STUDIES ON THE INTERACTION OF LOCAL ANAESTHETIC AGENTS WITH THE NOREPINEPHRINE TRANSPORTER

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by

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Studies on the interaction of local anaesthetic agents on the norepinephrine transporter.

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Intravenous regional guanethidine Bier's block (IVRGBB) is often used to treat complex regional pain syndrome type I (CRPS 1). Guanethidine is taken up via the neuronal norepinephrine transporter (NET) and displaces norepinephrine (NE) from release vesicles. Clinically, a local anaesthetic, usually prilocaine is co-administered to reduce discomfort during administration. The efficacy of IVRGBB for CRPS 1 has been questioned. As cocaine is a known, potent inhibitor of NET, it is hypothesised that other local anaesthetic agents may act similarly and hence reduce the efficacy of IVRGBB.

The local anaesthetic agents cocaine, tetracaine, dibucaine, procaine, bupivacaine prilocaine and lidocaine all inhibited the uptake of [^{3}H]NE (as a surrogate for guanethidine) into both SH-SY5Y human neuroblastoma and HEK293 cells expressing the recombinant human NET (293-hNET) with pIC_{50} values ranging from 6.81 (cocaine) to 2.89 (lidocaine). Of particular interest was prilocaine with a pIC_{50} of 3.72 and 3.19 in SH-SY5Y and 293-hNET cells respectively. As prilocaine is estimated to reach around 1mM in IVRGBB, such values would make a reduction in guanethidine uptake likely. Local anaesthetics were also found to displace the specific NET label [^{3}H]nisoxetine in a concentration dependent manner with pK_i values ranging from 5.53 (cocaine) to 2.21 (lidocaine). Values for prilocaine were 3.16 and 3.07 in SH-SY5Y and 293-hNET cells respectively. There was a positive correlation between pIC_{50} (uptake) and pK_i (binding) suggesting a link between uptake inhibition and specific uptake site interaction. In *ex vivo* electrically field stimulated mouse vas deferens, prilocaine (1mM), procaine (300μM) and cocaine (30μM) reduced the efficacy of guanethidine block by accelerating twitch response recovery. Furthermore, the effects of prilocaine appeared to be competitive as the inhibition was partially overcome by increasing the concentration of guanethidine.

Based on the results of this thesis, I would strongly advise that until further clinical research is carried out, guanethidine and local anaesthetic agents should not be coadministered in IVRGBB.

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List of abbreviations

5-HT	5 hydroxytryptamine (serotonin)
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
AV	atrio-ventricular
B_{max}	maximum binding
BP	blood pressure
BSA	bovine serum albumin
cDNA	complementary deoxyribose nucleic acid
CNS	central nervous system
COMT	catechol-o-methyl transferase
CRPS I	complex regional pain syndrome type I
CRPS II	complex regional pain syndrome type II
DAT	dopamine transporter
DMEM	Dulbecco's modified eagle medium
DMI	desipramine
DPM	disintegrations per minute
DRG	dorsal route ganglion
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EFS	electrical field stimulation
EGTA	ethyleneglycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
FBS	foetal bovine serum
G418	geneticin
GABA	γ-aminobutyric acid
GI	gastro-intestinal
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
HEK	human embryonic kidney
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
hNET	human norepinephrine transporter
Ig	Immunoglobulin
IVRBB	intravenous regional Bier's block
IVRGBB	intravenous regional guanethidine Bier's block
K_d	dissociation constant
kDa	kilo Daltons
K_i	inhibition constant
K_m	equilibrium constant
MAO	monoamine oxidase
MAZ	mazindol
MEM	minimum essential medium
mRNA	messenger ribose nucleic acid
mVD	mouse vas deferens
n	number of experiments
N	number of binding sites
NDPK	nucleoside diphosphate kinase
NE	norepinephrine
NET	norepinephrine transporter

NSB	non-specific binding
NSU	non-specific uptake
PAGE	poly-acrylamide gel electrophoresis
PEI	polyethyleneimine
PGI_2	prostaglandin I_2
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
RSD	reflex sympathetic dystrophy
SA	sino-atrial
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SERT	serotonin transporter
SMP	sympathetically maintained pain
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TENS	transcutaneous electrical nerve stimulation
TMD	transmembrane domain
TTX	tetrodotoxin
VMAT	vesicular monoamine transporter

CHAPTER 1

General Introduction.

1.1.1 Autonomic nervous system

The autonomic nervous system (Gibbins, 1990) has two main divisions; sympathetic and parasympathetic (see fig 1.1). The enteric nervous system is also considered to be a division but is not of concern here (Furness & Costa, 1987). The autonomic nervous system conveys the output from the central nervous system to the rest of the body except for the motor innervation of skeletal muscle and neuroendocrine output. It is largely outside the influence of voluntary control. The main functions of the autonomic nervous system are control of:

- contraction and relaxation of smooth muscle
- all exocrine and certain endocrine secretions
- heartbeat
- control of intermediary metabolism

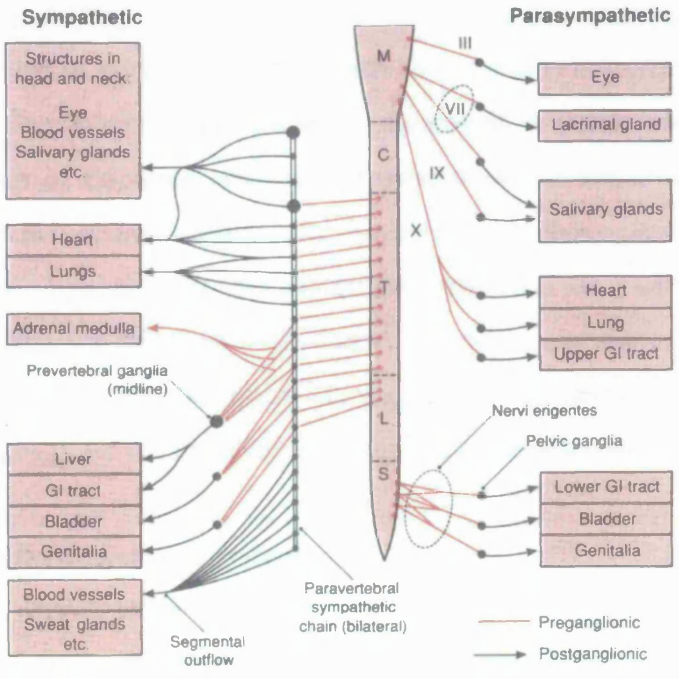


Figure 1.1 Basic plan of the mammalian autonomic nervous system (M = medullary; C = cervical; T = thoracic; L = lumbar; S = sacral) from Rang, Dale and Ritter, 1995. NB. In both systems, the transmitter at the ganglion is acetyl choline. At the effector junction in the sympathetic and parasympathetic nervous system the transmitter is NE and ACh respectively. (exception, sweat glands have cholinergic synapses)

The main difference between autonomic and somatic efferent pathways is that the autonomic consists of two neurons arranged in series, whereas in the somatic a single motoneuron connects the central nervous system to the skeletal muscle fibre. The two neurons involved in the autonomic pathway are the preganglionic and postganglionic fibres, synapsing in an autonomic ganglion. Autonomic ganglia lie outside the CNS and contain the nerve endings of the preganglionic fibres and the cell bodies of the postganglionic fibres.

1.1.2 Sympathetic nervous system

Sympathetic preganglionic neuron cell bodies are located in the lateral horn of the grey matter of the thoracic and lumbar segments of the spinal cord, and the fibres leave the spinal cord in the spinal nerves as thoracolumbar sympathetic outflow. Just outside the spinal cord they leave the spinal nerve as filaments that run to the paravertebral chain of sympathetic ganglia lying bilaterally to the spinal column. Postganglionic sympathetic axons rejoin the spinal nerves and most reach their peripheral destinations via the spinal nerve branches. The others, which travel to abdominal or pelvic viscera, have their cell bodies in a group of unpaired prevertebral ganglia in the abdominal cavity. The only exception to this two-neuron arrangement, as mentioned above, is the innervation of the adrenal medulla. Sympathetic postganglionic endings or 'varicosities' synthesise norepinephrine (NE), the principal neurotransmitter, from L-tyrosine. This is present in the bloodstream and is a dietary constituent but can also be synthesised from phenylalanine in the liver. The pathway for the synthesis of norepinephrine is shown in figure 1.2.

1.1.3 Parasympathetic nervous system

Parasympathetic nerves leave the CNS in two separate regions. The cranial outflow consists of preganglionic fibres from the oculomotor, facial, and glossopharyngeal cranial nerves. The vagus nerve carries fibres to the abdominal and thoracic viscera. The parasympathetic ganglia are in close proximity to their respective target organs with the postganglionic fibres being very short in comparison to those of the sympathetic nervous system. Parasympathetic fibres destined for the abdominal and pelvic viscera leave the spinal cord in a bundle of nerves known as the nervi erigentes. These fibres spread to their respective ganglia supplying target tissues such as the bladder, rectum and genitalia. The

pelvic ganglia carry both sympathetic and parasympathetic fibres and the two divisions are not anatomically distinct in this area.

1.1.4 Autonomic nervous system overlap

In some areas (visceral smooth muscle of the gut and bladder and the heart) the sympathetic and parasympathetic systems produce opposite effects, but there are others where only one division of the autonomic system operates. Sweat glands and most blood vessels have only sympathetic innervation whilst ciliary muscles in the eye have only parasympathetic innervation. Bronchial smooth muscle has only parasympathetic (constrictor) innervation but its tone is sensitive to circulating epinephrine acting upon the constrictor innervation rather than directly upon the smooth muscle. However, in the salivary glands, the two systems have similar effects.

It is incorrect to think of the two branches of the autonomic nervous system as physiological opponents. Each has its own physiological function and can be more or less active in a particular organ a tissue depending upon the conditions. The role of the autonomic nervous system is to control specific functions from moment to moment (Janig & MacLachlin, 1992). The popular concept of 'rest & digest' and 'fight or flight' analogies is a crude oversimplification.

1.1.5 Neurotransmission and reuptake in sympathetic nervous system

Subsequent to an action potential, vesicles containing NE fuse with the plasma membrane in an intracellular calcium ($[Ca^{2+}]_i$ dependent manner. Once released from postganglionic synaptic terminals, NE has a number of possible fates. There are several subtypes of adrenergic receptors with which it may interact, β and α, having various effects (table 1.1). α_2 receptors are found on presynaptic terminals and perform a 'negative feedback' role. In conditions of excess NE, G_i coupled α_2 receptors act to inhibit further NE release by reducing calcium influx via second messenger systems. In contrast, α_1 receptors are stimulatory in nature and are generally present on the postsynaptic membrane, e.g. vascular smooth muscle (figure 1.3). NE is removed from the synapse by two possible mechanisms, neuronal norepinephrine transporter (NET) or uptake$_2$. Neuronal NET (formerly designated

as uptake$_1$) has the highest affinity for NE (K_m 0.5μM, V_{max} 17 ± 0.71 pmol min^{-1} mg^{-1}, Gu *et al.*, 1993), compared with uptake$_2$ (250 ± 80μM, 53pmol min^{-1} mg^{-1}, Fiebig & Trendelenburg, 1978). Uptake$_2$ is the extraneuronal NE transporter. Once back in a varicosity, NE may enter a vesicle via the vesicular monoamine transporter (VMAT) or become catabolised by monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT). In non-neuronal cells, NE is simply catabolised as they do not possess release mechanisms.

Figure 1.2 Steps in the synthesis of dopamine, norepinephrine and epinephrine. The enzymes involved at each stage are shown in italics.

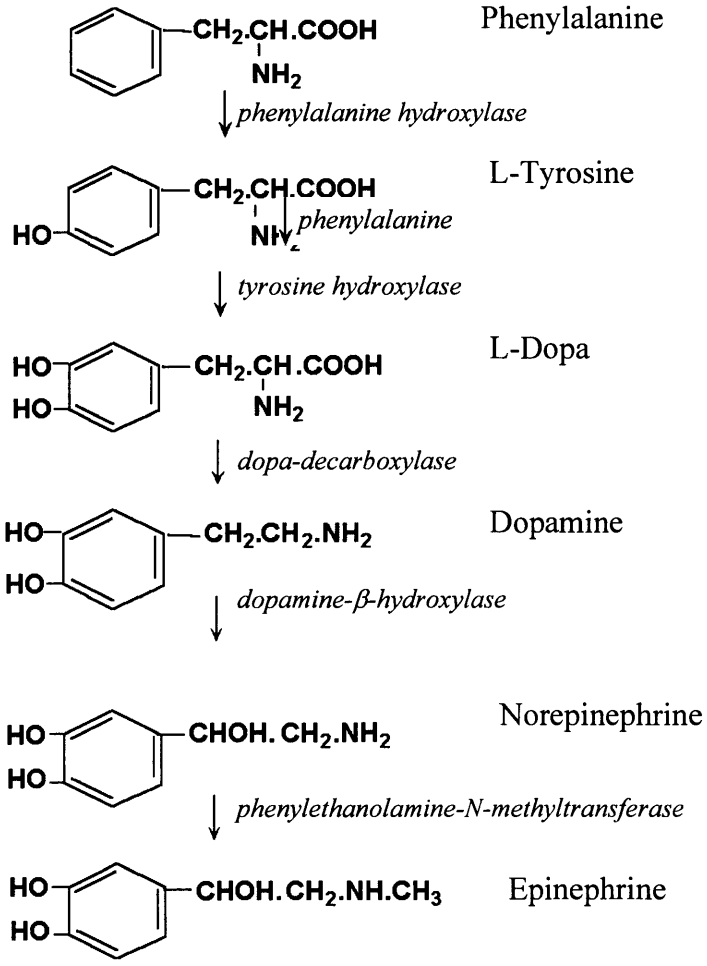


Table 1.1 Actions of the autonomic nervous system. From Rang, Dale and Ritter, 1995.

Organ	Sympathetic	Receptor	Parasympathetic	Receptor type
Heart				
SA node	rate ↑	β_1	rate ↓	M_2
Atrial node	force ↑	β_1	force ↓	M_2
AV node	automaticity ↑	β_1	cond. vel. ↓	M_2
			AV block	M_2
Ventricular muscle	automaticity ↑	β_1	No effect	
	force ↑			
Blood vessels				
Arterioles				
coronary	constriction	α		
muscle	dilatation	β_2	no effect	
viscera	}			
skin	} constriction	α	no effect	
brain	}			
erectile tissue	} constriction	α	dilation	M_3
salivary gland	}	α		
veins	constriction	α	no effect	
	dilation	β_2		
Viscera				
Bronchi				
smooth muscle	no symp. inne. dilated by circ. epinephrine	β_2	constriction	M_3
glands	no effect		secretion	M_3
GI tract				
smooth muscle	motility ↓	α_2, β_2	motility ↑	M_3
sphincters	constriction	α_2, β_2	dilatation	M_3
glands	no effect		secretion	M_3
			gastric acid ↑	M_1
Uterus				
pregnant	contraction	α	variable	
non-pregnant	relaxation	β_2		
Male sex organs	ejaculation	α	erection	? M_3
Eye				
pupil	dilatation	α	constriction	M_3
ciliary muscle	relaxation (slight)	β	contraction	M_3
Skin				
sweat glands	secretion	α	no effect	
pilomotor	piloerection	α	no effect	
Salivary glands	secretion	α, β	secretion	M_3
Lacrimal glands	no effect		secretion	M_3
Kidney	renin secretion	β_2	no effect	
Liver	glycogenolysis gluconeogenesis	α_2, β_2	no effect	

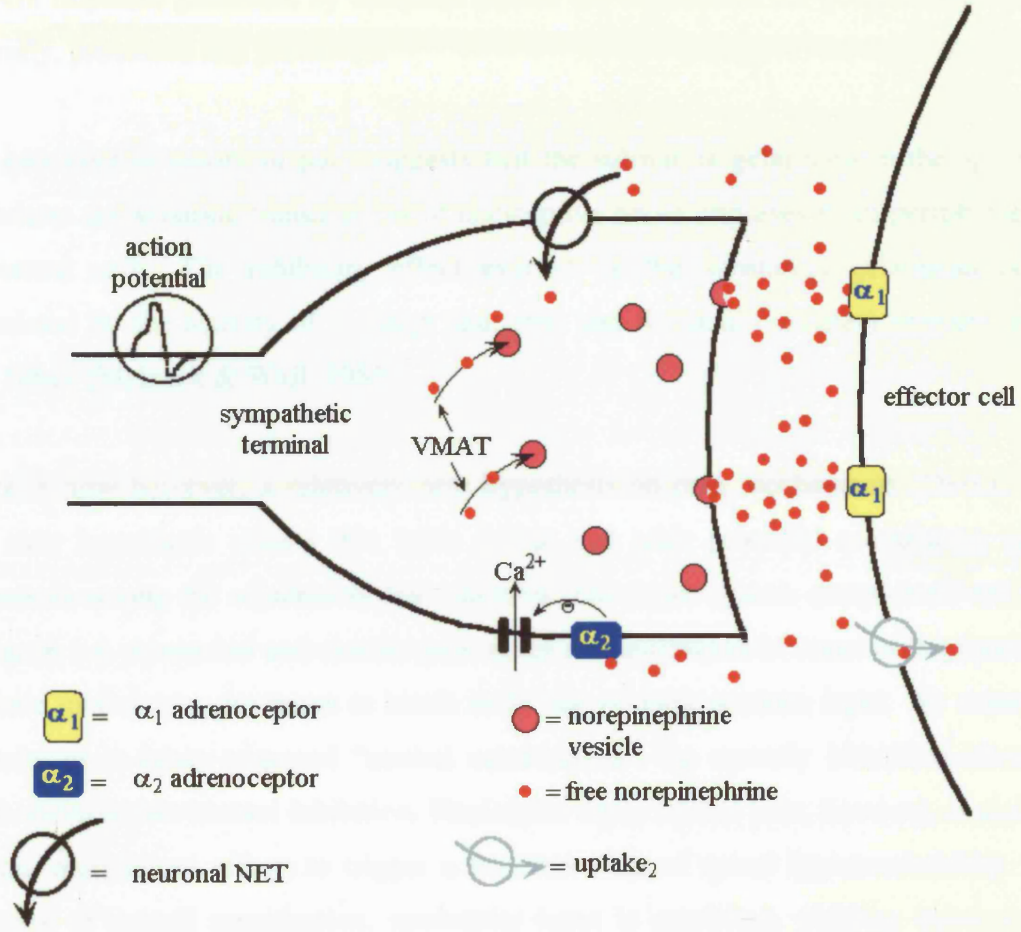


Figure 1.3 Schematic showing representation of release and reuptake of norepinephrine in sympathetic nerve endings. Also shown are the α adrenergic receptors, postsynaptic excitatory α_1 and presynaptic α_2 inhibitory. α_2 receptors provide negative feedback by inhibiting Ca^{2+} influx via 2nd messenger systems.

1.2 The Pain System

The pain system encodes information on the intensity, location and dynamics of strong, tissue-threatening stimuli. It is normally presented as a serial, "bottom up" system, where afferent impulses generated by adequate stimuli are encoded in the periphery, propagated centrally, processed and perceived.

The gate control theory of pain suggests that the substantia gelatinosa in the spinal cord modulates the synaptic transmission of nociceptive nerve impulses from peripheral fibres to central cells. The inhibitory effect exerted by the substantia gelatinosa is itself modulated by the activity of L (large diameter) and S (small diameter) primary afferent pain fibres (Melzack & Wall, 1965).

There is now however, a relatively new hypothesis on pain mechanisms (Devor, 1996). The new hypothesis retains this basic layout and adds powerful modulatory (gating) influences among the adjacent system modules. The major system components are shown in Figure 1.4. Abnormal and chronic pain states are understood in terms of the functioning of these modulatory processes as much as by the primary noxious input. An example of modulation is injury-triggered "central sensitisation", the recently identified obverse of touch-evoked gate-control inhibition. Nociceptor input signals pain, however, it also has a second, modulatory effect: to trigger a transient state of spinal hyperexcitability. In the presence of central sensitisation, nociceptor input is amplified, yielding increased pain (hyperalgesia). Low-threshold touch inputs are also amplified, so that light non-noxious touch evokes pain (allodynia). The fact that Aβ fibre mediated touch input can trigger pain is rather controversial, as Aβ fibres have long been thought to evoke touch and vibration, whereas Aδ and C input signals pain and temperature. Another implication of this "new synthesis" is that pain following tissue injury with inflammation, and pain following nerve injury (neuropathic pain) are basically alike. In both, pain results from nerve impulses arising abnormally in the periphery and from amplification of their central effects by the spinal hyperexcitability state that they, themselves, induce.

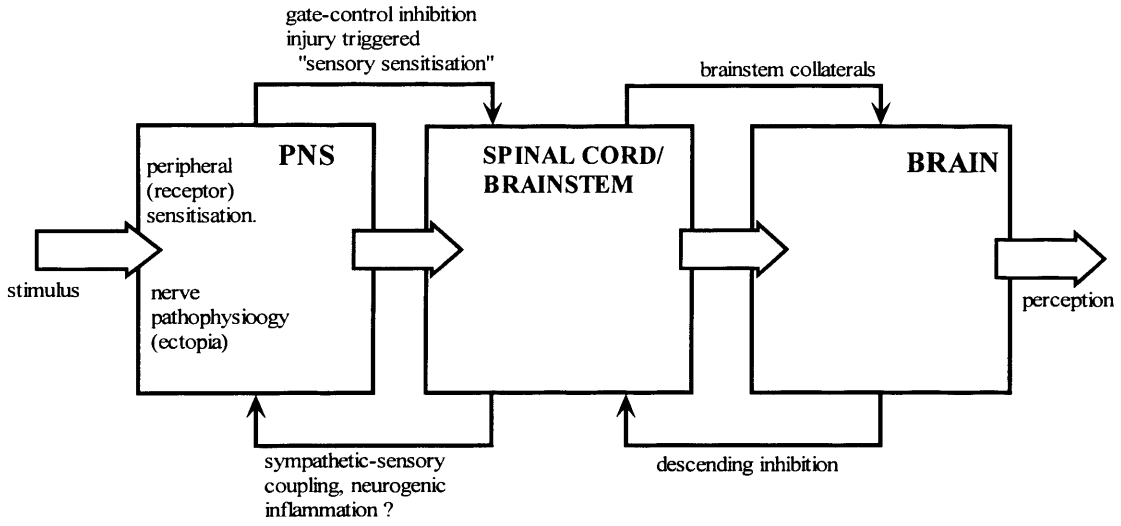


Figure 1.4 Block diagram indicating the major components of the pain system, and modulatory relations among them. This thesis is mainly concerned with the left side of the diagram. (from Devor, 1996).

1.3.1 Reflex Sympathetic Dystrophy & Complex Regional Pain Syndrome Type 1

Reflex sympathetic dystrophy (RSD) and its related disorders have been given several different titles over the years including; algodystrophy, post-traumatic dystrophy, sympathetic-maintained pain syndrome, causalgia, Sudeck's atrophy, and more recently, complex regional pain syndrome type I (CRPS type I). The syndrome has a complex and varied symptomatology, including persistent painful, swollen, discoloured, and abnormally sensitive limbs, (allodynia) most often developing after an injury of some kind, the pain being of unexpected severity (Gordon, 1996).

The sympathetic nervous system can be associated with pain in two ways. First it shows both generalised and specific localised reactions in response to noxious, tissue damaging events under control of the mesencephalon, hypothalamus, and suprahypothalamic brain structures and are understood as components of the confrontational defence "fight or flight" reaction (Bandler and Shipley, 1994; Janig, 1995). These pre-programmed elementary behaviours and their association with the endogenous control of analgesia enable the organism to cope with dangerous situations that are always associated with pain or impending pain. More localised and selective reactions of the sympathetic nervous system are organised within the spinal cord and periphery. They are associated with

somatosympathetic, viscerosympathetic and viscerovisceral reflexes as opposed to the adrenocortical system under the control of the hypothalamohypophyseal axis (Janig, 1985).

The sympathetic nervous system can be involved in pain following tissue damage with and without any obvious nerve lesion. This is sometimes followed by diffuse burning pain and hyperalgesia, which can sometimes be relieved by sympathetic efferent blockade. This pain can be correlated with changes in peripheral blood flow, sweating, trophic changes in skin, hair, nail fascia and bone growth, and movement including an increase in physiological tremor. These changes are thought to be related either directly or indirectly to the sympathetic nervous system. When the pain associated with these changes is relieved by sympathetic blockade it is deemed sympathetically maintained pain (SMP). However, to avoid the mechanistic assumptions of such terminology a descriptive term "complex regional pain syndrome" (CRPS) has been adopted (Janig & Koltzenburg, 1991). The following observations on patients with pain after peripheral trauma strongly support the hypothesis that efferent sympathetic nervous activity can be involved in pain generation.

- Pain is sometimes relieved following sympathetic blocks (by local anaesthetics applied to paravertebral ganglia (Bonica, 1990), or by other sympatholytic procedures such as intravenous regional guanethidine biers block (IVRGBB) (Hannington-Kiff, 1974b), or intravenous phentolamine (Arner, 1991; Raja *et al.*, 1991; Campbell *et al.*, 1992).
- Pain can be rekindled or enhanced by an alpha-adrenergic agonist applied to the affected extremity (e.g. iontophoretically through the skin or injected into the skin in patients with superficial burning pain and hyperalgesia) (Wallin *et al.*, 1976; Torebjork, 1990; Davis *et al,.* 1991; Wahren *et al.*, 1995). Injection of epinephrine around chronic nerve-end neuromas can elicit or enhance pain in humans (Chabal *et al.*, 1992).
- Guanethidine injected intravenously into the affected extremity initially elicits pain that is presumably generated by NE released from postganglionic terminals (Blumberg & Janig, 1994).
- Continuous electrical stimulation of decentralised thoracic sympathetic ganglia in conscious causalgic patients who had surgery reproducibly elicited the tingling and burning pain after latencies of 420 seconds (Walker & Nulsen 1948).

A descriptive and operational definition of RSD, now complex regional pain syndrome type I (CRPS I) was adopted at the VIth World Congress on Pain (Janig & Koltzenburg 1991). The term 'Reflex Sympathetic Dystrophy' was considered inappropriate mainly because it implies that the sympathetic nervous system is causally involved, and secondly that the clinical phenomenology occurs as a result of a reflex activation of sympathetic neurons. The syndrome formerly known as causalgia was designated as complex regional pain syndrome type II (CRPS II). The essential difference of the neuronal mechanisms of CRPS I and CRPS II is that the first may develop after trivial trauma without nerve lesion, the latter develops from a major nerve lesion. The criteria for CRPS I are the inclusion of regional pain (spontaneous and evoked) and other sensory changes following a noxious event. The pain is associated with changes in skin colour, skin temperature, abnormal sweating, oedema, and sometimes motor abnormalities. The noxious event may include minor trauma (e.g. distortion, bruising, or skin lesions), bone fracture, operations (e.g. of carpal tunnel, Dupuytren's contracture), and other lesions (e.g. shoulder trauma, myocardial infarction, or even contralateral cerebrovascular lesion.) An important feature is that the symptoms are often disproportionate to the inciting event and the site of the lesion at the limb does not determine the location of symptoms (Blumberg, 1988; Blumberg & Janig, 1994). Spontaneous pain is a prominent feature in most patients.

RSD poses two fundamental paradoxes. Firstly, the positive symptoms, including pain, with (partial) nerve injury when transection of a sensory axon would be expected to reduce sensations. Secondly, the involvement of sympathetic (efferent) system in the symptomatology of RSD. If the sympathetic system is an efferent system whose primary functions are to regulate autonomic target organs (e.g. blood vessels and glands), how do efferent fibres come to affect afferent (sensory) functions.

Pain in RSD appears to be a combined result of: the remodelling of membrane electrical properties in afferent neurones following axon injury, and altered signal processing in the spinal cord, triggered by the injury. "Sympathetic-sensory coupling" occurs in at least three different levels along the afferent neurone. The nerve injury site, the neuronal cell body in the dorsal root ganglion (DRG), and in peripheral tissue affected (i.e. partially denervated) by the nerve injury (Devor *et al.*, 1994).

Beginning within hours of axotomy, many injured afferents develop a global hyperexcitability that is expressed as spontaneous firing, and abnormal sensitivity to a broad range of depolarising stimuli: physical chemical and metabolic. The "Na^+ channel hypothesis of ectopia" attempts to account for this global hyperexcitability. Briefly, the model proposes that the neural mechanism that normally regulates local membrane channel and receptor density is disrupted following a lesion. As a result there is an ectopic increase in the level of voltage sensitive Na^+ channel protein and perhaps other membrane bound channel and receptor molecules. This is sufficient to initiate hyperexcitability and occurs in experimental nerve injury sites (Matzner & Devor, 1992; Devor *et al.*, 1993). Nerve injury leads to the disruption of regulatory factors from Schwann cells and elimination of many downstream targets of Na^+ channel insertion. Consequent "damming up" of these channels occurs in neuroma endbulbs and sprouts. Channel expression is also likely to be affected by nerve injury.

Sympathetic-sensory coupling is most likely to be chemical rather than physical (i.e. neurotransmitter(s)-mediated). Two mechanisms have been proposed, the direct and indirect coupling hypotheses. The direct coupling hypothesis suggests that NE released from sympathetic endings acts on α-adrenoceptors in the membrane of injured afferents, resulting in depolarisation, sensitisation and impulse firing (Devor, 1983). This hypothesis is based on the observations that sympathetic efferent stimulation evokes ectopic afferent activity, which is blocked by α-adrenoceptor antagonists (Devor & Janig, 1981; Blumberg & Janig, 1984; Habler *et al.*, 1987). The indirect coupling hypothesis holds that the NE released does not act directly on afferents, but rather on presynaptic α-adrenoceptors (probably α_2) on the sympathetic efferent endings themselves (Levine *et al.*, 1986). This triggers the release of prostanoids (e.g. PGI_2) which, in turn, sensitise and excite afferents and induce hyperalgesia. Indeed, research shows that α_2 responses are predominant in injured afferent endings of ligated and cut nerves, DRG neurons and other nerve preparations (Chen Y *et al*, 1996; Sato & Perl, 1991; Perl, 1994; Xie *et al.*, 1995; Sato *et al.*, 1993; Levine *et al.*, 1986; Khasar *et al.*, 1995; Taiwo *et al.*, 1990; Tracey *et al.*, 1995).

