained by altering the rate and/or volume of ventilation. Sodium bicarbonate was given intravenously where necessary to correct base deficits.

Rectal temperature was monitored with a thermistor and maintained at 37 ± 1°C using a heating blanket controlled by a feedback circuit (Harvard Bioscience Ltd USA). Cats had their bladders catheterized to allow for the continuous flow of urine.
3.3b Pneumothorax
All animals were given a pneumothorax in order to minimise respiratory-related movement artifacts which would have hampered the recording procedures. An incision was made in the skin overlying the middle ribs, midway between the rib heads and sternum. The muscle and subcutaneous fat were cleared from the ribs using blunt dissection or cauterisation. A pair of scissors was pushed through the muscle and pleura between two ribs into the thoracic cavity. To allow passage of air, the hole between the ribs was kept open by passing a thread around one of the ribs and attaching it tightly to a pole positioned near the animal so that the rib cage was pulled open (cats) or by introducing a piece of polyethylene tubing through the hole into the thoracic cavity (rats). Following the pneumothorax, an end-expiratory pressure of 2-3 cm \( H_2O \) was applied to the expiratory line to prevent atelactasis.

3.4 Electrode manufacture
(i) Five and seven-barrelled electrodes for iontophoresis
Electrodes were made from five or seven pieces of filamented glass capillary tubing (Clark Electromedical Instruments, internal diameter 1.2 mm; length 10 cm) which were fitted into a collar of brass tubing and glued. Electrodes were pulled on a vertical puller (Narishige Scientific Instrument Labs, Japan). The glass was heated, twisted in a 180° turn and allowed to drop 2-3 mm before cooling the glass and pulling the electrode. The bottom electrode was discarded and the tip of the top electrode was broken back to a tip diameter of 2-3 \( \mu m \) under a binocular microscope fitted with a graticule using a polished glass rod. The barrels of the non-tapered end were gently heated and splayed so that drugs could be introduced into a barrel without contaminating the drugs in the other barrels.

One of the barrels was filled with 4M sodium chloride solution which had been filtered using a millipore system (Millipore SA, France, filter holder swinnex-15). A silver wire was placed into the barrel and the resistance of the electrode was
tested. Electrodes with resistances of between 3-6MΩ were kept for recording purposes, any others were discarded, since electrodes with high resistances led to difficulty in locating neurones and passing current while, with low resistance electrodes, units were difficult to isolate. The other barrels were filled with the prepared and filtered drugs just prior to their use in each experiment.

(ii) Seven-barrelled electrodes for microinjection
Electrodes were prepared and pulled in the same way as the recording electrodes but both top and bottom electrodes were used if suitable. The end of the electrode was pinched off using watchmakers forceps to a tip diameter of 50-60 μm and the ends of the barrels were bent back as above.

The central barrel of the electrode was filled with metal to enable electrical stimulation in the spinal cord. The metal used was an equal mixture by weight of Woods metal (BDH Chemicals Ltd.) and Indium pellets (Aldrich Chemicals Co. Inc.) which were heated to their combined melting points (70°C) and then drawn up into polyethylene tubing which had an internal diameter just less than the glass capillaries. Once the tubing had been cut away from the metal, a 1 cm length was placed in the central barrel of the electrode and pushed as near to the bottom as possible with a piece of tinned copper wire (24 swg; RS Components Ltd). The metal was gently heated on a hot plate so that the end melted and could be pushed to the tip of the electrode with the wire. The wire was then pushed into the metal and the electrode checked under a binocular microscope for breaks in the metal and tested to see whether there was continuous circuit through the electrode.
CHAPTER 4

EFFECT OF IONTOPHOРЕSED $\alpha_1$ADRENOCEPTOR AGONISTS
ON SYMPATHETIC PREGANGLIONIC NEURONAL ACTIVITY
IN THE RAT
INTRODUCTION

The general introduction reviewed the many iontophoretic investigations which had studied the possible influence of catecholaminergic innervation of the IML on sympathetic preganglionic neuronal activity in the cat and rat. These studies all observed that adrenoceptor agonists iontophoresed in the vicinity of SPNs in vivo caused decreases in the firing rate of these neurones which were mediated by $\alpha_2$-adrenoceptors. However, in experiments carried out in the cat and rat spinal cord slice, adrenaline and noradrenaline caused both excitatory and inhibitory effects on SPNs, the former being mediated by $\alpha_1$-adrenoceptors. This indicated that $\alpha_1$-adrenoceptors are present in the spinal cord of the cat and rat and are involved in mediating excitatory input onto SPNs. Furthermore, in the previous chapter, the presence of $\alpha_1$-adrenoceptor binding sites were demonstrated anatomically in the IML of the cat. In order to determine whether these $\alpha_1$-adrenoceptors are involved in sympathetic control in vivo, this study iontophoresed selective $\alpha_1$-adrenoceptor agonists in the vicinity of antidromically indentified SPNs in the rat.

4.1a Selective $\alpha_1$-adrenoceptor agonists

Two agonists were used in this study, methoxamine and phenylephrine, both of which have been shown to be selective for $\alpha_1$-adrenoceptors (Furchgott, 1970; Starke, Endo and Taube, 1975; Besse and Furchgott, 1976; Drew, 1976; Wikberg, 1978). Both these drugs have been applied iontophoretically in other studies and were shown to be excitatory. Iontophoresis of methoxamine and phenylephrine in the vicinity of cerebral cortical neurones increased the firing rate of these neurones (Bevan, Bradshaw and Szabadi, 1977). The potency of methoxamine was observed to be less than that of phenylephrine in this and a later study (Bradshaw, Pun, Slater and Szabadi, 1981), an effect which seemed to be a genuine biological action of the drug since the relative mobilities of the drugs were similar when tested in vitro. The latency to onset of the action of methoxamine was significantly greater than that of phenylephrine and noradrenaline and was assumed to be a characteristic of the lower potency of the drug. In addition, the time course of the effect of methoxamine was longer than
both phenylephrine and noradrenaline (Bradshaw et al. 1981). This was attributed to the fact that methoxamine shows little affinity for the uptake mechanism involved in the reabsorption of catecholamine into the presynaptic terminal (Trendelenberg, Maxwell and Pluchino, 1970) and is therefore present in the synaptic cleft for longer than other catecholaminergic agonists.

4.1b Alfuzosin as a selective $\alpha_1$-adrenoceptor antagonist

The $\alpha_1$-adrenoceptor antagonist used in these studies was alfuzosin. This is a relatively new, selective $\alpha_1$-adrenoceptor antagonist produced by Synthelabo. Intravenous alfuzosin selectively blocked the pressor response to intravenous cirazoline (an $\alpha_1$-adrenoceptor agonist) in pithed rats (Cavero, Lefevre-Borg and Manoury, 1984a) and to intravenous phenylephrine in man (Davies, Guinebault, Johnson, Seymour, Sinclair and Warrington, 1986). In addition, the affinity of alfuzosin for displacement of $[^3H]$prazosin binding was forty times higher than that for displacement of $[^3H]$clonidine (an $\alpha_2$-adrenoceptor agonist) binding (Cavero, Galzin, Langer, Lefevre-Borg, Manoury and Pimoule, 1984b). Intravenous infusions of alfuzosin caused a decrease in sympathetic preganglionic nerve activity presumably due to action on central $\alpha_1$-adrenoceptors (Ramage, 1986a). This antagonist is much more soluble in water or saline than prazosin which is normally dissolved in lactic acid (see Ramage, 1986a). The solubility of alfuzosin and its selectivity for $\alpha_1$-adrenoceptors were the important factors in choosing this antagonist for the iontophoretic study.

4.1c A possible presynaptic site of action for $\alpha_1$-adrenoceptor ligands

Recent studies have indicated that $\alpha_1$-adrenoceptors involved in sympathoexcitation are not located exclusively on the postsynaptic terminal. Koss and co-workers (Ito, Hey and Koss, 1988; Koss, Hey and Ito, 1990) studied the sympathetic-cholinergic electrodermal response to hypothalamic stimulation and demonstrated that this response was reduced by intravenous injections of the $\alpha_1$-adrenoceptor antagonist prazosin. Spinalisation, or the intravenous injection of the $\alpha_2$-adrenoceptor antagonist yohimbine, significantly blocked the depressant effects of prazosin. This indicated that prazosin may act indirectly by blocking
presynaptic $\alpha_1$-adrenoceptors which, when normally activated, tonically reduce the amount of neurotransmitter released from a descending inhibitory supraspinal pathway. Spinalisation, or blockade of the receptor mediating the inhibitory response, removed the tonically active descending inhibitory pathway and the effect of prazosin was no longer seen. Since yohimbine blocked the effect of prazosin, it was suggested that the inhibitory neurotransmitter was a catecholamine, acting at postsynaptic $\alpha_2$-adrenoceptors. Therefore, in some experiments, rats were spinalised between the first and second thoracic vertebrae to observe if the changes in the firing rate of an SPN due to iontophoresis of the $\alpha_1$-adrenoceptor agonists were maintained under these conditions.

4.1d Are there differential inputs onto subpopulations of SPNs?

There is now evidence to indicate that subpopulations of SPNs may be differentially controlled by descending supraspinal pathways (see general introduction). Subpopulations of SPNs display different physiological characteristics, such as ECG- or respiratory-related firing patterns (see Jänig and Szulczyk, 1980). Therefore, it may be possible to relate the response of a neurone to an agonist to its physiological characteristics. In a recent study by Gilbey and Stein (1991) the physiological characteristics of SPNs in the upper lumbar segments of cat spinal cord were studied in relation to their responses to iontophoresed 5-HT. It was found that SPNs which had their activity decreased by iontophoresed 5-HT were more likely to be excited by noxious stimulation of the hind limb, while those SPNs which were excited by 5-HT had their firing rate decreased or were unaffected by noxious stimulation. However, there was no correlation between the ECG- or respiratory-related firing patterns of SPNs and their response to 5-HT.

In this study SPNs were tested for their ECG and respiratory-related activities and the axonal conduction velocities were calculated to see whether those SPNs which were affected by iontophoresis of either methoxamine or phenylephrine had different firing patterns and/or conduction velocities to those SPNs which were not affected by these agonists. In this way it may be possible to determine
whether there is a relationship between the characteristics of an SPN and its response to iontophoresed $\alpha_1$-adrenoceptor agonists.
METHODS

4.2a Surgical Procedures

(i) Stabilisation of the cord
Iontophoretic experiments involved recording extracellularly from single SPNs in the spinal cord. In order to facilitate recording from these neurones for long periods of time, the spinal cord was fixed rigidly to minimise movement. After initial surgery, the rat was moved to a recording table and its head was fixed in a stereotaxic head frame. A string was attached to the tail and tied to a horizontal bar which was positioned to the rear of the animal. The string was shortened until the tail and body of the rat were stretched slightly. Incisions were made in the skin on either side of the vertebral column near the pelvic girdle. Using blunt dissection, the muscle on both sides of the column was cleared and the vertebrae were clamped. The metal clamp was lifted (so that the rat was suspended at this point) and rotated backwards until the vertebral column was completely horizontal.

(ii) Exposure of the nerves
A mid-dorsal incision from the shoulder to the neck was made in the skin. The cutaneous maximus muscle overlying the vertebral column was cauterised on both sides so that the left scapula could be retracted partially. The acromiotrapezius and spinotrapezius muscles on the left side were cauterised and a thread passed through the bone muscle and skin of the scapula and tied to fully retract the scapula. Metal retractors were used to hold the scapula away from the vertebral column.

(a) The cervical sympathetic nerve
The left cervical sympathetic nerve runs within a bundle of nerves (including the vagus) near the left common carotid artery. Two to three centimetres of the nerve were dissected clear and placed across bipolar platinum stimulating electrodes. The identity of the nerve was confirmed by electrical stimulation (3 V; 30 Hz frequency and 1 ms pulse width) which caused pupillary dilatation. The left vagus nerve was sectioned (the right vagus having been exposed and sectioned after
(b) The phrenic nerve
The phrenic nerve can be located running first perpendicular to, and then parallel
to the vertebral column as it travels caudal to the thoracic cavity. The nerves of
the brachial plexus were cut and retracted to reveal the phrenic nerve. The nerve
was then isolated and cleared of connective tissue, cut peripherally and the central
deend wrapped around platinum bipolar recording electrodes. The phrenic and the
cervical sympathetic nerves were then covered with warm paraffin oil.

(iii) Exposure of the spinal cord
The mid-dorsal incision made for exposure of the nerves was extended caudally,
and the muscle and skin on the right side of the vertebral column reflected. The
first to third thoracic segments of the cord were identified by the large spinous
process on the second thoracic segment and the muscle overlying these vertebrae
was cleared. A laminectomy was carried out to expose the dorsal surface of the
cord at the second thoracic segment using fine bone rongeurs (taking care not to
damage the lateral venous sinuses) to expose the dorsal surface of the spinal cord.
The dura mater was opened using fine forceps and deflected under a binocular
dissecting microscope. A clamp was placed on the third thoracic vertebra and
lifted slightly so that the exposed cord was horizontal. The cord was then covered
with warm paraffin oil.

(iv) Spinalisation
For spinalisation, the mid-dorsal incision from shoulder to neck was extended
rostrally to the skull. Connective tissue overlying the parietal and interparietal
areas of the skull was removed using bone scrapers. The cutaneous maximus
muscle was separated from the skull and cut along the vertebral column to reveal
the first and second cervical vertebrae. Overlying muscle was scraped away from
these vertebrae to expose the spinal cord between the first and second vertebrae.
A pair of scissors could be placed between the vertebrae to transect the spinal
cord. Bleeding was stopped by packing cotton wool balls tightly around the area
of transection. Once bleeding ceased, it was possible to check whether the spinalisation was complete.

**4.2b Stimulation of the cervical sympathetic nerve**
The cervical sympathetic chain was stimulated with a single rectangular pulse (1 ms pulse width, 1-4 V, 1 Hz) delivered by a Digitimer programmer (D 4030) and an isolated stimulator (DS2, Digitimer Ltd.; see figure 4.1).

**4.2c Preparation of drugs and iontophoresis**
Drugs used were methoxamine HCl; phenylephrine HCl; glutamic acid (all Sigma); alfuzosin HCl (Synthelabo). All drugs were made up in either 0.9% saline or distilled water and the pH of each solution adjusted when necessary using 0.1M sodium hydroxide or 0.1M hydrochloric acid to increase the solubility of the drug and optimise the degree of ionisation. This allowed ejection or retention of the drugs using an electrical current (see table 4.1 for the solvent and pH used with each compound). Each drug was drawn up into a syringe which was attached via a millipore filter system to a narrow gauge cannula. The cannula was fed carefully into the barrel of the microelectrode and the drug expelled so that the barrel was filled without risk of contaminating the other barrels.

Iontophoresis of drugs was carried out using a Neurophore Drug Ejection Unit (Medical Systems Corporation; figure 4.1). Drugs were retained within the barrels by passing specific retaining currents for each compound (see table 4.1 for values). During ejection of drugs, current balancing was achieved through an automatic circuit incorporated into the Neurophore unit. Some effects were still seen that seemed to be due to the passing of current rather than an effect of the drug itself (see results section). To control for these artifacts, saline was ejected at the same current and polarity as the drug under test.
4.2d Data collection
Sympathetic preganglionic neuronal activity was recorded using a high impedance headstage (NL 100, Neurolog Ltd) and amplified using an A.C. preamplifier (gain 1-10K, NL 104, Neurolog Ltd). The signal was then filtered (band width 500 Hz-1.5 KHz, NL 125, Neurolog Ltd) and fed into a tape recorder (Racal 7DS).

Phrenic nerve activity was also amplified (gain 1-10 K), filtered (band width 100Hz-1.5KHz) and fed into the computer. Also recorded on tape were blood pressure, tracheal pressure and electrocardiogram (ECG; see figure 4.1).

4.2e Recording and identification of neurones
Five-barrelled microelectrodes were advanced into the spinal cord just lateral to the dorsal root entry zone using an electronic microdrive (Nanostepper, type B) while the cervical sympathetic chain was stimulated at rate of 1 Hz. The stimulus was triggered by the Digitimer which also produced a TTL pulse at the same time to trigger the computer and tape recorder. Extracellular neuronal activity was recorded through one barrel of the electrode, and after amplifying and filtering, was fed into the computer. A signal averager programme (Cambridge Electronic Design Ltd) was used to average the neuronal activity which occurred in the 200 ms after the stimulus to the cervical sympathetic chain. Antidromically evoked spikes occurred at a constant latency after stimulation therefore, on an average of 20 sweeps on the computer, any sympathetic preganglionic neurones which were being driven by the stimulation showed on the trace as a distinct event above the noise (see figure 4.2). The electrode was advanced into the cord until it was sufficiently near to one SPN to obtain a good neuronal signal to noise ratio. The neurone was further confirmed as being antidromically activated using standard criteria (see results section).

Many of the SPNs which were identified by antidromic stimulation had no ongoing activity. If the neurone was quiescent, glutamate was iontophoresed until a steady neuronal firing rate was obtained and this current was maintained throughout the experimental procedure.
**TABLE 4.1**

Drug solutions used for iontophoretic experiments
<table>
<thead>
<tr>
<th>DRUGS</th>
<th>SOLVENT</th>
<th>CONC.</th>
<th>pH</th>
<th>RETAINING CURRENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHOXAMINE</td>
<td>DIST H₂O</td>
<td>0.05 M</td>
<td>4.5</td>
<td>-15 nA</td>
</tr>
<tr>
<td>PHENYLEPHRINE</td>
<td>SALINE</td>
<td>0.05 M</td>
<td>5.0</td>
<td>-15 nA</td>
</tr>
<tr>
<td>GLUTAMATE</td>
<td>DIST H₂O</td>
<td>0.2 M</td>
<td>7.4</td>
<td>+20 nA</td>
</tr>
<tr>
<td>ALFUZOSIN</td>
<td>DIST H₂O</td>
<td>0.01 M</td>
<td>7.4</td>
<td>-15 nA</td>
</tr>
<tr>
<td>SALINE</td>
<td>-</td>
<td>-</td>
<td>4.5-5.0</td>
<td>-15 nA</td>
</tr>
</tbody>
</table>
FIGURE 4.1.

Block diagram of the experimental arrangement for iontophoretic experiments.
4.2f Data analysis (on- and off-line)

All data analysis was carried out using an interface and software supplied by Cambridge Electronic Design Ltd. in conjunction with an IBM-compatible microcomputer.

(i) Measurement of axonal conduction velocity

For each SPN that was recorded, an axonal conduction velocity was estimated. The latency of the antidromic spike evoked by stimulation of the sympathetic chain was measured and at the end of the experiment, the distance between the stimulating electrodes and the position of the microelectrode in the cord was measured. The conduction velocity was then calculated as

\[
\text{conduction distance} \quad \text{latency of response}
\]

(ii) Effects of drugs on sympathetic preganglionic neuronal activity

Sympathetic preganglionic neuronal activity was fed into a spike processor (Digitimer D130) which generated TTL pulses which were delivered to a computer. The TTL pulses were obtained by setting a window on the spike processor so that only activity within the window triggered the computer thus removing the possibility of counting noise or artifacts as neuronal activity. In addition, if there were other neurones firing in the background, the window could be set to eliminate such spikes. In this way, any changes in the firing rate of the antidromically identified SPN alone could be observed. The numbers of spikes occurring in the window were counted in 1-10 second bins and a rate histogram generated. Drugs were iontophoresed using a Neurophore system which was fitted with an analog output. This provided a voltage which was proportional in magnitude and polarity to the actual current passed through the barrel of the microelectrode. This output was delivered to the computer and the tape to mark exactly when a drug was being ejected.

For some neurones, interspike interval histograms were also constructed to
determine the effect of \( \alpha_1 \)-adrenoceptor agonists on the firing pattern of these neurones. The window was set so that each spike triggered the computer. The time between consecutive spikes was captured in 100 bins (each of 100 ms) and this value corresponded to the interspike interval. These histograms were constructed for each neurone both before and after iontophoresis of the agonist.

(iii) Phrenic-triggered Histograms
Phrenic-triggered histograms were produced to study whether SPNs displayed any respiratory-related activity. Phrenic nerve activity was fed into an integrator (Neurolog NL 703) to be rectified and smoothed (time constant 100 ms). This output was fed into an interface (Neurolog NL 515) which generated a TTL pulse when integrated phrenic nerve activity reached a preset level. The TTL pulse generated was then used to trigger the computer. Neuronal activity was fed into a spike processor as before and a post-stimulus time histogram constructed so that neuronal firing occurring in the 2-6 second time period after the computer was triggered was captured in 30-50 ms bins.