1.3.2 Diagnosis of CRPS

The diagnosis of CRPS is not only by identification of the above clinical symptoms. It is important to determine whether and which components of pain may be related to sympathetic activity. There exists a number of diagnostic tests for sympathetically maintained pain (SMP). These tests include sympathetic blocks with local anaesthetics, guanethidine block and phentolamine test.

Temporary sympathetic blocks may be performed by applying local anaesthetics ipsilaterally to the stellate ganglion or the lumbar paravertebral sympathetic ganglia, respectively (Bonica, 1990). A complete sympathetic block is assumed when the temperature of the ipsilateral finger or toe tips increases to ≥35°C. Temporary relief of pain indicates that the efferent sympathetic system may be involved in the generation of pain.

Blockade of the sympathetic nervous system using regional guanethidine may be used as a diagnostic test to prove SMP (see 1.4.2).

Arner (1991) and Raja *et al.* (1991) independently introduced the phentolamine test as a tool for diagnosis of CRPS. As phentolamine is an α-adrenoceptor antagonist (α_1, α_2), it relieves the pain induced by norepinephrine release from postganglionic terminals. During intravenous infusion of phentolamine, pain is measured continuously by visual analogue scale. However, phentolamine is only a short acting antagonist. If pain is reduced, the sympathetic nervous system is likely to be involved in the generation of pain (Arner, 1991). A potential problem with this technique is that it cannot be effectively blinded because intravenous administration of phentolamine causes a temporary drop in blood pressure (BP) coupled with a compensatory tachycardia. Perhaps regional administration using a suprasystolic pneumatic cuff would be more appropriate and could be blinded in a clinical study with normal saline.

1.4.1 Types of sympathetic block

Table 1.2 shows the treatments that have been recommended for CRPS I over the past 135 years (from Schwartzman and McClellan, 1987).

Cold, wet compresses
Hot wax applications
Anti-inflammatory agents
Radiation therapy
Ultrasound to the stellate ganglion
Hypnosis
Electroacupuncture
Immobilisation with cast or splint
Calcitonin
Thalamotomy
Physical therapy
Corticosteroids
Transcutaneous nerve stimulation
Propranolol
Phenoxybenzamine
Bier block with guanethidine sulphate (+/- local anaesthetic)
Bier block with reserpine (+/- local anaesthetic)
Bier block with lidocaine hydrochloride and corticosteroids
Paravertebral sympathetic ganglion block
Continuous paravertebral sympathetic ganglion block
Periarterial sympathectomy
Paravertebral sympathetic ganglionectomy

Table 1.2 shows the treatments that have been recommended for CRPS I over the past 135 years. All therapies that have proved effective are aimed at blocking the effects of sympathetic hyperactivity. Other treatments may have limited successes but casting or immobilisation has been proven to exacerbate the problem (Fam & Stein, 1981; Goldner 1980). In contrast, physical therapy alone has been shown to be effective in the treatment of CRPS I (Schumacker & Abramson, 1949; Bernstein *et al.*, 1978). Exercise (table 1.2) is directed toward improving the mobility of the affected extremity. However, due to the level of pain, adequate pain relief should be obtained before the activity. Transcutaneous electrical nerve stimulation (TENS) is postulated to relieve pain by an artificially generated barrage of nerve impulses in large axons (Szeinfeld & Palleres, 1983). Various studies indicate that prolonged treatment with high dose corticosteroids may be beneficial in the treatment of CRPS I. Corticosteroids should be considered for patients who refuse or cannot tolerate treatments that block sympathetic activity.

Phenoxybenzamine, an α antagonist, has been used as an effective treatment for acute (less than 6 weeks, treated with 40-120mg for six to eight weeks) CRPS I. However, orthostatic hypotension was a major reported side effect due to the inability to control blood pressure by peripheral vasoconstriction (Ghostine *et al.*, 1984).

Homans (1940) was the first to describe the use of paravertebral sympathetic ganglion blockade to treat CRPS. In the late 1980's, paravertebral sympathetic ganglion block was the most widely recommended treatment for CRPS (Schwartzman & McClellan, 1987). Overall, serial sympathetic ganglion blocks lead to definite, if transient, improvement in most patients and are probably more effective than administration of systemic corticosteroids. To achieve prolonged paravertebral sympathetic blockade, indwelling catheters have been utilised for infusion of local anaesthetic (Betcher *et al.*, 1953).

Periarterial sympathectomy has been used (Rasmussen & Freedman, 1946) successfully to treat CRPS but is less effective than paravertebral ganglionectomy. Surgical paravertebral ganglionectomy is recommended for those patients who only show transient relief with ganglion blocks (DeTakats & Miller, 1943; Spurling, 1930). Erdemir *et al.* (1982) suggest that in patients with CRPS of the lower extremities, epidural sensory blocks should be performed to determine the exact level of ganglionectomy required for complete pain relief.

1.4.2 Bier's blocks

Bier's block is a technique used for regional anaesthesia. The limb is elevated and isolated from the systemic circulation by a tourniquet (or suprasystolic pneumatic cuff). An anaesthetic or other substance is then injected into the limb intravenously for 5 to 15 minutes, after which the tourniquet is removed. Poplawski *et al.* (1983) used Bier blocks with lidocaine and hydrocortisone sodium succinate in CRPS I patients. In the 28 subjects studied, 57% improved to the point where only occasional, if any, analgesics were required. Best results occurred in patients who had had symptoms for less than 9 months and most required a series of two to three injections for prolonged relief.

Intra-arterial reserpine (table 1.2) has been used effectively in the relief of pain and vasospasm associated with Raynaud's phenomenon, frostbite and CRPS (Chuinard *et al.*, 1981; Romeo *et al.*, 1970). Reserpine interferes with the storage of norepinephrine, thereby causing its gradual depletion in nerve endings. In contrast to guanethidine, reserpine inhibits uptake into the amine storage vesicles and has little or no effect upon the norepinephrine transporter (NET). However, due to its high lipid solubility it easily crosses the blood brain barrier causing central depression and behavioural effects such as, hypokinesis, hypothermia and sedation. Piloerection, miosis and diarrhoea are also common.

A method for providing specific regional sympathetic blocks was developed by Hannington-Kiff (1974b) who used Bier blocks performed with 10 to 20mg of guanethidine sulphate. Guanethidine displaces norepinephrine in presynaptic vesicles and prevents its re-uptake (Maxwell, 1982). Excellent pain relief has been reported for this by many authors (Glynn *et al.*, 1981; Tabira *et al.*, 1983; Loh *et al.*, 1980). However, the results of guanethidine treatment vary greatly with pain relief lasting from 12-36 hours but can be as long as 6 months. Repeated treatments are recommended with studies ranging from one to greater than 20 blocks administered over a period of 1 day to several months (Tanelian, 1996). Kingery (1997) claims that there is consistent evidence that guanethidine and reserpine IVRBB's are ineffective analgesics for CRPS. Like Bier's block with local anaesthetic, the prognosis is generally more positive when symptoms have lasted less than 6 months.

The procedure itself involves exsanguination of the affected limb before inflation of a suprasystolic pneumatic cuff. Intravenous infusion of combined guanethidine and prilocaine follows with the cuff maintained for 20 minutes before being slowly released. In upper extremities, 10mg guanethidine in 30ml 0.5% prilocaine and lower extremities, 20mg in 50ml of 0.5% prilocaine is infused. Local anaesthetic is included in order to reduce the discomfort that guanethidine infusion evokes.

1.5 Guanethidine

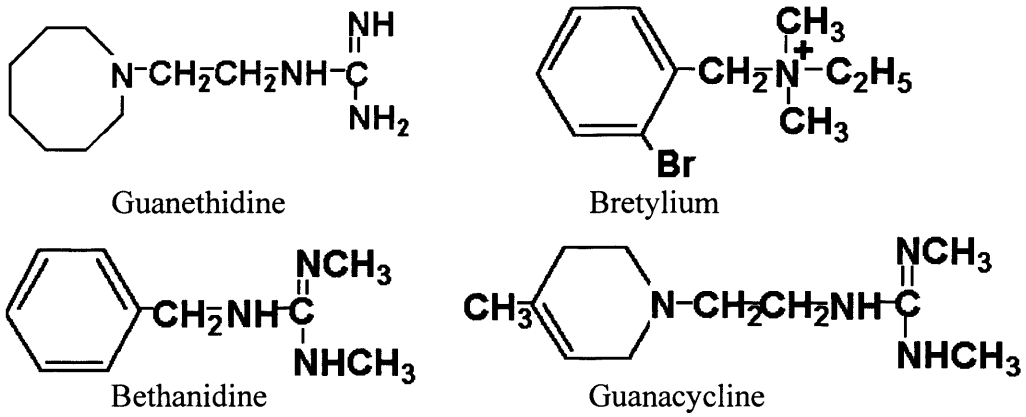


Figure 1.5 Structure of guanethidine and some other adrenergic neuron-blocking agents.

Figure 1.5 shows the structure of guanethidine and some of its related adrenergic neuron blocking drugs. It is well established that guanethidine accumulates in adrenergic neurons via the norepinephrine transporter (Iversen, 1967;1971). Once inside the cell, guanethidine becomes sequestered into the amine storage vesicles. This sequestration into the vesicles displaces norepinephrine where it is either removed via oxidative deamination by monoamine oxidase (Maxwell & Wastila, 1977; Giachetti & Shore, 1967) or is simply released into the synapse. This release is sufficient, following intravenous administration of guanethidine to lead to sympathomimesis in many effector cells. Guanethidine caused a rise in the [^{3}H]-norepinephrine in the splenic outflow following its injection into the cat splenic artery. However, this action is short in comparison to the duration of subsequent neurone blockade (Hertting *et al.*, 1962). The main difference between guanethidine and its related compounds like bretylium and debrisoquine is that it does not inhibit MAO. The intraneuronal norepinephrine displaced by these compounds is therefore preserved for re-uptake into vesicles, with the result that depletion is much less marked than with guanethidine (Boura & Green, 1984). It is estimated that guanethidine accumulates within the adrenergic nerve endings as compared with its concentration in the extracellular space by a ratio of 1,000 to 3,000:1 (Hausler, 1971). This fact coupled with the observation that guanethidine is a relatively weak local anaesthetic agent (Bein, 1960), supports the idea that it may produce a depressant effect on the adrenergic terminals via a local anaesthetic action (Boura *et al.*, 1960). Chronic guanethidine treatment (50mg per kg body weight per day for ten days) promotes selective and extensive degeneration of postganglionic

sympathetic neurons in the rat, presumably as a result of its detrimental effect upon intracellular respiration (Burnstock *et al.,* 1971; Eranko & Eranko, 1971). Guanethidine (5-25μM) was found to inhibit mitochondrial electron transport in rat brain, heart and liver mitochondrial preparations (Ferreira *et al.*, 1984).

1.6 Twelve membrane spanning transporters

1.6.1 Norepinephrine transporter

The norepinephrine transporter is a member of the 12 membrane spanning sodium ion dependent gene super-family (Worrall & Williams, 1994). These transporters are the principal means by which neurotransmitters in the synaptic cleft are removed, with the exception of acetylcholine, which is inactivated by synaptic enzymatic degradation. NET is widely distributed throughout the mammalian brain and is inhibited by tricyclic antidepressants, amphetamines and cocaine. Studies on tissue slices, synaptosomes, cultured cells and resealed membranes show that NETs require millimolar concentrations of extracellular Na^+ (Iversen & Kravitz, 1966; Bogdanski & Brodie, 1966; Sammet & Graefe, 1979; Harder & Bönisch, 1985; Bönisch & Harder, 1986) and millimolar concentrations of Cl^- (Sánchez-Armáss & Orrego, 1977; Friedrich & Bönisch, 1986; Ramamoorthy *et al.* 1993*a,b*) in order to function. There is a transport coupling ratio of $1Na^+$:$1Cl^-$:$1NE^+$ (Friedrich and Bönisch, 1986; Ramamoorthy *et al.*, 1993*a,b*). This co-transport explains the influx of catecholamines against amine concentration gradients (Stein, 1986; Kanner & Schuldiner, 1987; Lauger, 1991) coupling NE movement to the energetically favourable influx of Na^+ and Cl^- down their respective concentration gradients at a fixed stoichometry. This suggests a single positive charge translocation inwards for each cycle. Inward fluxes have been demonstrated in clonal cell lines with both NE and importantly guanethidine (Galli *et al.*, 1995). The hNET (human norepinephrine transporter) has also been shown to have channel modes of conduction blocked by cocaine and antidepressants (Galli *et al.*, 1995). Pacholczyk *et al.,* (1991) made an important breakthrough, employing an expression cloning strategy to isolate the cDNA from the human NET.

In order to sequence the gene responsible for NET proteins, expression cloning was performed. This is a complex process involving insertion of complementary DNA (cDNA) libraries from cells expressing NET into a bacterial vector plasmid, selection for the

protein required and then sequence analysis of the gene responsible. In the case of hNET expression cloning, the following protocol was used (Pacholczyk *et al.*, 1991).
Pools of clones from a human SK-N-SH cell (Biedler *et al.,* 1973) cDNA library were transfected into COS-1 cells and transfectants expressing the norepinephrine transporter were identified. The norepinephrine analogue m-iodobenzylguanidine ([^{125}I]mIBG) (Buck *et al.,* 1985) is accumulated intracellularly by SK-N-SH cells through the norepinephrine transporter (Richards & Sadee, 1986) allowing autoradiographic visualisation of transporter expressing transfectants. DNA was rescued from positive COS-1 transfectants by Hirt lysis (Hirt, 1967) and the resulting plasmid pools re-screened and subdivided until a single clone was obtained. Sequence analysis of the cloned cDNA predicts a protein of 617 amino acids with a relative molecular mass of ~69KDa. The transporter was subsequently stably transfected into HEK-293 (human embryonic kidney) cells to give the 293-hNET (human norepinephrine transporter) clonal cell line (Galli *et al.*, 1995).

Hydropathy analysis predicts 12 membrane spanning domains, cytoplasmic N- and C-termini and a large extracellular loop containing three potential glycosylation sites (figure 1.6). The sequence was found to have similarities (48% homology) with the GABA (γ-aminobutyric acid) transporter (Pacholczyk *et al.*, 1991). Chimera and site-directed mutagenesis studies place critical residues for substrate stereospecificity and binding in the transmembrane domains (TMDs) nearest to the NH_2 and COOH terminus, whereas the fundamental mechanisms governing transport appear to lie between TMDs 5 and 9 (Giros *et al.,* 1994; Buck & Amara 1994; 1995). NET and serotonin transporter (SERT) chimeras with interchanged NH_2 and COOH termini (tail chimeras) maintained antagonist recognition. However, a chimera formed within the NET TMD1, just a few amino acids distal to the site utilised by a functional NET chimera bearing a SERT NH_2 terminus, loses NE transport activity (Moore & Blakely, 1994). NET antibodies verify that both the tail and the TMD1 chimera are synthesised at equivalent levels and migrate appropriately in SDS-PAGE (Poly-acrylamide gel electrophoresis). These findings implicate residues of TMD1 in folding, plasma-membrane targeting or NE recognition. A NET mutation in TMD 9 has been associated with familial orthostatic intolerance, reinforcing the contribution of NETs to noradrenergic signalling (Shannon *et al.*, 2000). As much as 80-90% of released NE may be recaptured and re-released, which suggests a role for NETs in synaptic economy (Schomig *et al.*, 1989). Specific NET antibodies have been produced

and utilised to identify 54 and 80-kDa bands in immunoprecipitation and immunoblots of stably transfected cells as recognised by a NET specific antibody, N430 (Melikian *et al.*, 1994). The two bands identified in the cell line studies correspond to a biosynthetic intermediate form of hNET (54kDa) and the mature N-glycosylated hNET protein (80kDa), apparent from the progression of label in 'pulse-chase' studies (Melikian *et al.*, 1996). In rat brain tissue, only a single 80kDa band was found, suggesting the predominance of fully processed forms of NET in native tissue (antibody specificity to both rodent and human forms) (Kippenberger *et al.*, 1999).

Kitayama *et al.* (1992) presented mutagenesis data suggesting a critical role for conserved TMD1 Asp and TMD7 serine residues in DATs (dopamine transporters) for dopamine and/or cocaine recognition. Mutagenesis of the TMD1 Asp residue is also found to reduce or greatly reduce NE or 5-HT transport respectively in NET and SERT (Melikian *et al.*, 1993). Charge-charge interactions between the TMD1 Asp residue and the protonated NH_2 group of catecholamines, and hydrogen bonding of catechol OH groups with TMD7 serine residues are proposed sites for catechol recognition. This model is analogous to that suggested for catecholamine recognition by β-adrenergic receptors (Strader *et al.*, 1989). Antibody experiments verify that NET and SERT mutations eliminating or reducing function do not do so because of an inability to synthesise transporter protein, suggesting structural changes affecting ligand recognition (Blakely *et al.*, 1994).

N-glycosylation to the 80kDa form of hNET appears to promote physical maturation and surface targeting of biogenic amine transporters. Data from Tate & Blakely (1994) suggest that N-glycosylation itself may play little or no role in direct ligand recognition, but rather sugar addition may increase the probability that transporter proteins fold properly and/or are protected from degradation so that they can be efficiently trafficked to the plasma membrane.

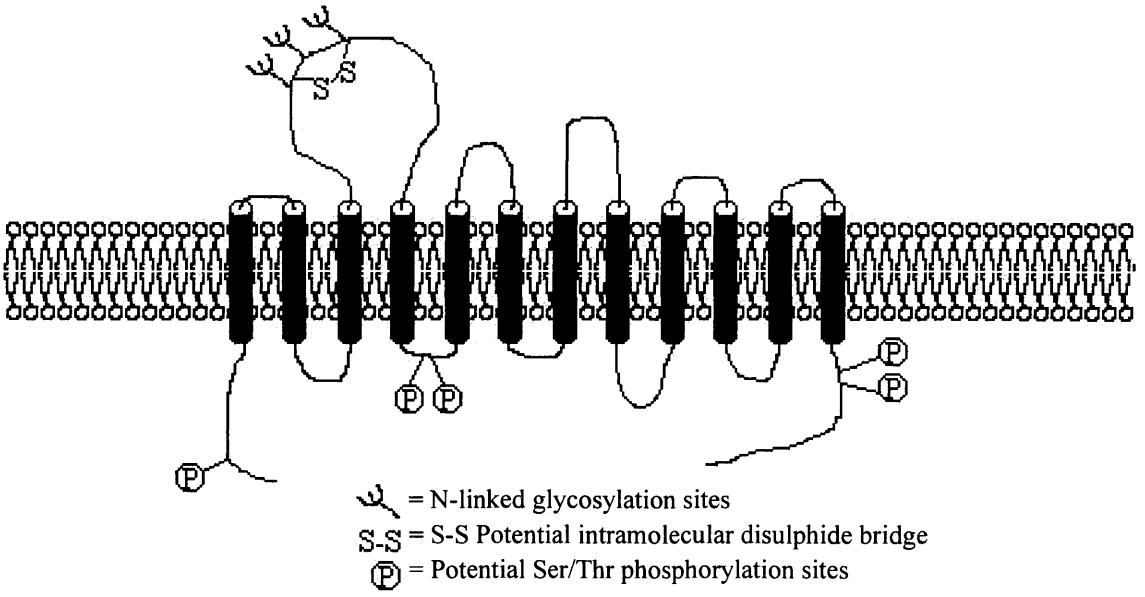


Figure 1.6 Predicted topological structures of plasma membrane NETs. NET is predicted to span the membrane with 12 transmembrane domains (TMDs). NET bears a large hydrophilic loop between TMD3 and TMD4, within which are the only canonical sites for N-linked glycosylation. These glycosylation sites have recently been shown to be glycosylated in NET in transfected cells (Melikian *et al.*, 1994). Potential sites for serine or threonine protein phosphorylation are also indicated. Figure adapted from Blakely *et al.* (1994).

Table 1.3 Properties of some transporter proteins that have been cloned and sequenced.

Transporter (substrate)	Tissue	Amino Acids	M_r (kDa)	Glycosylation sites	Reference
GAT1 (GABA)	human brain	599	67.00	3	Nelson *et al.*, 1990
NET (NE)	human brain	617	69.00	3	Pacholczyk *et al.*, 1991
SERT (serotonin)	rat brain	630	70.17	2	Blakely *et al.* 1991
DAT (dopamine)	rat brain	619	69.00	4	Shimada *et al.*, 1992
DAT (dopamine)	bovine brain	693	75.00	3	Usdin *et al.*, 1991
choline	rat spinal cord	635	70.63	2	Mayser *et al.*, 1992
glycine	rat brain	573	64.00	2	Guastella *et al.*, 1992
glutamate	rat brain	573	64.00	2	Pines *et al.*, 1992

1.6.2 Uptake 2

As well as neuronal norepinephrine uptake by NET, there exists an extraneuronal norepinephrine transporter. This transporter is conventionally known as $uptake_2$ (neuronal NET was formerly known as $uptake_1$). Extraneuronal uptake by the rat heart was shown to be a saturable process obeying Michaelis-Menten kinetics. The K_m was high (250μM) and may be interpreted as indicating that extraneuronal uptake does not play any important role in the removal of norepinephrine from the extracellular space, when the concentration of the amine is low. Once taken up by $uptake_2$, the norepinephrine is broken down by catechol-o-methyltransferase (COMT) and MAO to 3-methoxy-4-hydroxymandelic acid (Fiebig & Trendelenburg, 1978).

1.6.3 The vesicular monoamine transporter

Prior to secretion, monoamines are concentrated from the cytoplasm into vesicles by vesicular monoamine transporters (VMAT). These transporters can also carry non-physiological compounds such as guanethidine (Henry *et al.*, 1994). VMAT proteins are predicted to possess 12 transmembrane segments, with both ends lying on the cytoplasmic side. They possess N-glycosylation sites in a putative luminal loop and phosphorylation sites in cytoplasmic domains. Two homologous but distinct genes for VMAT have been cloned from rat, bovine and human adrenal glands. It is thought that the two subtypes are addressed specifically to large secretory granules and small synaptic vesicles reflecting distinct physiological roles of each type of vesicle. VMATs act as electrogenic antiporters of protons and monoamines, using a proton electrochemical gradient generated by a vacuolar ATP-dependent H^+ pump (V-ATPase) on the same membrane. In chromaffin granules, VMAT is very efficient, developing catecholamine gradients of five orders of magnitude (Henry *et al.*, 1994).

1.7 SH-SY5Y cells

SH-SY5Y cells are human neuroblastoma cells derived from the sympathetic nervous system and therefore form an *in vitro* model of sympathetic postganglionic neurons. These cells express several properties of mature sympathetic neurones without the need to add

additional chemicals to the culture medium (Vaughan *et al.*, 1995). Examples of sympathetic characteristics expressed by SH-SY5Y cells are, $[Ca^{2+}]_i$ dependent release of NE (Sudhof & Jahn, 1991), uptake of NE (Murphy *et al.*, 1991a), depolarisation by K^+, veratridine or activation of nicotinic receptors (Murphy *et al.*, 1991b; Vaughan *et al.*, 1993), functional L- and N-type Ca^{2+} channels (McDonald *et al.*, 1994), active μ-opioid receptors and α_2-adrenoceptors (Lameh *et al.*, 1992) and κ_3-opioid receptors (Cheng *et al.*, 1995).

1.8 Other Drugs acting on NET

1.8.1 Nisoxetine

Until 1990, uptake sites for NE in the brain were not extensively studied compared to uptake sites for 5 hydroxytryptamine (5-HT, or serotonin), due in part to a lack of available radioligands for the NE uptake site (Tejani-Butt *et al.*, 1990). [^{3}H]desipramine ([^{3}H]DMI) (Langer *et al.*, 1981; Lee *et al.*, 1982; Rehavi *et al.*,1982) and [^{3}H]mazindol ([^{3}H]MAZ) (Javitch *et al.*, 1984) have been used to measure uptake sites for norepinephrine in the brain of the rat. However [^{3}H]DMI exhibits a high non-specific binding and also binds to a low affinity site (Lee *et al.*, 1982; Backstrom *et al.*,1989), whereas [^{3}H]MAZ also binds to uptake sites for dopamine (Javitch *et al.*, 1984). [^{3}H]nisoxetine binding is Na^+ dependent (Tejani-Butt, 1992). This dependence is in common with [^{3}H]MAZ (Lee *et al.*, 1982; Rehavi *et al.*, 1982) and [^{3}H]DMI (Javitch *et al.*, 1984). Nisoxetine is displaced by potent inhibitors of NE uptake, (DMI, MAZ and maprotiline) (Tejani-Butt, 1992). Compounds which potently and selectively inhibit uptake of 5-HT (fluoxetine) or dopamine (GBR 12909) greater than that of NE yield K_i values of nisoxetine displacement from rat cerebrocortical membranes of >1μM (Lee *et al.*, 1982, Backstrom *et al.*, 1989). This suggests that specific binding of nisoxetine is associated with NE uptake sites and not 5-HT or DA uptake sites. In comparison with [^{3}H]DMI, [^{3}H]nisoxetine has much lower non-specific binding. At double it's K_d concentration, nisoxetine has about 80% specific binding whereas [^{3}H]DMI is less than 40%. This high selectivity and low non specific binding makes it the radioligand of choice for studies of uptake sites for NE (Tejani-Butt *et al.*, 1990).

1.8.2 Imipramine

Imipramine is a member of the tricyclic antidepressants group of drugs including amitryptiline, nortryptiline and desipramine. It exerts its antidepressant effect by preventing the removal of released amines from the extracellular space by uptake transporters. It is not specific to NET as it also inhibits SERTs. It is a useful drug for determining the non-specific binding and uptake of amines as it displaces from and inhibits monoamine uptake transporters. The tricyclics get their name from their 'three ringed' structure (Baldessarini, 1990) (figure 1.7).

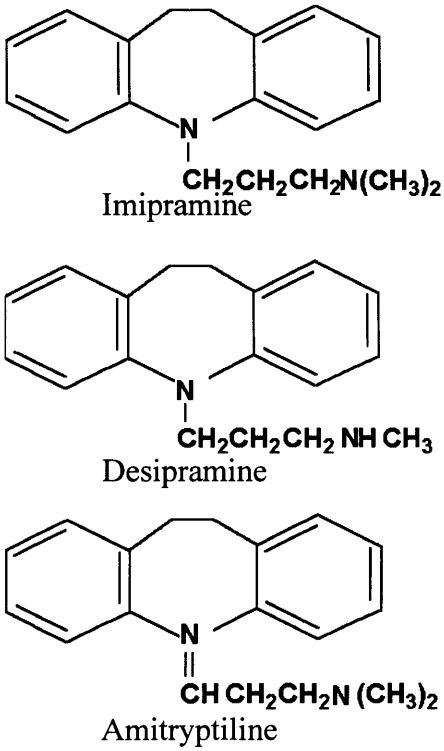


Figure 1.7 Structures of the common tricyclic antidepressant agents imipramine, desipramine and amitryptiline.

1.8.3 Cocaine

Cocaine (figure 1.8) is a naturally occurring substance present in the leaves of *Erythroxylon coca,* a plant indigenous to South America, where extracts of the leaves were taken to produce a sense of wellbeing and euphoria (Van Dyke & Byck, 1982). Cocaine is a local anaesthetic (see section 1.9), but it also inhibits the reuptake of norepinephrine therefore prolonging its action. Clinical effects include mydriasis, increased heart rate and blood pressure, and pale skin (Gay, 1982). As cocaine also blocks norepinephrine uptake in the CNS, this action is responsible for the euphoria produced (Van Dyke & Byck, 1982). Its sympathomimetic and local anaesthetic effects have made it useful as a topical

anaesthetic which also reduces bleeding in nasal surgery (Albers, 1990). It is this inhibition of norepinephrine uptake which forms the main hypothesis of this thesis (see 1.10).

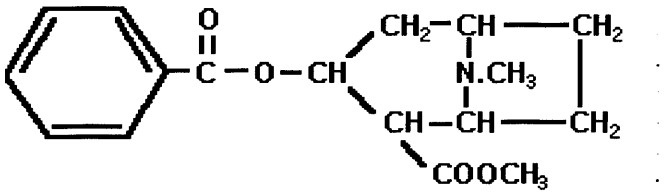


Figure 1.8 Chemical structure of the local anaesthetic and norepinephrine uptake inhibitor cocaine.

1.9.1 Local anaesthetic pharmacology

Local anaesthetic agents cause a reversible block of the conduction of impulses in the peripheral nervous system. Strictly speaking, 'anaesthesia' implies loss of consciousness whereas 'analgesia' refers to pain control. When the term 'local' is applied to anaesthesia, no loss of consciousness is implied. A more correct term would be 'local analgesia' but 'local anaesthesia' is in universal common use.

The first local anaesthetic agent was the ester cocaine, and was introduced to clinical practice by Koller (1884), who described its topical anaesthetic properties for ophthalmological use. In England the dentist William Hunt first injected cocaine in 1886. The problems associated with cocaine; addictiveness, variable efficacy (as a plant extract subject to climatic variations), and its tendency to cause tissue sloughing led to the development of a synthetic injectable local anaesthetic, procaine. Procaine was synthesised in 1905 by Einhorn followed by amethocaine chloroprocaine and in 1943, Lofgren synthesised lidocaine, the first of a new class of amide local anaesthetics, (cited in Atkinson, 1987).

Most of the clinically useful local anaesthetics have a similar basic structure consisting of a lipophilic aromatic portion and a hydrophilic amine group linked by an intermediate chain. The linkage can be ester or amide and each forms a distinct chemical group (figure 1.9), Those with an amino-ester linkage include procaine, tetracaine, benzocaine, chloroprocaine and cocaine and are ester derivatives of para-aminobenzoic acid. Those with the amide

linkage include lidocaine, mepivacaine, prilocaine, bupivacaine, etidocaine and dibucaine. Their molecular masses range from 220 to 288 (Courtney & Strichartz, 1987). Ester type local anaesthetic agents generally have relatively short duration of anaesthesia because they are unstable in solution and are readily metabolised by plasma and tissue esterases which are ubiquitous. In contrast, amide type local anaesthetics are metabolised exclusively by liver amidases. Recovery from anaesthesia by amide agents is by redistribution from the injection site into the circulation. Amide type local anaesthetics have virtually replaced esters as injectable agents. However, some esters such as benzocaine and cocaine have limited use as topical agents. Esters have limitations due to their propensity to be highly allergenic and also interfere with the bacteriostatic action of sulphonamides.

The pharmacological actions of local anaesthetics are:

- reversible block of neural conduction in nerve fibres and nerve endings.
- relaxation of smooth muscle (e.g. vasodilatation).
- reversible block of skeletal muscle end-plate transmission (usually clinically insignificant).
- inhibitory or antiarrythmic actions on the heart.
- mixed features of both stimulation and depression of the central nervous system due to a common action of local anaesthetic drugs on cell membranes.

Important properties and considerations when using local anesthetics are; reversibility, rapidity of onset, stability, penetration, duration, freedom from unwanted effects and metabolism.

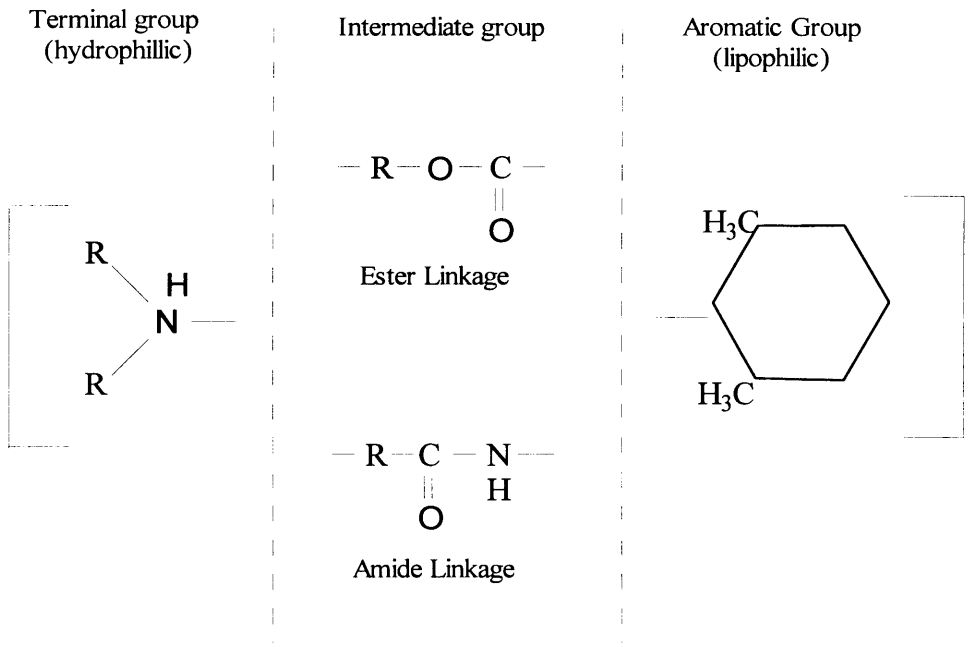


Figure 1.9 Molecular configuration of local anaesthetics. NB. Carbon atoms shown in the aromatic group are integral to the ring structure.

Three distinct sites of action have been proposed where local anaesthetics might produce their effect upon Na^+ conductance in excitable membranes (Ritchie, 1975).

1. On the membrane surface, involving alteration of the fixed negative charge and hence transmembrane potential, without change in resting intracellular potential.

2. Within the membrane matrix, involving its lateral expansion, thereby causing distortion of the sodium channel.

3. Specific binding sites within the sodium channel.

The specific binding site theory seems compatible with most experimental findings (Covino, 1980): e.g.,

- Benzocaine, which is completely non-ionised, is active.
- Ionised forms act upon the internal surface of the axonal membrane.
- Some optical isomers show differential activity.
- Varying frequency of nerve stimulation can alter the level of local anaesthesia.

Furthermore a model of a single site of action has been developed involving three conformational states of the sodium channel (figure 1.10).

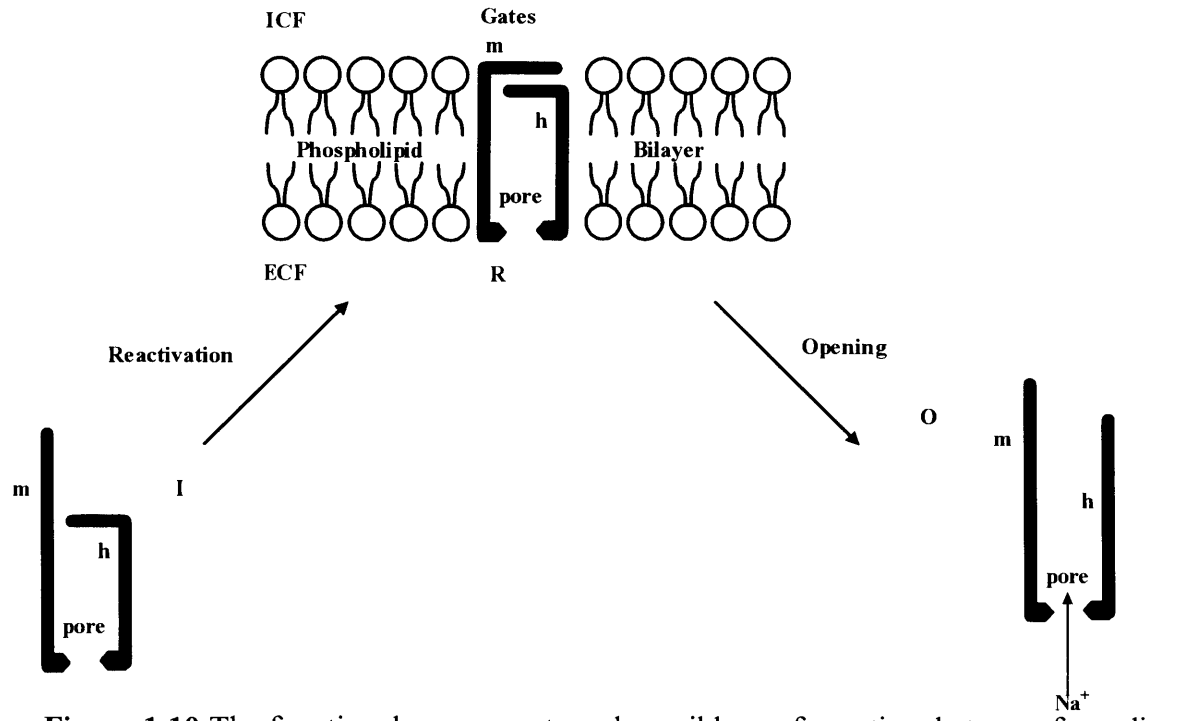


Figure 1.10 The functional components and possible conformational states of a sodium channel. R, resting state; ICF, intracellular fluid; ECF, extracellular fluid; m, activation gate; h, inactivation gate (Hille, 1977).

1 Resting state (R), in which sodium activation and inactivation gates (m and h respectively) are closed and which predominates before nerve stimulation.

2 An open state (O), where both gates are open allowing free flow of ions. This is the main state during depolarisation.

3 An inactive state (I) where only the inactivation gate (h) is closed immediately following stimulation and the activation gate (m) is open. This state underlies the initial phase of repolarisation and the refractory period.

Local anaesthetics have a higher affinity for Na^+ channels in their inactivated state and prevent electrical conduction in the open state. Non-ionised, lipophilic molecules of local anaesthetic are capable of binding in all three states via diffusion through the lipid membrane. Ionised hydrophilic species are only able to gain access to the binding site through the open h-gate when the channel is in the open state (Hille, 1977).

An increase in the frequency of nerve stimulation has been shown to enhance the level of block of local anaesthetics on sodium conductance (Strichartz, 1973; Courtney, 1975). If

an *in vitro* nerve preparation is treated with a local anaesthetic at low concentration and stimulated at low frequency, a constant decrease in conductance develops. Increasing the frequency of stimulus with the same concentration of local anaesthetic the degree of block will increase until a steady state of "frequency dependent" block is reached. This phenomenon can be explained by Hille's modulated receptor theory (Hille, 1977). The drug binds to the channel in the O and I states with greater affinity than in the R state. Frequent stimulation makes the association with O and I state channels more likely.

The relevance of frequency dependent block in clinical anaesthesia has not been established, yet it would follow that more active neurons would be blocked more effectively e.g. pain fibres during surgery. However, in a blinded study by Stevens *et al* (1996) of brachial plexus blocks with and without high frequency transcutaneous electrical nerve stimulation of the radial and ulnar nerves for 15 minutes, no significant differences between groups could be demonstrated. There is good reason to believe that this phenomenon contributes to the relative cardiotoxicity of some local anaesthetics. For example, the antiarrhythmic effect of lidocaine can be accounted for by its 'fast in, fast out' block of sodium channels in the myocardium. In contrast, bupivacaine's 'fast in, slow out' effect increases the risk of cardiac depression and arrhythmogenesis (Clarkson & Hondeghem, 1985). In addition, the R(+) isomer of bupivacaine produces a more pronounced frequency-dependent block than the S(-) isomer (Vanhoutte *et al.*, 1991), which could contribute to differences in the lethal doses of the isomers observed in animals (Aberg, 1972; Luduena *et al.*, 1972).

There are many physical characteristics of local anaesthetics which, in some way affect local anaesthetic action, these are described below;

1.9.2 Molecular weight

Molecular weights of local anaesthetic agents range from 220 to 288 (Courtney & Strichartz, 1987). These differences affect aqueous diffusion coefficients but will only be small as these are related to the inverse of the square root of the molecular weight. Dural permeability is claimed to be more dependent upon molecular weight than lipophilicity (Moore *et al.*, 1982; Bernards & Hill, 1991). Molecular weight might also be relevant to the movement of local anaesthetics in the sodium channel of the nerve membrane.

1.9.3 Lipid solubility

Local anaesthetic potency is directly related to lipid solubility *in vitro*. As 80% of the nerve's structure is lipid, a more lipid soluble a molecule is more readily absorbed into the membrane than one that is less lipid soluble. However, sequestration by non-specific tissue components and fat near the site of action also modulate anaesthetic profile. *In vivo* studies have shown that these potencies are not necessarily reflected clinically. The difference *in vivo* is thought to be due to the differential vasodilatory effects of individual agents. Lidocaine, which is more potent than prilocaine or mepivacaine in *vitro* is less potent *in vivo.* This is due to its potent vasodilator properties and hence it is 'washed out' more quickly than other agents, effectively giving it a shorter duration of anaesthetic effect (Srichartz *et al.,* 1990). Lipid solubility is measured as octanol:buffer distribution coefficients and shows good rank-order correlation with *in vitro* partition into rat sciatic nerve and human extradural and subcutaneous fat (Rosenberg *et al.*, 1986; Strichartz *et al.* 1990).

1.9.4 Ionisation

The ionisation of local anaesthetics is related to pKa. Higher pKa values promote ionisation at physiological pH that would be expected to delay diffusion, thereby prolonging onset of action (Ritchie & Greenguard, 1966). Esters generally have higher pKa values (8.6-9.3) than amides (7.8-8.2) making them more ionised and hence slower diffusing. Ionisation is also relevant to the stability, solubility and activity of local anaesthetics and their equilibrium distribution in various body compartments. Due to the differing stabilities of local anaesthetic agents, they are dispensed in solutions of differing pH. Ester types are dispensed in relatively acidic solutions so that they exist in the more stable and soluble ionised form (as low as pH2.8 c.f. pH4.4-6.4 for amides). Due to the lower pH of the ester solutions, there remains less of the non-ionised drug available, further reducing the delay in nerve blocking. Further alkalinisation of the amides would be expected to shorten the latency of block.

1.9.5 Protein binding

Protein binding determines the duration of action of a local anaesthetic. Its affinity for binding to plasma proteins is an estimate of its ability to bind to the site within the sodium

channel, the rationale being that a highly protein bound drug will stay attached for longer. However the vasodilatory effect of the individual drug must also be considered when comparing *in vivo* results of duration of anaesthesia (Strichartz *et al.,* 1990).

1.9.6 Aqueous solubility

The aqueous solubility of a local anaesthetic is related directly to the extent of ionisation (and therefore decreases as pH is raised) and related inversely to lipid solubility (Ritchie & Greenguard, 1966). Benzocaine, which lacks an amino group attached to the carbon chain, is almost insoluble in water. This makes benzocaine useful only in topical anaesthesia. The intrathecal injection of the less aqueous-soluble agents is advised to be less than 2% as animal studies show that morphological effects of precipitation on the spinal cord are apparent only above this concentration (Adams *et al.,* 1974,1977).

1.10 Thesis Aims

The fundamental hypothesis behind the work described in this thesis is that the inclusion of local anaesthetic agents in IVRGBB may inhibit the uptake of guanethidine by NET and therefore reduce the efficacy of treatment. A variable efficacy of intravenous regional guanethidine Bier's block (IVRGBB) has been reported (Kingery, 1997). The rationale for this hypothesis is the observation that the local anaesthetic agent cocaine is a known potent inhibitor of NET. In an attempt to support this hypothesis the following experiments are planned.

1. Measurement and characterisation of [^{3}H]norepinephrine uptake in SH-SY5Y and 293-hNET cells and their use as "*in vitro*" model systems expressing endogenous (SH-SY5Y) and recombinant (293-hNET) human NETs. Examination of potential inhibitory effects of a range of local anaesthetic agents upon uptake will be made.

2. Measurement and characterisation of the binding of [^{3}H]nisoxetine to SH-SY5Y and 293-hNET cell membranes to measure hNET expression levels. Examination of any competitive displacement by local anaesthetic agents will be made and compared with the effects on [^{3}H]NE uptake.

3. Determination of relative hNET expression levels in SH-SY5Y and 293-hNET cells by polyacrylamide gel electrophoresis (PAGE), western blotting and immunochemiluminescence.

4. Determination of any competitive effects of local anaesthetic agents with guanethidine using an *ex vivo* model of sympathetically innervated tissue, the electrical field stimulated (EFS) mouse vas deferens.

CHAPTER 2

Materials and Methods.

2.1 Sources of reagents

Amersham Life Sciences (Bucks, UK) L-[7,8-^{3}H]norepinephrine (36-42 Ci mmol^{-1}) and [N-*methyl*-^{3}H]nisoxetine (85 Ci mmol^{-1}), enhanced chemiluminescence kit.

Fisher, (Loughborough, UK), sodium chloride, potassium chloride, calcium chloride, magnesium sulphate, glucose, potassium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, glycine, glycerol, methanol, propan-2-ol, Folin & Ciocalteau's reagent, Optiphase safe, glacial acetic acid, and tissue culture flasks.

Life Technologies (Uxbridge, UK) Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, L-glutamine, penicillin plus streptomycin, fungizone, trypsin-EDTA, geneticin (G418).

Safeway, Hayes (Middlesex, UK) low fat dried milk.

Schleicher & Schuell, (Dassell, Germany) nitro-cellulose paper.

Sigma Chemical Company Ltd (Dorset, UK). Imipramine.HCl, lidocaine.HCl, prilocaine.HCl, bupivacaine.HCl, tetracaine.HCl, dibucaine.HCl, procaine.HCl, cocaine.HCl, pargyline.HCl, L-ascorbic acid, tetrodotoxin, EDTA, EGTA, Tris-HCl, Tris base, Triton-X, acrylamide/bis acrylamide, PEI, BSA, TEMED, sigma protein markers (electrophoresis), bromophenol blue, ammonium persulphate, SDS, tween 20, sodium deoxycholate, soybean trypsin inhibitor, *o*-phenanthroline, leupeptin, iodoacetamide phenylmethylsulfonyl fluoride. Goat anti-mouse horseradish peroxidase conjugated IgG.

USB, Amersham, (Ohio, USA) HEPES

MAb Technologies Inc. (USA) mouse anti-hNET monoclonal antibody.

2.2 Buffer composition

Harvest buffer HEPES buffered saline with 4mM EDTA, pH7.4 with 10M NaOH.

Krebs-HEPES (mM) Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), $H_2PO_4^-$ (1.2), SO_4^{2-} (1.2), Glucose (11.7) and HEPES (10), pH7.4, with 10M NaOH. Pargyline (0.2) and ascorbic acid (0.2) were included in norepinephrine uptake experiments. Pargyline, an inhibitor of monoamine oxidase was included to prevent the subsequent catabolism of norepinephrine after uptake and ascorbic acid was present to prevent its oxidation (figure 2.1).

Homogenising buffer (radioligand binding studies) 50mM Tris buffer, 120mM NaCl, 5mM KCl, pH7.4 with 10M HCl.

Incubating buffer (radioligand binding studies) 50mM Tris buffer, 300mM NaCl, 5mM KCl, pH7.4 with 10M HCl.

Western blotting

a) Electrophoresis buffers

Solubilisation buffer 10mM Tris, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate, supplemented with 1mg ml^{-1} soybean trypsin inhibitor, 1mM *o*-phenanthroline, 1μg ml^{-1} leupeptin, 1mM iodoacetamide and 250μM phenylmethylsulfonyl fluoride (PMSF).

Loading buffer 62.5mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue.

Running buffer 25mM Tris-base, 250mM glycine, 0.1% SDS, pH8.3.

Polyacrylamide Running Gel 8% 4.6ml H_2O, 2.7ml 30% acrylamide/bis acrylamide in 29:1 ratio, 2.5ml 1mM Tris base, pH8.8, 100μl 10% SDS, 100μl 10% ammonium persulphate, 6μl N,N,N',N'-Tetramethylethylenediamine (TEMED).

Polyacrylamide Stacking Gel 5% 1.4ml H_2O, 333μl 30% acrylamide/bis acrylamide in 29:1 ratio, 250μl 1mM Tris base, pH6.8, 20μl 10% SDS, 20μl 10% ammonium persulphate, 2μl TEMED.

b) Immunoblotting Buffers

Transfer buffer 50mM Tris-base, 39mM glycine, 0.04% SDS, 20% methanol, pH8.3.

Blocking solution 5% low fat milk in 20mM Tris-HCl, 0.5M NaCl, 0.5% Tween 20.

Mouse vas deferens buffer Krebs solution (mM) NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, Glucose 10 (Mg^{2+}-free, pH7.4)

2.3 Cell culture

SH-SY5Y human neuroblastoma cells (obtained from JL Biedler, Sloane-Kettering Institute for Cancer Research, Rye, NY, USA), passages 83-110, were routinely maintained in minimum essential medium (MEM) supplemented with L-glutamine 2mmol l^{-1}, penicillin 100iu ml^{-1}, streptomycin 100μg ml^{-1}, fungizone 2.5 μg ml^{-1} and 10% (v/v) foetal calf serum in 5% CO_2/humidified air at 37°C. Stock cultures were fed twice weekly and a day prior to experimentation, and passaged weekly (Atcheson *et al.*, 1993; Lambert *et al.*, 1996). The cell monolayers were harvested for sub-culture by treatment with 0.05% trypsin-0.02% EDTA, and cultured in 80cm^2, 175cm^2 tissue culture flasks or 525cm^2 triple flasks and grown until confluent (6-10 days).

293-hNET cells (passages 2-14) were routinely maintained in high glucose (4500mg l^{-1}) Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) foetal bovine serum, L-glutamine 2mmol l^{-1}, penicillin 100iu ml^{-1}, streptomycin 100μg ml^{-1} and stock, non-experimental cultures were maintained in media supplemented with 250μg ml^{-1} geneticin (G418) (Galli *et al.*, 1996). 293-hNET cells were otherwise treated in the same way as SH-SY5Y cells.

G418 is an inhibitor of protein synthesis which is inactivated by aminoglycoside-3'-phosphotransferase by phosphorylation. The resistance gene for G418 is present on the plasmid that encodes for the recombinant hNET. Therefore, cells not transfected with, (or losing) the hNET plasmid will lack the G418 resistance gene and will not survive, hence hNET expression is maintained.

2.4 Cell Harvesting

Cells for [^{3}H]NE uptake experimentation were harvested with harvest buffer (section 2.2) washed twice with, and re-suspended in Krebs-HEPES buffer, pH7.4, after centrifugation at 440g for 2min at 4°C. Cells were kept on ice and used within 2 hours of harvesting.

2.5 Membrane preparation

Cell membranes were prepared by harvesting SH-SY5Y and hNET cells as described but suspending in homogenising buffer (section 2.2) and homogenising with an 'Ultra Turrax

T25' three times for 5 seconds at 13,500rpm at 4°C in 25ml of homogenising buffer. Membranes were centrifuged at 20374g for 10 minutes at 4°C, then washed with 25ml homogenising buffer. The resulting pellet was then re-homogenised and re-suspended twice more. The resulting suspension was placed on ice until required.

2.6 Measurement of [^{3}H] norepinephrine ([^{3}H]NE) uptake in whole cell suspensions.

Assays were performed in 0.5ml volumes of Krebs-HEPES buffer, pH7.4 supplemented with ascorbic acid (200μM) as an antioxidant, and pargyline (200μM) at 37°C in 5ml plastic tubes. (figure 2.1)

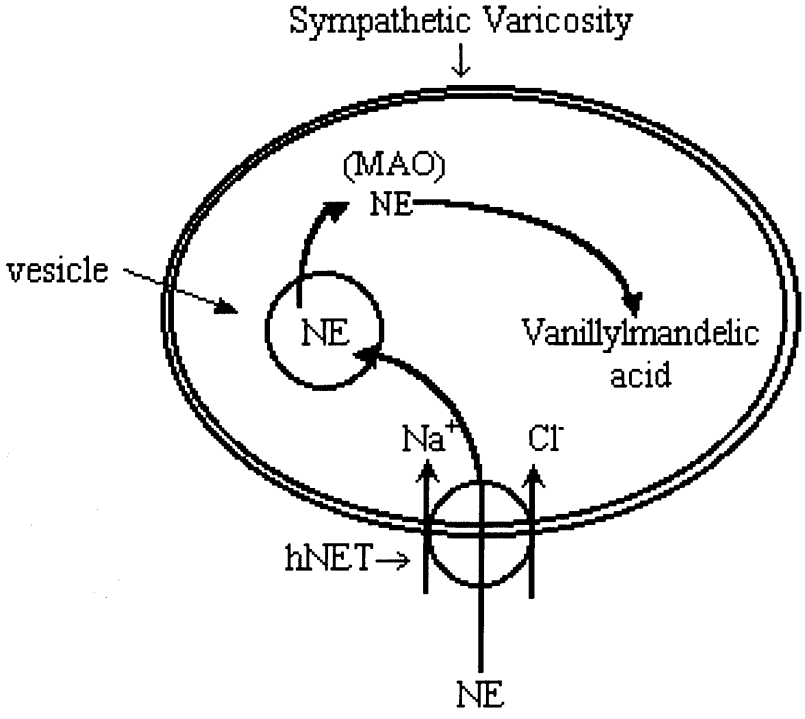


Figure 2.1 Diagrammatic representation of the sympathetic nerve varicosity. hNET (human norepinephrine transporter) takes up extracellular norepinephrine (NE) co-transported with Na^+ and Cl^- ions down their concentration gradients. NE is incorporated into vesicles. Any extravesicular NE is catabolised to vanillylmandelic acid by monoamine oxidase (MAO) present on mitochondrial membranes. Pargyline is an inhibitor of MAO and is included in buffer in uptake experiments to prevent metabolism of [^{3}H]NE.

2.6.1 Time Course of [^{3}H]NE uptake

A time course was first determined in the presence of [^{3}H]NE (0.5μCi tube^{-1}, ~25nM) (Atcheson *et al*, 1993) by termination at 0,1,2,3,4,5,8,10,13,15 and 20 minutes by rapid

vacuum filtration using a Brandell cell harvester onto Whatman GF/B filters and washed with three 4ml aliquots of ice cold Krebs-HEPES buffer (table 2.1). Filter bound radioactivity was extracted overnight in Optiphase Safe scintillant, and measured by liquid scintillation spectroscopy. Non specific uptake was determined in the presence of the hNET inhibitor, imipramine (10μM).

Table 2.1 Typical protocol for [^{3}H]NE uptake time course using ~25nM [^{3}H]NE. Assay volume 0.5ml. NSU= (non-specific uptake).

	Volumes (μl)			
	Cell prep.	Imipramine (50μM)	[^{3}H]NE	Buffer
Total	100	-	100	300
NSU	100	100	100	200

2.6.2 Concentration response curves

After determination of uptake time course parameters, cells were pre-incubated with or without the compound under investigation for 30mins prior to addition of a fixed concentration of [^{3}H]NE as above then termination at 4 and 5 minutes for hNET and SH-SY5Y cells respectively. Local anaesthetic agents and their concentrations were; tetracaine, procaine, dibucaine, bupivacaine, prilocaine, lidocaine, ($3x10^{-8}$ to 10^{-2} M) and cocaine ($3x10^{-10}$ to 10^{-4} M).

The dependency of uptake upon Na^+ concentration was investigated by reducing buffer Na^+ concentration, maintaining tonicity with equimolar choline. Uptake was measured at 0,28.7,57.3,86,114.6 and 143.3mM sodium concentrations.

Table 2.2 Typical addition protocol for concentration response curves of local anaesthetic agents using ~0.5nM [^{3}H]NE for uptake. Assay volume 0.5ml

	Volumes (µl)				
	Cell prep.	Imipramine	[^{3}H]NE	Buffer	Local Anaesthetic
Total	100	-	100	300	-
Compound	100	-	100	200	100
NSU	100	100	100	200	-

2.6.3 Tetrodotoxin sensitivity

In SH-SY5Y cells the effects of Na^+ channel blockade with tetrodotoxin (TTX, 900nM) (Ritchie & Rogart, 1977) on the response to prilocaine ($3x10^{-8}$ to 10^{-2} M) was measured (table 2.3). In these studies TTX and prilocaine were included in the 30min pre-incubation. 900nM was in excess of the concentration required to block all Na^+ channels (Guo, 1987). TTX studies were only carried out in SH-SY5Y cells because of their neuronal and hence excitable nature.

Table 2.3 Addition protocol for concentration response curve of prilocaine with and without tetrodotoxin (TTX) 0.5nM [^{3}H]NE. Assay volume 0.5ml.

	Volumes (µl)					
	Cell prep.	Imipramine	[^{3}H]NE	Buffer	Prilocaine	TTX
Total	100	-	100	300	-	-
Prilocaine	100	-	100	200	100	-
Prilocaine +TTX	100	-	100	100	100	100
NSU	100	100	100	200	-	-

2.7 Principle and theory of radioligand binding studies

Radioligand binding is a technique used to measure the binding characteristics of a receptor, channel or carrier and relies on having a specific radioligand for that site. Binding is quantifiable by virtue of the fact that the ligand has been labelled by incorporation of a radioactive isotope into its molecular structure.

The main radioligand binding protocols employed in this thesis are isotopic dilution to determine K_d and B_{max} and displacement studies where the affinity values (K_i) of unlabelled compounds are determined. These are discussed in greater detail later in this chapter.

2.7.1 Saturation binding

In this type of experiment, increasing concentrations of radiolabelled ligand (usually high affinity antagonists) are used, up to a concentration at which all the available binding sites for that ligand become occupied (figure 2.2). This is termed the B_{max}, or the maximum specific binding of a ligand, often expressed in units of fmol ligand mg protein^{-1}. The relationship between B_{max} and the number of binding sites (N) is influenced by the number of binding sites possessed by each target molecule/receptor. Also from this type of experiment, the equilibrium dissociation constant is calculated (K_d). The K_d is termed as the concentration of ligand which allows an equal amount of receptors bound to ligand as receptors unbound. Or more simply, the concentration which yields half maximum binding.

Equilibrium is reached when new ligand-receptor complexes are formed at equal rates to the dissociation of the ligand receptor complexes; these two values are termed, k_{on} and k_{off}.

$$\text{Ligand} + \text{Receptor} \underset{K_{on}}{\overset{K_{off}}{\rightleftharpoons}} \text{Ligand-Receptor complex}$$

$$K_d \text{ equilibrium} = k_{off}/k_{on} = [L][R]/[LR]$$

When the ligand occupies half the receptors, the concentration or amount of unoccupied receptors equals the concentration or amount of occupied receptors: [R]=[LR].

This can only be true when K_d equals [L]. i.e. the K_d is the concentration of ligand which will bind to half the receptors at equilibrium.

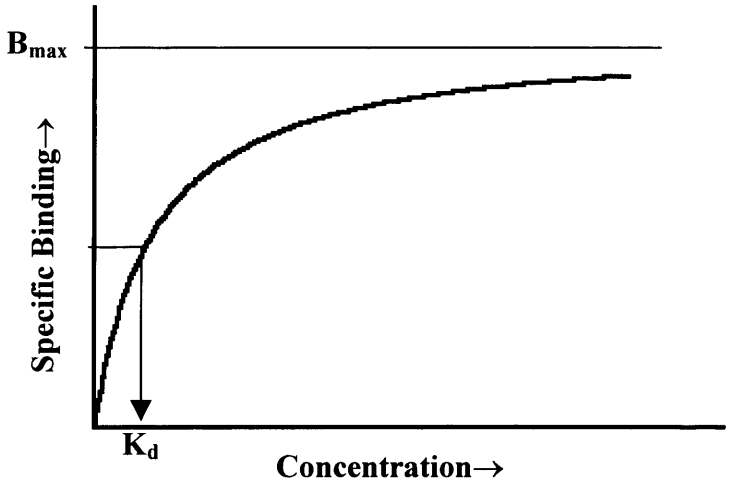


Figure 2.2 Example specific saturation curve or binding isotherm (figure modified from Graphpad Prism 2).

The curve shown in figure 2.2 is known as a rectangular hyperbola, binding isotherm, or saturation binding curve. The X-axis is the concentration of the ligand, and the Y-axis is the specific binding. Specific binding is zero initially, and increases to a maximum plateau value (B_{max}). B_{max} is the maximum number of binding sites, expressed in the same units as the Y-axis (e.g. radioactive disintegrations per minute (DPM), sites per cell, or fmol of ligand per mg of tissue). K_d is the equilibrium dissociation constant, expressed in the same units as the X-axis (concentration). When the drug concentration equals K_d, half the binding sites are occupied at equilibrium.

2.7.2 Non-specific binding

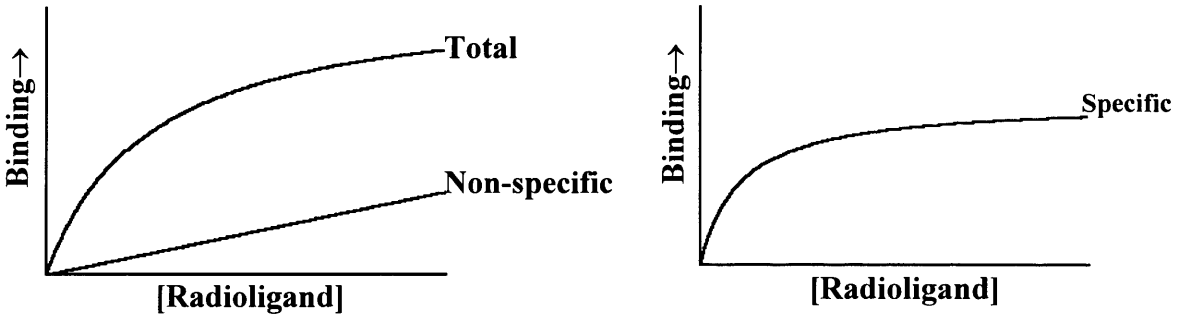


Figure 2.3 Specific binding is calculated by subtraction of non-specific binding from total binding. (figure taken from Graphpad Prism2).