(iv) ECG-triggered Histograms
ECG-triggered histograms were generated to study whether neurones exhibited cardiac-related activity. The ECG signal was passed through an interface and the R-wave was used to generate a TTL pulse which was then used to trigger a sweep of the computer. Neuronal activity was fed into a spike processor as before and a post-stimulus time histogram was set up so that neuronal activity occurring in the 300 ms period after the R-wave trigger was collected in 3-5 ms bins.

(v) Statistical analysis
In order to determine whether SPNs in animals with their neuraxis intact were significantly more likely to have ongoing activity than those neurones in spinalised animals, a 2x2 contingency table was constructed (Siegel, 1959). Median tests (Siegel, 1959) were performed to determine whether (i) the median conduction velocities of neurones which were excited by \( \alpha_1 \)-adrenoceptor agonists differed significantly from the values obtained for those neurones which were not affected
by these agonists and (ii) the median durations of the excitatory effects of iontophoresis of methoxamine and phenylephrine were significantly different. The G-test of independence (Sokol and Rohlf 1969) was used to test whether neurones which were excited by \( \alpha_1 \)-adrenoceptor activation were significantly more likely to have ECG or respiratory-related activity patterns. For each statistical test, differences were considered significant if \( p \leq 0.05 \).
RESULTS

4.3a Antidromic identification of SPNs
One hundred and twenty eight SPNs were identified antidromically in this study. The neurones were confirmed as being antidromically activated using standard criteria:

(i) The antidromic spike showed an all-or none response i.e. the neurone was not activated below a threshold voltage and once activated, the spike size remained constant through increasing stimulus voltages above the threshold value.
(ii) The latency of the antidromically evoked response was constant.
(iii) It was possible to cancel the antidromically activated spike with a spontaneous or glutamate-activated action potential (see figure 4.2).

These identified SPNs were found at depths of 411-1082 µm below the dorsal surface of the spinal cord. Of these neurones, 42 had ongoing activity and 73 could be activated by microiontophoresis of glutamate at currents of 0 (retaining current off) to 198 nA. The remaining quiescent neurones could not be activated and therefore were not studied further. The proportions of neurones with ongoing activity in rats with their neuraxis intact was compared to that observed in spinalised rats. Of 75 SPNs which were tested in rats with the neuraxis intact, 33 neurones (44 %) had ongoing activity and 42 (56 %) were driven by glutamate. Of 44 SPNs which were tested in spinalised rats, 10 neurones (23 %) had ongoing activity and the remaining 34 neurones (77 %) were activated by glutamate. It was observed that there was a significantly greater number of SPNs with ongoing activity in animals with intact neuraxes than in those which were spinalised (p<0.05).

4.3b Axonal conduction velocity
Axonal conduction velocities were calculated as described in the method section 4.2f(i). The mode class of conduction velocity was 0.5-0.6 m/s and the median conduction velocity was 0.55 m/s. The range of conduction velocities was 0.28 to
Antidromic identification of a sympathetic preganglionic neurone by collision testing using an orthodromic action potential (ongoing or glutamate driven) to trigger the antidromic stimulus delivered to the cervical sympathetic nerve.

(A) shows five superimposed traces of antidromic action potentials to illustrate the fixed latency of this response.

(B) the delay between the orthodromic spike and the stimulus to the cervical sympathetic nerve was reduced so that collision occurred between the orthodromic and antidromic spike.

(C) The delay between the orthodromic spike and antidromic stimulation was increased so that no collision occurred and the antidromic action potential reappeared.

Filled circles mark orthodromic spikes
Open circles mark antidromic spikes
Arrows mark the stimulus artifacts
1.73 m/s indicating that the fibres were unmyelinated. The distribution of velocities is shown in the histogram (figure 4.3.A). Separate histograms were constructed of the conduction velocities of neurones with ongoing activity and those which were quiescent (figure 4.3.B and 4.3.C.). There was little difference in the distribution of conduction velocities between the two groups, indeed both groups had a mode class of 0.5-0.6 m/s and median conduction velocities of 0.55 m/s which were the same values as those obtained for the pooled data.

4.3c ECG-related activity of SPNs (neuraxis intact)

Forty neurones were analyzed for ECG related activity using ECG- or blood pressure-triggered histograms constructed as described in the methods. ECG-related activity was used to indicate whether the neurones were under baroreceptor modulation (see Blumberg, Jänig, Rieckmann and Szulczyk, 1980). Of the 40 neurones, analyzed 16 had ongoing activity and the remainder were activated by iontophoresis of glutamate. For each SPN analyzed the computer was triggered and sympathetic preganglionic neuronal activity was accumulated over 500 double cardiac cycles (3 ms). In recent experiment, (Gilbey and Stein, 1991) it was found that the most reliable method of discerning whether the neurone displayed ECG-related activity was by visual examination of the histogram plots. Examples of neurones with very strong, strong, weak and no ECG related activity are shown in figure 4.4. Of the neurones analyzed, only 10 (4 with ongoing activity) showed very strong or strong ECG-related activity (see table 4.2 for characteristics of various neurones).

4.3d Phrenic-related activity (neuraxis intact)

In order to examine respiratory-related firing patterns of SPNs, the phrenic nerve which innervates the diaphragm was used as an indicator of central respiratory drive. The onset of a burst of activity in this nerve (which indicated the start of inspiration) was used to trigger the computer and histograms were constructed over 50 cycles (2-6 seconds each cycle) to study the respiratory-related activity of 17 SPNs. The animals were artificially respired at a much higher frequency than that of phrenic bursts and the vagi were cut. This dissociated the lung inflation
FIGURE 4.3.

Histograms of the estimated conduction velocities of upper thoracic SPNs projecting to the cervical sympathetic chain.

(A) Histogram showing the conduction velocities of all SPNs tested (n = 128).

(B) Histogram of the conduction velocities of those SPNs which were spontaneously active (n = 42).

(C) Histogram of the conduction velocities of those SPNs which had no ongoing activity (n = 86).

In each case the mode class of conduction velocity was 0.5-0.6 m/s.
ECG- (R-wave) or blood pressure-triggered histograms of sympathetic neuronal discharge taken over 500 double cardiac cycles (bin size 5 ms).

SPNs were categorised in four different ways according to their level of ECG- or BP-related activity and examples from each category are shown.

(A) SPN with very strong ECG-related activity (ongoing activity).
(B) SPN with strong BP-related activity (triggered from the systolic blood pressure; glutamate-driven).
(C) SPN with weak ECG-related activity (glutamate-driven).
(D) SPN with no ECG-related activity (glutamate-driven).
FIGURE 4.5.

Phrenic-triggered histograms of sympathetic neuronal discharge taken over 50 cycles (bin size 2-6ms).

SPNs were divided into three categories according to their respiratory-related activity and examples from each are shown.

(A) SPN with an inspiratory-related firing pattern (glutamate-driven).
(B) SPN with an expiratory-related firing pattern (ongoing activity).
(C) SPN which was not modulated by respiratory inputs (glutamate-driven).
cycle from central respiratory drive so that any related firing patterns were not a resultant effect of afferent feedback from the lungs. SPNs were classified according to the pattern of their respiratory-related discharge into 3 categories
(a) Those which were most likely to fire during the phrenic burst were classified as inspiratory-related (figure 4.5.A).
(b) Those SPNs which were most likely to fire during phrenic silence were classified as expiratory-related (figure 4.5.B).
(c) Those SPNs which showed no clear relationship to phrenic nerve discharge were classified as non-modulated by respiratory inputs (figure 4.5.C).

17 SPNs were analyzed for respiratory-related activity, 10 of which had ongoing activity while the rest were activated by glutamate. Of those SPNs tested, 7 had inspiratory-related activity, (3 with ongoing activity); 6 displayed expiratory-related activity (4 with ongoing activity) and the remaining 4 SPNs had no respiratory-related activity. Table 4.2 shows other characteristics of those SPNs with respiratory-related activity.

4.3e Effects of iontophoresed methoxamine
(i) Effects on SPNs in animals with their neuraxis intact.
Recordings were made from 21 sympathetic preganglionic neurones, 9 of which had ongoing activity, while the remainder were activated by iontophoresed glutamate. Methoxamine was iontophoresed at currents of 30-60 nA for up to 3 minutes and caused an increase in the firing rate of 13 neurones, 5 of which had ongoing activity. The remainder were unaffected by iontophoresis of methoxamine. The excitatory effect lasted for 1.67-16.2 minutes (median 6.8 minutes) and was slow in onset, taking up to 3 minutes to reach a peak. In many cases, a decrease in firing rate was observed during application of methoxamine. This seemed to be a current artifact since iontophoresis of saline had a similar inhibitory effect and there was no rebound excitation after the current control. An example of the excitation seen with iontophoresis of methoxamine onto an SPN in a rat with the neuraxis intact is shown in figure 4.6.
FIGURE 4.6.

Histogram of the firing rate of an SPN in a rat with intact neuraxis to show the effect of iontophoresed methoxamine (bin size 5 seconds).

In this, and subsequent, histograms of the firing rate of SPNs, firing rate of the neurone is shown on the Y axis with time in seconds on the X axis. The neurone was activated by 19 nA glutamate as shown by the hatched line below the histogram. Methoxamine was iontophoresed at 40 nA for 2 minutes (indicated by the black bar) and caused an initial decrease in firing rate of the SPN during current injection followed immediately by a large increase in firing rate. The firing rate returned to control levels after 975 seconds. Saline iontophoresed at the same current and for the same time caused only a decrease in the firing rate of the neurone with no subsequent increase.
(ii) Effects on SPNs in spinalised animals
In an attempt to show whether the excitatory effect of iontophoresis of methoxamine on SPNs was a direct effect or a result of interaction of methoxamine with tonically active descending supraspinal pathways, animals were spinalised between the first and second cervical vertebrae at least 3 hours before SPN recording commenced.

Recordings were made from nine SPNs, one of which had ongoing activity, the other eight were driven by glutamate. Methoxamine was iontophoresed at currents of 16-30 nA for up to 3 minutes and caused an increase in the firing rate of six of these neurones, all of which were driven by glutamate. These increases lasted 3.3-20.8 minutes (median 5 minutes) and once more any initial decrease in the firing rate of these neurones seemed to be a current artifact. Histogram showing the effect of methoxamine on the firing rate of an SPN in a spinalised rat are shown in figures 4.7 and 4.9. Figure 4.8 shows the raw neuronal activity of this neurone to show that methoxamine had no effect on the spike size during the increased firing.

4.3f Effects of iontophoresed phenylephrine
(i) Effects on SPNs in animals with their neuraxis intact
Recordings were made from 10 SPNs, 3 of which had ongoing activity, while 7 were activated by glutamate. Phenylephrine was iontophoresed at currents of 30 nA for 1-3 minutes and caused an increase in the activity of 7 of these neurones, 2 of which had ongoing activity. The duration of these excitatory effects ranged from 0.83-16.67 minutes with a median value of 3 minutes. This median value was significantly smaller than that obtained for methoxamine (0.01 > p > 0.001). The remaining 3 neurones were unaffected by phenylephrine. Saline iontophoresed at the same currents and for a similar time course had no effect on the firing rate of these neurones. An example of the excitatory effect of phenylephrine is shown in figure 4.10. The raw neuronal activity of this neurone is shown to demonstrate that phenylephrine had no effect on the size of the action potential (see figure 4.11).
FIGURE 4.7.

Histogram of the firing rate of an SPN in a spinalised rat to show the effect of iontophoresed methoxamine (bin size 5 seconds).

The neurone was activated by glutamate at 70 nA (hatched bar) and methoxamine was iontophoresed at 30 nA for 2 minutes (black bar). After an initial decrease in firing rate (due to a current effect) there was a very large increase in neuronal activity which lasted for 1250 seconds before returning to control levels. Once more saline was iontophoresed at the same current and for the same time period (black line) and caused only a decrease in firing rate with no ensuing increase.
FIGURE 4.8.

The raw neuronal activity of a single SPN which was excited by iontophoresis of methoxamine to demonstrate that spike size remained constant during iontophoresis of methoxamine and the increased firing rate. Iontophoresis of methoxamine and saline are indicated by the black bar.
FIGURE 4.9.

Histogram of the firing rate of an SPN in a spinalised rat to show the effect of iontophoresed methoxamine (bin size 5 seconds).

This SPN was shown in the previous histogram (see figure 4.7.) to be excited by iontophoresis of methoxamine. Once the firing rate had returned to control firing rate, the glutamate iontophoretic current was turned down to a level at which the SPN became quiescent (20 nA). Methoxamine was iontophoresed at 30 nA for 2 minutes and this caused the neurone to start firing again at a steady rate for around 600 seconds. When the effect of methoxamine had worn off and the neurone was once more quiescent, the glutamate current was turned up to the original level to show that it was still possible to activate the SPN.
**FIGURE 4.10.**

Histogram of the firing rate of an SPN in a rat with intact neuraxis showing the effect of iontophoresis of phenylephrine (bin size 5 seconds).

X and Y axis as figure 4.6. The neurone was activated by glutamate at 50 nA (hatched line) and phenylephrine was iontophoresed at a current of 30 nA for 1 minute. This caused a almost immediate increase in activity of the SPN an effect which lasted for 140 seconds. Iontophoresis of saline caused only a slight increase in activity during the period of drug ejection.
FIGURE 4.11.

The raw neuronal activity of an SPN in a rat with its neuraxis intact which was excited by iontophoresis of phenylephrine.

Iontophoresis of phenylephrine is marked by the black line. The effect of saline on raw spike activity is also shown.
(ii) Effects on SPNs in spinalised animals

The effect of phenylephrine was examined on 7 neurones in spinalised rats, 2 of which had ongoing activity. Iontophoresis of phenylephrine at currents of 30 nA for 1-3 minutes caused an increase in the firing rate of 6 neurones, 1 of which had ongoing activity (see figure 4.18 for an example). These effects lasted from 1.83-7.5 minutes with a median value of 3.6 minutes (this value is significantly less than that obtained for methoxamine; 0.05 > p > 0.01).

4.3g Effect of \(\alpha_1\)-adrenoceptor agonists on the firing pattern of SPNs

Four SPNs which were tested for their response to either methoxamine or phenylephrine had their firing pattern and/or their firing rate altered by iontophoresis of these agonists. The neuronal firing changed from a tonic pattern of activity to a burst pattern. An example of this effect on the firing pattern of an SPN is shown in figure 4.12 in histogram form with the raw neuronal activity shown in figure 4.13. Phenylephrine had little effect on the overall firing rate of this SPN but caused the neurone to start firing in bursts. If larger bin sizes (10 seconds) were used in the rate histogram this effect of phenylephrine was masked (figure 4.14). The effect of phenylephrine on this neurone was also analyzed using interval histograms to show the distribution of the delays between action potentials. The two distributions of intervals between spikes before and after phenylephrine are shown in figure 4.15 and the change in firing pattern is clearly shown by the difference in the distributions. Interval histograms were also constructed for an SPN which was excited by iontophoresis of phenylephrine but there was no change in firing pattern (see figure 4.16). Here the shape of the two distributions of intervals between spikes are similar but the increased firing rate of the neurone after phenylephrine has shifted the whole distribution of spike intervals to the left. Another example of a change in the firing pattern of an SPN with iontophoresis of an \(\alpha_1\)-adrenoceptor agonist is shown in figure 4.8. Both the firing rate and pattern of activity of this neurone was affected by methoxamine so that the increases in activity were in part due to bursts of firing.
FIGURE 4.12.

Histogram of the firing rate of an SPN in a spinalised rat to show the effect of iontophoresis of phenylephrine (1 second bins).

This SPN was spontaneously active. Phenylephrine iontophoresed at 30 nA for 1 minute caused a change in the firing pattern of this SPN, so that bursts of action potentials were seen rather than tonic activity. This effect lasted for 345 seconds before activity returned to the original firing pattern.
FIGURE 4.13.

Raw neuronal activity of the same SPN as in figure 4.12 which had its firing pattern changed from tonic to burst firing.
FIGURE 4.14.

Histogram of the firing rate of the same SPN as the previous histogram (10 second bins).

Phenylephrine was iontophoresed at 30 nA for 1 minute as mentioned above. The increased bin size masks the change in firing pattern of the SPN so that phenylephrine does not appear to be having an effect on the activity of this SPN.
Figure 4.15.

Interval histograms of an SPN where phenylephrine had little effect on the firing rate of the neurone but caused a change in the firing pattern (300 sweeps; bin size 100 ms; SPN as the 2 previous histograms).

(A) Before phenylephrine.
The times between spikes fall into a normal distribution suggesting regular firing of the neurone.

(B) After phenylephrine.
The large peak at the beginning of the histogram corresponds to the intervals between spikes within a burst of activity ("intraburst" spike intervals). The large spread of values at longer time intervals correspond to the times between bursts ("interburst" spike intervals).

These histograms show clearly the difference in the firing pattern of this SPN in the presence of phenylephrine.
A

No. of intervals

B

No. of intervals
FIGURE 4.16.

Interval histograms of an SPN where phenylephrine caused an increase in the firing rate of this neurone but the firing pattern of the neurone was not changed (300 sweeps; bin size 100 ms).

(A) Before phenylephrine.
The times between peaks fall into a normal distribution, suggesting regular firing of the neurone.

(B) After phenylephrine.
The shape of distribution of the times between spikes has not changed suggesting that the neurone is still firing regularly. However, the whole distribution is shifted to the left since phenylephrine has caused the neurone to fire more therefore the times between spikes are less.
4.3h Effects of iontophoresis of alfuzosin on the excitation of SPNs by phenylephrine

The effect of iontophoresis of the $\alpha_1$-adrenoceptor antagonist alfuzosin was studied on 4 SPNs (2 in spinalised rats) which had previously been shown to be excited by iontophoresis of phenylephrine. Alfuzosin was iontophoresed at currents of 30-60 nA for 2-3 minutes before application of phenylephrine. Phenylephrine was then iontophoresed at the same current that had previously caused excitation of the neurones (30 nA). In 2 cases this subsequent iontophoretic application of phenylephrine had no effect on the firing rate of SPNs (figure 4.17). In the other 2 SPNs some antagonism of the effect of phenylephrine was seen. In 1 experiment some recovery of the effect of phenylephrine on the firing rate of SPNs was seen after 30 minutes (figure 4.18). In this experiment iontophoresis of alfuzosin seemed to decrease the ongoing activity of the neurone so the current of glutamate was increased to restore the original firing rate of the neurone. As the effect of alfuzosin wore off the control firing rate remained at the new level despite more glutamate now being ejected.

4.3i Is there a relationship between the characteristics of an SPN and its response to iontophoresis of $\alpha_1$-adrenoceptor agonists?

SPNs recorded from rats with their neuraxis intact were tested where possible for ECG-related and respiratory-related firing patterns. The conduction velocity of those SPNs (in both spinalised rats and those with an intact neuraxis) which were excited by iontophoresis of $\alpha_1$-adrenoceptor agonists was also recorded. This was carried out in an attempt to show whether neurones that were excited by iontophoresis of either methoxamine or phenylephrine had different physiological characteristics to those that were unaffected by the agonists. The distribution of conduction velocities of those neurones that were excited by iontophoresis of methoxamine or phenylephrine is shown in figure 4.19.A. Also displayed is the histogram of conduction velocities of those SPNs that were unaffected by those drugs (figure 4.19.B). In a previous study on the functional characteristics of lumbar SPNs, it was demonstrated that the conduction velocities of those SPNs which converge onto postganglionic neurones supplying skeletal muscle were
FIGURE 4.17.

Histogram of the firing rate of an SPN in a rat with intact neuraxis showing the effect of alfuzosin on the excitatory effects of phenylephrine (bin size 5 seconds).

This neurone was activated by 4 nA glutamate (hatched line). Phenylephrine was iontophoresed at 30 nA for 2 minutes (black line) and caused an initial decrease (during current ejection) then a large increase in the firing rate of the neurone. Once activity had returned to control levels alfuzosin was iontophoresed at 30 nA for 2 minutes (black line). One minute after the alfuzosin iontophoresic current was stopped, phenylephrine was once more iontophoresed with the same parameters as before. The resultant increase in the firing rate of the SPN was very much reduced from the response seen before alfuzosin. Alfuzosin was once more iontophoresed at 30 nA for 2 minutes and after this period phenylephrine was applied with the same parameters as before. This time phenylephrine had very little effect on the firing rate of the neurone, causing only the decrease in firing rate during current injection. The fact that alfuzosin had no effect on the decrease in firing rate suggests that it is a current artifact rather than a genuine effect of the phenylephrine.
FIGURE 4.18.