In addition to binding to the receptors, radioligand also binds to other sites termed non-specific sites. Non-specific binding is detected by measuring radioligand binding in the presence of a saturating concentration of an unlabeled ligand that binds to the sites/receptors. Under those conditions, all of the receptors will be occupied by the unlabeled drug so the radioligand can bind only to non-specific sites. To calculate the specific radioligand binding to receptors, the non-specific binding at a particular concentration of radioligand is subtracted from the total binding at that concentration.

Generally the unlabeled compound at a concentration equal to 100 times its K_d for the site/receptor is sufficient to obtain non-specific values.

Non-specific binding usually has a linear relationship with the concentration of radioligand (within the range it is used) (figure 2.3).

2.7.3 Isotopic dilution

When direct saturation cannot be performed to yield a complete curve because of (1) high K_d (low affinity) and (2) the amount of radioligand required exceeds 'financial means', isotopic dilution can be performed. In this case a fixed concentration of radiolabelled ligand, at a concentration about 10 fold lower than the K_d is added to each assay with varying concentrations of the same unlabelled compound. Clearly, as the concentration of unlabelled compound increases, the labelled compound is diluted, giving a lower value for bound DPM (units of radioactivity, figure 2.4). As the two compounds are essentially identical, and have the same affinity, the actual activity bound figure multiplied by the ratio of total compound concentration to labelled compound equates to the binding if all of the compound present was radiolabelled. This method assumes that only a small fraction of either ligand binds (confirmed by comparing total DPM of radioligand sample added with total bound DPM). In other words the method assumes that free concentration equals the concentration added (N.B. concentration added = ['hot']+['cold']). The technique also assumes that there is no co-operative binding of the chosen ligand (figure 2.4). Total ligand bound can be calculated by the formula below.

Total Bound = Radioligand Bound (fmol mg protein^{-1}) x [Total]/[Radioligand]

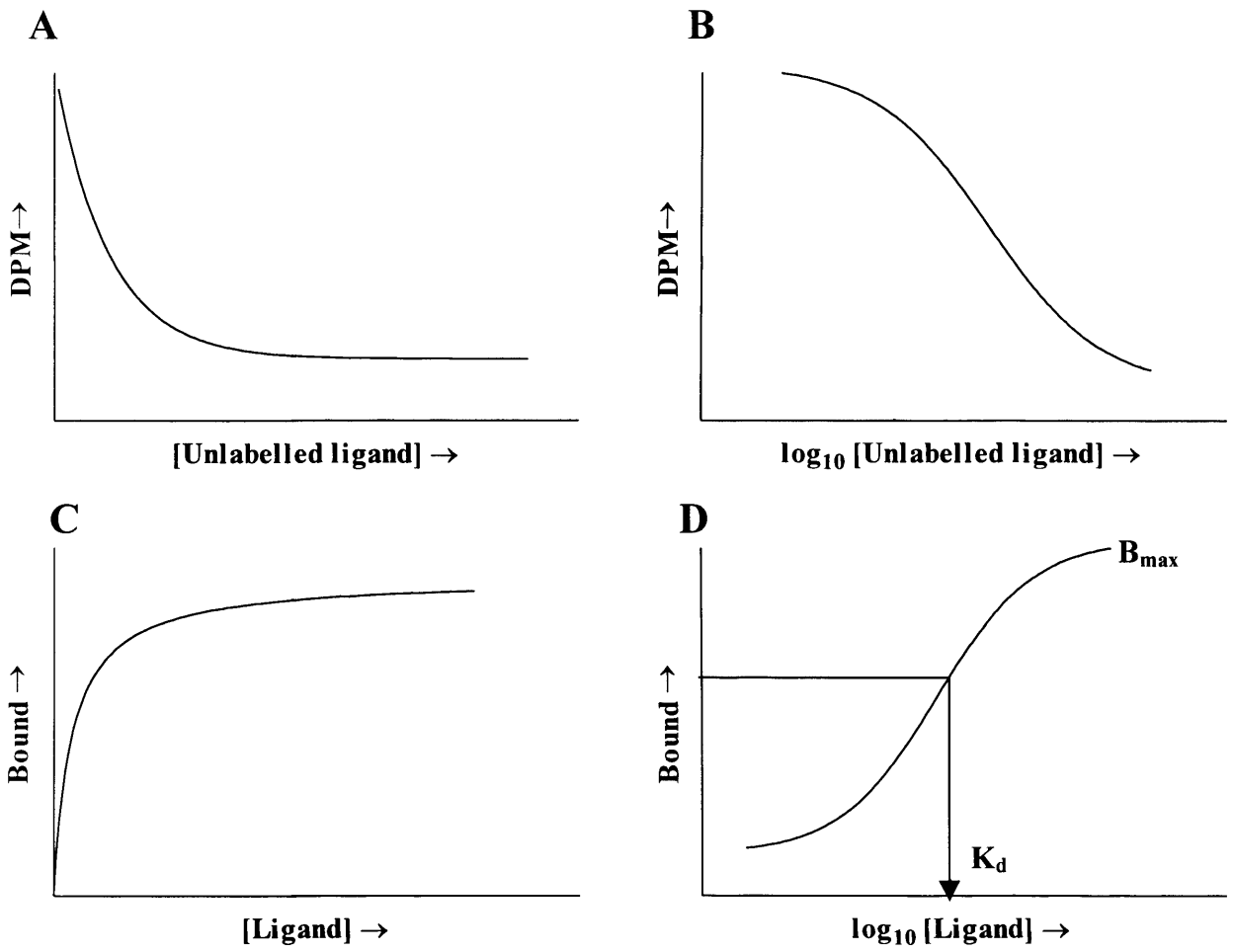


Figure 2.4 Examples of curves produced in isotopic dilution binding assays. A. Relationship between DPM bound and linear concentration of unlabelled ligand giving an inverse rectangular hyperbole. B. The same DPM against unlabelled ligand as in A but with a log concentration X axis giving a sigmoid curve. C. The same data as shown in A and B but converted into fmol mg protein^{-1} on the Y axis and linear ligand concentration on the X axis. D. The isotopic dilution curve converted as in C but with a logarithmic scale on the X axis. This format is used to calculate individual pK_d and B_{max} values using Graphpad Prism (see section 4.4.3).

2.7.4 Displacement binding studies

When the K_d of the radiolabelled compound is known, the affinities of other unlabelled compounds can be determined by displacement binding. A known constant concentration of radioligand is included in each assay, then varying concentrations of unlabelled compound added. As the concentration of this unlabelled compound increases, the amount of radioligand bound decreases as it is displaced. As direct saturation cannot be performed because the competing ligand is not measurably radioactive itself, radioligand must also be present to label the sites. A higher concentration of this radioligand will take a larger concentration of unlabeled drug to compete for the binding, shifting the displacement curve to the right. (figure 2.5). After equilibrium is reached and bound compound separated and counted, the affinity constant (K_i) is calculated from the Cheng and Prusoff equation:

$$K_i = \frac{IC_{50}}{(1+(L/K_d))}$$

Where the K_i is the affinity of the receptor for the competing drug, IC_{50} is the concentration which gives 50% displacement of radioligand, L is the concentration of radioligand in the assay and K_d is the dissociation constant of the radioligand (Cheng and Prusoff 1973).
The subscript '$_i$' is used to indicate that the competitor inhibits radioligand binding. K_i can be interpreted as an equilibrium dissociation constant, usually abbreviated K_d. It is the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors.

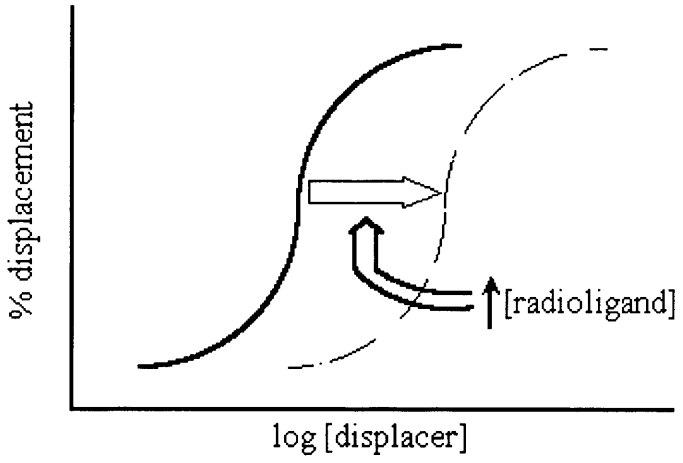


Figure 2.5 Representation of sigmoid plot of displacement of radioligand. An increase in radioligand concentration shifts the curve to the right. The Cheng-Prusoff equation shifts this curve back to a position giving values equivalent to if no radioligand is present. (diagram modified from Cembala, 1999).

2.7.5 Transporter versus G protein coupled receptor binding.

Binding experiments in these studies are concerned with ligands binding to the hNET. Many studies are however concerned with G protein coupled receptors (GPCR), whose affinities for agonist compounds vary according to the concentration of guanine nucleotides present in the assay. Therefore, in the case of GPCR binding, it is preferable to use a ligand which does not have an agonist action upon the receptor. However, a non-agonist ligand is not always available and steps must be taken to determine the affinity of both high and low affinity binding sites by manipulating the concentration of guanine nucleotides within the assay. Since our major concern here is with the binding of ligands to hNET, a transmembrane transporter, guanine nucleotide modification is of no consequence.

2.8 [^{3}H]nisoxetine binding studies.

2.8.1 Time course

The binding of [^{3}H]nisoxetine to the uptake recognition site on NET (Tejani-Butt, 1992) was measured in membranes prepared from SH-SY5Y and 293-hNET cells (see 2.5). Assays were performed at 4°C in 250µl of incubation buffer, pH7.4 containing ~70-150µg membrane protein tube^{-1} for 293-hNET membranes and [^{3}H]nisoxetine ~0.5-1nM for up to 300mins (Tejani-Butt *et al.*, 1990). Preliminary experiments showed low expression of hNET in SH-SY5Y membranes, therefore higher protein concentrations (150-300µg tube^{-1}) and higher ligand concentrations (2-4nM) were used. B_{max} and K_d were determined by isotope dilution ([^{3}H]nisoxetine, with unlabelled nisoxetine).

A binding time course (table 2.4) was first performed to determine the time required for equilibrium to be achieved. Bound and free [^{3}H]nisoxetine were separated by vacuum filtration on Whatman GF/B filters, which were extracted and counted as in uptake studies (table 2.4). Non-specific binding was defined in the presence of 10µM nisoxetine.

Table 2.4 Typical protocol for [^{3}H]Nisoxetine binding time course using ~0.5/~4nM [^{3}H]Nisoxetine depending upon cell type. Assay volume 0.25ml

	Volumes (µl)			
	Cell prep.	Nisoxetine	[^{3}H] Nisoxetine	Incubation Buffer
Total	50	-	50	150
NSB	50	50	50	100

2.8.2 Isotope dilution

In the case of nisoxetine isotopic dilution experiments (table 2.5), [^{3}H]nisoxetine (~0.5nM) was used with 0.03-300nM unlabelled nisoxetine. K_d and B_{max} for nisoxetine were determined in hNET cells only (see discussion 7.2).

2.8.3 Displacement studies

Local anaesthetic concentration response curves (Table 2.6) were calculated using a fixed concentration of [^{3}H]nisoxetine (~0.5/~4nM dependent upon cell type) with prilocaine, lidocaine, cocaine, procaine, tetracaine, bupivacaine and dibucaine (10^{-2} to $3x10^{-10}$M). In some experiments imipramine was included. Effects upon [^{3}H]nisoxetine binding were measured as above.

Table 2.5 Typical addition protocol for isotopic dilution experiments using ~0.5/~1nM (293-hNET) [^{3}H]nisoxetine. Assay volume 0.25ml.

	Volumes (µl)				
	Cell prep.	Nisoxetine [excess]	[^{3}H]- Nisoxetine	Incubation Buffer	unlabelled nisoxetine
Total	50	-	50	150	-
Dilution	50	-	50	100	50
NSB	50	50	50	100	-

Table 2.6 Typical addition protocol for concentration response curves of local anaesthetic agents using ~0.5/~4nM [^{3}H]nisoxetine. Assay volume 0.25ml.

	Volumes (µl)				
	Cell prep.	Nisoxetine [excess]	[^{3}H]-Nisoxetine	Incubation Buffer	Compound
Total	50	-	50	150	-
Compound	50	-	50	100	50
NSB	50	50	50	100	-

2.9 SDS Poly-acrylamide gel electrophoresis and immunoblotting. (see section 5.3)

2.10 Lowry protein assay.

Protein was determined according to the method of Lowry *et al.* (1951), which relies on two basic assay principles; 1 Reaction of protein with copper in alkali, 2. Reduction of Folin's reagent by copper-treated protein.

Protein samples were diluted 1/25 in 0.5ml volumes 0.1M NaOH. A series of bovine serum albumin (BSA) standard solutions were made up in 0.1M NaOH at concentrations of 0, 50, 100, 150, 200, 250µg ml^{-1}. Reagents A, B, and C (where A = $NaHCO_3$ in 0.1M NaOH, B = 2% $CuSO_4$ and C = 2% Na^+ K^+ tartrate) in a 100:1:1 ratio were added in 2.5ml volumes to each (0.5ml) sample and standard of known concentration. Following incubation at 22°C for 10min, 250µl of Folin & Ciocalteu's reagent (1:4) dilution (in distilled water) was added. Tubes were vortexed and incubated at 22°C for 30min. Absorbance was read at 750nm on a Corning spectrophotometer. A standard curve was generated using linear regression on Graphpad Prism and unknown concentrations of protein extrapolated (figure 2.8).

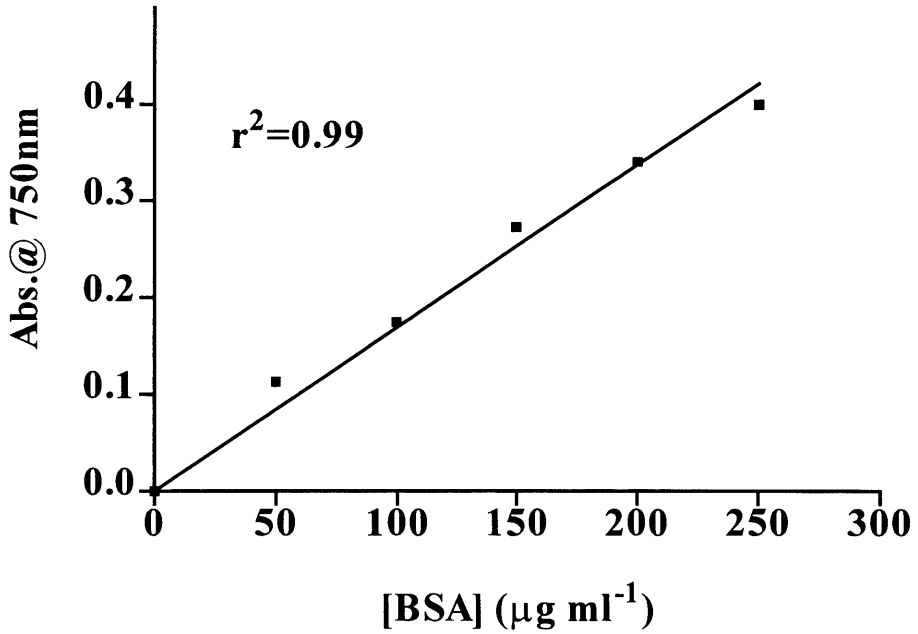


Figure 2.8 Typical Lowry assay protein standard curve. Protein concentration is directly proportional to the absorbance of light at a λ of 750nm in the concentrations shown here.

2.11 Mouse vas deferens experiments

These experiments were performed at the University of Ferrara, Department of Pharmacology under the supervision of Dr Girolamo Calo and Ms Daniella Rizzi. The studies were performed in order to test the hypothesis of local anaesthetic agents interfering with the normal sympatholytic actions of guanethidine in *ex vivo* innervated tissue.

Mouse vas deferens (mVD) tissue was prepared according to Hughes *et al.* (1975). from male Swiss mice (25-30g). The mice were first stunned then decapitated before dissecting out the vas deferens. The tissues were tied at each end with fine cotton thread, then suspended in 5 ml organ baths containing Krebs solution pH7.4, bubbled with 95% O_2 and 5% CO_2 (see section 2.2). The temperature was set at 33°C and a resting tension of 0.3g was applied. The mVD were continuously stimulated by two silver wire electrodes with supramaximal voltage (30V) rectangular pulses of 1ms duration and 0.05Hz. Electrically evoked contractions were measured isotonically by strain gauge transducers (Basile 7006).

Responses were recorded by two-channel 'Gemini' recorders (model 7070). After an equilibration period of ~60 min the contractions induced by electrical field stimulation (EFS) were stable. At this time, the effects of various concentrations of prilocaine were examined in order to determine the lowest concentration giving a complete block of the twitch response to EFS. Prilocaine was kept in contact with the tissue for 15 min. The tissues were then washed for ~1 hr to examine the reversibility of the effect of the local anaesthetic. In other experiments, various concentrations of guanethidine were added and allowed to act on the tissue for 15 minutes before washing in order to determine the lowest concentration that gave a consistent inhibition of twitch response reversible by 1 hour of washing. The tissues were washed every 15 minutes after the initial wash for a period of 1 hour to examine the recovery from any effects of the drug being examined (figure 2.9).

Following determination of required local anaesthetic concentrations, the effects of a combination of prilocaine and guanethidine were examined. Prilocaine and guanethidine were added at 1mM and 3μM respectively and allowed to act for 15 minutes before washing and recording as described previously. To examine a potential competitive inhibition of guanethidine uptake by prilocaine, the same experiment was performed with a higher concentration of guanethidine (10μM). A significant depression in relative recovery rate would indicate substrate (guanethidine) concentration dependence and therefore competitive inhibition.

Other local anaesthetic agents (procaine, cocaine) were also investigated individually and in co-application with guanethidine at concentrations likely to show inhibition of uptake as estimated from binding studies. Procaine and cocaine were chosen so that an amide, an ester and a compound already well known to inhibit NE could be examined.

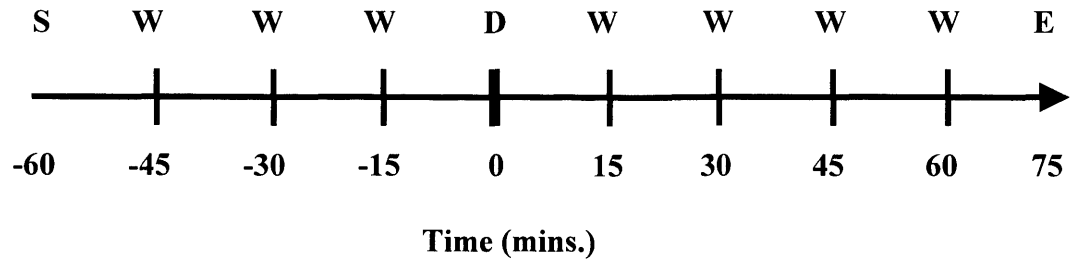


Figure 2.9 Time line of mouse vas deferens protocol. **S**= start, **W**= wash, **D**= drug **E**= end ±addition.

2.12 Data Analysis

1. Data are presented as mean ± SEM values for n individual experiments assayed once.
2. Computer-assisted curve fitting of individual curves was performed using Graphpad Prism 2 to calculate log half maximum inhibition of uptake and ligand binding (pIC_{50}) assuming:
    a. A variable slope sigmoid curve.
    b. 100% maximum where appropriate.
3. In binding studies IC_{50} values are corrected for the competing mass of [^{3}H]nisoxetine according to Cheng & Prusoff (1973) (section 2.3).
4. For the mouse vas deferens experiments the data are presented as mean percentage of control EFS-induced twitch ± SEM values for n individual experiments as described in figure legends.
5. Statistical comparisons were made where appropriate by Students' *t* test and analysis of variance using Graphpad Prism 2. Bonferroni correction was applied after multiple *t* tests as appropriate. Differences were considered significant when $p<0.05$.

CHAPTER 3

Characterisation of uptake of [^{3}H]Norepinephrine by SH-SY5Y and 293-hNET cells and interaction of local anaesthetic agents.

3.1 Introduction

Norepinephrine (NE) is the main neurotransmitter contained and released in sympathetic neurones. NE is taken up by norepinephrine transporters (NET) present on sympathetic nerve membranes where it is incorporated into vesicles by the VMAT ready for release by exocytosis (Henry *et al.,* 1994). The sympatholytic drug guanethidine produces its effect by being taken up by hNET and subsequently displaces the releasable stores of NE from vesicles via VMAT resulting in sympatholysis (Giachetti & Hollenbeck, 1976; Maxwell 1982). It was hypothesised that local anaesthetic agents may inhibit the uptake of [^{3}H]NE and hence guanethidine by an interaction with the hNET.

The effects of anaesthetic agents on two simple cellular systems will be investigated in this chapter. SH-SY5Y cells are a model of human sympathetic neurones expressing hNET and, as an excitable cell is capable of release when stimulated. 293-hNET cells are human embryonic kidney cells transfected with, and stably expressing the recombinant hNET protein.

An interaction of local anaesthetic agents with uptake of [^{3}H]NE may support the hypothesis of prilocaine reducing the efficacy of IVRGBB (intravenous regional guanethidine Bier's block) in the treatment of CRPS I (complex regional pain syndrome type I).

3.2 Aims

The aims of this chapter are to provide a basic characterisation of the uptake of [^{3}H]NE by SH-SY5Y and 293-hNET cells. Once this is established, the effects of various local anaesthetic agents upon [^{3}H]NE uptake will be investigated. If local anaesthetic agents are found to inhibit uptake as hypothesised, IC_{50} values will be determined for each compound. Comparison of such values with concentrations used in clinical practice will be made.

3.3 Methods

For details of chemicals, cell culture and harvesting, uptake protocols and analysis of data the reader is directed to chapter 2.

3.4 Results

3.4.1 [^{3}H]NE Uptake time course

The uptake of [^{3}H]NE by SH-SY5Y cells was time dependent and linear up to 10 minutes with maximum specific uptake occurring at 20 minutes amounting to 1647±44 fmol mg protein^{-1}. At 5 minutes, non-specific binding amounted to ~45%. In 293-hNET cells, uptake was also time dependent and maximum specific uptake occurred at 10min. Uptake was linear until 4 mins and maximum uptake for hNET cells was 1129±167 fmol mg protein^{-1}. At 4 mins, non-specific uptake amounted to ~23% (figure 3.1). After uptake in 293-hNET cells had reached a maximum at 10 minutes, subsequent values for uptake were lower. Maximum uptake values (at 25nM [^{3}H]NE) for each cell line were significantly different ($p<0.05$). All further experiments were terminated at 4 minutes in 293-hNET cells and 5 minutes in SH-SY5Y cells.

3.4.2 Sodium dependence and imipramine sensitivity of uptake.

Specific [^{3}H]NE uptake into both cell types was sodium dependent. With decreasing Na^+ concentration the amount of uptake reduced (figure 3.2). Imipramine completely inhibited uptake in both cell lines with pIC_{50} values of 7.18 ± 0.11 and 6.69 ± 0.14 for SH-SY5Y and hNET cells respectively (figure 3.3 and table 3.1). These values were significantly different ($p<0.05$). This supports the assumption that specific uptake occurs via hNET, a member of the 12 transmembrane spanning, sodium and chloride dependent transporters gene super-family (Worrall & Williams, 1994).

3.4.3 Effects of local anaesthetic agents on [^{3}H]NE uptake in SH-SY5Y cells

Specific uptake of [^{3}H]NE into SH-SY5Y cells was inhibited in a concentration dependent manner by all local anaesthetic agents examined, with pIC_{50} values as shown in table 3.1. In addition all local anaesthetics produced a complete inhibition of specific uptake. The ester type local anaesthetics, cocaine> tetracaine> procaine (figure 3.4) were more potent than the amides dibucaine> bupivacaine> prilocaine> lidocaine (figure 3.5).

3.4.4 Effects of local anaesthetic agents on [^{3}H]NE uptake in hNET cells

Specific uptake of [^{3}H]NE into 293-hNET cells was inhibited in a concentration dependent manner by all local anaesthetic agents examined with pIC_{50} values as shown in table 3.1. In addition all local anaesthetics produced a complete inhibition (100%). The ester type local anaesthetics, cocaine> tetracaine> procaine (figure 3.6) were more potent than the amides dibucaine> bupivacaine> prilocaine> lidocaine. (figure 3.7)

It is notable that in both SH-SY5Y and 293-hNET cells, the inhibition curves for the amide, bupivacaine were steeper than those of all other agents, particularly so in hNET cells with a slope factor of 3.6 which is significantly greater than the range from 0.48 to 1.47 for all other agents. The reason for this steep curve is unclear.

3.4.5 Correlation between pIC_{50} values between cell lines

When pIC_{50} values for each cell line for all compounds investigated are plotted (figure 3.8), a strong positive correlation (r^2=0.998) is achieved. This suggests that the inhibition of uptake by local anaesthetic agents and imipramine is likely to be due to an action at a similar site on each cell line.

3.4.6 Effects of Na^+ channel blockade by TTX upon [^{3}H]NE uptake in SH-SY5Y cells

Local anaesthetic agents inhibit voltage sensitive Na^+ channels (Butterworth & Strichartz, 1990; French *et al.*, 1998; Ritchie, 1975) and this may be responsible for the inhibition of uptake in SH-SY5Y cells. To test this hypothesis voltage sensitive Na^+ channels were blocked with 900nM TTX then a concentration response curve determined for the inhibition of [^{3}H]NE uptake for prilocaine (used in IVRGBB). There was no difference ($p>0.05$) between pIC_{50} values in the absence (3.72±0.07) and presence (3.82±0.07) of TTX. (figure 3.9)

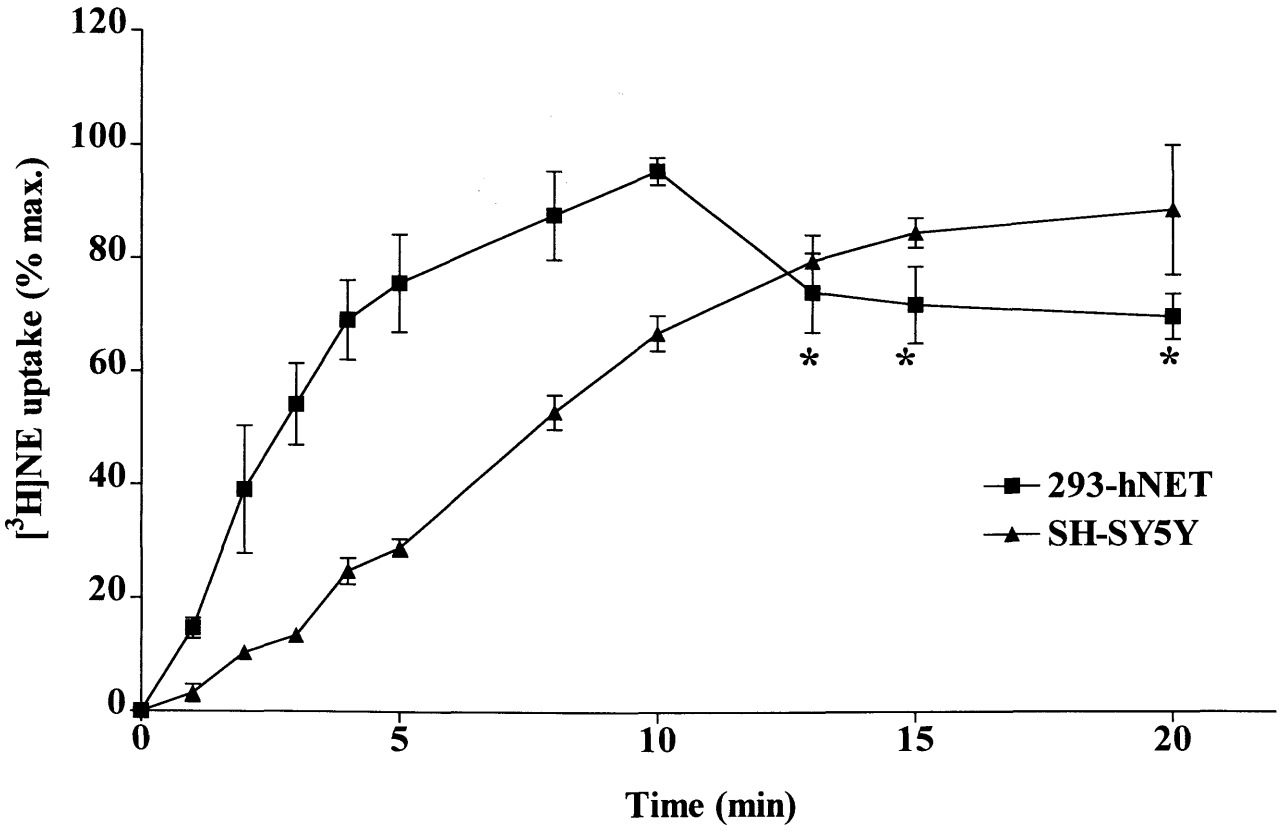


Figure 3.1 Time dependence of uptake of [^{3}H]NE by both SH-SY5Y and 293-hNET cells. The uptake values are shown as percentage of maximum uptake achieved. Data are mean±s.e.mean, n=6. Whole curves were significantly different. *= significantly different to time=10min. in 293-hNET cells ($p<0.05$).

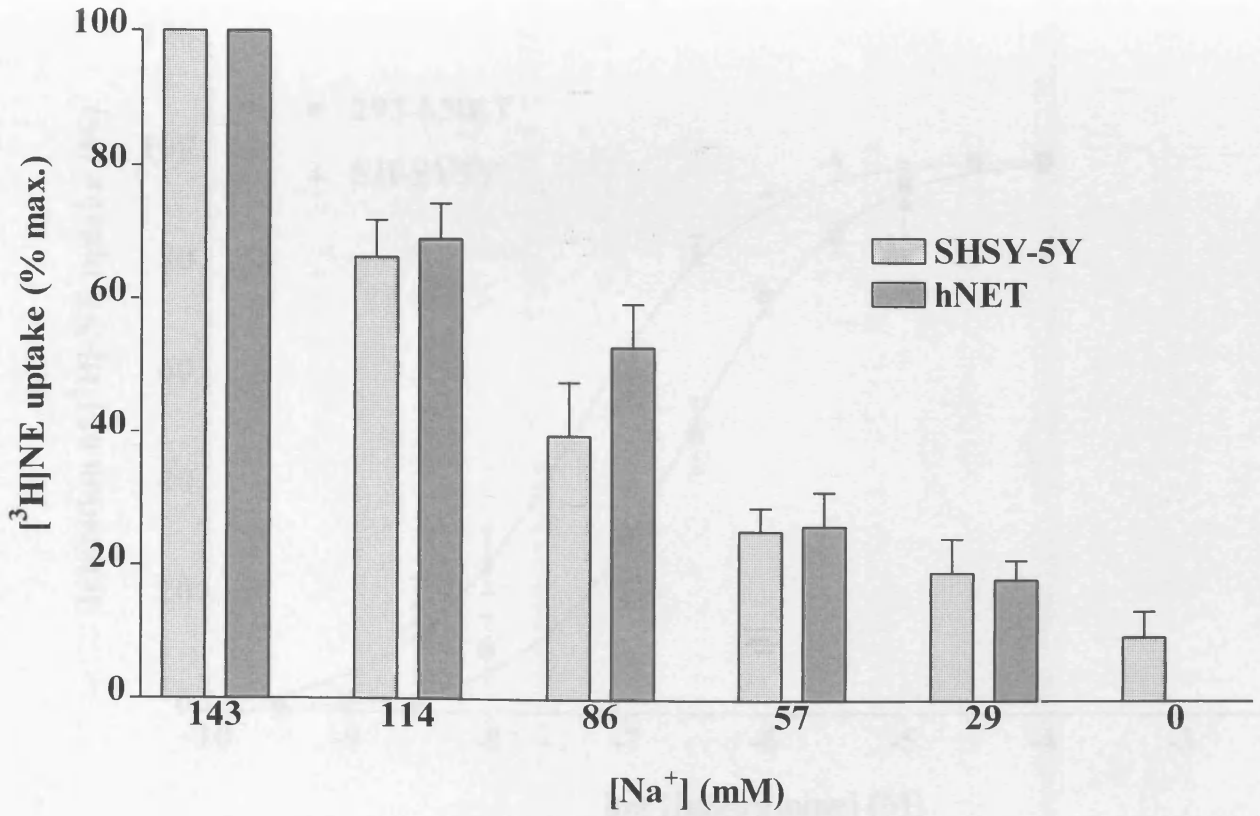


Figure 3.2 Sodium concentration dependence of [^{3}H]NE uptake in SH-SY5Y and 293-hNET cells, characteristic of 12 membrane spanning transporters. Data are mean ± s.e.mean, n=6.

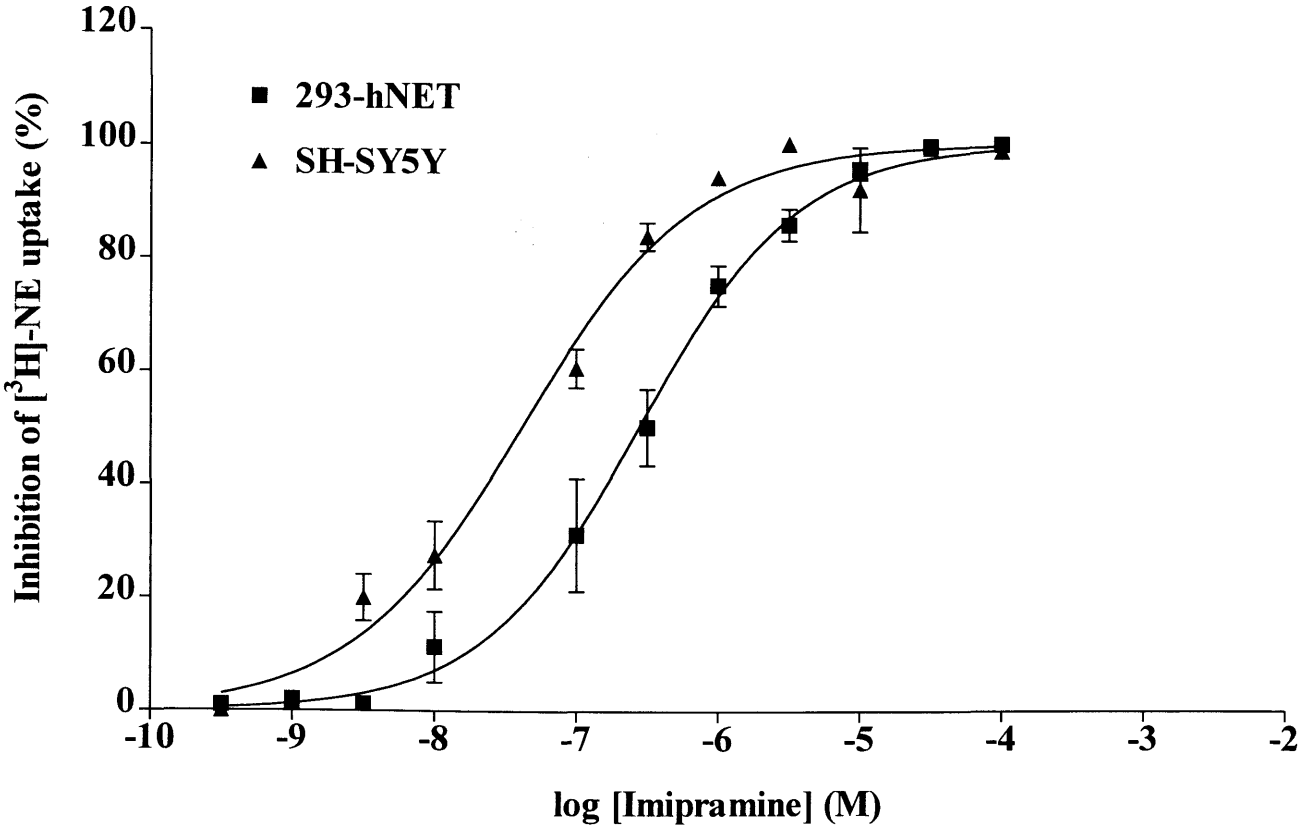


Figure 3.3 Log concentration response curve of inhibition of [^{3}H]NE uptake by imipramine as a percentage of specific uptake at 5 minutes in SH-SY5Y cells and 4 minutes in 293-hNET cells (mean±s.e.mean, n=4-7).

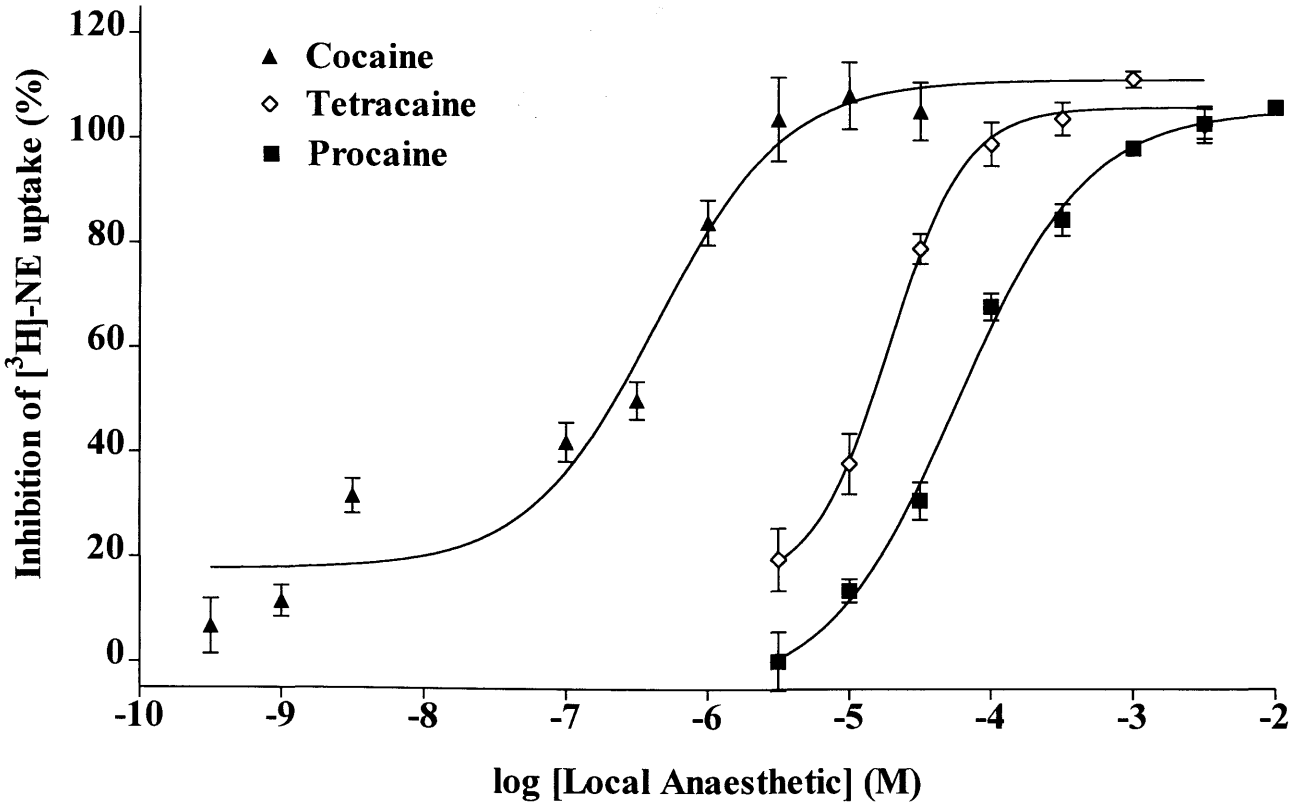


Figure 3.4 Log concentration response curve of ester type local anaesthetic agents showing their inhibition of [^{3}H]NE uptake as a percentage of specific uptake at 5 minutes in SH-SY5Y cells (mean±s.e.mean, n=4-8).

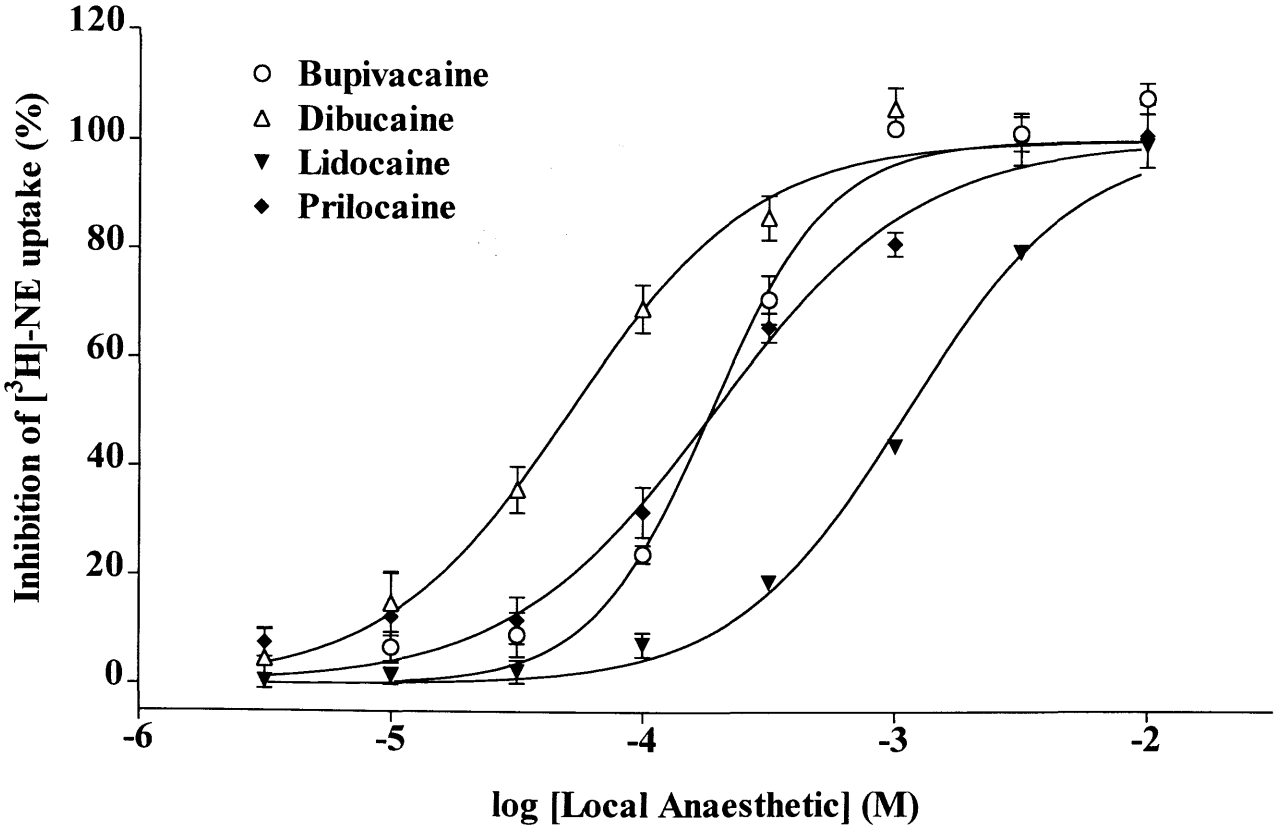


Figure 3.5 Log concentration response curve of amide type local anaesthetic agents showing their inhibition of [^{3}H]NE uptake as a percentage of specific uptake at 5 minutes in SH-SY5Y cells (mean±s.e.mean, n=4-8).

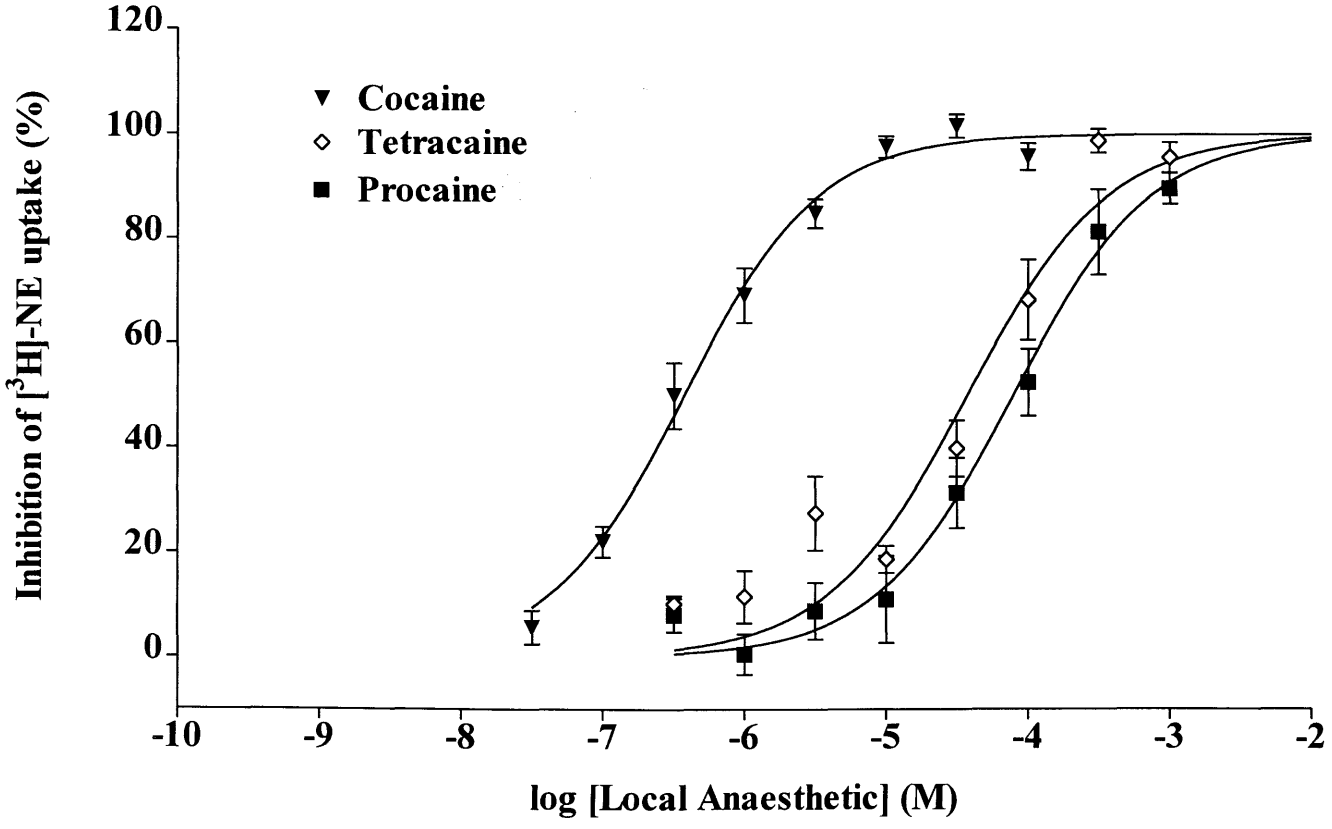


Figure 3.6 Log concentration response curve of ester type local anaesthetic agents showing their inhibition of [^{3}H]NE uptake as a percentage of specific uptake at 4 minutes in 293-hNET cells (mean±s.e.mean, n=6-9).

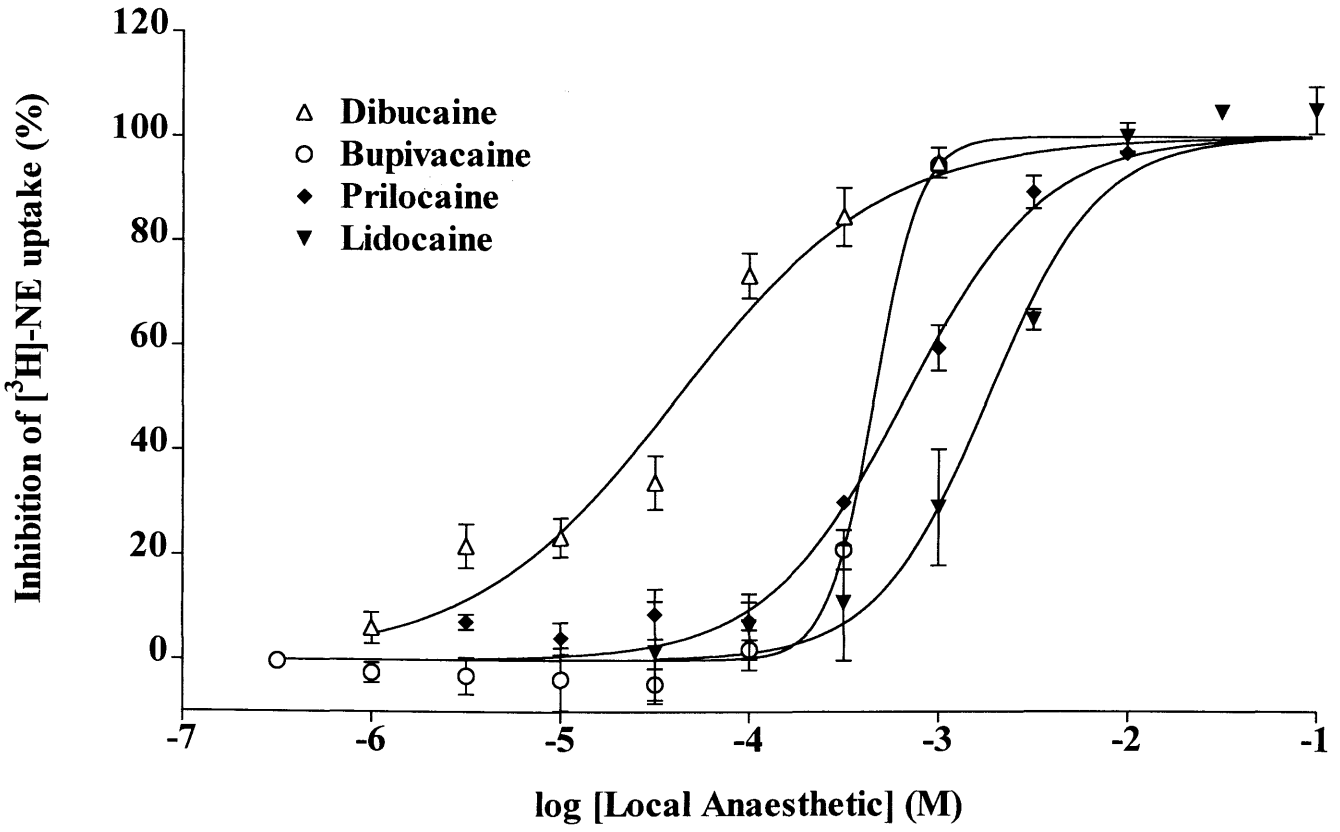


Figure 3.7 Log concentration response curve of amide type local anaesthetic agents showing their inhibition of [^{3}H]NE uptake as a percentage of specific uptake at 4 minutes in 293-hNET cells (mean±s.e.mean, n=4-9).

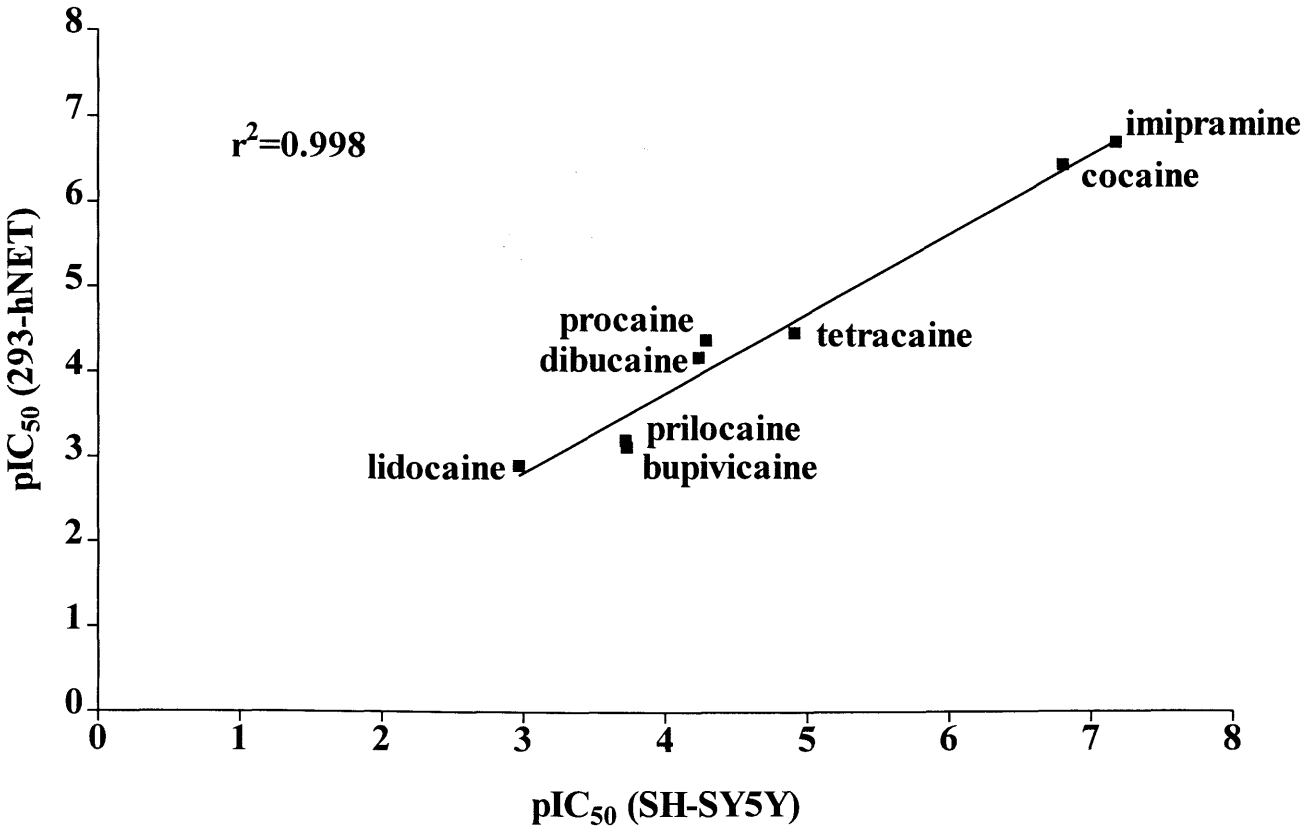


Figure 3.8 Graph showing the correlation between pIC_{50} values for [^{3}H]NE uptake inhibition by local anaesthetic agents and imipramine in SH-SY5Y and 293-hNET cells at 5 and 4 minutes respectively. r^2=0.998 ($p<0.05$). Values shown are mean n=(4-9).

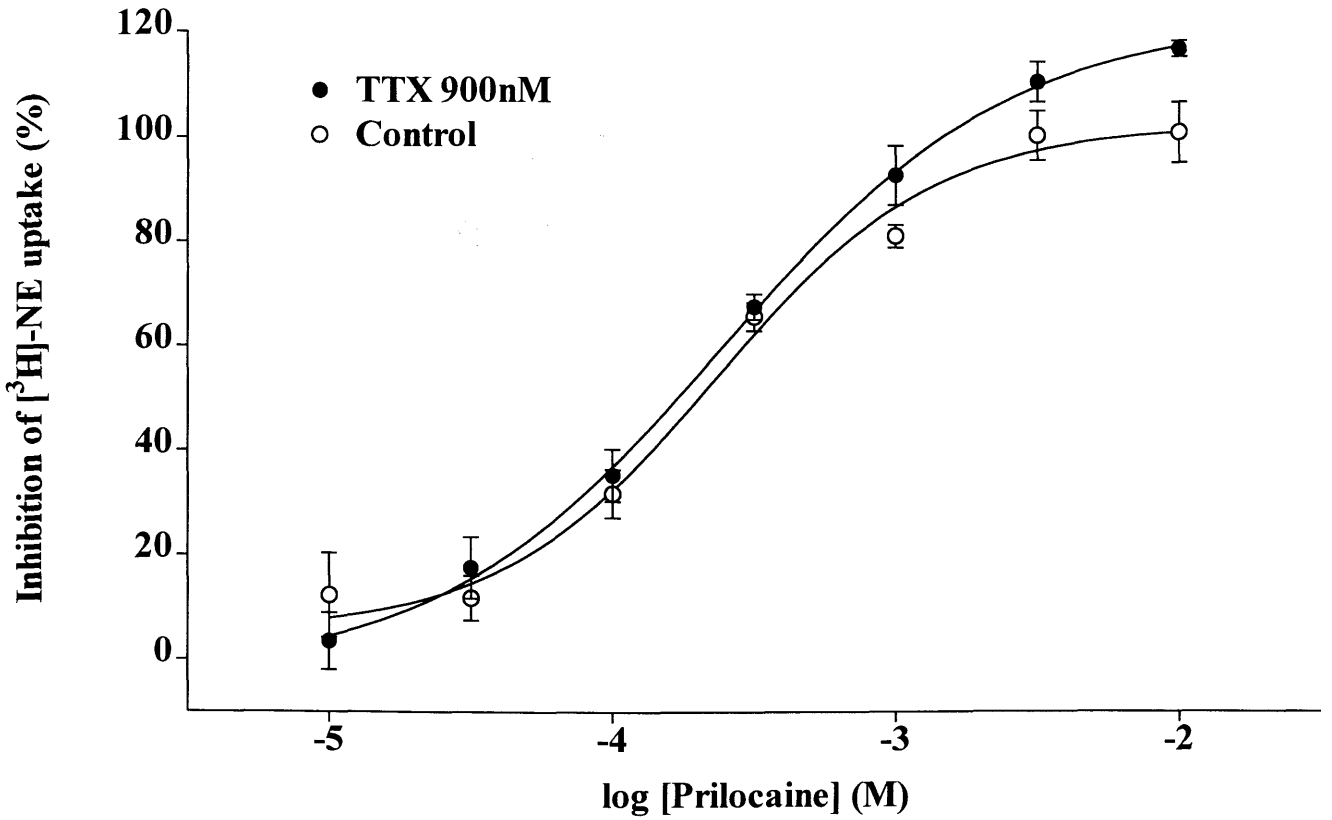


Figure 3.9 Log concentration response curves of prilocaine in the absence and presence of TTX showing the inhibition of [^{3}H]NE uptake as a percentage of specific uptake at 5 minutes in SH-SY5Y cells (mean±s.e.mean, n=7-8).

Table 3.1 pIC_{50} values for inhibition of [^{3}H]NE uptake in SH-SY5Y human neuroblastoma and 293-hNET cells by local anaesthetic agents and imipramine. Data are mean ± s.e.mean (n=4-9). There were some small but statistically significant differences between SH-SY5Y and hNET cells (figure 3.8). Significance refers to unpaired, two-tailed Student's t test.

Compound	pIC_{50} SH-SY5Y	pIC_{50} hNET	p	Ester/Amide
Imipramine	7.18±0.11	6.69±0.14	<0.05	-
Cocaine	6.81±0.01	6.43±0.09	<0.05	E
Tetracaine	4.91±0.04	4.45±0.09	<0.05	E
Procaine	4.24±0.04	4.16±0.02	>0.05	E
Dibucaine	4.29±0.08	4.36±0.05	>0.05	A
Bupivacaine	3.73±0.03	3.11±0.14	<0.05	A
Prilocaine	3.72±0.07	3.19±0.05	<0.05	A
Lidocaine	2.97±0.01	2.89±0.07	>0.05	A

3.5 Discussion

This chapter clearly demonstrates that SH-SY5Y and 293-hNET cells are capable of the uptake of [^{3}H]NE and that this can be blocked by a range of local anaesthetic agents.

Na^+ dependence is a characteristic of the 12 transmembrane spanning Na^+ and Cl^- dependent transporter gene super-family of which NET is an example (Worrall &Williams 1994). In this chapter, Na^+ dependence of both SH-SY5Y and 293-hNET was essentially identical. In addition imipramine sensitivity of uptake into both cell lines was comparable (~7.18 c.f. ~6.69) indicating that the endogenous and recombinant transporter are likely to be the same protein. However there was a difference in absolute uptake (at 25nM [^{3}H]NE) with SH-SY5Y cells taking up a maximum of 1647±44 fmol mg protein^{-1} and 293-hNET 1129±167 fmol mg protein^{-1}. The rates of uptake (at 25nM [^{3}H]NE) were also significantly different, 95.3±5.2 and 194.6±19.7 fmol mg protein^{-1} min^{-1} in SH-SY5Y and 293-hNET cells respectively.