Histogram of the firing rate of an SPN in a spinalised rat to show the effects of iontophoresis of alfuzosin on the excitatory effects of phenylephrine (bin size 5 seconds).

This neurone was activated by 41 nA glutamate (shown by the hatched line). Phenylephrine was iontophoresed at 30 nA for 2 minutes and caused a decrease in activity corresponding to the current ejection then a large increase in the firing rate of the neurone. After recovery from this effect, alfuzosin was iontophoresed at 30 nA for 2 minutes. This decreased the ongoing activity of the neurone therefore the current of glutamate was increased to 58 nA. Phenylephrine was iontophoresed using the same parameters and caused only a decrease in the firing rate of the SPN with no subsequent increase. Phenylephrine was then applied at regular intervals until a recovery of the excitatory effect was seen.
FIGURE 4.19.

(A) Histogram of the conduction velocities of those neurones which were excited by iontophoresis of either methoxamine or phenylephrine. The distribution is similar to that of all the SPNs tested and the mode class is 0.5-0.6 m/s.

(B) Histogram of the conduction velocities of those SPNs which were not affected by iontophoresis of either methoxamine or phenylephrine. The distribution is similar to that seen in (A), suggesting that there is no relationship between the response of a neurone to $\alpha_1$-adrenoceptor activation and their conduction velocities.
TABLE 4.2

A table of the characteristics of SPNs and their response to iontophoresis of methoxamine and phenylephrine.
<table>
<thead>
<tr>
<th>EFFECT OF $\alpha_1$-ADRENOCEPTOR AGONISTS</th>
<th>INCREASE IN FIRING RATE</th>
<th>NO EFFECT ON FIRING RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>ONGOING DISCHARGE/GLUTAMATE DRIVEN</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>CONDUCTION VELOCITY (MEDIAN &amp; RANGE)</td>
<td>0.55 m/s (0.35-1.3)</td>
<td>0.56 m/s (0.43-0.73)</td>
</tr>
<tr>
<td>ECG-RELATED ACTIVITY MODULATED</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ECG-RELATED ACTIVITY NON-MODULATED</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>ECG-RELATED ACTIVITY NOT TESTED</td>
<td>5</td>
<td>3</td>
</tr>
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<td>0</td>
</tr>
<tr>
<td>RESPIRATORY-RELATED DISCHARGE NOT TESTED</td>
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<td>6</td>
</tr>
</tbody>
</table>
significantly different from those of SPNs converging onto postganglionic neurones supplying hairy skin (Jánig and Szulczezyk, 1980). Statistical analysis showed that the median values of conduction velocities for those neurones excited by $\alpha_1$-adrenoceptor agonists (0.55 m/s) and those not affected (0.56 m/s) were not significantly different ($p>0.7$).

A table of the characteristics of SPNs and their response to iontophoresis of either phenylephrine or methoxamine was compiled in order to show any correlation between the two (table 4.2). Statistics were carried out to determine whether SPNs which were excited by $\alpha_1$-adrenoceptor agonists were significantly more likely to show ECG- or phrenic-related activity than those neurones which were unaffected by the agonists. There was found to be no significant difference between the two groups of neurones ($p>0.1-0.3$).
DISCUSSION

4.4a Effect of $\alpha_1$-adrenoceptor agonists on the firing rate of SPNs

This study has shown that iontophoresis of the selective $\alpha_1$-adrenoceptor agonists methoxamine and phenylephrine in the vicinity of antidromically identified SPNs can cause increases in the firing rates of these neurones. Iontophoresis of these agonists was never associated with a decrease in the firing rate of SPNs. In a number of experiments, an initial decrease in the firing rate of these neurones was observed, an effect which was a current artifact since the same effect was also seen with iontophoresis of saline. This decrease masked the onset of the excitatory effects of the drugs so that it was often difficult to see exactly when the drugs started to have an effect on the SPNs. However, in some cases a long latency to onset of the increase in firing rate was observed with these drugs. This may be a reflection of the distance of the microelectrode from the receptors at which the effects of the drugs are mediated. Neuroanatomical studies suggest that $\alpha_1$-adrenoceptors are located on the dendrites rather than the soma of SPNs (Milner et al. 1988, see general introduction), therefore the agonists may have to diffuse some distance to reach the $\alpha_1$-adrenoceptors.

Iontophoresis of both methoxamine and phenylephrine caused long-lasting increases in the firing rate of SPNs. The excitatory effects of iontophoresed methoxamine lasted significantly longer than those observed with iontophoresed phenylephrine in both spinalised rats and those with their neuraxis intact. This may be due to the fact that methoxamine shows little affinity for the uptake mechanism through the neuronal membrane and is not a substrate for either catecholamine O-methyltransferase or monoamine oxidase, both of which are enzymes involved in the breakdown of catecholamines (Trendelenberg, 1972).

The fact that both drugs caused increases in firing rates of SPNs which were long-lasting in comparison to effects seen with iontophoresis of other drugs (for example 5-HT; Gilbey and Stein, 1991) may be related to the mechanism of action of $\alpha_1$-adrenoceptor agonists. Recent in vitro experiments have suggested
that activation of $\alpha_1$-adrenoceptors caused slow depolarisations of SPNs with a rise time of 2-3 seconds and a decay time of 10-20 seconds (Yoshimura et al. 1987a). This effect seemed to be due to a decrease in a calcium-dependant potassium conductance since different extracellular potassium concentrations resulted in changes in the amplitude of the slow depolarisation, while calcium-free medium abolished the depolarisation (Nishi, 1989). Research suggests that effects of $\alpha_1$-adrenoceptors may be mediated by a second messenger system. Activation of these receptors caused an increase in turnover of phosphotidylinositol which in turn activates protein kinase C via diacylglycerol and increases intracellular calcium (Tolbert, White, Ospry, Cutts and Fain, 1980; Berridge, 1984). Baraban, Snyder and Alger (1985) have shown that applications of phorbol esters (which stimulate protein kinase C) onto hippocampal pyramidal cells can cause blockade of calcium-dependant potassium channels in these neurones. Phorbol esters were also shown to mimic the slow depolarisation of SPNs when applied to cat spinal cord slices (Nishi, 1990). Therefore it seems likely that activation of $\alpha_1$-adrenoceptors causes an increase in protein kinase C which somehow leads to closure of calcium-dependant potassium channels and production of a slow depolarisation. The involvement of a second messenger system in mediating this depolarisation may explain the long duration of effects seen with $\alpha_1$-adrenoceptor agonists.

4.4b Antagonism of the effects of phenylephrine

As it is difficult to obtain stable recordings from SPNs for long periods of time, the long time course of action of methoxamine excluded its use in antagonist studies. The shorter time course of action of phenylephrine allowed a study of the selectivity of the phenylephrine response using an $\alpha_1$-adrenoceptor antagonist on the same SPN.

In 4 SPNs the effect of iontophoresis of phenylephrine was decreased or abolished by prior iontophoretic application of the $\alpha_1$-adrenoceptor antagonist alfuzosin. Alfuzosin had little effect on the glutamate evoked firing of these neurones. This provides further evidence that the effect seen with iontophoresis of phenylephrine
is due to selective activation of $\alpha_1$-adrenoceptors.

4.4c Effect of $\alpha_1$-adrenoceptor agonists on the firing pattern of SPNs
Iontophoresis of either methoxamine or phenylephrine was associated with a change in the firing pattern of four SPNs so that the neurone fired in bursts. This change in firing pattern was also accompanied by an increase in the activity of three of the neurones (two of which were recorded from spinalised rats). These observations are particularly interesting since Yoshimura et al (1987c) reported that application of noradrenaline onto SPNs in the cat spinal cord slice induced rhythmic bursting in 30% of those neurones tested. These bursts of firing were superimposed on oscillations in the membrane potential which were characterised by the appearance at regular intervals of large (10 mV) amplitude depolarising waves of approximately 1 second duration. These oscillations persisted in the presence of tetrodotoxin and disappeared when the membrane was hyperpolarised which suggested that the rhythm was generated endogenously by the SPN rather than imposed on the neurone by synaptic input. The adrenoceptor involved in mediating these effects was not established in these experiments. However, in vitro experiments carried out in the rat hypothalamus have demonstrated that application of noradrenaline, methoxamine or phenylephrine induced burst activity in supraoptic nucleus neurones, an effect which is blocked by prazosin (Randle, Bourque and Renuad, 1984; 1986). This burst activity may be due to modulation of a transient potassium current, the A current (Randle et al. 1986). More work is necessary on the effects of $\alpha_1$-adrenoceptor agonists on the firing pattern of SPNs to discern the mechanism underlying this burst activity, however, it is interesting that burst firing can be observed in the in vivo preparation.

4.4d Other iontophoretic studies in the cat and rat
The iontophoretic experiments described in this thesis have been carried out in the rat. This laboratory has also looked at the effects of iontophoresed methoxamine on the firing rate of SPNs in the cat (Marks, Stein, Dashwood and Gilbey, 1990). Methoxamine was iontophoresed in the vicinity of 14 SPNs in the second and third lumbar segments of the intact cat spinal cord and caused an
increase in activity in eight of these neurones. Interestingly, in this study methoxamine caused a decrease in the firing rate of three of these neurones, an effect which was never seen in the rat. There are a number of possible explanations for this observation. Methoxamine may be acting on inhibitory interneurones or as an antagonist to tonically active descending excitatory pathways; methoxamine has high affinity but low activity at \( \alpha_1 \)-adrenoceptors and therefore may be acting as a competitive antagonist against the endogenous ligand (Ariëns, Simonis and Van Rossum, 1964). These observations in the cat support the autoradiographic results in the previous chapter which demonstrated a high concentration of \([^3\text{H}]\text{prazosin}\) binding sites in the cat intermediolateral cell column.

A recent investigation by Lewis and Coote (1990b) studied the effects of iontophoresed adrenaline and noradrenaline onto single SPNs in a similar preparation to the one used in these experiments. They showed that these endogenous catecholamines caused both increases and decreases in the firing rate of SPNs in the rat. These results are in contrast to those observed in previous studies involving iontophoresis of these agonists onto SPNs in the cat and pigeon (see general introduction). The different results observed in the rat compared with previous experiments may reflect a genuine species difference in the type, number or distribution of \( \alpha \)-adrenoceptor located on SPNs. Alternatively, the discrepancies may be due to the level of ongoing activity in the SPNs studied. Lewis and Coote (1990b) found that SPNs which were excited by catecholamines were more likely to display a high basal firing rate than those neurones which were inhibited. In previous iontophoretic studies the basal firing rates of SPNs tested was low which could explain why applications of adrenergic agonists caused only inhibitions of these neurones in earlier studies. This study also observed that noradrenaline and adrenaline induced burst firing in some of the SPNs under investigation. Once more, it was not established which adrenoceptors were involved in mediating these responses.

The results obtained in this thesis and those investigations of Lewis and Coote
(1990b) demonstrate that iontophoresed $\alpha_1$-adrenoceptor agonists or catecholamines increase the firing rate of SPNs. Previous iontophoretic experiments have shown $\alpha_2$-adrenoceptor mediated decreases in the firing rate of SPNs and in the in vitro preparation, both excitatory and inhibitory effects have been seen with applications of catecholamines (see general introduction). It would therefore be interesting to determine whether single SPNs in vivo can exhibit both $\alpha_1$- and $\alpha_2$-adrenoceptor mediated effects. This would be possible using iontophoresis of the non-selective catecholamines to cause either a decrease or an increase in firing rate of the neurone and then iontophoresing selective $\alpha_1$- or $\alpha_2$-adrenoceptor antagonists to attempt to block the observed response and unmask the opposite effect on the firing rate. This has been carried out in the in vitro preparation (see Yoshimura et al. 1987b) and it was observed that for some SPNs, superfusion of noradrenaline caused a slow ipsp which could be blocked with yohimbine to reveal an underlying slow epsp. These experiments would give an indication of whether $\alpha_1$- and $\alpha_2$-adrenoceptors co-exist on a single SPN.

4.4e Site of action of $\alpha_1$-adrenoceptor agonists

These results indicate that the firing rate of SPNs can be increased by activation of $\alpha_1$-adrenoceptors which are located on or near the neurone. In some experiments, rats were spinalised between the first and second cervical vertebrae, thereby removing the possibility of methoxamine or phenylephrine interacting with tonically active descending supraspinal pathways. Under these circumstances, iontophoresis of both $\alpha_1$-adrenoceptor agonists still caused increases in the firing rate of the majority of SPNs tested. These increases were very similar to those seen in rats with their neuraxis intact. This indicated either a direct effect of the $\alpha_1$-adrenoceptor agonists on postsynaptic receptors on the SPN or on antecedent neurones within the spinal cord, or both. In support of a direct effect on SPNs, Yoshimura et al. (1987a) showed that the $\alpha_1$-adrenoceptor-mediated slow depolarisations of SPNs persisted in the presence of tetrodotoxin. This drug specifically blocks sodium channels (Narahashi, Moore and Scott, 1964) so that axon and terminal membranes are inexcitable, therefore any effect of a drug
which persists under these circumstances is presumed to be due to a direct action at postsynaptic receptors. These results are in contrast to observations by Koss and coworkers (Ito et al. 1988; Koss et al. 1990) that spinalisation blocked the depressant effects of prazosin on the sympathetic cholinergic response to hypothalamic stimulation. This indicates that there are different mechanisms involved in the $\alpha_1$-adrenoceptor-mediated excitations of neurones in the two systems.

These observations indicate that at least a proportion of the excitatory effect of iontophoretically applied methoxamine and phenylephrine is due to activation of $\alpha_1$-adrenoceptors located on the postsynaptic membrane of SPNs. However this does not rule out the possibility of other presynaptic sites of action. Alpha$_1$-adrenoceptors may be located on other excitatory terminals which synapse on SPNs - activation of these putative receptors may facilitate release of an excitatory neurotransmitter to increase the firing rate of an SPN. Alternatively, $\alpha_1$-adrenoceptors may be located presynaptically on inhibitory neurones and when activated may result in a reduction in the release of the inhibitory neurotransmitter so that the neurone is disinhibited and fires more. These sites of action of $\alpha_1$-adrenoceptor agonists are at this stage purely speculative but cannot be ruled out as components of the excitatory effect of methoxamine and phenylephrine.

4.4f Is there a relationship between the characteristics of SPN and its response to iontophoresis of $\alpha_1$-adrenoceptor agonists?

The firing patterns of SPNs were studied in animals with their neuraxis intact. Neurones were analyzed for baroreceptor modulation and respiratory-related activity.

ECG-triggered histograms were constructed to show whether a neurone was under baroreceptor modulation. Of the 40 SPNs which were analyzed in this way, only 10 showed strong or very strong degrees of ECG-related activity (see figure 4.4 for examples). This percentage of baroreceptor-sensitive SPNs was very similar
to that observed in a previous study on SPNs in the T2 segment which innervate the cervical sympathetic chain (30%; Gilbey et al. 1982b). Aortic nerve stimulation depressed the activity of 50% of cervical sympathetic neurones in the rat, which indicated that a higher proportion of these neurones were influenced by baroreceptor activation (Numao and Gilbey, 1987). This may be due to the difference in the techniques used to show whether a neurone was baroreceptor-sensitive.

Respiratory-related firing patterns of SPNs were also studied using phrenic-triggered histograms. SPNs were categorised into three groups according to the pattern of respiratory-related activity. A high proportion of SPNs exhibited respiratory-related activity which reflected that seen in a previous study by Gilbey et al. (1986) who were recording from SPNs in the same segment of the rat. The ratios of SPNs displaying either expiratory or inspiratory-related activity was slightly different in the two studies; in the previous study, 30.5% had inspiratory-related activity and 46% had expiratory-related activity compared with 41.2% (inspiratory-related) and 35.3% (expiratory-related) in this study. This may be due to a difference in the partial pressures of CO₂ within arterial blood, although care was taken to keep these within a narrow range which was almost the same for the two investigations.

SPNs with ongoing activity showed very similar firing patterns to those neurones which were activated by iontophoresis of glutamate. This was consistent with the observations by Gilbey et al. (1986) and suggests that SPNs which do not fire spontaneously receive subthreshold inputs which are similar to those received by SPNs with ongoing activity. These results also confirm those observations of Lipski, Coote and Trzebski (1977) who showed that the antidromic latencies of SPNs varied with respect to central respiratory drive and suggested that these changes were due to changes in soma excitability.

Those SPNs which were analyzed for respiratory and ECG-related activity were used to assess whether certain physiological characteristics of SPNs (i.e. different
firing patterns, conduction velocity and the ongoing level of excitability of the neurone) could be correlated to the response of the neurone to iontophoresis of \(\alpha_1\)-adrenoceptor agonists. Table 4.2 shows the physiological characteristics of those SPNs which were tested for their response to iontophoresis of either methoxamine or phenylephrine. SPNs which were excited by methoxamine or phenylephrine were as likely to exhibit ECG- or respiratory-related activity as those SPNs which were unaffected by these agonists. In addition there was no significant difference in the conduction velocities of the two groups. Therefore, it seems that qualitatively, the effect of \(\alpha_1\)-adrenoceptor agonists on an SPN is not related to its physiological characteristics. The number (31 neurones) of SPNs tested in this way was reasonably small, therefore it may be that if the sample size had been larger, a pattern of responses exclusive to those SPNs which are excited by \(\alpha_1\)-adrenoceptor activation may emerge. In addition, it may be necessary to study a larger number of characteristics for each neurone to see whether there is a characteristic common only to those SPNs which are excited by \(\alpha_1\)-adrenoceptor activation. Gilbey and Stein (1991) investigated the ECG-, and respiratory-related activity of lumbar SPNs and their response to noxious heat applied to the hind limb. They were not able to show a relationship between the ongoing firing patterns of SPNs and their response to iontophoresis of 5-HT. However, those SPNs which were inhibited by noxious heat were more likely to excited by iontophoresis of 5-HT, while SPNs which had their firing rate increased by heat applied to the hind limb were inhibited by 5-HT.

Hence it may be important to study many more properties of SPNs and their various inputs to be able to group SPNs according to certain characteristics and their response patterns to application of different drugs.
CHAPTER 5

EFFECT ON CARDIAC SYMPATHETIC NERVE ACTIVITY
OF PHENYLEPHRINE MICROINJECTED INTO THE IML OF THE CAT
INTRODUCTION
To date, experiments which have examined the role of $\alpha_1$-adrenoceptors of the IML in the control of sympathetic activity have concentrated on the effects of agonists and antagonists on single SPNs both in vitro and in vivo (see general introduction and Chapter 4).

However, although these results provide evidence of an $\alpha_1$-adrenoceptor-mediated excitatory input onto antidromically identified SPNs in the IML of the spinal cord, it is impossible to determine the functional identity of these neurones and their target organs. This is especially important since patterned peripheral autonomic responses accompany many simple animal behaviours (see Kircheim, 1976). These patterned responses result from the selective innervation of subgroups of SPNs (see Cabot, 1990).

5.1a Is the location of SPNs in the spinal cord related to their function?
Most SPNs (not adrenal) synapse in sympathetic ganglia which are remote from their target organs. An SPN therefore cannot be functionally identified on the basis of its axonal projection to its postganglionic target. SPNs innervating any one postganglionic nerve are situated in a number of different segments in the cord (see general introduction), thus it is impossible to define the function of SPNs based on their location in the spinal cord. Recently developed techniques, involving the use of transsynaptic tracers such as pseudorabies virus (Strack and Loewy, 1990) and herpes simplex virus type 1 (Wesselingh et al. 1989), are now being used to study the spinal origins of SPNs innervating selective target organs. Strack and Loewy (1990) injected pseudorabies virus into the ear and the eye, which labelled postganglionic neurones in the superior cervical ganglion. These postganglionic neurones receive input from topographically organised SPNs within the spinal cord. The study demonstrated that SPNs innervating the eye arose predominantly from the first and second thoracic segments, while those which innervate the ear were mainly found in the fourth and fifth thoracic segments. These studies confirmed the observations of Lichtman, Purves and Yip (1979) who stimulated the upper thoracic ventral roots and used the degree of
vasoconstriction of the pinna and the iris as well as pupillary dilatation to
demonstrate the segmental arrangement of SPNs innervating these regions.
However, in both studies it was observed that SPNs innervating either organ could
be located in the same segment. Therefore it is impossible to deduce the target
organ of any one SPN on the basis of the segmental location of that neurone.