Local anaesthetic agents block voltage sensitive Na^+ channels and are used clinically in pain management (Lee & Wildsmith 1998; Mather & Cousins 1979). Structurally, local anaesthetics are composed of three moieties; lipophilic, intermediate ester or amide linkage and an aromatic (lipophilic) group (see 1.9.1) These agents are classified into ester or amide forms based on the link in the intermediate group (Tucker, 1983). In general local anaesthetic potency is related to lipid solubility, speed of onset is related to amount of ionised drug and duration of action is related to protein binding (Tucker & Mather, 1980). There is good evidence that cocaine, a prototypical local anaesthetic inhibits the re-uptake of NE (Trendelenburg, 1959) and this property is useful for the production of vasoconstriction in, for example, nasal surgery to reduce bleeding (Albers, 1990). It seems likely that this depression of NE uptake is not limited to cocaine and may indeed be a common characteristic of local anaesthetic agents but until very recently there was no data available (Sato *et al.*, 2000). Indeed, the results of this chapter show a clear interaction of a range of local anaesthetic agents with NE uptake and this has important clinical implications (see below).

A range of local anaesthetic agents covering the ester and amide classes inhibited the uptake of [^{3}H]NE in both 293-hNET and SH-SY5Y cells with a good correlation between

the two cell types (r^2=0.998, figure 3.8) indicating that the pharmacology of the transporter (with respect to local anaesthetic inhibition) is the same. Ester type local anaesthetic agents were in general more potent than the amide forms (figure 3.9). Of particular interest is the inhibition by prilocaine which is most commonly used in IVRGBB and the IC_{50} (0.69-0.85mM) which is close to that which might be expected clinically. Based on a simple bench study (estimation of arm volume by water displacement) in volunteers of varying body size, it is estimated that during IVRGBB 50mls of prilocaine (0.5% or 19.5mM) local anaesthetic (containing guanethidine) injected into an arm would on average be diluted around 17 fold. Assuming homogenous distribution this would yield an 'in the arm' concentration of around 1.15mM. This value is in good agreement with that presented in this study of 0.19-0.65mM.

Cocaine > Tetracaine > Dibucaine > Procaine > Bupivacaine > Prilocaine > Lidocaine

Figure 3.9 Rank order of potency of local anaesthetic agents for inhibition of [^{3}H]NE uptake in SH-SY5Y and 293-hNET cells.

It is clear that local anaesthetic agents inhibit the uptake of [^{3}H]NE so we further probed this response and hypothesised potential interaction with voltage sensitive Na^+ channels (although this would only be of relevance in the neuronal SH-SY5Y cell line). In SH-SY5Y cells, pre-incubation with TTX (900nM), a highly potent and selective biotoxin blocker of Na^+ channels, in excess of its maximum effective concentration (Ritchie, 1975), did not modify the pIC_{50} of prilocaine for inhibition of [^{3}H]NE suggesting that this agent does not inhibit uptake via interaction with Na^+ channels.

In summary it has been shown that a range of local anaesthetic agents inhibit the uptake of [^{3}H]NE into SH-SY5Y human neuroblastoma cells and HEK-293 cells expressing the recombinant hNET. It is notable that the findings are in agreement with those of Sato *et al.* (2000) in COS cells. These data have important clinical implications in IVRGBB for the treatment of CRPS1 where guanethidine and local anaesthetic are co-administered. Our data imply that the latter would inhibit uptake of the former. A reduction in guanethidine uptake by prilocaine could be detrimental to the efficacy of IVRGBB. As the local anaesthetic agents clearly inhibit the uptake of [^{3}H]NE, it is hypothesised that they may

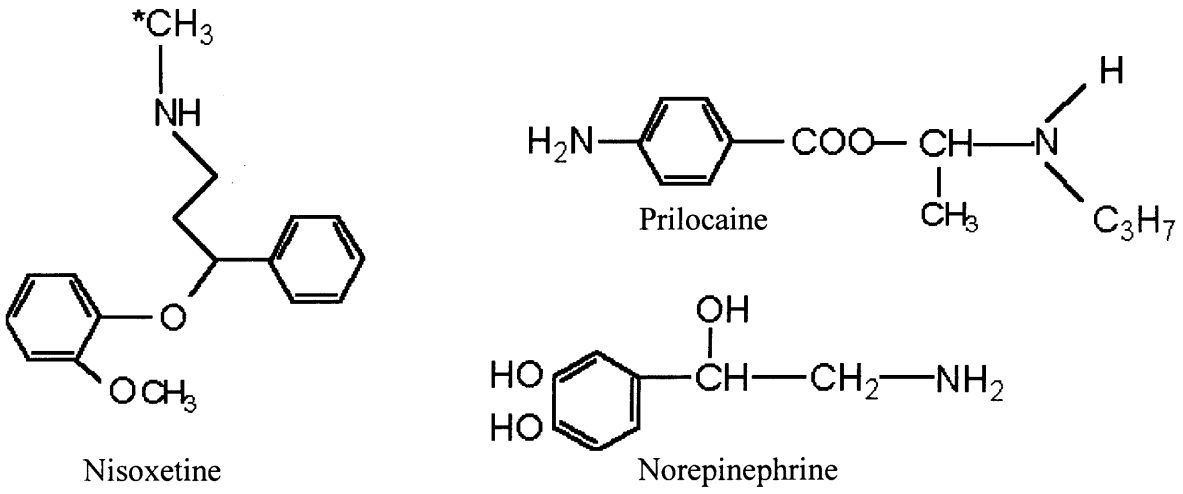
interact at the site of uptake and this site is probed further in the next chapter using radioligand binding techniques.

CHAPTER 4

Binding of [^{3}H]nisoxetine to SH-SY5Y and 293-hNET cell membranes and displacement by local anaesthetic agents.

4.1 Introduction

Nisoxetine is a potent and selective inhibitor of norepinephrine uptake with little or no affinity for a range of neurotransmitter receptors (Wong & Bymaster, 1976; Richelson & Nelson, 1984; Richelson & Pfenning, 1984). [^{3}H]nisoxetine (figure 4.1) has been demonstrated to be a suitable radioligand for the labelling of norepinephrine re-uptake sites in rat brain homogenates and tissue slices (Tejani-Butt *et al.,* 1990; Tejani-Butt, 1992). Several compounds have been used to label the hNET, e.g. the cocaine analogue [I^{125}]3β-[4-iodophenyl]tropan-2β-carboxylic acid methyl ester (Galli *et al.*, 1996), [^{3}H]desipramine, [^{3}H]mazindol (Lee & Snyder, 1981; Langer *et al.*, 1981; Rehavi *et al.*, 1981; Lee *et al.*, 1982; Javitch *et al.*, 1984) and (R)-[^{3}H]tomoxetine (Gehlert *et al.*, 1995). All of these compounds have K_d values in the range of 1-30nM. [^{3}H]nisoxetine has high affinity and selectivity (1.5nM, Cheetham *et al.*, 1996; rat cortical membranes). Chapter 3 indicates an interaction of local anaesthetic agents with hNET. This chapter further investigates this interaction by probing the potential displacement by local anaesthetics of [^{3}H]nisoxetine from the NE uptake sites on cell membranes. Indeed, many compounds which displace [^{3}H]nisoxetine from cortical cell membranes also inhibit NE uptake into synaptosomes (Cheetham *et al.*, 1996, see Chapter 7).



Nisoxetine

Prilocaine

Norepinephrine

Figure 4.1 Structures of nisoxetine, the amide local anaesthetic prilocaine and norepinephrine for comparison. Asterisk indicates the site of incorporation of the tritium label.

4.2 Aims

The aims of this chapter are firstly to characterise the binding of [^{3}H]nisoxetine to SH-SY5Y and 293-hNET cell membranes. Secondly, the effects of various local anaesthetic agents upon the binding of [^{3}H]nisoxetine will be determined to establish if the inhibition of [^{3}H]NE uptake is due to competition at the uptake site. From competition binding studies, K_i values will be calculated for each compound. Any competitive inhibition of [^{3}H]nisoxetine binding will be compared with the local anaesthetic concentrations encountered clinically to determine their relevance to the hypothesis that 'co-administration of local anaesthetic with guanethidine may reduce the efficacy of treatment by the former inhibiting uptake of the latter'.

4.3 Methods

For details of chemicals, cell culture, harvesting, binding protocols and analysis of data, the reader is directed to chapter 2.

4.4 Results

4.4.1 Time course of [^{3}H]nisoxetine binding to equilibrium

In 293-hNET cell membranes, the binding of [^{3}H]nisoxetine (~0.5-1nM) at 4°C was time dependent reaching a maximum at around 2 hours (Figure 4.2). At 3 hours non-specific binding amounted to around 5% and total binding was ~1% of total [^{3}H]nisoxetine added. All subsequent binding studies were therefore incubated for 3 hours to equilibrium.

4.4.2 Determination of K_d and B_{max} for [^{3}H]nisoxetine by isotopic dilution

Isotopic dilution experiments indicated that the binding of [^{3}H]nisoxetine (~0.5-4nM at 4°C) to 293-hNET membranes was concentration dependent and saturable (Figure 4.3) which, when analysed using a semi-log plot (Figure 4.4) yielded B_{max} and pK_d values of 5934±475fmol mg^{-1} protein and 8.41±0.05 respectively. Attempts to obtain a saturation curve with SH-SY5Y cells using isotopic dilution were unsuccessful, as specific binding was low even at high (300µg $tube^{-1}$) membrane protein concentrations. In a typical experiment non-specific binding amounted to >70%. All further studies with SH-SY5Y

cells were therefore conducted under conditions identical to those used for 293-hNET cells and the K_d assumed to be the same. This assumption is not unreasonable as K_d values of 1.5 ± 0.1nM and 7.57 ± 1.06 for rat frontal cortex membranes and SH-SY5Y cell membranes respectively have been reported by others (Cheetham *et al.*, 1995; Zavosh *et al.*, 1999).

4.4.3 Example of isotopic dilution calculation.

To get a value of specifically bound drug in an isotopic dilution experiment several steps must be taken to convert from 'raw' disintegrations per minute.

The following is stepwise example of an actual value from an isotopic dilution experiment in 293-hNET cells.

1. Raw dpm=1364, first the corresponding value for non-specific binding is subtracted (567dpm) giving 797.
2. Division of 797 by the DPM nM^{-1} (94,350) value calculated from assay volume and specific activity of the radiolabelled nisoxetine gives 0.008447nM bound ml^{-1}
3. 0.008447 is multiplied by 1,000 to convert from into fmol bound ml^{-1} , 8.447.
4. 8.447 is multiplied by the assay volume in ml (0.5) giving a value of 4.2235fmol bound $tube^{-1}$.
5. 4.2235 is divided by the amount of protein in mg per assay tube (0.078mg) giving 54.15 fmol bound mg $protein^{-1}$. This is the actual value bound.
6. The final step is to multiply 54.15 by the ratio of total nisoxetine concentration to radiolabelled nisoxetine (50.6nM : 0.582nM = 86.9). This gives a final converted value of 4705 fmol mg $protein^{-1}$. This calculation is performed for each concentration added.

4.4.4 Displacement of [^{3}H]nisoxetine by imipramine

As was expected for a known uptake inhibitor (Hollister & Claghorn, 1993), imipramine also displaced specific [^{3}H]nisoxetine binding from both SH-SY5Y and 293-hNET cell membranes, in a concentration dependent manner to a maximum displacement (to the same degree as unlabelled nisoxetine i.e. leaving only non-specific binding) (figure 4.5).

4.4.5 Competitive displacement of [^{3}H]nisoxetine from 293-hNET cell membranes by local anaesthetic agents.

Specific binding of [^{3}H]nisoxetine to 293-hNET was displaced in a concentration dependent manner by all local anaesthetic agents examined (figures 4.6 (esters) and 4.7 (amides)) with pK_i values as shown in table 4.1. In addition, all local anaesthetic agents showed a complete displacement of specific [^{3}H]nisoxetine binding. As with uptake, the ester type local anaesthetics, cocaine> tetracaine> showed greater affinity for displacement than the amides, dibucaine> bupivacaine> prilocaine> lidocaine. However procaine did not fit the general pattern with a K_i value of 3.27±0.06 which was between bupivacaine and prilocaine (table 4.1).

4.4.6 Competitive displacement of [^{3}H]nisoxetine from SH-SY5Y cell membranes.

Specific binding of [^{3}H]nisoxetine to SH-SY5Y was displaced in a concentration dependent manner by all local anaesthetic agents examined (figures 4.8 (esters) and 4.9 (amides)) with K_i values as shown in table 4.1. Due to low specific binding, the data were more variable than in 293-hNET cells. As with uptake, the ester type local anaesthetics procaine> cocaine> tetracaine> showed lower K_i (higher affinity) values for displacement than the amides dibucaine> bupivacaine> prilocaine> lidocaine. It is noteworthy that, contrary to the other local anaesthetics, cocaine did not displace specific [^{3}H]nisoxetine by 100% but reached a maximum displacement of 77±13% (n=4). An explanation cannot be offered for this but it may suggest isoforms of the transporter for which cocaine is selective leaving a fraction still bound to [^{3}H]nisoxetine. However, such subtypes have not previously been reported.

4.4.7 Correlation of K_i values between cell lines

There was a small (~6 fold) but statistically significant ($p<0.05$) difference in pK_i values for imipramine in both cell lines (SH-SY5Y, 6.36±0.20; 293hNET, 7.14±0.07). There was a significant difference ($p<0.05$) between pK_i values for the two cell types for cocaine, tetracaine, procaine, dibucaine and lidocaine (table 4.1). There was a significant positive correlation ($r^2=0.79$) between pK_i values in SH-SY5Y and 293-hNET cells (figure 4.10).

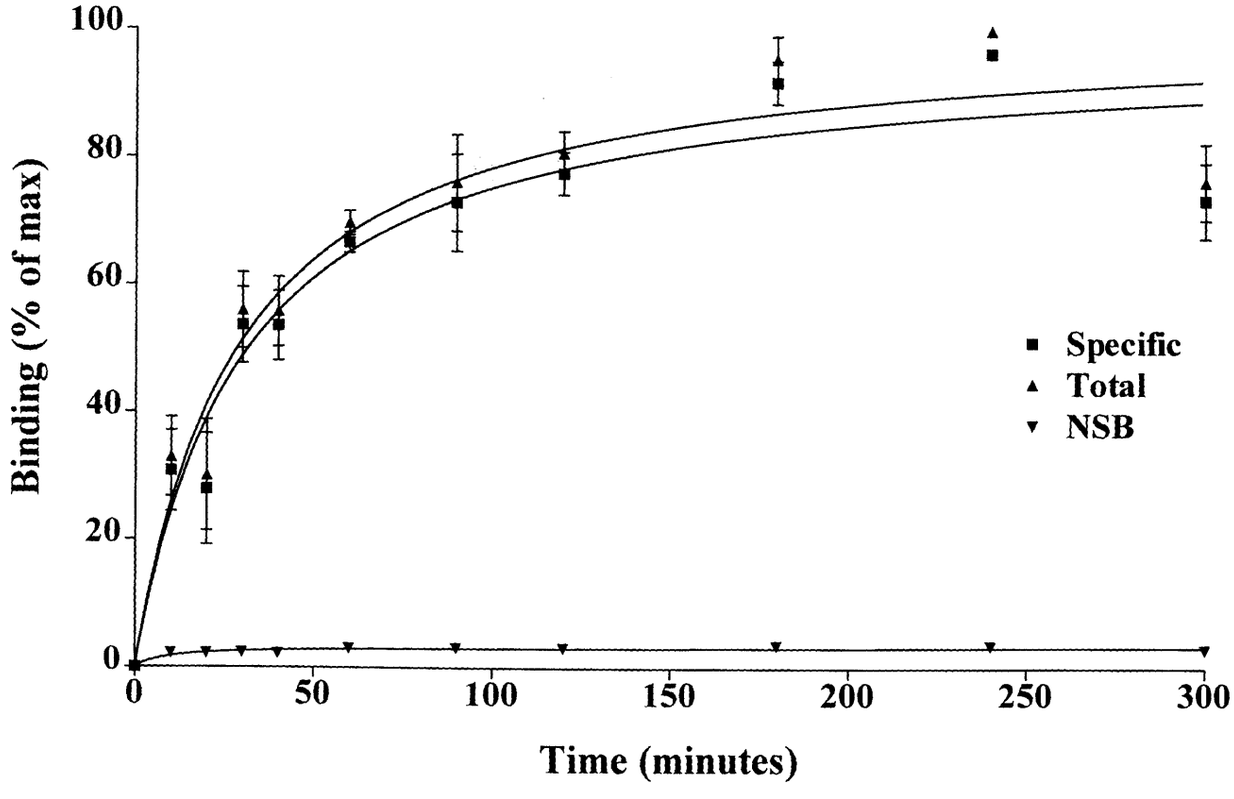


Figure 4.2 Time dependent binding of [^{3}H]nisoxetine (0.5~1nM, 4°C) to 293-hNET cell membranes. Data are shown as percentage of maximum binding as maximum values varied with age of cells. Maximum specific binding was 509.8 ± 48 fmol mg protein^{-1}. (Data are mean ± s.e.mean. n=4)

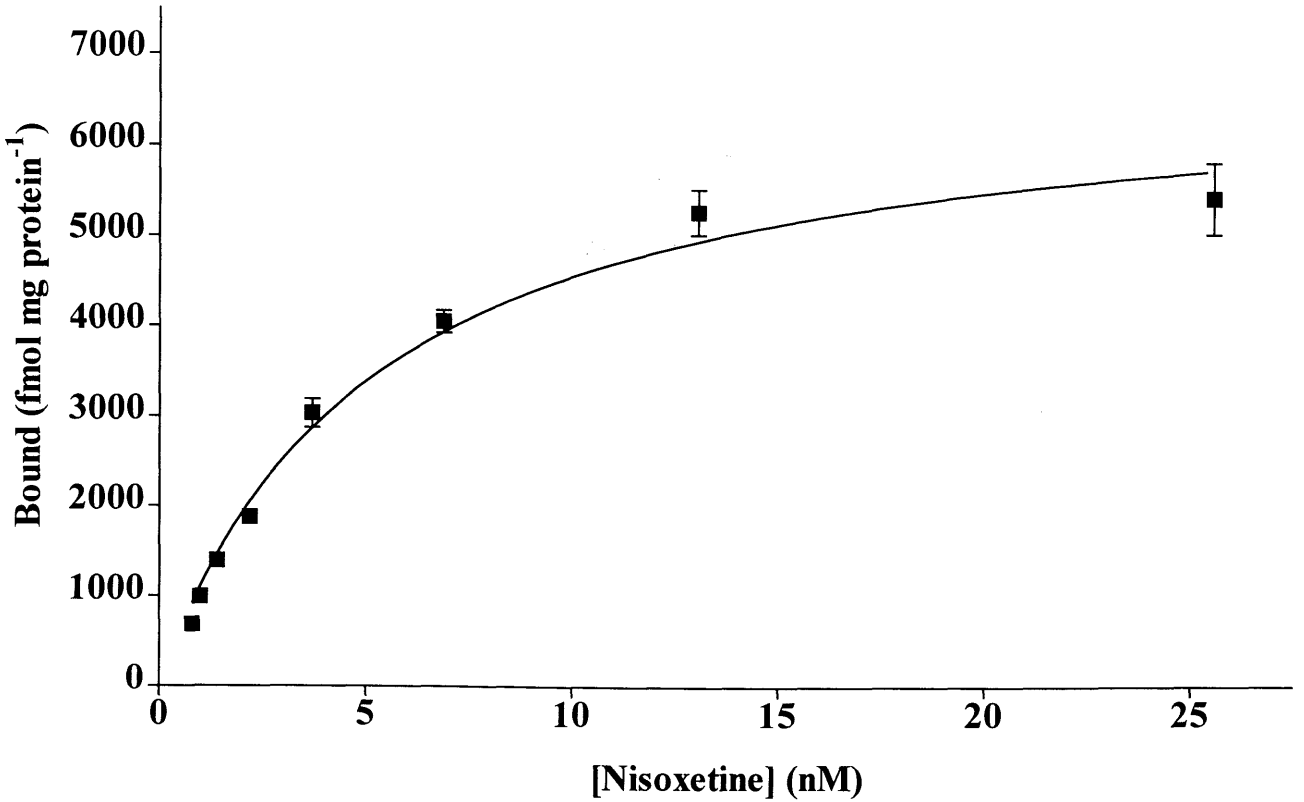


Figure 4.3 Concentration dependent binding of [^{3}H]nisoxetine to 293-hNET membranes. This curve was generated from isotopic dilution data hence greater s.e.mean values at higher nisoxetine concentration where the dilution factor is greater. Analysis of this curve yielded B_{max} and K_d of 6862 ± 284 fmol mg protein^{-1} and 5.16 ± 0.55, respectively (n=8).

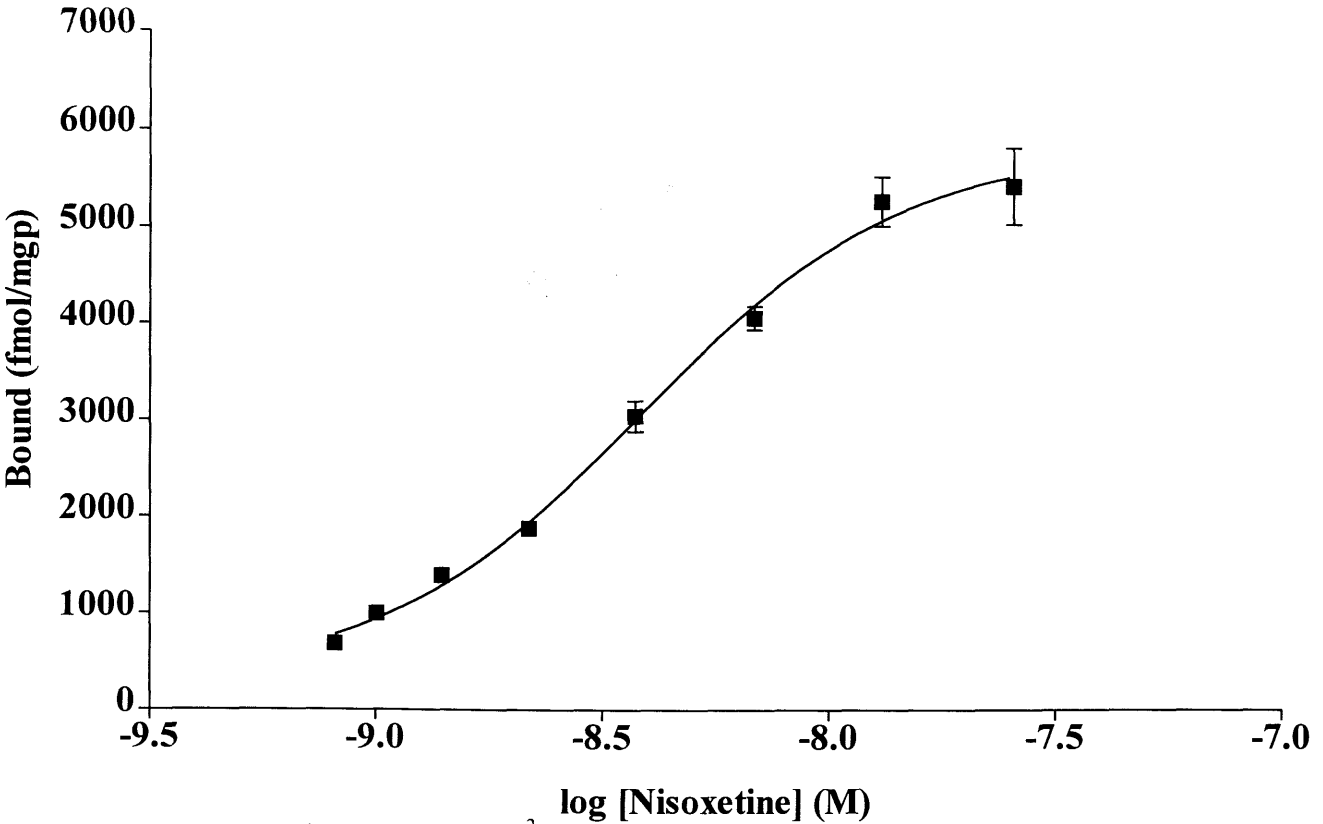


Figure 4.4 Sigmoid plot of [^{3}H]nisoxetine binding calculated from isotopic dilution data as in Figure 4.3. B_{max} and pK_d values were calculated by non-linear regression using Graphpad Prism2 as 5934±475fmol mg protein^{-1} and 8.41±0.05 respectively, slope factor 1.48±0.34. (Data are mean ± s.e.mean of values obtained from each individual experiment, n=8)

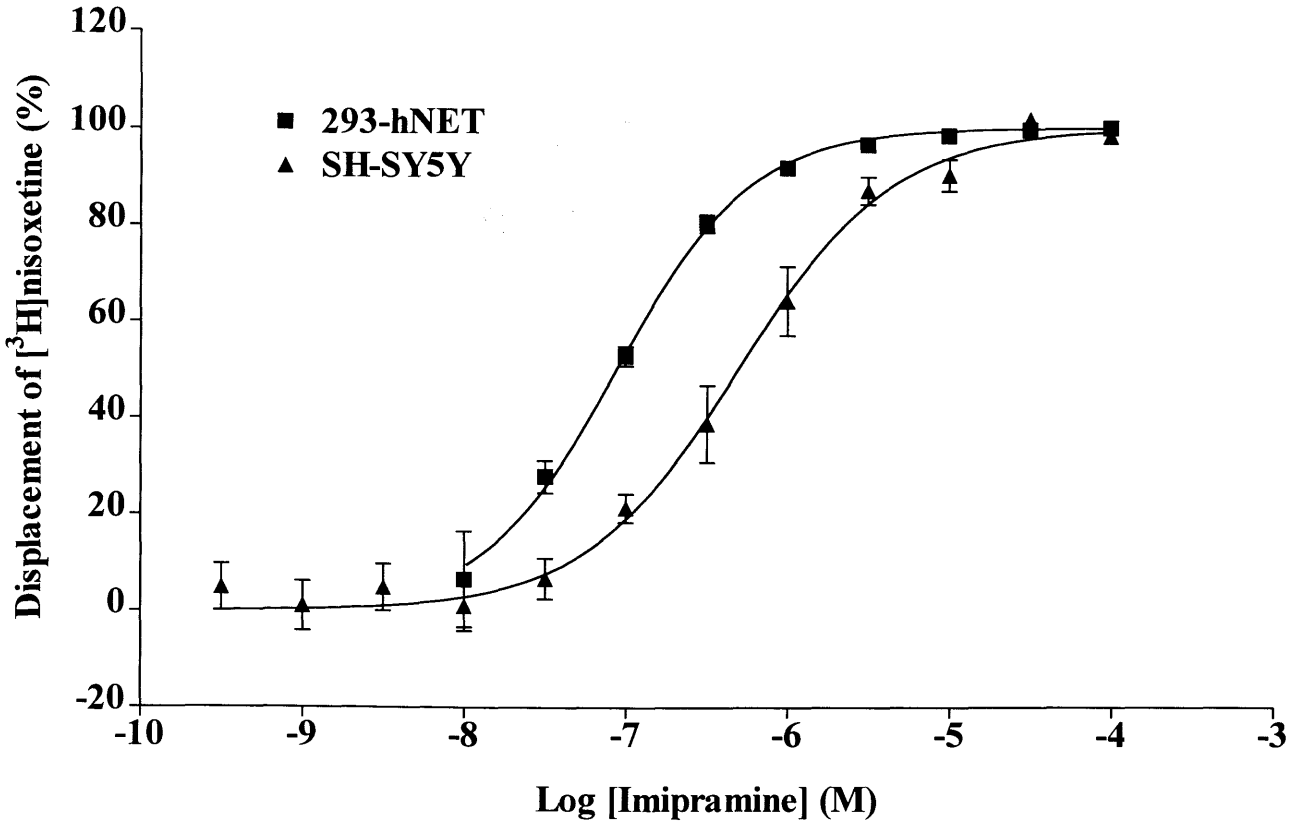


Figure 4.5 Log displacement curves of [^{3}H]nisoxetine by imipramine from 293-hNET and SH-SY5Y membranes. (Data are mean ± s.e.mean, n=5-6). The top of the curves were set to a constant 100%.

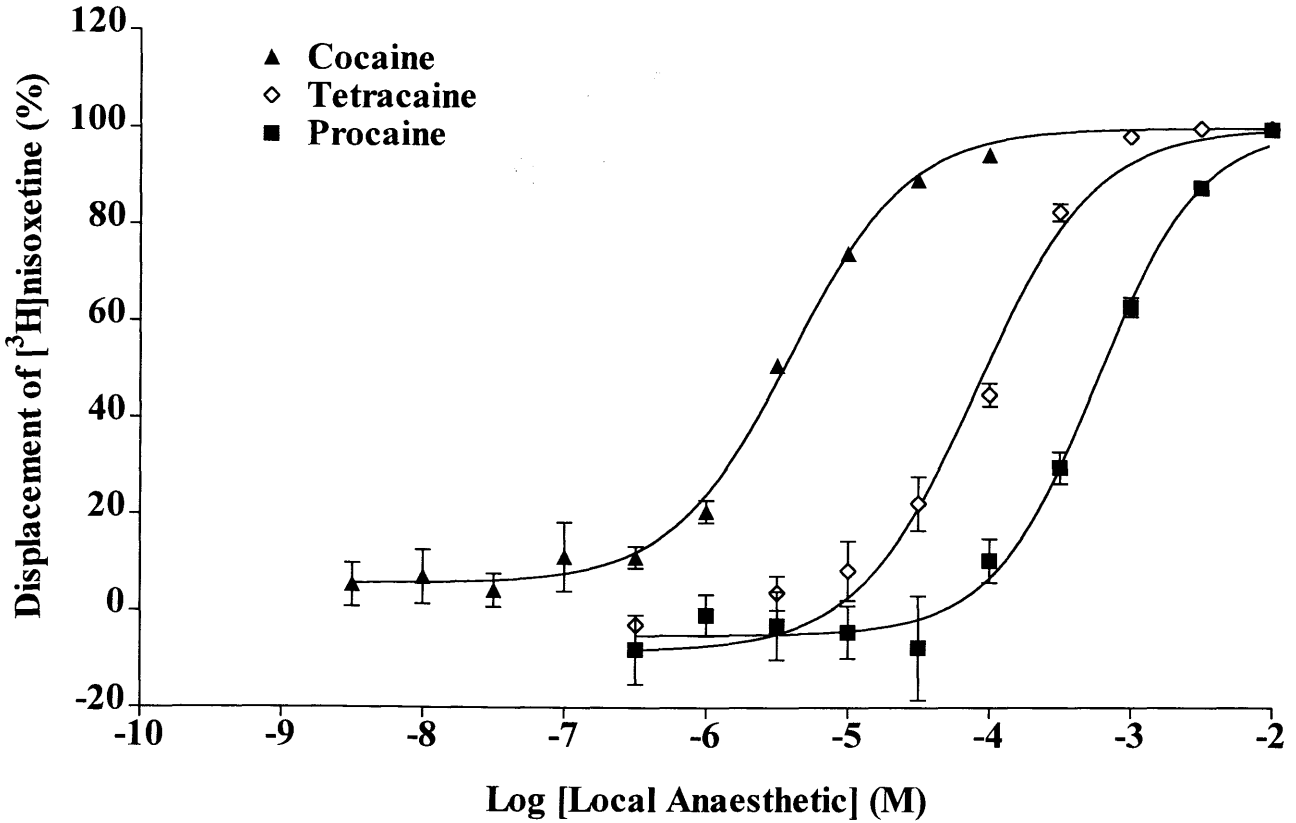


Figure 4.6 Log displacement curves of [^{3}H]nisoxetine by ester type local anaesthetic agents from 293-hNET membranes. (Data are mean ± s.e.mean, n=4-5). Top of curves were set to a constant 100%.