5.1b Relationship of the physiological characteristics of SPNs to their function
Recent research has attempted to relate the physiological characteristics of an
SPN to its functional role. Jänig and Szulczyk (1980) recorded from single
preganglionic fibres in the left lumbar sympathetic trunk and studied their
response patterns to various inputs. Since SPNs exhibited similar response
patterns to postganglionic neurones which project to specific targets, they could
be characterised on the basis of these patterns. However, this technique could
not characterise the many SPNs in this part of the cord (73% of their study)
which were quiescent under normal circumstances. Since there is a high
proportion of silent SPNs in other areas of the cord in both rat and cat (see
general introduction), only a small and biased group of SPNs could be
characterised. In addition, this technique of recording does not enable application
of drugs onto the somata or dendrites of the SPN giving rise to the recorded fibre
to look at the inputs onto this neurone. To overcome this difficulty, Gilbey and
Stein (1991) recorded extracellularly from antidromically identified SPNs to study
the physiological characteristics of these neurones. Respiratory-related activity
and baroreceptor modulation (using ECG-triggered histograms) of the neurones,
their conduction velocities, and their responses to noxious stimulation and
iontophoresis of 5-HT were studied. They found that some neurones (including
those that had been quiescent but were made to fire by iontophoresis of
glutamate) had similar response profiles to those described by Jänig and Szulczyk
(1980). In addition, neurones that were inhibited or not affected by noxious
stimulation were excited by iontophoresis of 5-HT, while those that were excited
by noxious heat were inhibited by 5-HT. This indicated that the effect of 5-HT
on the discharge of an SPN was related to the physiological characteristics of that
neurone. However, the classification of neurones by Gilbey and Stein (1991) was
tentative and many neurones had different response profiles which did not fit into any of the categories described by Jänig and Szulczkyk (1980).

5.1c The present study
In the present study, recordings were made from a postganglionic sympathetic nerve which is known to project to specific target organs. It was then possible to study the effect, on SPNs which innervated that nerve, of microinjection of the $\alpha_1$-adrenoceptor agonist phenylephrine into the IML of the spinal cord. If SPNs innervating the postganglionic nerve were excited by activation of $\alpha_1$-adrenoceptors, then an increase in the postganglionic nerve activity may be observed.

5.1d The functional role of the inferior cardiac nerve
The aim of the experiments was to gain some insight into the effect on cardiac nerve activity of activating $\alpha_1$-adrenoceptors in the region of the IML, therefore to achieve this recordings were made from the left inferior cardiac nerve (ICN). The ICN was chosen as it is known to project mainly to the heart (McKibben and Getty, 1968), and to a lesser degree, the oesophagus and pulmonary vessels (Gonella, Neil and Roman, 1979). Electrical stimulation of the left ICN of cats caused a large (43 beats per minute) increase in heart rate (Kamosinska, Nowicki and Szulczyk, 1989). Stimulation of the right ICN caused an even larger increase in heart rate (67 beats per minute) suggesting an asymmetry in the innervation of the heart between right and left sides of the cord. In the dog (Levy, Ng and Zieske (1966) stimulation of the left stellate ganglion caused a small increase in heart rate and a large increase in left ventricular systolic pressure. In addition, Sundaram, Murugaian and Sapru (1989) microinjected glutamate into the left IML and observed increases in contractility but very little change in heart rate.

5.1e Which SPNs innervate postganglionic neurones in the ICN?
HRP studies have shown that postganglionic neurones in the cat stellate ganglion (which send projections into the ICN) are innervated by SPNs with axons in the C8-T9 white rami, with the major input arising from axons in the T1 and T2 rami
(Chung et al. 1979; Oldfield and McLachlan, 1981; Pardini and Wurster, 1984). However, the main input to the postganglionic ICN itself cannot be determined using retrograde tracers since these will not cross the synapse within the ganglion. Studies involving electrical stimulation of each of the white rami and recording from the ICN have demonstrated that the major input to the ICN from the spinal cord arises from the third thoracic white ramus (Szulczyk and Szulczyk, 1987). Indeed, electrical stimulation of the left T3 white ramus also caused the largest increase in heart rate (Kamosinska et al. 1989). Rubin and Purves (1987) reported that neurones sending axons into a single white ramus are confined to a corresponding segment of the spinal cord. Therefore an efficacious drug microinjected into the left IML of the third thoracic segment of the spinal cord should produce the most marked changes in left ICN activity (without a great effect on heart rate).

In particular the present experiments examined the effect on ICN activity of phenylephrine (a selective α₁-adrenoceptor agonist) microinjected into the left IML of the third thoracic segment and investigated whether this effect could be blocked by microinjection of the selective α₁-adrenoceptor antagonist alfuzosin at the same site.
METHODS

5.2a Surgical procedures

(i) Exposure of the inferior cardiac nerve (ICN)

The initial surgery to expose the ICN was carried out on the dissecting table with the animal lying on its right side. A dorsal midline incision was made from below the eighth thoracic segment rostrally to the lower cervical segments. The cutaneous maximus muscle was cauterised along the length of the left side of the vertebral column and the connective tissue between the scapula and the rib cage was removed so that the scapula could be reflected. The muscle overlying the seventh cervical to fifth thoracic vertebrae was scraped away to reveal the whole of the dorsal aspect of the vertebrae. The spinalis dorsi and the longissimus dorsi were carefully removed by cauterisation to reveal the rib heads. Any relatively large arteries within the muscle block were ligated. The second to fourth rib heads were cleaned and the serratus ventralis muscle overlying these ribs was carefully removed. The second to fourth ribs were lifted individually away from the rib cage so that it was possible to scrape the intercostal muscle away from each of the ribs until a cotton thread could be passed behind the rib. Extreme care was taken to ensure that the pleura remained intact. However, if the pleura was perforated, a piece of muscle was glued over the tear to prevent subsequent leakage of paraffin oil into the thoracic cavity. Once each rib was freed from surrounding muscle and connective tissue, the cotton thread was knotted as near to the base of the rib as possible. This thread was used to pull the rib away from the chest cavity and pleura and bone cutting forceps were used to remove the rib and rib head. The cut ventral end of the rib was covered with bone wax to prevent bleeding from the bone.

Removal of the ribs exposed the sympathetic chain (which courses parallel to the vertebral column) and the stellate ganglion. Once these had been fully exposed and identified, the first to third white rami could be identified from their entry points into the stellate ganglion and the sympathetic chain. The first and second white rami and the chain caudal to the third thoracic ramus were cut. The ICN
could be traced from its exit point at the caudal end of the stellate ganglion. The nerve courses parallel to the ribs before travelling caudally alongside the vagus. The nerve was cleared as far caudally as possible, cut peripherally and desheathed by removing the surrounding connective tissue. The animals were then transferred to the recording table where the nerve was subsequently wrapped around bipolar platinum recording electrodes and bathed in a pool of warm paraffin.

(ii) Stabilisation of the cord
After transferral to the recording table, the head of the cat was positioned in stereotaxic head frame. The lumbar cord was stabilised by lateral clamps at the iliac crests. The cord was further stabilised by metal clamps attached to the lateral processes of the seventh cervical and the fifth thoracic vertebrae.

(iii) Exposure of the cord.
This was carried out in a similar way to that described for the rat. The muscle either side of the first to fourth vertebrae was retracted with threads. The second to fourth thoracic vertebrae were removed using bone cutting forceps, taking care to avoid tearing the lateral sinuses or damaging the spinal nerves. The dura mater was cut along its length, reflected and the cord covered in warm paraffin oil. Just prior to lowering the electrode into the cord, the pia mater was carefully removed using a hyperdermic needle.

(iv) Spinalisation (two experiments)
The fifth cervical vertebra was exposed of muscle in the same way as described above. A laminectomy was carried out at this level to expose the fifth cervical segment of the spinal cord. The dura mater was cut open and the spinal cord carefully transected avoiding the arteries lying under the cord. The bleeding was stopped by tight packing of the area with cotton wool balls. Once bleeding had stopped, it was possible to check whether the transection was complete. After transection, Tyrodes (4 ml/kg) was given intravenously at intervals of about one hour to support systemic blood pressure at around 90 mmHg.
FIGURE 5.1.

Line diagram showing the experimental design.

The inferior cardiac nerve was isolated, cut and the central end wrapped around bipolar recording electrodes. The signal was amplified, filtered and displayed as raw nerve activity or integrated over ten second periods and the integrated activity displayed on a chart recorder. The first and second white rami and the thoracic chain caudal to the third thoracic white ramus were cut and a laminectomy was performed to expose the third thoracic segment. A seven-barrelled glass microelectrode was advanced into the spinal cord just lateral to the dorsal root entry zone until electrical stimulation caused a short latency, low threshold response in the ICN (see figure 5.2.). At this point in the spinal cord (later verified histologically as the IML); drugs were microinjected in 100 nl volumes to study the effect of various drugs on cardiac nerve activity (ICN activity).
inferior cardiac nerve

T1 white ramus

T2 white ramus

amp

filter

T3 white ramus

stimulation

sympathetic chain
5.2b Electrical stimulation

The IML of the spinal cord was electrically stimulated through the central barrel of a microelectrode using single square wave pulses (10-40 μA, 1 ms pulse width, at 1Hz; see figure 5.1). A Digitimer Programmer (D 4030) was used to trigger an isolated stimulator (Neurolog DS2) which in turn triggered a stimulus isolator unit (Neurolog NL 800). This provided a constant current source to the central barrel of the microelectrode and the circuit was completed by attaching a crocodile clip from the stimulus isolator to the animal. The current passed was monitored on an oscilloscope (Tektronix) and set at the desired level.

In one experiment, the peripheral end of the cut T3 ventral root was placed on bipolar stimulating electrodes and the rootlet was electrically stimulated to determine the latency of the evoked potential in the ICN.

5.2c Preparation and microinjection of drugs

Drugs used were L-phenylephrine HCl (10-80 mM); L-glutamic acid (40 mM); 5-HT bimaleate (10 mM); (all Sigma); alfuzosin HCl (10 mM; Synthelabo) and saline or artificial cerebrospinal fluid.

All drugs were dissolved in saline or artificial cerebrospinal fluid and pH of the solution adjusted to 7.4 using 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The drugs were filtered and each barrel of the microelectrode was half filled with solution so that the meniscus could be viewed under the microscope. Once the electrode was placed in the holder, plastic tubing was fitted tightly over the end of each barrel and attached either to 50 ml syringe for manual pressure ejection of the drugs or to the outlet of a pneumatic pump module (PPM-2) of the Neurophore system. For the latter, pressure was generated by attaching the input of the module to a nitrogen cylinder. The pressure used to expel drugs (between 10-30 psi) and the time of ejection (1-10 seconds) was regulated using the manual pressure regulator on the module so that the volume of drug injected was controlled precisely. The drugs were injected in 1-3 cycles with 10 seconds
between each cycle.

It was possible to calculate the volumes of drugs injected by measuring the movement of the fluid meniscus through a binocular microscope fitted with a graticule (viewed at x 40 magnification). The cross sectional area of the glass tubing was calculated as 1.07 mm² (since the internal diameter of the tubing was 1.17 mm). Each division of the graticule viewed at x 40 magnification was equal to 0.024 mm so that every time the fluid meniscus moved one division on the graticule it was equivalent to 25 nl of drug being injected.

5.2d Data collection and analysis
Inferior cardiac nerve activity was recorded using a high impedance headstage (Neurolog NL 100). Activity was amplified using an AC preamplifier (gain 1K-10K) and filtered (band width 80Hz-2KHz).

ICN activity was then analysed in three different ways:-
(i) ICN activity was fed into a computer for signal averaging during electrical stimulation of the IML.
(ii) ICN activity was fed into a solid state integrator (Medical Electronics, RFHSM) and integrated over ten second periods to observe and quantify any changes in ICN activity due to microinjection of drugs.
(iii) ICN activity was stored on tape (Racal 7DS) for offline analysis. Also stored on tape were the blood pressure, tracheal pressure and ECG.

(i) Electrical stimulation of the IML
A signal averager programme (Cambridge Electronic Design Ltd) was used to study the effects of electrical stimulation of the cord on ICN activity. The IML was stimulated through the central barrel of the microelectrode. The digitimer programmer was used to trigger both the stimulation of the cord and computer so that the average evoked response of the ICN to stimulation of the IML was determined over 20 sweeps.
(ii) ECG-triggered averages of ICN activity

ECG-triggered averages of ICN activity were constructed by computer using the signal averager package (CED Ltd.). In some cases, ICN activity was rectified and smoothed (Neurolog NL 703) using a 20 ms time constant, otherwise, raw cardiac nerve activity was averaged. The ECG signal was passed through an interface and the R-wave was used to generate a TTL pulse which triggered the sweep of the averager. ICN activity which occurred in the 500 ms period after the trigger was averaged over 150 double cardiac cycles to see if the activity was related to the ECG pattern. Blood pressure was also averaged over this time period.

(iii) Effect of microinjection or intravenous administration of drugs on ICN activity

The effect of microinjection of drugs into the IML on ICN activity was studied by comparing the raw ICN activity and integrated ICN activity before and during drug injection. ICN activity was fed into a solid state integrator (Medical Electronics, RFHSM), integrated over 10 second periods and the output displayed on a pen recorder (TDM PAR 1000). At the end of each experiment the animal was killed with an overdose of sodium pentobarbitone and background noise recorded. This noise level was used at the beginning of analysis to set the integrator level. The effect of intravenous administration on integrated ICN activity of sodium nitroprusside and phenylephrine was also determined at the beginning of the experiment. Blood pressure was shown on the same trace to demonstrate the latency between changes in blood pressure and resulting changes in ICN activity.

Changes in ICN activity seen on microinjection of drugs were evaluated from the integrated traces. They were measured by calculating the difference between the mean control activity before injection of drug and the peak increase in ICN activity for each drug. Changes in ICN activity were considered significant if the increase or decrease in activity was more than 20% different from control activity. Alfuzosin was considered to have antagonised an action of a drug if the increase
in ICN activity due to microinjection of a drug was less than 50% of the response to that drug before alfuzosin. The effect of the drug on raw ICN activity was also shown in each case.

The numbers given for each drug response refer to the number of animals in which the effect was seen. The data obtained was treated in the same way as results obtained from iontophoretic studies, i.e. statistical tests were not carried out on pooled data since between experiments the change in ICN activity due to microinjection of a drug was highly variable (see discussion at end of chapter).

ECG activity was fed into a rate meter (Neurolog NL250) to obtain a measure of heart rate. This was analysed to determine whether microinjection of the drugs had any effect on heart rate. Blood pressure was monitored throughout the experiments and the effects of the drugs on blood pressure was observed. The mean heart rate and blood pressure (with the standard deviation) was calculated before and after microinjection of phenylephrine. The paired T-test was used to determine whether there was a significant difference between the values obtained before and after microinjection.

5.2e Histological verification of the position of the electrode
At the end of the experiment the injection site was lesioned electrically using anodal current (200 μA D.C. current for 30 seconds) and the cord removed and fixed in formal saline. The cord was frozen and 100 μm sections were cut on a freezing microtome (Leitz) and mounted onto acid cleaned microscope slides. The sections were stained in neutral red solution and dehydrated using 50%, 70% and 100% alcohol. The slides were placed in 3 solutions of histoclear for at least 30 minutes in total and were mounted with coverslips. The sections were viewed using an Olympus Vanox-T microscope to verify the position of the electrode within the cord and photographed.
RESULTS

5.3a Positioning the electrode in the IML

The effect on ICN activity of microinjecting phenylephrine into the IML was studied at sites at which electrical stimulation through the central barrel of the microelectrode evoked a short latency (20-30 ms), low threshold (10-40 μA, 1 ms pulse width) response in the ICN (Figure 5.2.A). The low threshold and short latency of response (latency similar to that seen when stimulating the peripheral cut end of the ventral roots: Figure 5.2.B) indicated that the tip of the electrode was positioned in or near a pool of SPNs innervating the ICN. In some experiments (n=12) 100 nl of glutamate was microinjected at this site and always produced an increase in ICN activity (see Figures 5.5 and 5.8).

5.3b Baroreceptor modulation of ICN activity

Two techniques were used to determine whether the ICN was influenced by arterial baroreceptors.

(i) Effect of intravenous administration of nitroprusside and phenylephrine

A bolus injection of sodium nitroprusside was given intravenously (4 μg/kg) to lower blood pressure. The median decrease in blood pressure for these experiments was 23 mmHg (range 10-60 mmHg; n = 8). This decrease in blood pressure led to a large increase in ICN activity presumably by unloading of the baroreceptor reflex (median 59%, range 25-200%) which lasted up to 1 minute beyond the duration of the effect on blood pressure. In five cases, the bolus injection caused an initial increase in blood pressure due to volume loading and a corresponding transient decrease in ICN activity was observed. An example of the effect of injection of sodium nitroprusside on ICN activity is shown in figure 5.3. On two occasions, phenylephrine (5 μg/kg) was given intravenously and caused a 50 mmHg increase in blood pressure in each case. This led to a 60-67% decrease in ICN activity.
FIGURE 5.2.

(A) Response in the ICN evoked by electrical stimulation of the IML (10 \( \mu \)A current, 1 ms pulse width) through the central barrel of a seven-barrelled microelectrode.

(B) Response in the ICN evoked by stimulation of the third thoracic ventral root. The latencies of responses were very similar.
FIGURE 5.3.

Effect of a bolus dose of nitroprusside on blood pressure and ICN activity.

Top trace: ICN activity integrated over 10 second periods

Bottom trace: Blood pressure.
The bar represents 1 minute.

At the arrow a bolus injection of nitroprusside was given intravenously. This caused an decrease in blood pressure of 40 mm Hg and an increase in ICN activity of 200%. This increase had a slightly delayed onset compared to the decrease in blood pressure.
(ii) ECG-triggered averages of ICN activity

ECG-triggered averages of ICN activity were constructed by computer to analyse the influence of arterial baroreceptors on ICN activity since ECG related activity is indicative of baroreceptor modulation (Blumberg et al. 1980). Control ICN activity from each experiment was analysed off-line by triggering the computer to average the raw or the rectified and smoothed ICN activity (using a 20 ms time constant) over 150 double cardiac cycles. Blood pressure was also averaged over this time period to determine the relationship of ICN activity to systolic pressure. ICN activity showed strong ECG related activity in all experiments and an example of this is shown in figure 5.4 A (rectified and smoothed ICN activity) and 5.4 B (raw ICN activity).

5.3c Effect of microinjection of phenylephrine on ICN activity.

In order to establish a working range of concentrations for microinjection of phenylephrine, two experiments were carried out in which 10 mM, 20 mM, 40 mM and 80 mM concentrations of phenylephrine were injected at fifteen minute intervals. These resulted in a dose related increase in ICN activity (see Figure 5.5) and for subsequent experiments 10 mM and 40 mM phenylephrine were used. If no effect was seen with microinjection of 10mM phenylephrine then 40mM was used (in spinalised cats, up to 80 mM phenylephrine was microinjected).

Using these concentrations, 100 nl of phenylephrine microinjected into the IML caused an increase in ICN activity in 15/17 experiments. These increases ranged from 22-259% (median = 75%) and lasted 1.67-23 minutes (median = 3.5 minutes; see Table 5.1). Heart rate and blood pressure were also monitored during microinjection of drugs and throughout the experiments. The mean heart rate and blood pressure before microinjection of phenylephrine were 192.2 beats per minute (standard deviation, 13.6; n=14) and 110.4 mm Hg (standard deviation, 16.7; n=14), respectively. Immediately after microinjection of phenylephrine, during the increase in ICN activity, mean heart rate and blood pressure were 192.36 beats per minute (standard deviation, 15.38; n=14) and

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FIGURE 5.4.

(A) ECG-triggered average of rectified and smoothed ICN activity (20 ms time constant) over 150 double cardiac cycles (500 ms).

The ECG trace and blood pressure are also shown over the same time period to show the relationship between the 3 averages. ICN activity had a strong correlation with ECG activity and blood pressure shown by the two peaks in rectified and integrated activity occurring in the double cardiac cycle. As a comparison, the computer was triggered by noise to show the lack of correlation of ICN activity over the same time period.

(B) ECG-triggered average of raw ICN activity over 150 cardiac cycles (500 ms; bottom trace).

Also shown are ECG and blood pressure.
FIGURE 5.5.

The effect on ICN activity of microinjection of 100 nl of 40 mM glutamate and 10 mM, 20 mM and 40 mM phenylephrine

Top trace: ICN activity integrated over ten second periods (bar represents 2 minutes).

Bottom trace: The expanded trace at the bottom shows more clearly the effects of the microinjected drugs on raw ICN activity (bar represents 2 seconds).

Arrows indicate microinjection of drugs. 40 mM glutamate caused a 250% increase in ICN activity, while 10, 20 and 40 mM phenylephrine caused increases in ICN activity of 62%, 90% and 140%, respectively
112.7 mm Hg (standard deviation, 14.12; n = 14). Using the paired T-test, these values were not significantly different from those obtained before phenylephrine (p >> 0.2 in both cases).

Microinjection of saline or ACSF into the IML had little or no effect on ICN activity; in the cases where an increase in ICN activity was observed it was immediate and ICN activity returned quickly to control levels. This effect was presumably a pressure artifact and was also observed on occasions with microinjection of drugs (see Figure 5.9).

In two experiments, cats were spinalised at the fifth cervical level. In one experiment, neither glutamate or phenylephrine caused an increase in ICN activity. However, in the other experiment, microinjection of 80 mM phenylephrine caused a 78% increase in ICN activity. Microinjection of ACSF was without effect (see figure 5.6).

5.3d Histological verification of the injection sites
At the end of each experiment, the site of injection of the drugs was marked by an electrical lesion (200 μA DC current for 30 seconds) and the cord was removed and sectioned to show the position of the electrode histologically. Figure 5.7.A. is a photograph of a spinal cord section stained with neutral red to show the neuropil and lesion in the IML. Figure 5.7.B. is a line drawing of a cat spinal cord section showing the sites at which phenylephrine was microinjected. Sites at which phenylephrine caused an increase in ICN activity are shown by the closed circles. Injection of phenylephrine into the cord at two sites, one lateral and one medial to the IML (marked by the open circles) had no effect on ICN activity.

5.3e Effect of alfuzosin on phenylephrine response
Experiments were carried out to determine whether the response to phenylephrine could be blocked by microinjection of alfuzosin, a selective α₁-adrenoceptor antagonist (see Cavero et al. 1984a and b). Alfuzosin (100 nl, 10
FIGURE 5.6.

The effect of microinjection of phenylephrine on ICN activity in the spinalised cat.

Top trace: Integrated ICN activity over ten second periods.

Bottom trace: Expanded trace of raw ICN activity.

40 mM glutamate was microinjected and caused a 106% increase in ICN activity. Injection of ACSF caused only a transient rise in activity due to the pressure of the injection. 80 mM phenylephrine when microinjected caused a 78% increase in ICN activity, an effect which lasted 1.17 minutes.
FIGURE 5.7.

(A) Photograph of a section of cat spinal cord to show a site of microinjection of drugs.

The cord was stained with neutral red to show the neuropil. The track of the electrode to the IML can be seen with the lesion positioned in the IML.

(B) A line drawing of the cat spinal cord showing sites at which microinjection of phenylephrine caused an increase in ICN activity.

Each site is marked by a closed circle, sites at which one or more injections were made are shown by the overlapping circles. All these sites are positioned in or around the IML. Injections of phenylephrine made medial or lateral to the IML (marked by the open circles) had no effect on ICN activity.
TABLE 5.1

Summary of the effects of microinjection of various drugs on ICN activity
<table>
<thead>
<tr>
<th>DRUGS INJECTED</th>
<th>EFFECT ON INFERIOR CARDIAC NERVE ACTIVITY</th>
<th>EFFECT ON INFERIOR CARDIAC NERVE ACTIVITY IN THE PRESENCE OF ALFUZOSIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHENYLEPHRINE</td>
<td>22-259% INCREASE (MEDIAN 75%, N = 15) DURATION 1.7-23 MIN (MEDIAN 3.5 MIN)</td>
<td>0-36% INCREASE (MEDIAN 0%, N = 6) DURATION 1.2 MIN (N = 1)</td>
</tr>
<tr>
<td>GLUTAMATE</td>
<td>80-350% INCREASE (MEDIAN 233%, N = 12) DURATION 1.7-5.3 MIN (MEDIAN 2.3 MIN)</td>
<td>78-350% INCREASE (MEDIAN 240%; N = 5) DURATION 0.8-5 MIN (MEDIAN 3.2 MIN)</td>
</tr>
<tr>
<td>5-HT</td>
<td>20-120% INCREASE (MEDIAN 49%, N = 12) DURATION 1.2-10 MIN (MEDIAN 2.8 MIN)</td>
<td>30-107% INCREASE (MEDIAN 80%; N = 3) DURATION 1-15 MIN (MEDIAN 3.2 MIN)</td>
</tr>
</tbody>
</table>
mM) was microinjected into the IML in 6 experiments where phenylephrine had previously caused an increase in ICN activity and subsequent responses to injection of phenylephrine were either reduced to less than 50% of the control response or abolished (see Table 5.1). In three of these experiments, alfuzosin was microinjected while activity was still elevated due to microinjection of phenylephrine and this abolished the increase and blocked the effects of further injections of phenylephrine. Recovery from the alfuzosin block was observed in two experiments (see Fig. 5.8 for an example of this).

5.3f Selectivity of the alfuzosin antagonism
5-hydroxytryptamine (5-HT) and glutamate, both of which have been shown previously to cause excitation of SPNs (see Coote, 1988) were used to test the selectivity of the antagonism of the phenylephrine response by alfuzosin (see table 5.1). The effect of microinjection of 100 nl of 40 mM glutamate and 10 mM 5-HT was examined in 12 and 6 cases, respectively. Glutamate caused a large increase in activity (range 80-350%, median 233%) in all 12 cases, an effect which lasted 1.67-5.33 minutes (median 2.33 minutes). In 5 cases when alfuzosin was microinjected, a subsequent dose of glutamate still caused large increases in ICN activity (range 78-350%, median 240%). In two of these experiments glutamate was microinjected before and after alfuzosin and caused an increase in ICN activity which was not affected by microinjection of alfuzosin. 5-HT when microinjected caused an increase in ICN activity in 5 of 6 preparations (range of increase 20-120%, median 49%). Alfuzosin failed to antagonise the increase in ICN activity elicited by 5-HT (n=3). In one experiment, alfuzosin was shown to antagonise the increase in ICN activity due to microinjection of phenylephrine while having no effect on the excitation seen on microinjection of 5-HT (Fig. 5.9). A subsequent dose of phenylephrine after 5-HT still had little effect on ICN activity suggesting that the antagonist action of alfuzosin had not worn off. Table 5.1 summarizes the effects of the various drugs on ICN activity.
FIGURE 5.8.

Effect of alfuzosin on phenylephrine response.
Top trace: Integrated ICN activity over ten second periods.

Bottom trace: Expanded trace of raw ICN activity.

40 mM glutamate was first microinjected into the IML and caused a large increase in ICN activity. 10mM phenylephrine was microinjected at 6 minute intervals and resulted each time in an increase in ICN activity (range 127-180%, duration 80-170 seconds). At the arrow, 100 nl of 10 mM alfuzosin was microinjected which abolished the increase in ICN activity and returned activity to below base-line levels. A subsequent dose of phenylephrine caused only a 36% increase in ICN activity. Forty minutes after alfuzosin microinjection of phenylephrine still caused a much diminished increase in ICN activity however at 80 minutes after alfuzosin there was a recovery of the phenylephrine effect, microinjection of 100 nl phenylephrine resulted in a 233% increase in ICN activity over base-line activity.
FIGURE 5.9.

Illustration of selective blockade of the phenylephrine response by alfuzosin

Top trace: Integrated ICN activity over ten second intervals

Bottom trace: Raw nerve activity on a different time scale to show the effects of drugs on ICN activity.

10 mM 5-HT and 40 mM phenylephrine were microinjected and both resulted in increases in ICN activity (120% and 259% respectively). Alfuzosin was microinjected while ICN activity was still elevated due to phenylephrine and this returned firing to below base-line levels. A subsequent injection of phenylephrine had no effect on ICN activity, the initial increase in ICN activity is an injection artifact since microinjection of the same volume of saline caused the same short-lasting increase in ICN activity. The effect of microinjection of 5-HT however was little affected by alfuzosin (107% increase in ICN activity). This lack of effect of alfuzosin on the excitatory response to 5-HT was unlikely to be due to the fact that the alfuzosin block had worn off since phenylephrine still had little effect on ICN activity.
5.4a Effect of microinjection of phenylephrine on ICN activity

This study has demonstrated that phenylephrine microinjected into the region of the IML of the third thoracic segment can cause an increase in the activity of SPNs which innervate the ICN. This increase was in the range of 22-259% with a median value of 75%. The range of increase was rather large but was to be expected for the following reasons:

(i) SPNs within the IML form a ladder type structure with clusters of neurones alternating with less populated patches (see general introduction). Retrograde HRP tracing studies on SPNs innervating the stellate ganglion alone in the cat have shown the clusters of SPNs to be further apart at a distance of about 1 mm (Chung et al. 1979). Therefore if the electrode is placed between two clusters of neurones, the effect of the drug microinjected will be smaller than if it was placed in the centre of a pool of SPNs.

(ii) Not all of the preganglionic neurones in the third thoracic segment innervate the stellate ganglion (Szulczyk and Szulczyk, 1987) and of those that do, only a proportion of these neurones will synapse onto postganglionic neurones in the ICN. Therefore the magnitude of the response will depend on whether the SPNs which are activated project to the ICN.

(iii) The levels of ongoing activity of SPNs innervating the ICN before injection of drugs were variable. If the ongoing activity was high then phenylephrine had less of an excitatory effect. This may be due to the fact that tonically active descending supraspinal inputs onto SPNs are responsible for a large part of the ongoing activity of these neurones and some of this may be mediated by $\alpha_1$-adrenoceptors (see general introduction). If a receptor is already occupied by an endogenous neurotransmitter from a tonically active neurone then the applied drug will have less available receptors at which to act and therefore the resultant response of the ICN will be smaller.

Although these results have shown that phenylephrine caused an increase in the overall activity of the ICN, it must be noted that this does not necessarily mean
that all SPNs which innervate neurones projecting through the ICN are excited by the $\alpha_1$-adrenoceptor agonist. SPNs may be excited, inhibited or not affected by $\alpha_1$-adrenoceptor activation with the majority being excited so that an overall increase is observed.

5.4b Effect of microinjection of phenylephrine on heart rate

In all experiments heart rate was analysed to determine whether phenylephrine (or any of the drugs) had an effect on heart rate. In all cases microinjection of phenylephrine was never associated with an increase in heart rate. Indeed, glutamate when microinjected also had no effect on heart rate even though increases in ICN activity of up to 350% were observed. Although previous observations have shown that stimulation of the ICN increases heart rate (Kamosinska et al. 1989), our observations were to be expected for two reasons:

(i) The ICN was cut peripherally, therefore any effect on heart rate of activating SPNs which innervate neurones projecting through the ICN was lost.

(ii) Other cardiac nerves were still intact and activation of SPNs which innervate neurones projecting through these nerves would be expected to increase heart rate. However, it must be noted that all microinjections were made into the left side of the IML. Previous studies have shown that the greatest effects on heart rate were observed with stimulation of the right pre- or post-ganglionic nerves innervating the heart or the right stellate ganglion (Levy et al. 1966; Kamosinska et al. 1989) or with microinjections of drugs into the right IML (Sundaram et al. 1989a; Sundaram, Murugaian, Krieger and Sapru, 1989b). Microinjections of drugs into the left IML of the rat had very little effect on heart rate (Sundaram et al. 1989a and b). These observations and the fact that the ICN was cut indicated that it was unlikely that an increase in heart rate would be observed.

5.4c Antagonism of the effect of phenylephrine with alfuzosin

The excitatory effect of phenylephrine could be antagonised by microinjection of the $\alpha_1$-adrenoceptor antagonist alfuzosin. This block was observed to be fully reversible - recovery of the effect of phenylephrine was observed about eighty
minutes after alfuzosin (see figure 5.8). The selectivity of this block was demonstrated using microinjections of glutamate and 5-HT, both of which have been shown to excite the majority of SPNs (Coote et al. 1981, Kadzielawa, 1983, Backman and Henry, 1983; McCall, 1983, Lewis and Coote, 1990a; Gilbey and Stein, 1991). Glutamate and 5-HT caused median increases in ICN activity of 233% and 49% respectively. Microinjection of alfuzosin did not antagonise the excitatory effect of microinjection of either of these drugs. The fact that alfuzosin failed to antagonise the excitation due to 5-HT is of importance since both 5-HT and α1-adrenoceptor agonists are thought to cause neuronal depolarisation through a decrease in resting K conductance (Yoshimura et al. 1986; Pape and McCormick, 1989). Therefore alfuzosin seems to be blocking the receptor itself rather than having an effect on the channels involved in mediating the response.

5.4d Other investigations

These results are in agreement with a recent study by Sundaram, Muragaian and Sapru (1991) who examined the effect of microinjection of noradrenaline and phenylephrine into the right IML on blood pressure and heart rate. They showed that microinjections of phenylephrine (0.1 pmol in 20 nl) or low concentrations (1-2 pmol in 20 nl) of noradrenaline caused increases in heart rate with little effect on blood pressure. If the α1-adrenoceptor antagonist prazosin was microinjected before noradrenaline there was no increase in heart rate suggesting that the effect of noradrenaline was being antagonised by prazosin.

There is, however, a large discrepancy in the concentrations of phenylephrine (0.5 μM in Sundaram et al. 1991 compared with 10-40 mM in the present study) used in the two studies to produce excitation of SPNs in the IML even though the concentrations of glutamate microinjected were similar (88.5 mM in Sundaram et al.'s experiments compared with 40 mM). This was surprising especially since we observed dose dependant increases in our response to phenylephrine and were able to block the increases in ICN activity due to phenylephrine with a selective antagonist. This suggested that the concentration of phenylephrine used in our study was not causing a nonselective increase in the excitability of the SPNs.
Equally the responses seen with concentrations used by Sundaram et al. (1991) were significant and could be blocked by prazosin. The differences might just represent a genuine species difference in the number, density or distribution of $\alpha_1$-adrenoceptors in the IML. Studies in the rat suggest that excitatory adrenergic inputs onto SPN (which may also represent the location of $\alpha_1$-adrenoceptors on SPN) are located on the dendrites rather than the soma of the neurone (Milner et al. 1988, see general introduction). The precise location of $\alpha_1$-adrenoceptors in the cat is not yet known although their presence has been demonstrated in the IML (see chapter 2). If they are located on the dendrites then a high concentration of phenylephrine may be necessary for sufficient amounts of drug to diffuse out to the dendrites. Another possibility for the discrepancy in the two results might be the different anaesthetics used in the two experiments.

5.4e Sites of action of phenylephrine

This preparation involved cutting the first and second thoracic white rami and the sympathetic chain caudal to the third thoracic white ramus. This removed the possibility of the effects of phenylephrine being due to activation of propriospinal pathways in the intermediate grey of the cord which project to segments rostral or caudal to T3 and innervate other neurones in the spinal cord (Matsushita, Ikeda and Hosoya, 1969). The increases in ICN activity were therefore due to increases in the firing rates of SPNs located in the third thoracic segment. Cutting the rami and the sympathetic chain also meant that the only input onto the ICN was through the T3 white rami. Therefore the level of activity in the ICN was governed by the level of activity of SPNs in the T3 segment alone. Any changes in ICN activity due to microinjection of drugs into the T3 segment were consequently much more prominent than if the activity of the ICN had been under control from SPNs in a number of different segments.

It was possible that the increases in ICN activity produced by phenylephrine were due to an action on antecedent neurones within the segment rather than a direct effect on the SPNs. Equally the effect of phenylephrine may be due to a presynaptic action on the terminals of an antecedent neurone, reducing the
amount of neurotransmitter released from a tonically active descending inhibitory input onto SPNs (a mechanism suggested by Koss and co-workers (Ito et al. 1988; Koss et al. 1990) for the effect of prazosin on a sympathetic-cholinergic response, see iontophoresis introduction). In two experiments, cats were spinalised at the fifth cervical level of the cord. Microinjection of phenylephrine into the IML of spinalised cats still caused an increase in ICN activity. It seems likely therefore that the excitation of SPNs produced by microinjection of phenylephrine was due to another mechanism other than that of disinhibition of the SPN. Was phenylephrine therefore acting directly on the SPN or on receptors on an interneurone at the spinal site to cause the excitation of SPNs? The response in the ICN seen with electrical stimulation of the IML was of a similar latency to the response evoked by stimulation of the ventral roots indicating direct stimulation of the SPNs (if a synapse was involved the response would have been longer and much more variable), however, injection of the drugs may spread to other areas. The fact that microinjection of phenylephrine into the cord at sites more lateral and medial to the IML had no effect on ICN activity suggests a limited spread of drug from the injection site. Furthermore, in support of a direct effect of \( \alpha_1 \)-adrenoceptor agonists on SPNs, application of noradrenaline onto SPNs in the tetrodotoxin treated cat spinal cord slice still caused depolarisation of these neurones, an effect which could be blocked by the \( \alpha_1 \)-adrenoceptor antagonist prazosin (Yoshimura et al. 1987a). Once more it must be noted that postsynaptic \( \alpha_1 \)-adrenoceptors may not be the only site of action of phenylephrine, other receptors may be located presynaptically and when activated may indirectly increase the firing rates of SPNs (see section 4.4e).

5.4f Advantages of the microinjection technique

These results have shown that microinjection of \( \alpha_1 \)-adrenoceptor agonists into the spinal cord allows discrete activation of receptors in the IML without spread of drug to other areas of the cord. The IML is identified using electrical stimulation to allow precise positioning of the electrode in the cord. This also helps to minimise the possibility that the drug is acting at sites other than the IML to cause excitation of SPNs. This technique has distinct advantages over other
techniques such as intravenous or even intrathecal injections where, although the receptor is involved in mediating a particular response is identified, the actual location(s) of these receptors is unknown. In addition, the response seen may be an overall result of activation of receptors at different sites. Intrathecal injection localises the drug to sites within the spinal cord, although there is a slight possibility of diffusion of the drug to brainstem regions. These injections involve implanting catheters in the spinal subarachnoid space so that drugs released from the catheters come into contact first with the dorsal horn. This makes it a useful technique for studying the effects of drugs on sensory systems in the cord since afferent inputs terminate in the laminae of the dorsal horn (see Brown, 1981). Indeed this technique is widely used in the study of modulation of nociceptive input (Yaksh and Rudy, 1977; Sullivan, Dashwood and Dickenson, 1987).

However, when studying the effects of intrathecally applied drugs on sympathetic outflow (see Shi et al. 1988), interpretation of the results is hampered by the fact that the drugs have to diffuse 500-2000 μm through the dorsal horn to reach the IML. On the other hand, iontophoretic studies only allow activation of a small group of receptors in any one area which is not enough to cause a response in a whole postganglionic nerve. With microinjection, the advantages of activating a sufficient number of receptors so that an effect can be seen in a specific nerve are combined with the ability to limit the availability of the drug to the area of interest.

In conclusion, this study has shown that microinjection of the α1-adrenoceptor agonist phenylephrine into the IML of the spinal cord increases the activity of SPNs innervating postganglionic neurones with their axons in the ICN. Since the ICN innervates the heart, these results indicate that SPNs involved in control of the heart are regulated in part by activation of α1-adrenoceptors in the IML.
CHAPTER 6
GENERAL DISCUSSION
These studies have demonstrated both the presence of $\alpha_1$-adrenoceptor ligand binding sites in the IML of the cat spinal cord and the fact that selective $\alpha_1$-adrenoceptor agonists applied into the IML increase the firing rate of SPNs in the rat and cat. In addition it has been observed that activation of these receptors can result in an alteration of the firing pattern of some SPNs so that the neurone exhibits burst firing.

6.1 The \textit{in vivo} preparation - the advantages of the technique and the necessity for further studies

Importantly, this is the first time that $\alpha_1$-adrenoceptor mediated excitations of SPNs have been observed \textit{in vivo}; they had only previously been observed in the spinal cord slice preparation (see general introduction). A great deal of pharmacological information can be obtained about the $\alpha_1$-adrenoceptor-mediated input onto SPNs in the \textit{in vitro} transverse slice preparation, since the receptors involved and the changes in ion conductances associated with receptor activation may be determined (see Nishi 1990). In addition, the effects of catecholamines on the membrane characteristics of SPNs can be studied. However, there are many advantages in using the intact animal when studying this catecholaminergic input onto SPNs, some of which are discussed in detail below.

6.1a Maintenance of synaptic inputs onto SPNs.