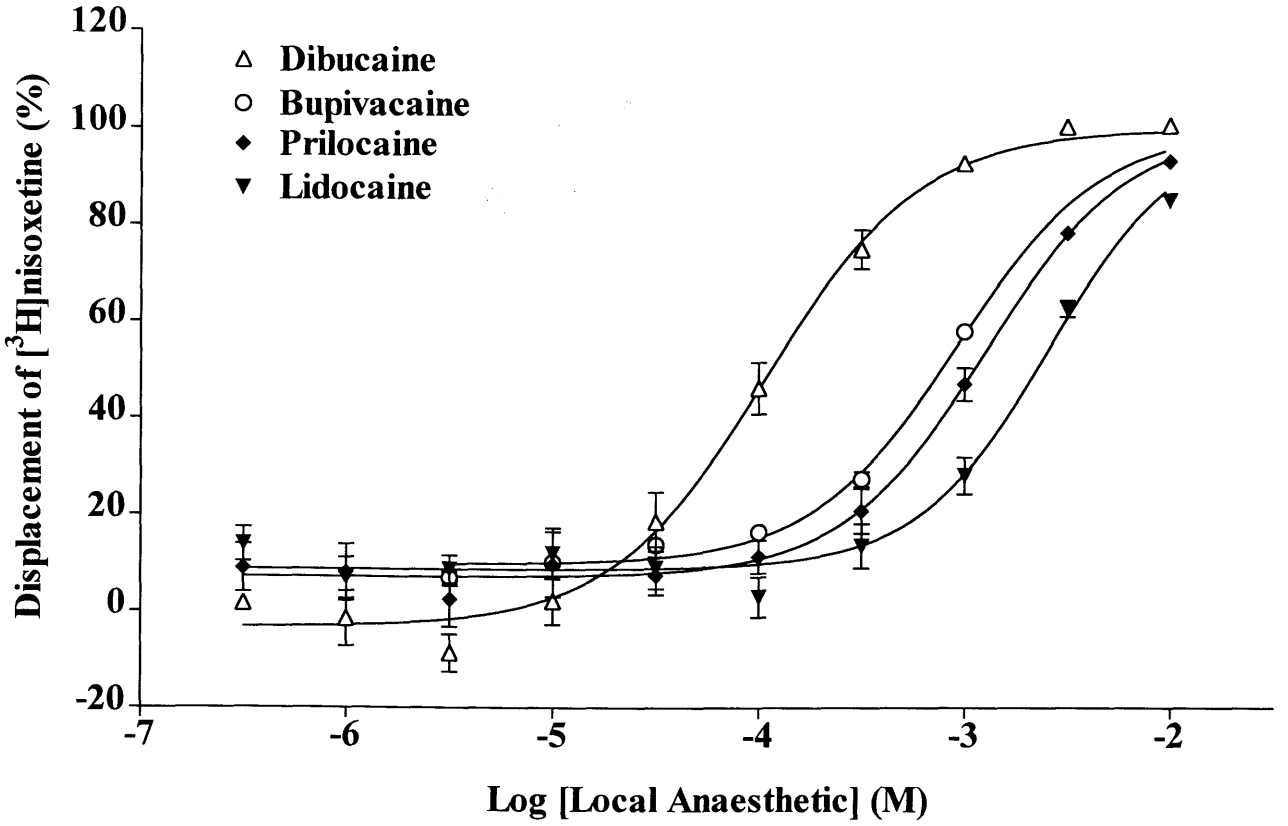


Figure 4.7 Log displacement curves of [^{3}H]nisoxetine by amide type local anaesthetic agents from 293-hNET membranes. (Data are mean ± s.e.mean, n=4-6) Top of curves were set to a constant 100%. Note that the highest possible concentration of bupivacaine was 1mM due to solubility problems, therefore its curve is extrapolated to 100% making the K_i value an estimate.

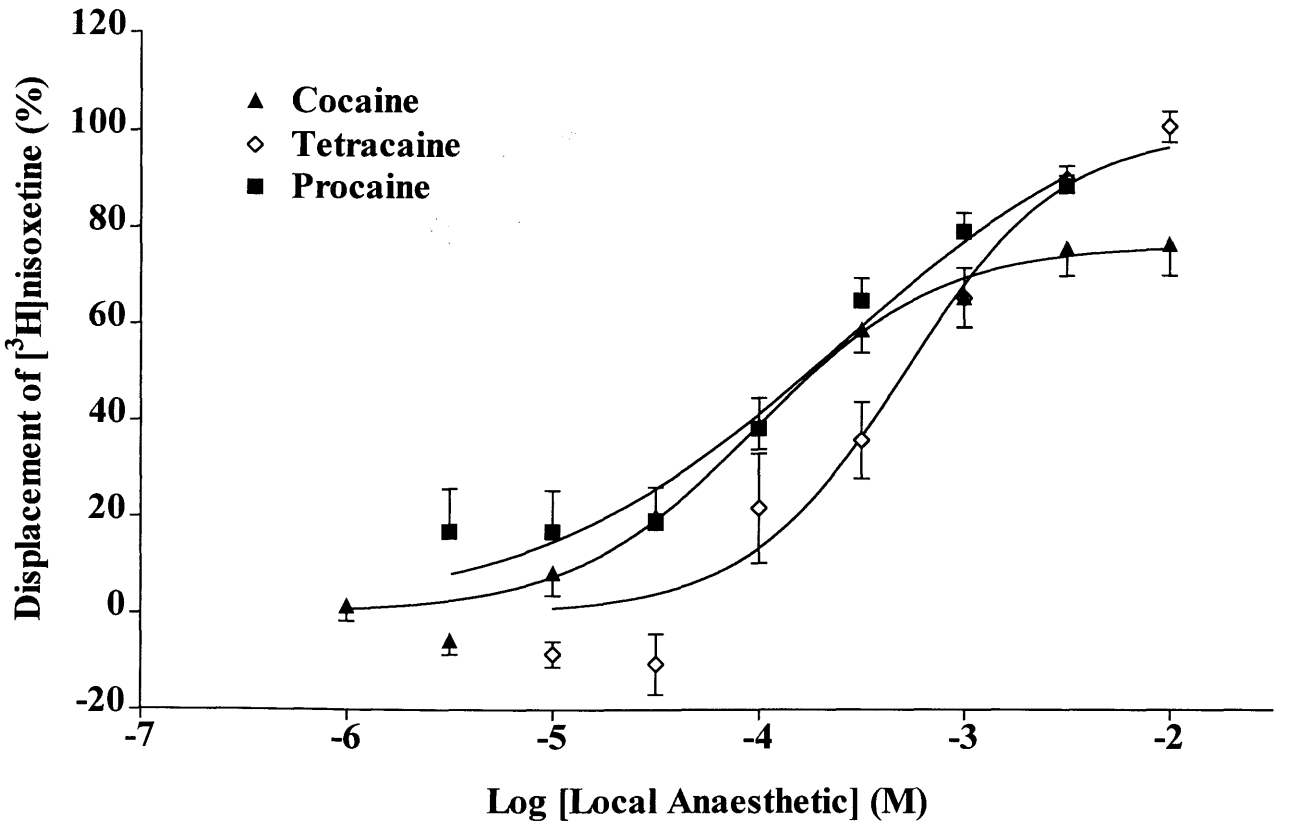


Figure 4.8 Log displacement curves of [^{3}H]nisoxetine by ester type local anaesthetic agents from SH-SY5Y membranes. (Data are mean ± s.e.mean, n=6-8). Top of curves were set to a constant 100% with the exception of cocaine which reached a maximum of 77±13%

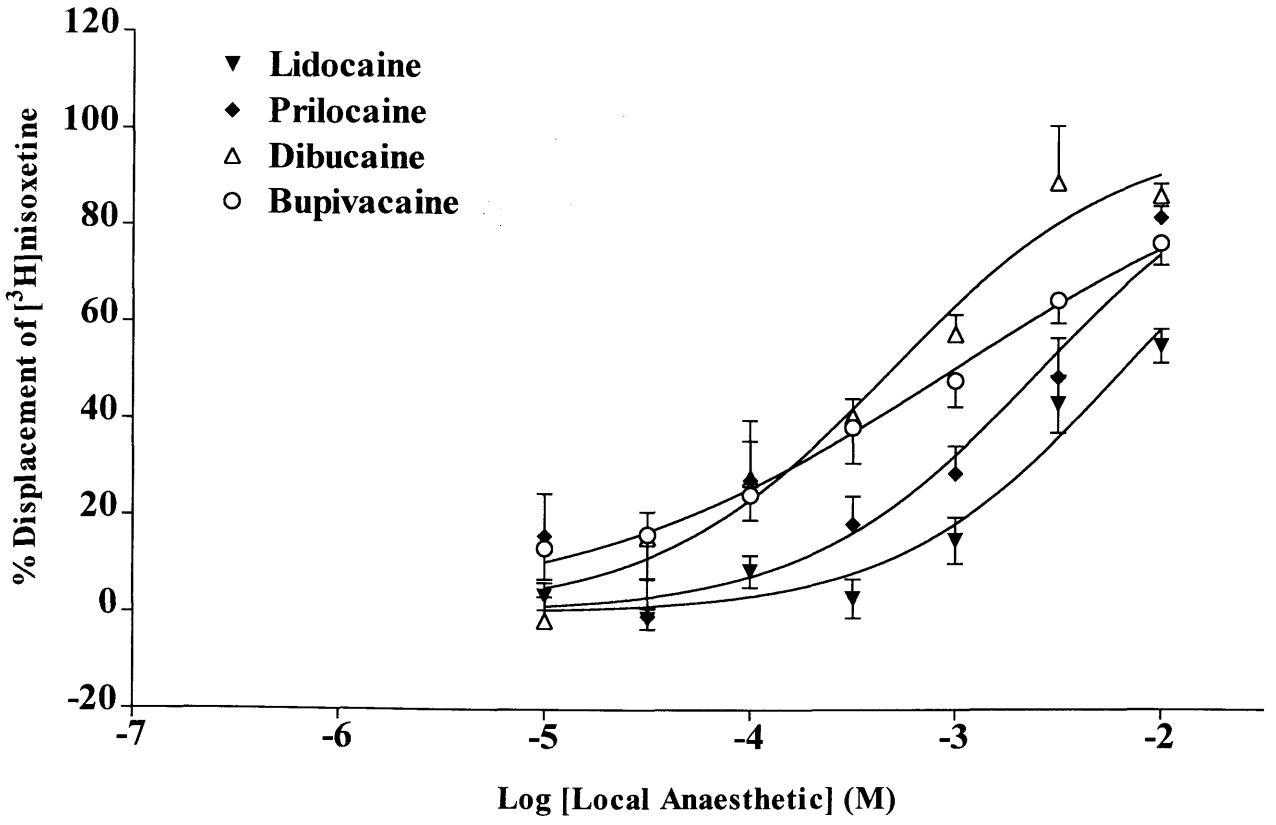


Figure 4.9 Log displacement curves of [^{3}H]nisoxetine by amide type local anaesthetic agents from SH-SY5Y membranes. (Data are mean ± s.e.mean, n=6-8). Top of curves were set to a constant 100%.

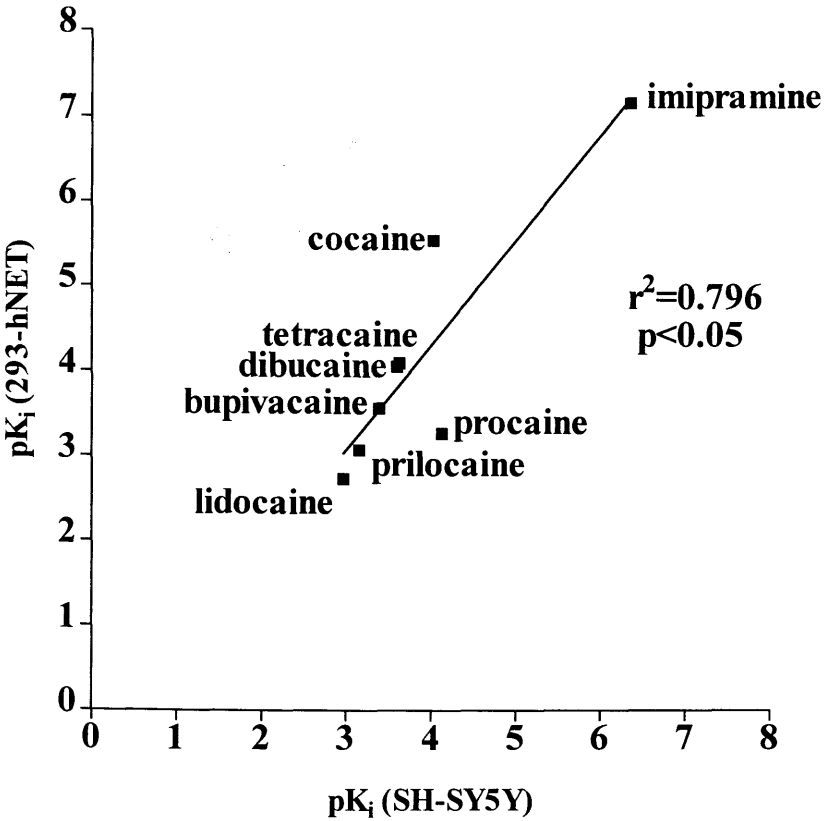


Figure 4.10 Correlation between pK_i values for local anaesthetic agents and imipramine in SH-SY5Y and 293-hNET cell membranes. r^2=0.796. Values shown are mean, n=4-8.

Table 4.1 pK_i values for displacement of [^{3}H]nisoxetine in SH-SY5Y and 293-hNET cell membranes by local anaesthetic agents and imipramine. pK_i values were calculated from IC_{50} values obtained from Graphpad Prism using the Cheng and Prusoff equation (see section 2.7) Data are mean+s.e.mean from (n=4-8). Significance refers to unpaired, two tailed Student's t test.

Compound	pK_i SH-SY5Y	pK_i 293-hNET	p	Ester/Amide
Imipramine	6.36 ± 0.20	7.14 ± 0.07	<0.05	-
Cocaine	4.03 ± 0.09	5.53 ± 0.01	<0.05	E
Tetracaine	3.63 ± 0.17	4.09 ± 0.07	<0.05	E
Procaine	4.13 ± 0.14	3.27 ± 0.06	<0.05	E
Dibucaine	3.60 ± 0.12	4.05 ± 0.10	<0.05	A
Bupivacaine	3.39 ± 0.20	3.56 ± 0.03	>0.05	A
Prilocaine	3.16 ± 0.14	3.07 ± 0.08	>0.05	A
Lidocaine	2.97 ± 0.09	2.73 ± 0.05	<0.05	A

4.5 Discussion

In this chapter it was demonstrated that [^{3}H]nisoxetine labels 293-hNET cell membranes in a time and concentration dependent manner reaching equilibrium at around 3 hours at 4°C. [^{3}H]nisoxetine is displaced almost completely by unlabelled nisoxetine and imipramine leaving non-specific binding of 1-5% (Tejani-Butt, 1992; Cheetham *et al.*, 1996).

Binding was also observed in SH-SY5Y cell membranes but was too low to reliably determine the K_d and B_{max} using isotopic dilution techniques. Attempts at estimating the B_{max} will be made from later experiments involving immunoblotting of cell extracts containing hNET protein (see chapter 6). In a typical experiment using ~0.5mg protein ml^{-1} and concentrations of [^{3}H]nisoxetine <K_d (determined in 293-hNET assay), ~800dpm specific binding was observed making; (a) isotope dilution unreliable and (b) direct saturation not feasible due to high concentrations required to achieve saturation. In a recent study (Zavosh, 1999), estimated the B_{max} of SH-SY5Y cell membranes to be ~20fmol mg protein^{-1} (with a K_d of ~2.6nM). Interestingly the B_{max} in parent SK-N-SH cell membranes

has been estimated at 2.2pmol mg protein^{-1} with a K_d of 6.7nM, a 100 fold higher B_{max} value than that of SH-SY5Y cells (Apparsundarum *et al.*, 1998). The discrepancy in B_{max} compared with uptake in our SH-SY5Y cells (essentially same uptake with widely differing B_{max}) is difficult to explain but may result from (a) differences in the membrane environment (neuronal c.f. non-neuronal), (b) the presence of NE storage vesicles in SH-SY5Y cells or (c) high expression of non-functioning transporters in 293-hNET cells like the 54-kDa biosynthetic intermediate which may contribute to ligand binding but not transport (Melikian, 1994). For a comparison with other studies with various ligands and tissues, see table 4.2. From these values, it appears that 293-hNET cells have a far greater B_{max} than most tissues and cells that express the transporter endogenously. That 293-hNET cells express more transporters is clearly a result of the transfection process. There also appears to be a trend toward tissues from rats having lower K_d values than those of cultured cell preparations.

It is also clear from this chapter that local anaesthetic agents, both ester and amide type, are capable of displacement of [^{3}H]nisoxetine from membranes prepared from SH-SY5Y and 293-hNET cells. This data supports the theory that local anaesthetics interact at the site of uptake to inhibit NE transport and hence presumably transport of other substrates like guanethidine. It seems that this is a common characteristic of all local anaesthetic agents studied and may have important clinical implications (chapter 7 Discussion).

It is notable that cocaine did not displace [^{3}H]nisoxetine by 100% but appeared to reach a maximum of ~80% (figure 4.8). An explanation cannot be offered for this, but it may suggest isoforms of the transporter for which cocaine is selective leaving a fraction still bound to [^{3}H]nisoxetine. However, such subtypes have not yet been reported. On the contrary, nisoxetine is considered to be a highly selective ligand for NET (Tejani-Butt, 1990).

Cocaine and procaine lie well away from the line of correlation between pK_i values in SH-SY5Y and 293-hNET cells where the other compounds correlate almost exactly (r^2=0.99), (figure 4.10). The reason for these discrepancies between cell lines are unclear but may be attributable to the differing lipid environment of the two cell membranes, i.e. neuronal and non-neuronal. Removal of imipramine from figure 4.10 also reduces the r^2 value from 0.79

to 0.4 showing limited correlation of the local anaesthetic displacement of [^{3}H]nisoxetine between cell lines.

In summary it has been shown that a range of local anaesthetic agents displace [^{3}H]nisoxetine, from its specific labelling site namely the NE re-uptake site on hNET in both SH-SY5Y and 293-hNET cells. It is also clear that SH-SY5Y cells express lower levels of hNET protein than 293-hNET cells. This will be investigated further in Chapter 5.

Table 4.2 B_{max} and K_d values obtained from other studies in various tissues with various NET ligands.

Ligand	B_{max} (fmol mg^{-1})	K_d (nM)	Tissue	Reference
[^{125}I]RTI-55	13,100 ± 600	14.20	293-hNET cell membranes	Galli *et al.*, 1995
[^{3}H]nisoxetine	~19,000	~5.00	293-hNET cell membranes	Zhu *et al.*, 1998
[^{3}H]nisoxetine	~400	4.40 ± 0.70	PC12 cell membranes	Zhu & Ordway, 1997
[^{3}H]nisoxetine	109 ± 6.4	0.70 ± 0.06	Rat cerebral cortex membranes	Tejani-Butt *et al.*, 1990
[^{3}H]nisoxetine	87 ± 4.0	1.02 ± 0.04	Rat frontal cortex homogenates	Cheetham *et al.*, 1996
R-[^{3}H]tomoxetine	~20	0.45 ± 0.10	Rat forebrain homogenates	Gehlert *et al.*, 1995
[^{3}H]nisoxetine	~28	0.88 ± 0.08	Rat forebrain homogenates	Gehlert *et al.*, 1995
[^{3}H]cyanoimipramine	225.5 ± 31.8	0.61 ± 0.13	Rat brain homogenates	Be ique *et al.*, (1998)
[^{3}H]nisoxetine	63.6 ± 5.9	0.76 ± 0.04	Rat brain homogenates	Be ique *et al.*, (1998)
[^{3}H]nisoxetine	1,240 ± 0.1	1.00 ± 0.09	Rat placenta homogenates	Shearman & Jerold, 1998
[^{3}H]nisoxetine	2,200 ± 130	6.70 ± 0.07	SK-N-SH cell membranes	Apparsandaram *et al.*, 1998
[^{3}H]nisoxetine	~20	7.57 ± 1.06	SH-SY5Y cell membranes	Zavosh *et al.*, 1998
[^{3}H]nisoxetine	1,050 ± 60	6.00 ± 0.17	SK-N-SH intact cells	Apparsandaram *et al.*, 1998
[^{3}H]nisoxetine	**5934 ± 475**	**3.89 ± 0.43**	**293-hNET cell membranes**	**This thesis, 2001**

CHAPTER 5

Comparison of hNET expression in 293-hNET and SH-SY5Y membranes by western immunoblotting.

5.1 Introduction

It was shown in the previous chapter that SH-SY5Y cells express hNET protein at much lower levels than in 293-hNET cells. As an actual value for B_{max} could not be determined from [^{3}H]nisoxetine binding experiments, an alternative method of assessment was considered. The current chapter involves the immunoblotting of cell extracts from both SH-SY5Y and 293-hNET cells. It is hoped that a comparison of expression density of hNET in the two cell lines can be achieved. The hNET proteins are separated by SDS (sodium dodecyl sulphate) PAGE (polyacrylamide gel electrophoresis). This method allows separation of proteins according to their molecular weight by differential migration through acrylamide gel. The denatured proteins become negatively charged after combination with SDS and smaller proteins migrate at a greater rate than larger ones, hence separation.

5.2 Aims

The aims of this chapter are to separate the hNET protein using polyacrylamide gel electrophoresis (PAGE) to visualise the glycosylated and non-glycosylated forms of hNET protein (Melikian *et al.,* 1994; see Introduction Chapter 1.6.1). The intention is to use samples of specific protein concentrations and optical densitometry to compare the expression density of hNET protein in each cell line.

5.3 Methods

5.3.1 SDS polyacrylamide gel electrophoresis background

Electrophoresis and blotting techniques employed in this chapter were developed from the method of Laemmli, 1970 using a 'trial and error approach'. Exact details of the final protocol used are shown in 5.4.4.

SDS poly-acrylamide gel electrophoresis is a technique which allows separation of proteins according to their molecular mass. SDS is an ionic detergent which binds to denatured protein molecules and causes them to be negatively charged in proportion to their size. The SDS-polypeptide complexes migrate through poly-acrylamide gel at a rate dependent on their size when subject to an electric field. Two types of gel are used for

separation. The stacking gel allows the formation of a boundary between Cl^- ions and glycine molecules, which allows the samples to be deposited on the surface of the running gel. The running gel is of a higher pH which results in the ionisation of the glycine molecules, freeing the samples to move and separate through the gel. After transfer from the gel to nitro-cellulose paper by electrical current, the proteins can be labelled with primary specific antibodies then visualised by a range of secondary antibody systems. In this case the visualisation technique was; a mouse anti-hNET primary antibody and a horseradish peroxidase conjugated, goat anti-mouse IgG, secondary antibody which interacted with enhanced chemiluminescence (ECL) reagent. The resulting luminescence was exposed to photographic film. Measurement of optical density of the resulting ECL band allows estimation of relative expression density in the two cell types, provided the original sample is of known protein concentration. Molecular mass of the detected proteins is determined by running an adjacent set of pre-stained molecular weight markers (figure 5.1 and 5.2).

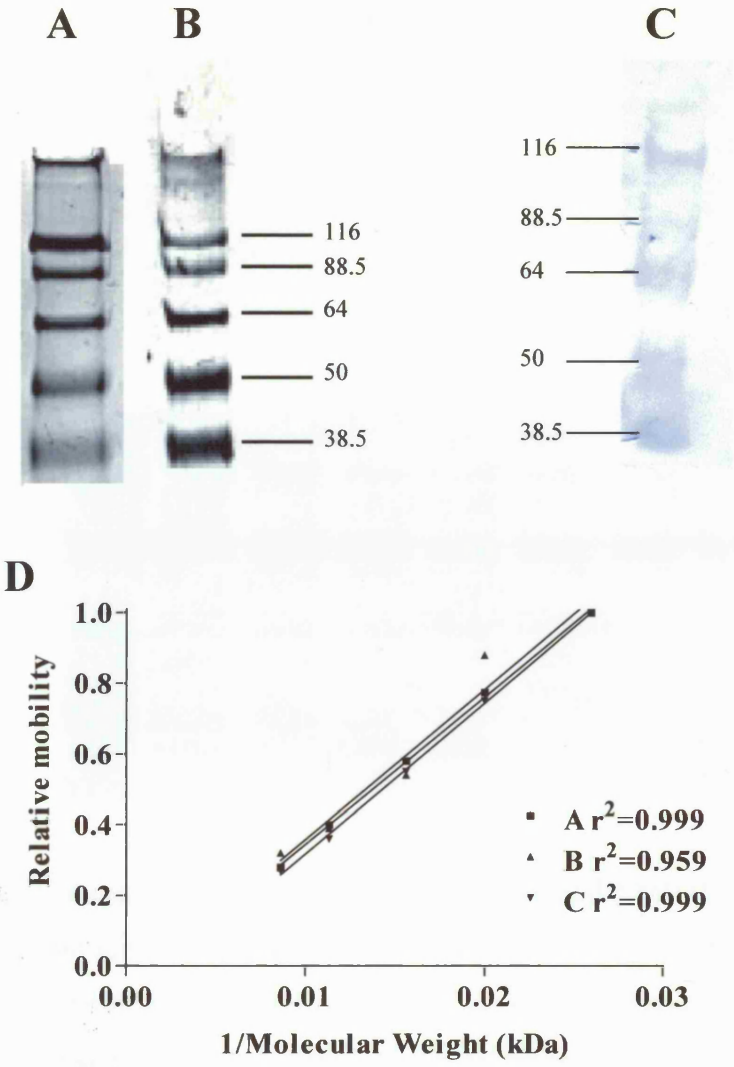


Figure 5.1 Protein standard separations. A. Sigma pre-stained SDS-PAGE standard solution electrophoresed on 10-27% SDS-PAGE according to the method of Laemmli (1970). B. Transfer of pre-stained markers (Sigma) from SDS-PAGE to a nitro-cellulose membrane using Sigma transfer buffer and semi-dry blotter. C. Transfer of pre-stained standard from SDS-PAGE to a nitro-cellulose membrane using standard transfer buffer and semi-dry blotter (this thesis). Numbers show molecular weights in kDa. D Graph showing the linear relationships in A, B and C of relative mobility against 1/molecular weight including r^2 values. (A and B were taken from Sigma-Aldrich product information, **www.sigma-aldrich.com**)

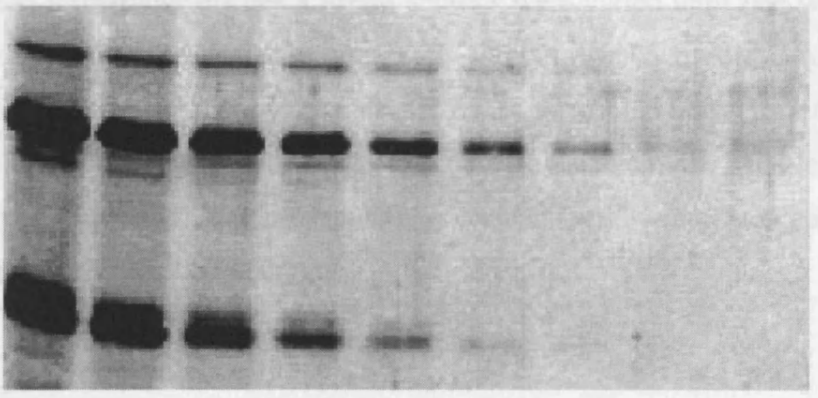


Figure 5.2 Enhanced Chemiluminescence. Storm model 860 (Fluorescence scanner) image of 2-fold serial dilutions of rat brain homogenate labelled by β-tubulin primary antibody, horseradish peroxidase conjugated secondary antibody and Hyperfilm ECL reagent. Left hand lane contains 10μl of neat rat brain homogenate in treatment buffer (2:5 ratio) and subsequent lanes to the right are 2 fold serial dilutions. Image and details acquired from Amersham Pharmacia Biotech product information (www.apbiotech.com {protein labelling and detection}).

Results 5.4

5.4.1 Verification of SDS polyacrylamide gel running

Initially this protocol was by 'trial and error', mainly by following the conditions and methods of Laemmli, (1970). First the gels were cast in the correct concentrations and loaded with samples. Problems encountered at this stage included air bubbles in the top layer (loading gel) when inserting the comb. This made some of the loading wells incomplete and unusable. It was also important to have made up the gels in the correct proportions, as occasionally instead of the samples progressing as a horizontal line of blue dye they spread throughout the gel. Current settings were varied until one which allowed the samples to progress satisfactorily in around 3½ hours was achieved. Greater currents than this could overheat the gel as the tank used was not cooled and was relatively small ($500cm^3$). As the gel was running, it was possible to see the dye in the samples progressing and also the separation of the pre-stained molecular weight markers, confirming the selection of the correct gel concentration.