In the general introduction it was noted that in the spinal cord slice preparation, the activities of SPNs are removed from the influence of supraspinal and intersegmental synaptic inputs onto the neurones, thus providing a very artificial situation for studying the effects of agonists on the activity of SPNs. In the intact animal, where other synaptic inputs are maintained, it is possible that an agonist which causes an effect on the firing rate or pattern of an SPN \textit{in vitro} has little or no effect in the intact animal (see general introduction). Results presented in this thesis infer that activation of $\alpha_1$-adrenoceptors increases the firing rate of SPNs, indicating that these receptors may be involved in controlling the activity of SPNs \textit{in vivo} (although it must be noted that anaesthetic was present in these
experiments which may affect normal synaptic input onto these neurones). The concept of α₁-adrenoceptors activating ion channels in SPNs which are also activated by other neurotransmitters (convergence of inputs; see Pape and McCormick, 1989) cannot be entirely ruled out in this system. Previous investigations have shown that 5-HT causes increases in the firing rate of SPNs (see section 5.4c) and it is conceivable that the excitatory effects of this agonist are due to enhancement of the activity of the same K⁺ channels that are involved in mediating the effects of α₁-adrenoceptor activation (see Pape and McCormick, 1989). It may interesting to observe whether a neurone which is excited by iontophoresed methoxamine or phenylephrine may fire more in the presence of selective 5-HT or other antagonists due to the availability of more ion channels.

6.1b Functional characterisation of neurones which are excited by α₁-adrenoceptor agonists

In the in vivo preparation, the physiological characteristics of SPNs which are excited by α₁-adrenoceptor agonists can be determined. This information may help to categorise neurones according to their different characteristics which may give some indication about the functions of the groups of neurones (see chapter 4). In the iontophoretic experiments, it was possible to determine whether single SPNs which were excited by activation of α₁-adrenoceptors had different physiological characteristics from those neurones which were unaffected by α₁-adrenoceptor agonists. SPNs were analyzed to determine whether they had respiratory-related or ECG-related firing patterns and the conduction velocity of the neurones was calculated. The physiological characteristics of SPNs which had their activity increased by α₁-adrenoceptor activation were not significantly different from those which were unaffected by α₁-adrenoceptor activation. This indicated that α₁-adrenoceptors mediate excitatory input onto a wide variety of SPNs regardless of their functional characteristics and the end organ innervated. However it may be necessary to study a wider range of physiological characteristics of the neurones (such as the effect of different sensory stimuli on the firing rate of a neurone) before a distinct pattern of characteristics unique to those neurones which are excited by α₁-adrenoceptor activation emerges (see
The results of the microinjection studies presented in this thesis showed that SPNs which innervate postganglionic neurones with axons in the ICN were excited by the $\alpha_1$-adrenoceptor agonist phenylephrine. This indicates that SPNs which control the heart have their firing rate increased by $\alpha_1$-adrenoceptor activation. From the results presented in this thesis, it seems that activation of $\alpha_1$-adrenoceptors has a primarily excitatory effect on SPNs, regardless of the function of those neurones. However, the effects of other agonists on the activity of SPNs may differ according to the end organ that is innervated by those neurones. Knowledge of the physiological characteristics of these neurones may lead to some functional categorization of the neurones which have their firing rate affected by these ligands.

6.1c Future experiments

There are now techniques which can be developed and used to determine the precise location of $\alpha_1$-adrenoceptors on SPNs in the spinal cord. Recently, immunocytochemical techniques have been used to localize a number of different receptors. This involves raising specific antibodies to receptors which are then used to detect these receptors at both the light and electron microscopic level. One of the first receptors to be studied in this way was the glycine receptor which was localised using antibodies against three different subunits of the receptor complex (Triller, Cluzeaud, Pfeiffer, Betz and Korn, 1985; Triller, Cluzeaud, Pfeiffer and Korn, 1986). They showed similar distributions of all three antibodies in the brain stem and spinal cord at the light and electron microscopic level. This technique also allows immunohistochemical labelling of both receptors and the neurotransmitters in the presynaptic terminals to elucidate which transmitters are released onto the receptor under study (Triller, Cluzeaud and Korn, 1987; Oberdorfer, Parakkal, Altschuler and Wenthold, 1987). A recent study in the rat and guinea pig looked at the distribution of glycine receptor immunoreactivity and GABA immunoreactivity in the IML of the spinal cord (Chiba and Semba, 1991). They observed a subpopulation of GABA immunoreactive axons which made synaptic contact with glycine receptor immunoreactive soma or dendrites, indicating the co-existence of GABA and
glycine in the IML. It must be noted that this study did not identify the glycine receptor immunoreactive neurones as SPNs. This technique is likely to become important in localising receptors in the CNS, however, before studies can be carried out in this way it is necessary to raise antibodies to each receptor under investigation. This problem is being approached using a number of different techniques, but production of antibodies which are suitable for immunocytochemical localisation of receptors is a lengthy process. Other studies have looked at the distribution of GABA/benzodiazepine receptors (Somogyi, Takagi, Richards and Mohler, 1989), β-adrenoceptors (Aoki, Joh and Pickel, 1987), nicotinic cholinergic receptors (Swanson, Simmons, Whiting and Lindstrom, 1987) and 5-HT₁A receptors (Sotelo, Cholley, El Mestikawy, Gozlan and Hamon, 1991). The α₁-adrenoceptor has been purified (Lomasney, Leeb-Landberg, Cotecchin, Regan, deBernardis, Caron and Lefkowitz, 1986) and antibodies have been raised to the receptor (Shreeve, Fraser and Venter, 1985) however, to date there have been no studies on the localisation of α₁-adrenoceptors in the CNS using immunocytochemistry.

There are some drawbacks associated with immunocytochemical localisation of receptors. One of the biggest problems is that an antibody to a receptor detects the protein antigen regardless of whether or not that antigen can bind a ligand. Immunocytochemical localisation of glycine receptors has revealed labelling of intracellular organelles in neurones of the medial nucleus of the trapezoid body (Triller et al 1985). These were only seen with antibodies to the larger subunits of the receptor complex and may be due to the fact that a significant proportion of the polypeptide making up the subunit is associated with the organelle but is not directly related to the receptor itself. False labelling of structures with immunocytochemistry can be identified as such if the sites are always intracellular but may cause misinterpretation of results if similar polypeptide sequences are found within the membrane. These problems can be overcome to some extent by using immunocytochemical localisation of receptors in conjunction with autoradiographic studies on the same receptor so that any labelling of regions with the immunocytochemical technique which are not also detected
auroradiographically should be interpreted with caution. Another limitation of this technique is that it is very difficult to quantify immunocytochemical labelling in tissue. Nevertheless, the information which could be obtained from studies such as these about the exact location of receptors would be of great value in future neurophysiological studies.

6.2 The role of the $\alpha_1$-adrenoceptor-mediated excitation of SPNs

The results presented in this thesis have demonstrated that activation of $\alpha_1$-adrenoceptor activation caused prolonged increases in the firing rate of SPNs. These observations are in agreement with in vitro experiments which observed that noradrenaline caused an $\alpha_1$-adrenoceptor mediated slow depolarisation which lasted several seconds (Yoshimura et al. 1987a). The possible second messengers involved in mediating this slow depolarisation have been discussed in chapter 4.

The exact role of this $\alpha_1$-adrenoceptor-activated slow depolarisation of SPNs, however, remains to be elucidated. It may be that activation of $\alpha_1$-adrenoceptors can itself bring the potential of the neurone to the threshold for firing. However, this input may be much more important in causing a prolonged, but subthreshold increase in the level of excitability of the neurone. This increased excitability means that other inputs onto the SPN may cause greater or more prolonged increases in the firing rate of the neurone.

It is known that catecholamines coexist with other neurotransmitters within spinally projecting neurones in the brainstem (see general introduction). In a recent immunohistochemical investigation, the glutamate synthesising enzyme phosphate activated glutaminase (PAG) has been observed to be extensively co-localised with PNMT in bulbospinal neurones of the RVLM (Minson, Pilowsky, Llewellyn-Smith, Kaneko, Kapoor and Chalmers, 1991). Furthermore, focal stimulation of the cat spinal cord slice elicited a fast epsp which was enhanced by a glutamate uptake inhibitor and depressed by the non-selective excitatory amino acid antagonist cis-2,3-piperidine decarboxylic acid (Inokuchi, Yoshimura, Yamada, Polosa and Nishi, 1992). It may be that glutamate or other neurotransmitters mediate the fast descending synaptic inputs onto SPNs and activation of the $\alpha_1$-adrenoceptors enhances the effect of these neurotransmitters. This mechanism may be involved
in the changes in the levels of excitability of the sympathetic nervous system seen in different behavioral states of the animal such as stress. The importance of the induction of burst firing in some SPNs due to \( \alpha_1 \)-adrenoceptor activation is discussed in the next section.

So far, the effect of \( \alpha_1 \)-adrenoceptors on the electrical activity of SPNs has been discussed. However, the possibility of a more long term effect on the SPN mediated by these receptors cannot be ruled out. This may involve a change in receptor density; ion channel density or state of activity; second messenger states or even may involve formation of new synapses so that the level of excitability of a neurone is increased more permanently. Recently it has been shown that glutamate agonists may participate in synaptogenesis during postnatal development through an action at metabotropic receptors (see Baskys, 1992). Such a role for \( \alpha_1 \)-adrenoceptors in the IML is purely speculative but may be of interest for future investigation. It must be noted, however that in the iontophoretic experiments, one neurone was studied where the glutamate current was turned down so that the neurone stopped firing. Methoxamine when applied increased the firing rate of this neurone, however when this effect of methoxamine had worn off, the glutamate current was turned back up to the original level and it was observed that this current had the same effect on the firing rate of the neurone as that seen prior to methoxamine. This is not supportive of a more long term effect of \( \alpha_1 \)-adrenoceptor activation however, it may be necessary to apply the \( \alpha_1 \)-adrenoceptor agonists for longer in order to observe any more long term effects.

6.3 Source of catecholaminergic input onto SPNs

Having established that activation of \( \alpha_1 \)-adrenoceptors increases the firing rate of SPNs the supraspinal source(s) of this input must be established. There are five main catecholaminergic cell groups in the midbrain and brainstem which project to the IML. These are the adrenergic C1; C2 and C3 cell groups of the medulla; the noradrenergic A5 region in the pons and the A11 group in the hypothalamus which is thought to be mainly dopaminergic. Dopaminergic neurones are unlikely
to cause an effect on a neurone via $\alpha_1$-adrenoceptors therefore the four former regions are likely candidates for causing $\alpha_1$-adrenoceptor mediated excitation of SPNs.

Recently the C2 cell group of the NTS-dorsal vagal motor nucleus complex and C3 cell group situated in the medial longitudinal fasciculus of the dorsal medulla have been demonstrated to project to the IML and may therefore be involved in the control of sympathetic activity (see general introduction). These specific neurones have not been studied to any great degree, however electrical or chemical stimulation of the dorsomedial medulla (which contains C3 neurones) caused large increases in blood pressure in rats, rabbits and cats (Goodchild and Dampney, 1985; Chai, Lin, Lin, Pan, Lee and Kuo, 1988; Lin, Wang, Kuo and Chai, 1989). Furthermore neurones in the cat dorsal medulla showed patterns of activity which were related to both inferior cardiac nerve activity and the cardiac cycle (Barman and Gebber, 1981; 1983). It must be noted however that the areas under study in these investigations included the C2 and C3 regions but were by no means restricted to these cell groups therefore the effects seen may be due to activation of noncatecholaminergic neurones. Once more it must be noted that there is evidence of co-localisation of neurotransmitters in neurones in these regions (e.g. NPY is localised in 75% of TH-containing neurones in the C2 region of the rabbit; Blessing, Howe, Joh, Oliver and Willoughby, 1986) and the effects observed in these studies may be due to release of more than one neurotransmitter.

One of the major noradrenergic projections to the IML is the A5 region of the pons. In the general introduction literature was reviewed which suggested that the A5 region may be involved in both sympathoexcitation and sympathoinhibition. There is evidence that approximately 90% of spinally projecting neurones in this region are catecholaminergic (Byrum, Stornetta and Guyenet, 1984). However, neurones in the A5 region express more than one neurotransmitter and catecholamines have been shown to coexist with enkephalin (but not NPY; Murukami et al. 1989); phosphate activated glutamate (PAG,
which may be labelling either glutamate or GABA since it is an enzyme involved in the synthesis of both neurotransmitters; Kaneko et al. 1990). The effects seen on stimulation of the A5 region may be due to release of more than one neurotransmitter onto the postsynaptic neurone or even onto antecedent neurones.

For many years it was assumed that the supraspinal catecholaminergic excitatory input onto these neurones originated from the C1 group of the RVLM. C1 neurones project to the spinal cord and adrenergic terminals are located on the postsynaptic SPN (see general introduction). Stimulation of the RVLM causes sympathoexcitation and increases in blood pressure and heart rate. However are these effects due to activation of the C1 group in the RVLM or other bulbospinal neurones located in the RVLM?

Pharmacological experiments have examined whether adrenoceptors are involved in mediating the pressor response to stimulation of the RVLM. Intrathecal injections of the α-adrenoceptor antagonist phentolamine or the β-adrenoceptor antagonists pindolol or propanolol had no effect on the pressor response to electrical stimulation of the RVLM (Conner and Drew, 1987; Mills, Minson and Chalmers, 1988). Furthermore, pretreatment with the PNMT inhibitor LY 134046 (which can decrease brain stem PNMT activity by 80% and hypothalamic adrenaline levels by 40%; Fuller, Hemrick-Luecke, Toomey, Horng, Ruffolo and Molloy, 1981) did not modify the tachycardia or vasodepressor response to stimulation of the RVLM (Conner and Drew, 1987), although this may be due to the fact that the levels of adrenaline are still sufficiently high to mediate the effects of RVLM stimulation. These experiments suggest that α-adrenoceptors are not important in mediating excitatory inputs from the RVLM, however the studies are by no means conclusive and further investigations are obviously necessary, especially in the light of the idea that the α1-adrenoceptor mediated input may have a neuromodulatory effect on the SPN with other neurotransmitters mediating the fast responses (section 6.3.). The intrathecal studies on the role of the C1 group in mediating the sympathoexcitatory responses
to stimulation of the RVLM used high frequency (100 Hz) stimulation for periods of 10 seconds. The fact that these studies failed to antagonise the vasopressor response to RVLM stimulation by intrathecal injections of adrenoceptor antagonists may be due to the fact that the system was being stimulated at such a suprathreshold level that blocking the slow depolarisation due to $\alpha_1$-adrenoceptor activation did not bring the membrane potential down to below threshold level to decrease the excitation observed. In addition, it may be that such supramaximal stimulation of the RVLM activated other descending excitatory supraspinal pathways that converge onto the same ion channels as those activated by adrenaline. In this case, blockage of the $\alpha_1$-adrenoceptors will remove the adrenergic excitation, which will free more channels for the other excitatory input to activate. Thus, any decrease in activity due to blockade of the $\alpha_1$-adrenoceptor mediated excitatory input onto SPNs may be masked by a further increase in the effect of the other excitatory input onto the SPN mediated by the same ion channels.

Researchers have also investigated whether spinally projecting pathways other than those adrenergic neurones may be involved in mediating the sympatoexcitatory effects of RVLM stimulation. Recent experiments have indicated a role for excitatory amino acids in mediating these effects. Intrathecal injections of excitatory amino acid antagonists and specific NMDA receptor antagonists or microinjections of these drugs into the IML blocked the vasopressor effects of electrical or chemical stimulation of the RVLM with little effect on the tachycardia (Mills, Minson, Pilowsky and Chalmers, 1988; Bazil and Gordon, 1991; Sundaram and Sapru, 1991). Similar results were also seen with iontophoretic applications of non-selective antagonists onto single SPN in the rat, although NMDA antagonists had no effect on the increase in firing rate due to stimulation of the RVLM (Morrison, Ernsberger, Milner, Callaway, Gong and Reis, 1989). It must be noted however, that application of these antagonists drastically reduced blood pressure indicating that those SPNs which are involved in the control of blood pressure had much lower levels of activity. It cannot be concluded whether the effects of the antagonists were due to selective blockade
of receptors which normally mediate the response to stimulation of the RVLM or whether there was a general decrease in the excitability of the neurones so that otherwise excitatory inputs onto these cells did not bring the membrane potential up to threshold for firing.

Experiments to date therefore have not been suitable for discerning which receptors are involved in mediating the sympathoexcitatory effects of RVLM stimulation. It may be that excitatory amino acids, adrenaline or other transmitters are released from terminals in the spinal cord upon stimulation of the RVLM. Autoradiographic binding techniques have shown the presence of both $\alpha_1$-adrenoceptor and kainate binding sites in the IML of the spinal cord (Morrison et al. 1988 and see chapter 3). Moreover, adrenaline-containing terminals have been shown to synapse directly onto identified SPNs in the spinal cord (Milner et al. 1988), while glutamate-containing terminals synapse directly onto unidentified neurones in the IML of the rat spinal cord (Morrison, Callaway, Milner and Reis, 1989). These inputs do not necessarily originate in the RVLM, although recent studies have shown that RVLM neurones synapse directly onto SPNs in the spinal cord (Zagon and Smith, 1990).

Focal stimulation of the cat spinal cord slice (which activates descending fibres in the dorsolateral funiculus) causes a complex response in SPNs which includes both fast and slow epsp (Nishi 1989). The fast epsp is mediated through excitatory amino acid receptors, thought to be primarily non-NMDA receptors however, NMDA receptors may also be involved (Inokuchi et al. 1992). The slow epsp is blocked by application of prazosin suggesting that the receptor involved is an $\alpha_1$-adrenoceptor. Therefore it may be that the C1 neurones are involved in a modulatory role while the excitatory amino acid-containing pathway may mediate the fast response to RVLM stimulation, a suggestion discussed in the section 6.3. Recently it has been suggested that there may be differential release of neurotransmitters which mediate fast synaptic events and neuromodulators from nerve terminals. Research has shown that release of neurotransmitters from large dense core vesicles only occurs if the cytoplasmic Ca$^{2+}$ levels reach a certain
level (Verhage, McMahon, Ghijsen, Boomsma, Scholten, Wiegant and Nicholls, 1991). This is only achieved if repetitive action potentials occur to induce a significant rise in the bulk cytoplasmic Ca\textsuperscript{2+} concentration. This is in contrast to release of neurotransmitter from small vesicles which are found in clusters around the active zones of the presynaptic terminals, near Ca\textsuperscript{2+} channels. It is proposed that in this case it is the increase in local Ca\textsuperscript{2+} concentration around the active zones which triggers release of neurotransmitter from these vesicles. Although these Ca\textsuperscript{2+} levels need to be higher than the concentration needed to cause exocytosis from large vesicles, the fact that they need only be transient increases around the active zone means that a single action potential is enough to cause release from small vesicles. Therefore neurotransmitters involved in mediating fast postsynaptic potentials will be contained in small vesicles while those transmitters with more of a neuromodulatory role will be located in large dense core vesicles. This view is supported by studies on the release of neurotransmitters from the postganglionic splanchnic nerves in pig (Lundberg, Rudehill, Sollevi, Fried and Wallin, 1989). In these terminals the fast neurotransmitter is noradrenaline while NPY has a neuromodulatory role. NPY is stored only in large dense-cored vesicles while noradrenaline is stored in both large and small vesicles. At low frequency stimulation noradrenaline was preferentially released but as the frequency was increased so more NPY was released. The pattern of stimulation was also important since regular and irregular bursts of stimulation caused a greater release of NPY than that seen when the frequency alone was increased. An electron microscopic study on the ultrastructure of PNMT-containing terminals in the IML has demonstrated the presence of both large dense-cored and small vesicles (Milner et al. 1988). Moreover, the enzyme PAG has been observed to colocalised with PNMT in spinally projecting neurones in the RVLM (Minson et al. 1991). Therefore the Cl group in the RVLM may release adrenaline from dense-cored vesicles only upon higher frequency stimulation. This fits in with the idea discussed in section 6.3 that adrenaline increases the excitability of the SPN under certain conditions such as stress so that other neurotransmitters such as glutamate have a greater excitatory effect on SPNs.
Therefore, in order to study the role of Cl and glutamate-containing neurones in the RVLM, it may be necessary to investigate the effect of stimulating the RVLM at different frequencies and patterns of stimulation on the release of different neurotransmitters. This would involve intracellular recording from SPNs which would still detect any changes in the cell membrane potential due to stimulation of the RVLM even if the membrane did not reach threshold for firing. Thus the problems previously encountered with excitatory amino acid antagonists decreasing the level of activity of the SPNs could be avoided. It may be possible to use the brainstem spinal cord preparation (see Smith, Liu and Feldman, 1988) in these investigations which combines the stability of an in vitro preparation with the bulbospinal projections still intact for recording intracellularly from SPNs. It must be noted, however, that in order to keep these preparations alive, only very young animals can be used and although there is evidence that PNMT-containing neurones do synapse onto SPNs at this early age (Bernstein-Goral and Bohn 1988a), the density of the PNMT fibres and the distribution of the terminals is very different to that observed in the adult rat Bernstein-Goral and Bohn 1988a and b). These investigations observed that there is a period of adrenergic hyperinnervation of SPNs during the neonatal period. This preparation is therefore not suitable to use as a model for studying the adrenergic inputs onto SPNs in the adult although results obtained using this preparation are of interest. It is possible to record intracellularly from SPNs in the adult in vivo preparation (see Dembowsky et al. 1985b), however these have a low yield of results due to problems with stability in the whole animal. It may be possible to gain some information about inputs onto SPNs from extracellular recordings in vivo if care was taken to control the stimulus parameters and using α₁-adrenoceptor agonists in conjunction with either applications of excitatory amino acids or stimulation of the RVLM to determine whether activation of α₁-adrenoceptors increases the effects of excitatory amino acid receptor activation.

It is particularly interesting to note the observations of Lundberg et al. (1989) that regular bursts of stimulation of the postganglionic nerve caused greater increases in the release of NPY from the postganglionic terminal than that seen with tonic
stimulation. This is worthy of note since, in the present studies, it was observed that iontophoresed methoxamine or phenylephrine induced burst firing in four neurones (see section 4.3g). Thus it may be that this change in firing pattern in the SPN mediated by \( \alpha_1 \)-adrenoceptors increases the release of a neurotransmitter or neuromodulator at the terminals of the neurone. This increases the activity of the postganglionic neurone which may lead to a greater effect on the end organ. Therefore, the role of \( \alpha_1 \)-adrenoceptors in this situation is a more subtle one, causing an indirect increase in end organ activity without necessarily increasing the mean firing rate of the SPN. It may be possible to study this further using a combination of iontophoretic techniques and HPLC analysis (see Lundberg et al. 1989), however the increase in neurotransmitter release from one neurone due to a change in the firing pattern of an SPN may be very difficult to detect and other more sensitive techniques for measuring neurotransmitter release may be required.

In conclusion, this study has shown that activation of \( \alpha_1 \)-adrenoceptors in the IML can increase in the firing rate and alter the firing pattern of SPNs. The supraspinal origins of the innervation responsible for their activation remain to be determined. It is also necessary to examine further the physiological importance of the \( \alpha_1 \)-adrenoceptor mediated input onto SPNs.


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[3H]Prazosin binding in the intermediolateral cell column and the effects of iontophoresed methoxamine on sympathetic preganglionic neuronal activity in the anaesthetized cat and rat*

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The autoradiographic localization of [3H]prazosin (α1-adrenoceptor ligand) binding sites was determined in cat spinal cord sections. High levels of [3H]prazosin binding were found in the intermediolateral cell column (IML) at thoracic and lumbar levels. The iontophoresis of the α1-adrenoceptor agonist methoxamine onto sympathetic preganglionic neurones (SPNs) in anaesthetized cats and rats caused excitation of 8 cat SPNs and 13 rat SPNs. These results suggest an excitatory role for some of the catecholaminergic innervation of the IML.

The intermediolateral cell column (IML) of the spinal cord, an area containing high concentrations of sympathetic preganglionic neurones (SPNs), is richly innervated by phenylethanolamine-N-methyl transferase (PNMT)-containing (and thus presumed adrenergic) neurones12,13. Recently PNMT-containing terminals have been shown to synapse directly onto SPNs in the rat9. The influence of this innervation on sympathetic neuronal activity has been an area of intense investigation over the last decade.

In vivo and in vitro studies have shown that applications of adrenoceptor agonists in the vicinity of antidromically identified SPNs cause α2-adrenoceptor-mediated inhibition of these neurones8,18. Indeed, in vitro autoradiographic binding studies have shown a high concentration of α2-adrenoceptor binding sites with a relative paucity of α1-adrenoceptor binding sites in the IML5. Recently, however, evidence of a direct α1-adrenoceptor-mediated excitation of SPNs has emerged from in vitro studies10,16,17.

This investigation re-examined the distribution of [3H]prazosin binding in the cat spinal cord using in vitro autoradiography and evaluated the effect of iontophoretic application of methoxamine (a highly selective α1-adrenoceptor agonist15) on the discharge of SPNs in the anaesthetized cat and rat.

Frozen 20-μm sections of cat spinal cord from the second thoracic to the third lumbar segment were prepared for autoradiography as described previously5 and incubated in 5 nM [3H]prazosin (22 Ci/mol; obtained from Amersham U.K.). The degree of [3H]prazosin binding to non-specific sites was established by incubating in the presence of excess (1–10 μM) unlabelled phentolamine. The sections were dried in a stream of cold air and apposed to [3H]Hyperfilm (Amersham). After an exposure time of 12 weeks, the film was developed using Kodak D19, fixed, rinsed and dried. The autoradiographs were photographed using a Nikon macro system. The sections were stained with 0.1% thionine to show histology of the underlying tissue and photographed using an Olympus Vanox-T system.

Iontophoretic studies were carried out on anaesthetized male rats (230–400 g) and female cats (2–3 kg). Rats were anaesthetized initially with sodium pentobarbitone (60 mg/kg i.p.) and cats were anaesthetized with a mixture of chloralose (80 mg/kg) and sodium pentobarbitone (12 mg), given i.v. Animals were paralysed with gallamine triethiodide (16 mg/kg in rat; 4 mg/kg in cat, given i.v.) and artificially ventilated on oxygen-enriched air. After neuromuscular blockade, anaesthesia was assessed by evaluating paw pinch and palpebral reflexes as the blockade wore off, and from the pupil size and recordings of blood pressure and heart rate. Additional doses of chloralose were given when necessary. Catheters were placed in the femoral artery and vein for monitoring blood pressure and blood gases, and for administration of drugs, respectively. All animals were given a pneumothorax and an end expiratory pressure of 2–3 mm H2O was applied to the expiratory line to

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methox. methox. cc: time

20 nA
glut. 25 nA

spikes

250 500 750 1000 1250 1500

methox amine iontophoresed at 20 nA (marked by the black bar) caused an initial decrease, then an increase in the firing rate of the neuron, an effect which lasted 150 s. This effect was fully repeatable. Iontophoresis of saline at the same current (cc, marked by the bar) caused only a decrease in firing rate of the neuron with no subsequent excitation. This suggests that the inhibition seen was a current effect rather than a genuine effect of methoxamine. B: histogram of the firing rate of a thoracic sympathetic preganglionic neuron (SPN) in rat. Firing rate of the neuron is shown on the Y-axis against time in seconds on the X-axis. The neuron was activated by 70 nA of glutamate as shown by the hatched line below the histograms. Methoxamine iontophoresed at 30 nA for 2 min (indicated by the black bar) increased the firing rate of the neuron. The firing rate returned to control values after 1250 s. Saline applied at the same current and time had no effect.

Fig. 1. Autoradiographic localization of \[^{3}H\]prazosin binding in the third thoracic segment of cat spinal cord. A: the underlying tissue of the cord stained with thionine. The IML is marked with an arrow. B: total \[^{3}H\]prazosin binding in the spinal cord. The grey matter has a high level of binding indicated by the density of grains. The IML has the highest level of binding shown by the darker spots. C: the degree of non-specific \[^{3}H\]prazosin binding in a section adjacent to (B) incubated in the presence of excess unlabelled phentolamine. Prevent atelectasis. Rectal temperature was maintained at 37 °C ± 1 °C using a heating blanket controlled by a feedback circuit. In some experiments rats were spinalized between the first and second cervical level. The base of the skull and the upper vertebrae were exposed and the muscle around the vertebrae removed to expose the spinal cord. The cord was cut between the vertebrae and bleeding controlled with gelatin sponge (Sterispon No.1).

The left cervical sympathetic chain in the rat and the left lumbar sympathetic chain in the cat were exposed and isolated for antidromic identification of SPNs\(^7\). In the rat, the chain was placed over bipolar platinum stimulating electrodes and covered with warm paraffin oil; in the cat stainless steel stimulating electrodes were
wrapped around the chain and then embedded in a vinyl polysiloxane impression material (Repisol, light body, De Trey). A laminectomy was carried out to expose the third thoracic segment of the spinal cord in the rat and the second or third lumbar segment in the cat, and the spinal cord was covered in warm paraffin oil. Extracellular recordings of the activity of SPNs were made through one barrel of a 5-barrelled glass electrode. The other barrels contained glutamate (pH 7-4), 2% Pontamine sky blue in 0.5 M sodium acetate (for current balancing), methoxamine (pH 4.5) and saline (pH 4.5) which was used for current controls. If the neurone did not have ongoing activity, glutamate was applied to activate the cell.

Sympathetic preganglionic neuronal activity, blood pressure and trigger pulses were stored on tape (Racal 7DS) for analysis. All data analysis was carried out using an interface and software supplied by Cambridge Electronic Design Ltd. in conjunction with an IBM compatible microcomputer. Sympathetic preganglionic neuronal activity was fed into a spike processor (Digitimer D130) which generated TTL pulses which were delivered to the computer via the interface.

Total and non-specific binding of [3H]prazosin was studied in cat spinal cord from the second thoracic to the third lumbar level. Generalized binding of [3H]prazosin, indicated by the grain density of the autoradiograph, was observed throughout the spinal cord, especially in the grey matter as shown in Fig.1. There was a much greater degree of binding in the IML compared with the rest of the grey matter at all levels of the spinal cord. Non-specific binding (shown at the third thoracic segment in Fig.1) was very low in all cases. Also shown in Fig.1 is the underlying tissue of the spinal cord at T3, stained with thionine. The IML is marked with an arrow and corresponds well with the area of highest levels of [3H]prazosin binding.

Recordings were made from 14 SPNs in the second and third lumbar segments of the cat spinal cord (3 which had ongoing activity and 11 which were activated with glutamate at currents of 7–25 nA). Methoxamine iontophoresed at currents of 20–100 nA in the vicinity of these neurones for two minutes caused an increase in the firing rate of 8 neurones, one of which had ongoing activity. Three neurones were inhibited (all of which were driven by glutamate) and three were not affected by iontophoresis of methoxamine (two with ongoing activity, one driven by glutamate). The excitatory effect which lasted 125–320 s was slow in onset and took up to 3 min to reach a peak (Fig.2A).

Recordings were made from 21 sympathetic preganglionic neurones in the second thoracic segment of anaesthetized rats with their neuraxes intact. Eight of these neurones had ongoing activity and the other 13 neurones were activated with glutamate.

Methoxamine was iontophoresed at currents of 30–40 nA for up to 3 min and caused an increase in the firing rate of 13 neurones, 5 of which had ongoing activity. The remainder were unaffected by methoxamine. The excitatory effect lasted for 100–975 s (Fig.2B), was slow in onset and took up to 3 minutes to reach a peak.

Methoxamine was iontophoresed in the vicinity of two thoracic neurones in anaesthetized rats spinalized between C1 and C2 at least 3 h before recording. Both neurones were glutamate-activated and their firing rate was increased by iontophoresis of methoxamine ejected at 30 nA for 2 min and the effects lasted for 365 and 1020 s.

Our results have shown the autoradiographic localization of a relatively high level of [3H]prazosin binding sites in the IML of the cat spinal cord. Furthermore, we have demonstrated that methoxamine can have an excitatory effect on sympathetic preganglionic neuronal discharge, indicating an excitatory role for α1-adrenoceptors in the IML.

High levels of [3H]prazosin binding indicated by the high grain densities on the autoradiographs were found throughout the grey matter of the spinal cord with a much higher density of grains in the IML at all levels from T2 to L3. These results are in contrast to a previous report [6]. The difference between the two experimental protocols was the longer exposure time of the slides to hyperfilm (12 weeks rather than 5) using [3H]prazosin with the same specific activity as before.

Prazosin is well known to have a high affinity for α1-adrenoceptors, however, recent evidence has suggested that prazosin binds to another site (α2B-adrenoceptor) for which it has a similar affinity as yohimbine [3]. Preliminary experiments have shown that unlabelled methoxamine displaces [3H]prazosin binding in the IML, suggesting that [3H]prazosin is binding to α1-adrenoceptor sites (Marks, unpublished observations).

We have also shown that iontophoresis of the α1-adrenoceptor agonist methoxamine in the vicinity of SPNs can cause an increase in the firing rate of these neurones. The long latency to onset seen after application of methoxamine may be due to the low potency of methoxamine [2] and also may be due to the fact that the receptors at which methoxamine is having its effect are located some distance from the tip of the microelectrode. The long time course of action may have at least two explanations: (1) methoxamine has a very low affinity for the amine uptake mechanism [14] and therefore may be removed slowly from its site of action, and (2) cellular mechanisms may be responsible for the long time course; α1-adrenoceptor activation of SPNs is known to cause a slow depolarising potential which involves a decrease in...
Ca²⁺-dependant K⁺ conductance and is mediated by cyclic AMP¹¹.

In some experiments, rats were spinalized between the first and second cervical vertebrae, thereby removing the possibility of methoxamine interacting with tonically active descending supraspinal pathways. Methoxamine iontophoresed in this preparation caused an increase in firing rate of SPNs similar to that seen in the rats with their neuraxes intact. This suggests either a direct effect of methoxamine on postsynaptic receptors on the SPN or on interneurones within the spinal cord, or both. In support of a direct action of methoxamine on SPNs, in vitro experiments have shown that the slow depolarising potential evoked by bath applications of noradrenaline and mediated via α₁-adrenoceptors (since the effect is abolished by prazosin) persists in the presence of TTX, suggesting a direct effect onto SPNs¹⁶,¹⁷. There are a number of possible explanations for the decrease in firing rate seen in 3 SPNs in the cat. For example, methoxamine may be acting on inhibitory interneurones or as an antagonist to tonically active descending excitatory pathways; methoxamine has high affinity but low activity at α₁-adrenoceptors and therefore may be acting as a competitive antagonist against the endogenous ligand³.

In summary, we have combined autoradiographic studies with neuropharmacological studies on the role of α₁-adrenoceptors in the IML of the spinal cord. Iontophoretic application of methoxamine in the vicinity of SPNs can cause an increase in the firing rate of these neurones in the anaesthetized rat (spinalized and neuraxis intact) and cat. These results complement the observations of Coote and Lewis⁴ and suggest that at least some of the catecholaminergic input to SPNs is excitatory and mediated by α₁-adrenoceptor activation.

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AUTORADIOGRAPHIC LOCALIZATION OF \( \alpha \)-ADRENOCEPTOR BINDING SITES IN THE INTERMEDIOLATERAL CELL COLUMN OF THE CAT.

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A previous study from this laboratory failed to show any significant \( \alpha \)-adrenoceptor binding in the intermediolateral cell column (IML) of the cat spinal cord (Dashwood, M.R. et al, Neuroscience, 15: 537-551, 1985). However, recent observations (Yoshimura, M. et al, Brain Research, 414: 138-142, 1987) have shown an \( \alpha \)-adrenoceptor mediated excitation of sympathetic preganglionic neurones in vitro. We therefore re-examined the distribution of \( \alpha \)-adrenoceptor binding sites in the IML of the cat.

Experiments were carried out as described previously (Dashwood et al) using [\(^3\)H]prazosin as the \( \alpha \)-adrenoceptor ligand, however, sections were exposed to hyperfilm for twelve weeks. The degree of binding to non-specific sites was established by incubating in the presence of excess phentolamine (10\( \mu \)M). Prazosin showed a marked binding in the IML at thoracic and lumbar levels. The difference between the previous results and those of the present study may be explained by the longer exposure times to the hyperfilm in the present case.

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KEY WORDS: (see instructions pg. 4)
1. Cardiovascular
2. Catecholamine
3. Autonomic
4. Spinal cord
α₁-Adrenoceptors in the intermediolateral cell column (IML) and their function in sympathetic control in the anaesthetized rat and cat

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Recently Yoshimura et al. (1987) have shown an α₁-adrenoceptor mediated excitation of sympathetic preganglionic neurones (SPNs) in cat spinal cord slices, an effect which is inconsistent with in vitro autoradiographic binding studies showing α₂-adrenoceptor binding sites in the IML of the cat but a relative paucity of α₁-adrenoceptors (see Dashwood et al. 1985). To resolve this inconsistency, the distribution of α₁-adrenoceptor binding sites in the IML was re-examined and methoxamine, a selective α₁-adrenoceptor agonist, ionophoresed in the vicinity of SPNs in vivo.

In vitro autoradiographic studies were carried out as described previously (Dashwood et al. 1985) using [³H]prazosin as the α₁-adrenoceptor ligand and incubating in the presence of excess phentolamine to establish the extent of binding to non-specific sites. However, the exposure time was increased to 12 weeks from the 5-week period used previously. Under these conditions, prazosin showed marked binding in the IML at thoracic and lumbar levels with very little non-specific binding.

Ionophoretic studies were carried out on anaesthetized rats (sodium pentobarbitone 60 mg/kg, i.p.) and cats (choralose 80 mg/kg, i.v.). Additional doses of chloralose were given where necessary in both species (assessed by evaluating paw-pinch and palpebral reflexes and from recordings of blood pressure). Animals were paralysed with gallamine triethiodide (16 mg/kg in rat; 4 mg/kg in cat), ventilated artificially, then prepared for recording from SPNs.

In rats, methoxamine (30–60 nA for 1–3 min) increased the firing rate of 6 of 9 thoracic SPNs tested. Similarly, in cats, methoxamine (20–100 nA for 2 min) excited 6 of 12 lumbar SPNs tested. The effect of methoxamine on SPNs with ongoing activity and those driven by glutamate was similar. The neurones showed a slow onset to firing, it taking up to 100 s to reach a maximal discharge.

These observations complement those of Coote & Lewis (1989) and indicate that some of the catecholaminergic innervation of the IML may transmit excitatory drive to SPNs.

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Microinjection of phenylephrine into the intermediolateral cell column elicits an increase in cardiac sympathetic nerve activity in the anaesthetized cat

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Previous studies from this laboratory support the idea that α1-adrenoceptors are involved in the transfer of excitatory drive to sympathetic preganglionic neurones (SPNs; see Marks et al. 1990). In the present study this possibility was further investigated by the microinjection of an α1-adrenoceptors agonist (phenylephrine) into the intermediolateral cell column (IML), observing the effect on cardiac sympathetic nerve activity and determining whether the effect could be blocked by a selective α1-adrenoceptor antagonist, alfuzosin (Cavero et al. 1984).

Experiments were carried out on anaesthetized (chloralose 80 mg/kg I.V. or sodium pentobarbitone 40 mg/kg I.P.), paralysed (vecuronium bromide 0.2 mg/kg) and artificially ventilated cats. Additional doses of chloralose were given when necessary (see Marks et al. 1990). Recordings were made from the central end of the left inferior cardiac nerve (ICN). A laminectomy was performed to expose segments T2–T4 of the spinal cord. A seven-barrelled microelectrode was advanced into the T3 segment of the spinal cord just lateral to the dorsal root entry zone until electrical stimulation through the central barrel caused a low threshold short latency response in the ICN. At this site drugs were micro-injected from the remaining six barrels using a pressure injection system. The location of the electrode was later verified histologically.

Micro-injection of 100 nl of 10 mM phenylephrine caused an increase in cardiac nerve activity (CNA) in 13/15 cases (range, 13–140%; median, 60%). In five experiments where alfuzosin was micro-injected after phenylephrine subsequent injections of phenylephrine had no effect; micro-injection of glutamate still caused an increase in CNA.

These results indicate that α1-adrenoceptors are involved in regulating those SPNs which control the heart.

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SUMMARY

1. In anaesthetized cats the effect of the α₁-adrenoceptor agonist phenylephrine, microinjected into the left intermediolateral cell column of the spinal cord at the third thoracic level, was studied on left inferior cardiac nerve activity.

2. Microinjection of 100 nl of 10 or 40 mM-phenylephrine caused increases in inferior cardiac nerve activity in fifteen out of seventeen experiments.

3. The microinjection of the α₂-adrenoceptor antagonist alfuzosin (100 nl of 10 mM) into the intermediolateral cell column antagonized the excitatory response elicited by phenylephrine.

4. Increases in inferior cardiac nerve activity produced by glutamate and 5-hydroxytryptamine microinjected into the intermediolateral cell column were not antagonized by alfuzosin.

5. It is concluded that activation of α₁-adrenoceptors in the region of the intermediolateral cell column can cause an increase in the firing rate of sympathetic preganglionic neurones which innervate postganglionic neurones projecting into the inferior cardiac nerve.

INTRODUCTION

The intermediolateral cell column of the spinal cord (the location of the majority of sympathetic preganglionic neurones) receives projections from numerous supraspinal neurones, many of which are catecholamine containing (see Coote, 1988). Recently, catecholamine and phenylethanolamine-N-methyl-transferase (PNMT: the enzyme involved in catalysing the conversion of noradrenaline to adrenaline) immunoreactive boutons have been shown to make synaptic contacts with sympathetic preganglionic neurones (Chiba & Masuko, 1987; Milner, Morrison, Abate & Reis, 1988). The influence of these neurones on sympathetic activity has been studied extensively and it is now clear that they may have diverse actions on sympathetic preganglionic neurones mediated by different adrenoceptors.

Autoradiographic studies have demonstrated the presence of both α₁- and α₂-adrenoceptor binding sites in the intermediolateral cell column of the cat (Dashwood, Gilbey & Spyer, 1985; Marks, Stein, Dashwood & Gilbey, 1990). Until recently, in vivo ionophoretic application of α-adrenoceptor agonists onto sympathetic pre-
ganglionic neurones had been shown to cause only decreases in sympathetic preganglionic neuronal activity, an effect mediated by $\alpha_2$-adrenoceptors (de Groat & Ryall, 1967; Guyenet & Cabot, 1981; Kadzielawa, 1983). However, experiments from this laboratory have shown that ionophoresis of the selective $\alpha_1$-adrenoceptor agonists, methoxamine and phenylephrine, in the vicinity of sympathetic preganglionic neurones in the rat and cat can cause an increase in their firing rate (Marks et al. 1990; S. A. Marks, unpublished observations). Additionally, in the rat ionophoresis of adrenaline and noradrenaline has been demonstrated to cause both decreases and increases in sympathetic preganglionic neuronal activity (Lewis & Coote, 1990). In vitro studies on both rat and cat spinal cord slices have further demonstrated that adrenaline and noradrenaline can cause both slow depolarizations and slow hyperpolarizations of these neurones, the effects being mediated by $\alpha_1$- and $\alpha_2$-adrenoceptors, respectively (Yoshimura, Polosa & Nishi, 1987a, b, c; Miyazaki, Coote & Dun, 1989). Other effects of sympathetic preganglionic neurone adrenoceptor stimulation have been reported: namely, shortening of the sympathetic preganglionic neurone action potential; suppression of the slow after-hyperpolarization; production of an after-depolarization and rhythmic membrane potential oscillations (see Nishi, 1990).

Although the above data provide information on the actions of adrenoceptor agonists on sympathetic preganglionic neurones in general, there is no information on their effect on sympathetic preganglionic neurones of known function; sympathetic preganglionic neurones cannot be identified on the basis of their axonal projections (see Gilbey & Stein, 1991) and sympathetic preganglionic neurones of any one segment innervate many organs (Langley, 1892; Njå & Purves, 1977; Strack, Sawyer, Hughes, Platt & Loewy, 1989).

The aim of the present experiments was to gain some insight into the effect on cardiac nerve activity of activating $\alpha_1$-adrenoceptors in the region of the intermediolateral cell column. To achieve this recordings were made from the left inferior cardiac nerve and drugs were microinjected into the left intermediolateral cell column of the third thoracic segment of the spinal cord. The inferior cardiac nerve was chosen as it is known to project to the heart (McKibben & Getty, 1968), and to a degree to the oesophagus and pulmonary vessels (Gonella, Neil & Roman, 1979) and electrical stimulation of this nerve causes a large increase in heart rate (Kamosinska, Nowicki & Szulczyk, 1989). Drugs were microinjected into the T3 segment as postganglionic neurones in the stellate ganglion, which send axonal projections into the inferior cardiac nerve, although receiving inputs from a number of white rami (Pardini & Wurster, 1984) receive their major one through the third thoracic white ramus (Szulczyk & Szulczyk, 1987). Furthermore, Rubin & Purves (1987) reported that neurones sending axonal projections into a single white ramus are confined to the corresponding segment of the spinal cord. Therefore an efficacious drug injected into the left intermediolateral cell column of the third thoracic segment of the spinal cord should produce a marked change in left inferior cardiac nerve activity.

In particular, the present experiments examined the effect on inferior cardiac nerve activity of phenylephrine (a selective $\alpha_1$-adrenoceptor agonist; Wikberg, 1978) microinjected into the left intermediolateral cell column of the third thoracic
segment and investigated whether this effect could be blocked selectively by microinjection of alfuzosin (a selective $\alpha_1$-adrenoceptor antagonist; Cavero, Galzin, Langer & Lefevre-Borg, 1984) at the same site.

A preliminary report of this work has been published (Marks & Gilbey, 1991).

**METHODS**

Experiments were carried out on twenty-six female cats (1.7–2.9 kg) anaesthetized with either an i.v. injection of $\alpha$-chloralose (80 mg kg$^{-1}$ + 12 mg sodium pentobarbitone) or an i.p. injection of sodium pentobarbitone (40 mg kg$^{-1}$). Supplementary doses of chloralose were given when necessary as judged from recordings of blood pressure and heart rate, and from pupil size and the cat's palpebral and paw reflexes. During data collection, animals were paralysed with an infusion of vecuronium bromide at a rate of 400 $\mu$g kg$^{-1}$ h$^{-1}$ (6 ml h$^{-1}$ in saline) and artificially ventilated on oxygen-enriched air after cannulation of the trachea. At regular intervals, the animals were allowed to recover from neuromuscular blockade and the depth of anaesthesia was maintained at a level at which both corneal and flexor responses were sluggish.

Catheters were placed in the femoral artery and vein for monitoring blood pressure and for administration of drugs, respectively. A catheter was placed in the bladder to allow continuous flow of urine. Animals were given a pneumothorax and an end-pressure was applied to the expiratory line to prevent atelectasis. End-tidal CO$_2$ was monitored continuously and arterial blood samples were taken periodically to check pH and blood gas tensions which were kept in the following range: pH, 7.35–7.45; $P_{a,CO_2}$, 30–40 mmHg; $P_{a,0_2}$, > 80 mmHg. An infusion of sodium bicarbonate was used to correct base deficits. Rectal temperature was maintained at 37 ± 1 °C with a heating blanket controlled by a feedback circuit.

Animals were placed in a stereotaxic frame and the lumbar cord was stabilized by lateral clamps at the iliac crests. The cord was further stabilized by clamps attached to the lower cervical and middle thoracic vertebrae.

The left stellate ganglion was exposed retropleurally by a dorsolateral approach removing the second, third and fourth ribs. The first and second thoracic white rami and the sympathetic chain caudal to the third thoracic white ramus were isolated and cut. The inferior cardiac nerve, which arises from the stellate ganglion, was isolated and cut. The nerve was desheathed and the central end wrapped around bipolar recording electrodes and bathed in a pool of liquid paraffin.

A laminectomy was performed to expose the third thoracic segment and the spinal cord was covered with warm paraffin oil. A seven-barrelled glass microelectrode assembly (50 $\mu$m tip diameter) was advanced into the spinal cord just lateral to the dorsal root entry zone until electrical stimulation (10–40 $\mu$A current, 1 ms pulse width) through the Woods-metal-filled central barrel caused a short latency response in the cardiac nerve (see Results section and Fig. 2A). At this site in the cord (depth 1450–1800 $\mu$m) drugs were injected in 100 nl volumes using a pressure injection system (Neurophore, Medical Instruments Inc.). These volumes were given using 1–10 s pulses (one to three pulses at 10 s intervals). The volume of drug injected was monitored by measuring the movement of the fluid meniscus directly through a microscope (x 40 magnification) fitted with a graticule. At the end of the experiment the injection site was lesioned electrically (200 $\mu$A DC current for 30 s) and the cord was removed and fixed in formal saline. The cord was frozen and 100 $\mu$m sections were cut on a freezing microtome. The sections were stained with Neutral Red and the position of the electrode was verified using a Vanox-T system microscope.

Figure 1A shows the experimental set-up.

**Drugs**

Drugs were dissolved in either saline or artificial cerebrospinal fluid (both pH 7.4). Drugs used were L-phenylephrine HCl (10–40 mm); L-glutamic acid (40 mm); 5-HT bimaleate (10 mm) (all Sigma); alfuzosin HCl (10 mm; Synthelabo) and saline or artificial cerebrospinal fluid (controls).

**Analysis of data**

Inferior cardiac nerve activity, blood pressure, tracheal pressure, ECG and trigger pulses were stored on tape (Racal 7DS) for off-line analysis. Inferior cardiac nerve activity was rectified and integrated over 10 s periods using a solid-state integrator. This was used in conjunction with raw
cardiac nerve activity in order to observe and quantify changes in nerve activity following microinjection of drugs. At the end of each experiment animals were killed with an overdose of sodium pentobarbitone and background noise recorded. This noise level was used at the beginning of analysis to set the integrator level.

Inferior cardiac nerve • T1 white ramus

Amp

Filter

T3 white ramus

Sympathetic chain

Fig. 1. A, line diagram showing the experimental set-up. B, a line drawing of the cat spinal cord showing sites at which microinjection of phenylephrine caused an increase in inferior cardiac nerve activity (each site is marked by a filled circle). Injections of phenylephrine made lateral or medial to the intermediolateral cell column (marked by the open circles) had no effect on inferior cardiac nerve activity.

The data obtained were treated in the same way as results obtained from ionophoretic studies, i.e. statistical tests were not carried out on pooled data since between experiments the change in inferior cardiac nerve activity due to microinjection of a drug and the duration of the effect seen were highly variable. This variability is to be expected for the following reasons: (i) the position of the electrode within the cord in relation to sympathetic preganglionic neurones will vary greatly between experiments; sympathetic preganglionic neurones form a ladder-type structure in the intermediolateral cell column with clusters of neurones about every 1 mm alternating with less-populated patches (Chung, Chung, Lavelle & Wurster, 1979), therefore if an electrode is positioned between two clusters of neurones the effect of the microinjected drugs will be smaller than if the electrode is placed in the centre of a pool of sympathetic preganglionic neurones; (ii) not all sympathetic preganglionic neurones in the third thoracic segment innervate postganglionic neurones projecting into the inferior cardiac nerve, therefore the magnitude and duration of the response will also depend on the numbers of sympathetic preganglionic neurones activated that project to the inferior cardiac nerve.

Changes in inferior cardiac nerve activity seen on microinjection of drugs were calculated as percentage change from controls, were measured by calculating the difference between the mean control activity and the peak increase in inferior cardiac nerve activity for each drug and were considered significant if the change was more than 20% from control. Alfuzosin was considered to have antagonized the action of phenylephrine (or any other drug) if it reduced the inferior cardiac nerve activity response by more than 50%.
The numbers given for each drug response refer to the number of animals in which the effect was seen.

RESULTS

Positioning the electrode in the intermediolateral cell column

The effect on inferior cardiac nerve activity of microinjecting phenylephrine into the intermediolateral cell column was studied at sites at which electrical stimulation evoked a short latency (20–30 ms), low threshold (10–40 μA, 1 ms pulse width) response in the inferior cardiac nerve (Fig. 2A). The low threshold and short latency of response (latency similar to that seen when stimulating the peripheral cut end of the ventral roots; Fig. 2B) indicated that the tip of the electrode was positioned in

![Image](https://example.com/image1.png)

Fig. 2. A, response in the inferior cardiac nerve evoked by electrical stimulation within the intermediolateral cell column. B, response in the inferior cardiac nerve evoked by stimulating T3 ventral root.

or near a pool of sympathetic preganglionic neurones innervating the inferior cardiac nerve. In some experiments \( (n = 12) \) 100 nl of glutamate was microinjected at this site and always produced an increase in inferior cardiac nerve activity (see Figs 3 and 4).

Effect of phenylephrine on inferior cardiac nerve activity

In order to establish a working range of concentrations for microinjection of phenylephrine, two experiments were carried out in which 10, 20 and 40 mM concentrations of phenylephrine were injected at 15 min intervals. These resulted in a dose-related increase in inferior cardiac nerve activity (see Fig. 3) and gave a working range of concentrations. For subsequent experiments 10 and 40 mM phenylephrine were used. If no effect was seen with microinjection of 10 mM phenylephrine then 40 mM was used.

Using these concentrations, phenylephrine caused an increase in inferior cardiac nerve activity in 15/17 experiments (median 75%) with a median duration of 3.5 min. Sites at which phenylephrine caused an increase in inferior cardiac nerve activity are shown in Fig. 1B. Injection of phenylephrine into the cord at two sites, one lateral and one medial to the intermediolateral cell column (marked by the open circles), had no effect on inferior cardiac nerve activity. Microinjection of saline or artificial cerebrospinal fluid into the intermediolateral cell column had little or no effect on inferior cardiac nerve activity; in the cases where an increase in inferior cardiac nerve activity was observed it was immediate and inferior cardiac nerve
activity returned quickly to control levels. This effect was presumably a pressure artifact and was also observed on occasions with microinjection of drugs (see Fig. 5).

**Effect of alfuzosin on phenylephrine response**

Experiments were carried out to determine whether the response to phenylephrine could be blocked by microinjection of alfuzosin, a selective $\alpha_1$-adrenoceptor antagonist. Alfuzosin was microinjected into the intermediolateral cell column in six experiments where phenylephrine had previously caused an increase in inferior cardiac nerve activity and the subsequent response to injection of phenylephrine was either abolished ($n = 5$) or reduced (Fig. 4). Recovery from the alfuzosin block was observed in two experiments (see Fig. 4).

**Selectivity of the alfuzosin antagonism**

5-Hydroxytryptamine (5-HT) and glutamate, both of which have been shown previously to cause excitation of sympathetic preganglionic neurones (see Coote, 1988), were used to test the selectivity of the antagonism of the phenylephrine response by alfuzosin. The effects of microinjection of 100 nl of 40 mM-glutamate and 10 mM-5-HT were examined in twelve and six cases, respectively. Glutamate caused a large increase in activity in all twelve cases (median 233%; see Figs 3 and 4). In five cases when alfuzosin was microinjected, a subsequent dose of glutamate caused large increases in inferior cardiac nerve activity (median 240%). In two of these experiments glutamate was microinjected before and after alfuzosin and caused an
increase in inferior cardiac nerve activity which was not affected by microinjection of alfuzosin. 5-HT when microinjected caused an increase in inferior cardiac nerve activity in five of six preparations (median 49%). Alfuzosin failed to antagonize the increase in inferior cardiac nerve activity elicited by 5-HT ($n = 3$). In one experiment,

**Fig. 4.** Example of the effect of alfuzosin (Alf) on a phenylephrine (Phe) response. The upper traces show inferior cardiac nerve activity integrated over 10 s periods. The lower traces show raw inferior cardiac nerve activity. Forty millimolar glutamate (Glu) was first microinjected and caused an increase in inferior cardiac nerve activity of 262%. Ten millimolar phenylephrine was microinjected at 6 min intervals and resulted each time in an increase in inferior cardiac nerve activity (range 127–180%). At the arrow 100 nl alfuzosin was microinjected and a subsequent injection of phenylephrine caused only a 36% increase in inferior cardiac nerve activity. Eighty minutes after the microinjection of alfuzosin there was a recovery of the phenylephrine effect. Arrows indicate microinjection of drugs.

alfuzosin was shown to antagonize the increase in inferior cardiac nerve activity due to microinjection of phenylephrine while having no effect on the excitation seen on microinjection of 5-HT (Fig. 5).

**DISCUSSION**

This study has demonstrated that the microinjection of phenylephrine into the region of the intermediolateral cell column of the third thoracic segment can cause an increase in the activity of sympathetic preganglionic neurones which innervate the inferior cardiac nerve. The excitatory effect of phenylephrine could be antagonized by microinjection of the $\alpha_1$-adrenoceptor antagonist alfuzosin and full recovery from this blockade was demonstrated in two experiments. The selectivity of this antagonism was demonstrated since alfuzosin did not antagonize the excitatory effects of microinjections of either 5-HT or glutamate. The fact that alfuzosin failed to antagonize the excitation due to 5-HT is of importance since both...
5-HT and \( \alpha_1 \)-adrenoceptor agonists are thought to cause neuronal depolarization through a decrease in resting \( K^+ \) conductance (Yoshimura, Polosa & Nishi, 1986; Pape & McCormick, 1989). \( \alpha_1 \)-Adrenoceptors are therefore likely to be involved in regulating cardiac function.

Fig. 5. Illustration of selective blockade of phenylephrine (Phe) response by alfuzosin (Alf). The upper traces show integrated nerve activity over 10 s intervals. The lower traces show raw nerve activity on a different time scale to show the effects of drugs on cardiac nerve activity. Ten millimolar 5-HT and 40 mM-phenylephrine were microinjected and both resulted in increases in inferior cardiac nerve activity (120 and 259\% respectively). Alfuzosin was microinjected while inferior cardiac nerve activity was still elevated due to phenylephrine. A subsequent injection of phenylephrine had no effect on inferior cardiac nerve activity. The initial increase in inferior cardiac nerve activity was an artifact since microinjection of the same volume of saline caused the same short-lasting increase in nerve activity. The effect of microinjection of 5-HT, however, was affected little by alfuzosin (107\% increase in inferior cardiac nerve activity) and was unlikely to be due to the fact that the alfuzosin block had worn off since phenylephrine still had little effect on inferior cardiac nerve activity.

The increases in inferior cardiac nerve activity were produced by phenylephrine exciting sympathetic preganglionic neurones located in the third thoracic segment as T1 and T2 white rami were cut and the sympathetic chain cut caudal to where it was joined by the T3 white ramus. However, was the increase in inferior cardiac nerve activity produced by phenylephrine due to an action of the drug directly on sympathetic preganglionic neurones or via antecedent neurones? The fact that microinjection of phenylephrine into the cord at sites more lateral and medial to the intermediolateral cell column had no effect on inferior cardiac nerve activity suggests a limited spread of drug from the injection site. Furthermore, in support of a direct effect of phenylephrine on sympathetic preganglionic neurones, application of noradrenaline onto sympathetic preganglionic neurones in the tetrodotoxin-treated cat spinal cord slice caused depolarization of these neurones, an effect which could be blocked by the \( \alpha_1 \)-adrenoceptor antagonist prazosin (Yoshimura et al. 1987a).
A recent study has demonstrated that microinjection, into the intermediolateral cell column of the rat, of noradrenaline (50–100 μM in 20 nl) and phenylephrine (5 μM in 20 nl) caused an increase in heart rate (Sundaram, Murugaian & Sapru, 1991). The noradrenaline effect was mediated by α₁-adrenoceptors since it was blocked by prazosin (an α₁-adrenoceptor antagonist). Although these results are in agreement with our observations that α₁-adrenoceptors in the intermediolateral cell column excite sympathetic preganglionic neurones that regulate the heart there is a large discrepancy in the concentrations of phenylephrine microinjected to produce the responses even through the concentrations of glutamate microinjected were similar (88-5 mM in Sundaram et al.’s experiments). These differences might represent a genuine species difference in the number and density of α₁-adrenoceptors in the intermediolateral cell column and/or the different anaesthetics used.

Physiological activation of these α₁-adrenoceptors in the intermediolateral cell column could result from the release of catecholamines from axon terminals arising from a number of supraspinal cell groups which have been shown to project directly to the intermediolateral cell column. These cell groups include C1 of the rostral ventrolateral medulla (Ross, Armstrong, Ruggiero, Pickel, Joh & Reis, 1981; Ross, Ruggiero, Joh, Park & Reis, 1984a), C2 in the nucleus of the tractus solitarius (NTS), C3 in the dorsomedial medulla near the NTS and the lateral tegmental field (Wesselingh, Li & Blessing 1989; Minson, Llewellyn-Smith, Neville, Somogyi & Chalmers, 1990), A5 and to some extent A1 in rat, and A1 and possibly the A7 in the cat (McKellar & Loewy 1982; Coote, 1985; Strack et al. 1989). In the past, functional studies have focused on the C1 cell group (Ross, Ruggiero, Park, Joh, Sved, Fernandez-Pardal, Saavedra & Reis, 1984a). However these studies do not give conclusive evidence that adrenergic neurones in this region are important in the control of sympathetic activity. Clearly, further studies are needed to discover the functional importance of the catecholaminergic inputs onto sympathetic preganglionic neurones.

In conclusion, it appears that activation of α₁-adrenoceptors in the region of the intermediolateral cell column can increase inferior cardiac nerve activity. The supraspinal origin(s) of the innervation responsible for their activation remains to be determined.

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