5.4.2 Determination of correct transfer conditions

Once the separation was complete, the gel was carefully removed from the glass plate 'sandwich' and plastic spacers. Noting its orientation it was prepared for blotting by placing in a 'sandwich' with three sheets of 'transfer buffer' (see section 2.2) soaked Whatman GF/B filter paper then nitro-cellulose membrane on the bottom and a further three sheets of Whatman GF/B filter paper on the top. The sandwich was placed in a 'semi-dry' blotter with the anode at the bottom. Various current settings and times of blotting were tested. Protein transfer was easily visualised by addition of Ponceau S solution (Sigma, UK) which remained bound to proteins when gently washed with distilled water leaving pink vertical bands on the nitro-cellulose corresponding to the sample migration.

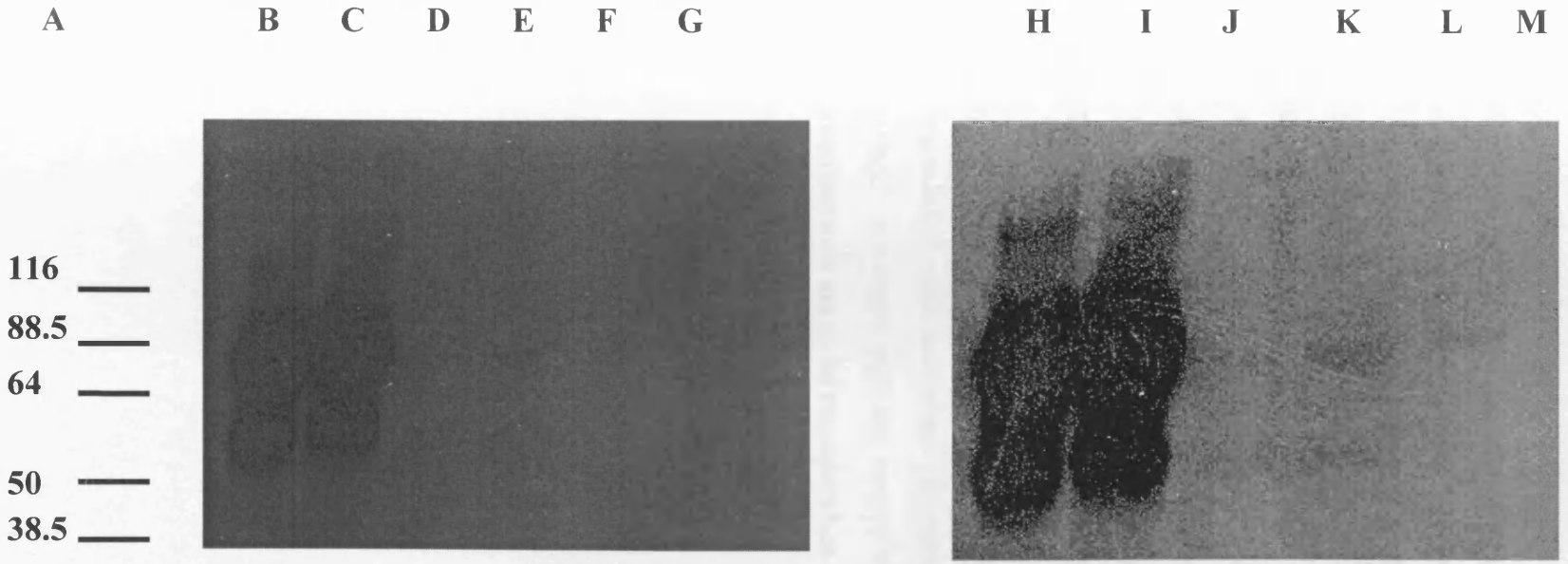


Figure 5.3 Typical western blots of an experiment where the appropriate protein concentrations had not yet been determined. A shows the positions of the pre-stained markers (sizes in kDa) from nitro-cellulose membrane. Lanes B and C contained 20µg of 293-hNET cell extract lanes, D and E contained 150µg of SH-SY5Y cell extract and lanes F and G contained 75µg of SH-SY5Y cell membrane. Lanes H to M were identical to Lanes B to G except the photographic film was exposed for 5min as opposed to 3 to visualise the feint chemiluminescence of the SH-SY5Y cell extracts. Background density appears different only in these manipulated images because the contrast has been adjusted to make the feint SH-SY5Y bands more visible. This was not necessary with films having the optimum protein concentrations.

5.4.3 Determination of correct protein and antibody concentrations for optimum visualisation.

In order to obtain clear hNET bands on the photographic film, several factors must be taken into account. As the expression level of hNET protein in 293-hNET cells is much greater than in SH-SY5Y cells (see chapter 4), it was necessary to dilute the samples to a concentration which would allow visualisation at a similar exposure time as SH-SY5Y cell samples. Several attempts at visualisation with too great a 293-hNET sample concentration resulted in films on which it was impossible to see clear bands from SH-SY5Y cells unless 293-hNET bands were overexposed (e.g. figure 5.3). Bands of comparable density were required in order to estimate the relative expression density of the two cell types. In preliminary blots, a concentration of 293-hNET 60 fold lower than that of the SH-SY5Y cells was eventually found to give satisfactory results.

Another variable for visualisation was the concentration of primary (mouse anti-hNET monoclonal) and secondary (horseradish peroxidase conjugated goat anti-mouse IgG) antibody incubated with the blotted membrane. The concentrations recommended by the manufacturers are to be considered as a guide and experimentation was necessary to find a suitable combination.

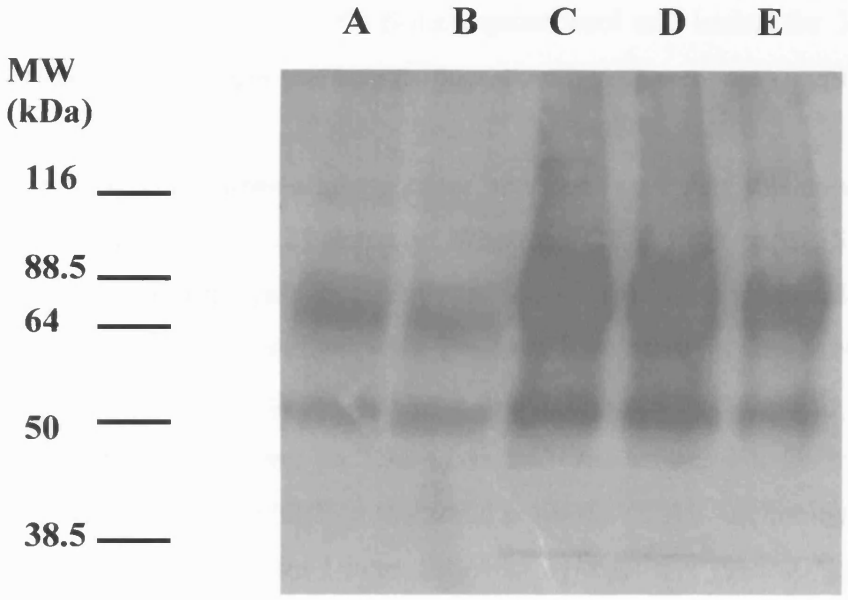


Figure 5.4 Typical Western blot (from n=3) for hNET expression in 293-hNET and SH-SY5Y cells. Lanes A and B are SH-SY5Y cell membranes (150μg protein), C and D 293-hNET cell membranes (5μg protein) and E, 293-hNET cell membranes (2.5μg). Immunoreactive species of 80kDa and 54kDa are shown. Positions and sizes in kDa of pre-stained molecular weight markers are shown on the left.

5.4.4 Final successful protocol for comparison of hNET expression density in SH-SY5Y and 293-hNET cells

Cells were harvested and homogenised as for binding experiments. The membranes were then solubilized in 200μl of ice cold solubilisation buffer (30 min, 4°C, Luckham rotatest orbital shaker, see section 2.2). Solubilized extracts were centrifuged (20,000g, 10 min, 4°C) and protein content of supernatants assessed according to Lowry *et al.*, (1951) (see section 2.10). After repeated experimentation (see above), 150μg of protein per lane for SH-SY5Y cell extract and 5 and 2.5μg per lane 293-hNET cell extract (a concentration

difference of 30 and 60 fold respectively) were determined (n=3, see figure 5.4) as suitable for visualising both proteins for comparison. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 10% bis-acrylamide running gels with 5% stacking gels (Laemmli, 1970). Prior to loading, solubilised extracts were combined with sample buffer containing 10% β-mercaptoethanol and boiled for 3 minutes. Boiled pre-stained molecular weight markers (Sigma, Poole, UK) were also included (figure 5.1).

Gels were placed onto nitro-cellulose paper between two treble thickness, pre-soaked (in transfer buffer, see section 2.2) sheets of Whatman GF/B filter paper. Separated proteins were transferred to nitro-cellulose using a semi-dry blotter in transfer buffer. Nitro-cellulose membranes were stained with Ponceau-S solution in 5% acetic acid to verify protein transfer. Blots were de-stained with dH_2O, blocked overnight in blocking solution at 4°C then extensively rinsed in TBS (Tris buffered saline, 4 x 10 mins.). Blots were incubated with mouse anti-hNET monoclonal antibody (MAb Technologies Inc.) at 1:250 dilution in blocking solution for 1 hour, followed by extensive rinsing. Secondary antibody (Horseradish peroxidase conjugated goat anti-mouse IgG, Sigma, Poole UK) was then added (1:1500 dilution in blocking solution, 1 hour, 22°C). Immunoreactive bands were detected by enhanced chemiluminesence (figure 5.2) An exposure time of 3 minutes was sufficient to visualise immunoreactive bands of both cell lines on the photographic film. Optical density of immuno-blotted bands on photographic film were assessed using Image Master system (Amersham, UK). The Image Master system uses a transmission scanner to transfer the film image into a digital computer image which can then be measured accurately for optical density.

Two major immunoreactive species of hNET, 80kDa (active transporter) and 54kDa (biosynthetic intermediate) were visualised using the specific monoclonal antibody. These data are consistent with other studies in cells expressing hNET (Melikian, 1994). No data involving immunoblotting with SH-SY5Y cells are currently available. Optical density scanning of the 80kDa species suggested a 160 fold lower expression of hNET in SH-SY5Y cells (taking into account relative dilution) compared with 293-hNET and confirmed the low degree of [^{3}H]nisoxetine binding.

5.5 Discussion

This chapter clearly confirms, as indicated in [^{3}H]nisoxetine binding (Chapter 4), that SH-SY5Y cells express the hNET protein at lower levels than in the recombinant 293-hNET cells, yet despite this, the SH-SY5Y cells take up NE with a similar or greater initial rate than 293-hNET cells. It is estimated that with the relative densities shown here coupled with the values for B_{max} for 293-hNET cells that the B_{max} for SH-SY5Y cells would be around 33fmol mg protein^{-1}. Using the protocol of [^{3}H]nisoxetine binding in chapter 4, this level of expression will be at the limits of detection (see section 7.2).

The two immunoreactive bands shown on the blots are in agreement with studies by other groups showing the two (80kDa and 54 kDa) forms of the hNET protein (Schroeter *et al.*, 2000; Melikian *et al.*, 1994). From immunoprecipitation and immunoblot analysis of LLC-NET Melikian *et al* 1994**,** (pig kidney epithelial cells with stably transfected recombinant NETs) revealed two immunoreactive hNET species of 80 and 54-kDa, the smaller of which is the biosynthetic intermediate of the larger form. Deglycosylation studies suggested that the nonglycosylated hNET protein, synthesised in the presence of tunicamycin (a glycosylation inhibitor), is a 46-kDa core protein that is rapidly degraded. The 80-kDa species is considered to be the active transporter which has an estimated 24-hr half life as it slowly disappears following tunicamycin treatment, coupled with reduced capacity for [^{3}H]NE transport. Note that these observations were in LLC-NET cells and not in the cells used in this thesis. Differences may occur between cell lines, for example, transfected HeLa cell extracts expressed only the 54-kDa form of NET, likely to be a result of cell-specific glycosylation patterns (Melikian, 1994). It was demonstrated that non-*N*-glycosylation of NET protein at its three consensus sites on the large hydrophilic extracellular loop between transmembrane domains (TMDs) 3 and 4 reduces protein stability, surface trafficking and substrate translocation but does not affect ligand recognition (Melikian, 1996). Therefore, it is likely that the B_{max} values estimated in chapter 4 reflect both forms (54 and 80kDa) of transporter.

CHAPTER 6

Effect of local anaesthetics, guanethidine and their co-application on the electrically stimulated mouse vas deferens.

6.1 Introduction

Vas deferens is an example of a sympathetically innervated tissue (Bentley & Sabine, 1963) and an ideal *ex vivo* model in which to investigate an interaction of local anaesthetics and guanethidine. The vas deferens has a three layered structure comprising an external areolar coat, a muscular coat of smooth muscle (both longitudinal and circular) and an inner mucous epithelial coat (Williams *et al.*, 1989) In organ bath conditions this tissue responds to electrical field stimulation (EFS) by producing reproducible and substantial contractions (Bigoni *et al.*, 1999).

The vas deferens is innervated by the sympathetic branch of the autonomic nervous system (from the pelvic plexus in man) utilising norepinephrine at the postganglionic neuroeffector (vas) synapse. In this system released NE activates post-synaptic α_1 receptors which are G_q coupled and produce an increase in $Ins(1,4,5)P_3$ formation in the muscle resulting in elevated $[Ca^{2+}]_i$ and contraction (Docherty, 1998). Norepinephrine is cleared from the synapse and the contractile signal is terminated by reuptake via NET and the recovered NE is re-incorporated into vesicles by vesicular monoamine transporters (VMATs) (Henry *et al.*, 1994), Figure 6.1.

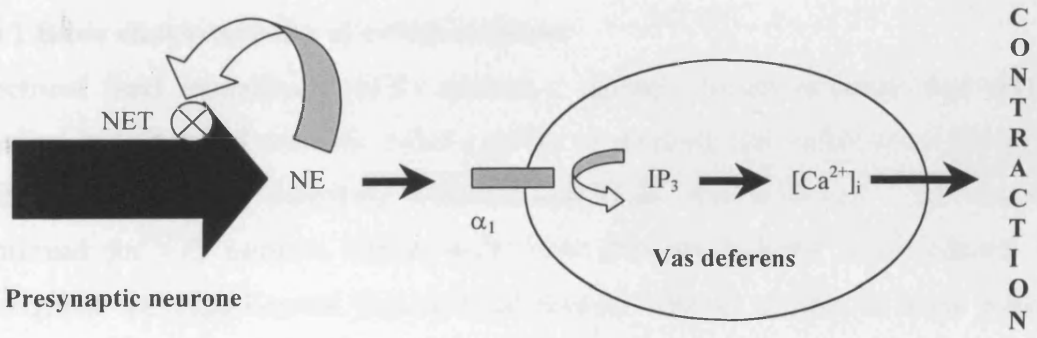


Figure 6.1 Schematic representation of NE release activating α1 adrenoceptors on vas deferens to produce smooth muscle contraction. (IP_3, inositol(1,4,5)trisphosphate)

In Chapters 3 and 4 a clear interaction of a range of local anaesthetic agents with NET was demonstrated both at the level of [^{3}H]NE uptake and [^{3}H]nisoxetine binding. Excluding a clinical study in man the next logical step is to proceed to an *ex vivo* sympathetically innervated tissue and in this respect the vas deferens, as described, represents an ideal model. In this system it will be possible to verify in an essentially intact tissue, the cellular data obtained previously.

6.2 Aims

The aims of this chapter are to test the hypothesis that local anaesthetic agents impair the uptake of guanethidine in the mouse vas deferens, a sympathetically innervated *ex vivo* preparation. Specifically;

1. To characterise the contractile response to EFS and confirm that the stimulus parameters used produce a neurogenic contraction.
2. To examine the effects of guanethidine (sympathomimetic) and local anaesthetic agents alone.
3. To determine whether local anaesthetic agents (amide prilocaine, ester, procaine and the known NET inhibitor cocaine) modify the actions of guanethidine.
4. To determine if the interaction is competitive.

6.3 Materials and methods

For full details of materials and methods and analysis of data the reader is referred to chapter 2.

6.4 Results

6.4.1 Basic characteristics of twitch response

Electrical field stimulation (EFS) elicited a biphasic twitch response that returned to baseline in less than 2 seconds. After a period of washing and stabilisation (45 minutes) a twitch response with relatively constant amplitude was achieved. This control state continued for >75 minutes (figure 6.2). Note that the baseline level reduced steadily throughout the experimental period in all tissues. This is evident in some tissues to a greater degree than others. In order to control for this declining baseline, an occasional reset was required (figure 6.3). EFS contraction with the parameters used (30V, 1ms) was clearly neurogenic and mediated by α_1 adrenoceptors as the contraction was readily blocked by tetrodotoxin (300nM) and prazosin (1μM), (data not shown). These data are in agreement with those of Hughes *et al.* (1975). It should be noted that the traces shown in the figures are scans of actual data and therefore hand-written annotations remain. Moreover, as the timescale for each trace has been slightly distorted in order to fit on these pages the curved vertical lines remain to indicate 5 minute intervals.

6.4.2 Effects of guanethidine and prilocaine

Prilocaine at 1 mM induced an immediate inhibition of twitch response that reached a maximum (100%) after 2 min. This inhibitory effect was rapidly reversible after washing (figure 6.3). Guanethidine (3μM) also inhibited EFS control twitch by 95±3% in 15 min (figure 6.4). However, in contrast to prilocaine this effect was only partially reversed after 1 hour of washing (33±12% of control values) When prilocaine and guanethidine were added in combination at 1mM and 3μM respectively (figure 6.5), a reversal of (80±13%) was observed after one hour which was greater ($p<0.05$) than the reversal produced by guanethidine (3μM) alone. These data are summarised in figure 6.6

Raising the guanethidine concentration to 10μM inhibited the EFS twitch response to 4±3% of control and was reversed to 8±3% after 1 hour of washing (figure 6.7). When prilocaine (1mM) was co-incubated with guanethidine (10μM) the EFS twitch was inhibited to 2±2% of control but reversed to 30±10% after 1 hour of washing, (figure 6.8). These data are summarised in figure 6.9. This reversal produced by the combination was greater ($p<0.05$) than that produced seen with guanethidine alone but was less than that seen with the lower (3μM) concentration of guanethidine. These data are suggestive of a competitive interaction between guanethidine and prilocaine.

6.4.3 Effects of guanethidine and procaine

Procaine (300μM) alone facilitated the twitch response to 152±14% of control but returned to control level 10 minutes after the first wash, from which point the trace remained stable (figure 6.10). When co-incubated with guanethidine (3μM) the EFS twitch was reduced to 24±4% of control and reversed to 77±7% of control after 1 hour of washing (figure 6.11). The reversal produced by the combination was greater than that produced by guanethidine alone ($p<0.05$). These data are summarised in figure 6.12.

6.4.4 Effects of guanethidine and cocaine

Cocaine (30μM) inhibited the EFS twitch response to 53±8% of control and was completely reversed by 1 hour of washing (figure 6.13). When co-incubated with guanethidine (3μM) the EFS twitch was reduced to 39±6% of control and was reversed to 86±10% after 1 hour of washing (figure 6.14). The reversal produced by the combination

was greater ($p<0.05$) than that produced by guanethidine alone. These data are summarised in Figure 6.15.

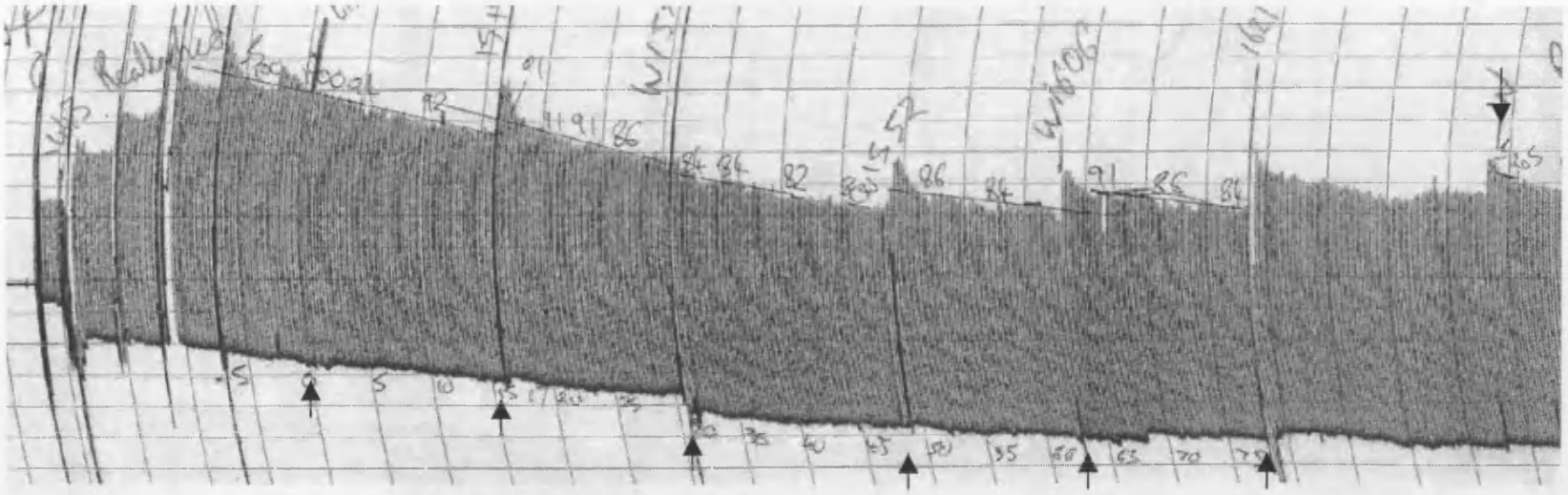


Figure 6.2 The trace shows the electrically stimulated twitch response of the mouse vas deferens. Vertical deflection in an upward direction indicates a contraction. The vertical curved lines on the recording paper are 5 minutes apart. The positions of the arrows indicate where the tissue was washed. This trace shows only control twitches in the absence of any drug treatment with washes every 15 minutes. Amplitude of twitch was variable between tissues and recording apparatus adjusted to view the maximum deflection and therefore any changes in response. Note that immediately after washing, the amplitude of the response increases temporarily due to the mechanical stimulus to which the tissue is subjected as the bath is emptied and refilled.

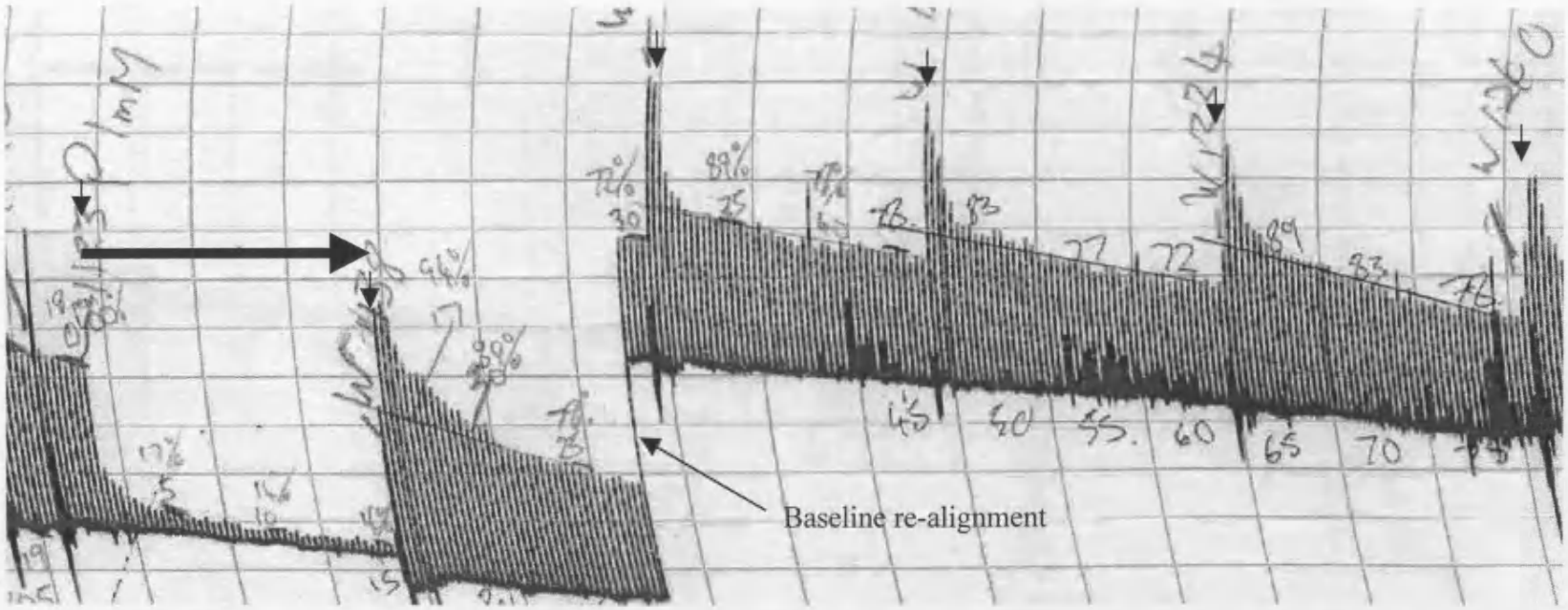


Figure 6.3 Typical trace showing the effect of prilocaine (1mM) on EFS twitch response. The horizontal arrow indicates the time of drug treatment and the arrows indicate washes of the tissue. The amplitude of the response immediately prior to time 0 (before drug addition) is considered to be the basal (100%) twitch response, against which all subsequent changes are compared.

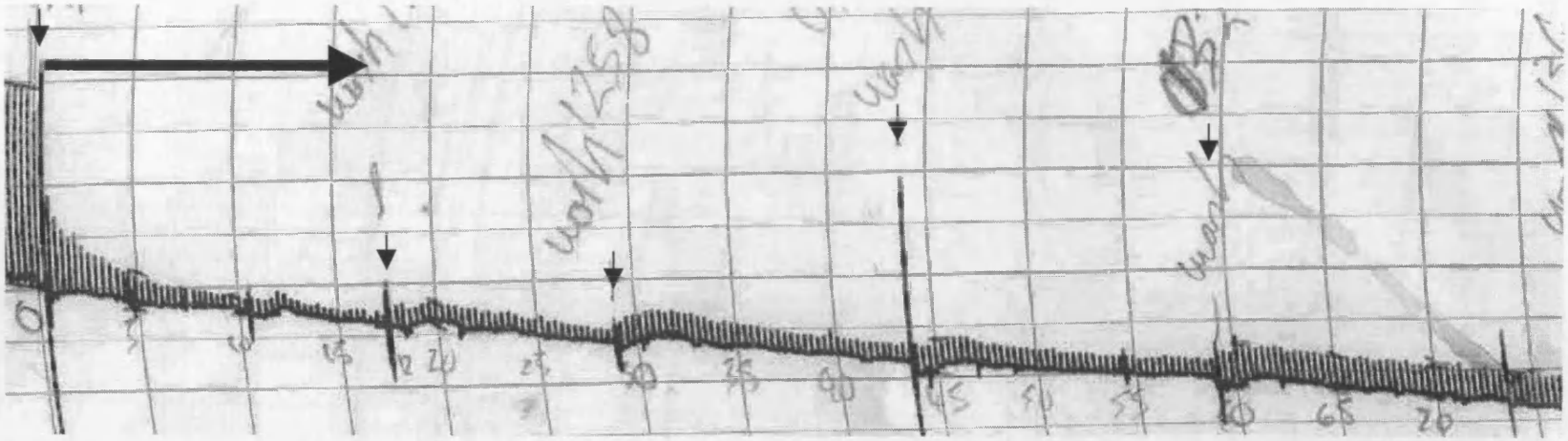


Figure 6.4 Typical trace of vas deferens EFS response and the effect of guanethidine (3μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

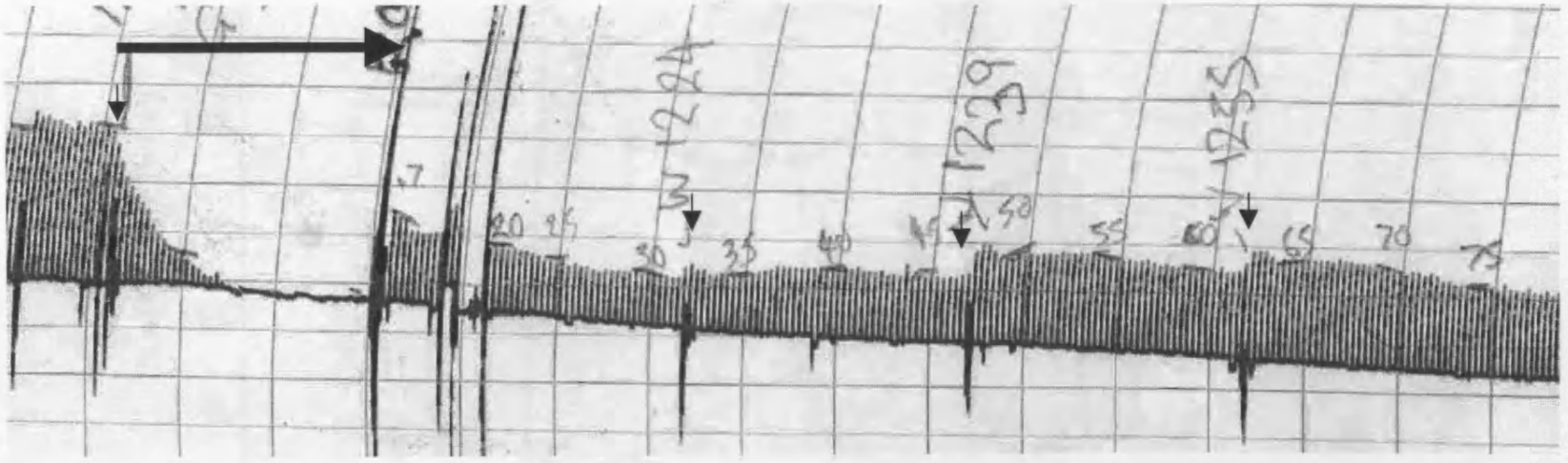


Figure 6.5 Typical trace of vas deferens EFS response and the effect of prilocaine (1mM) with guanethidine (3μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

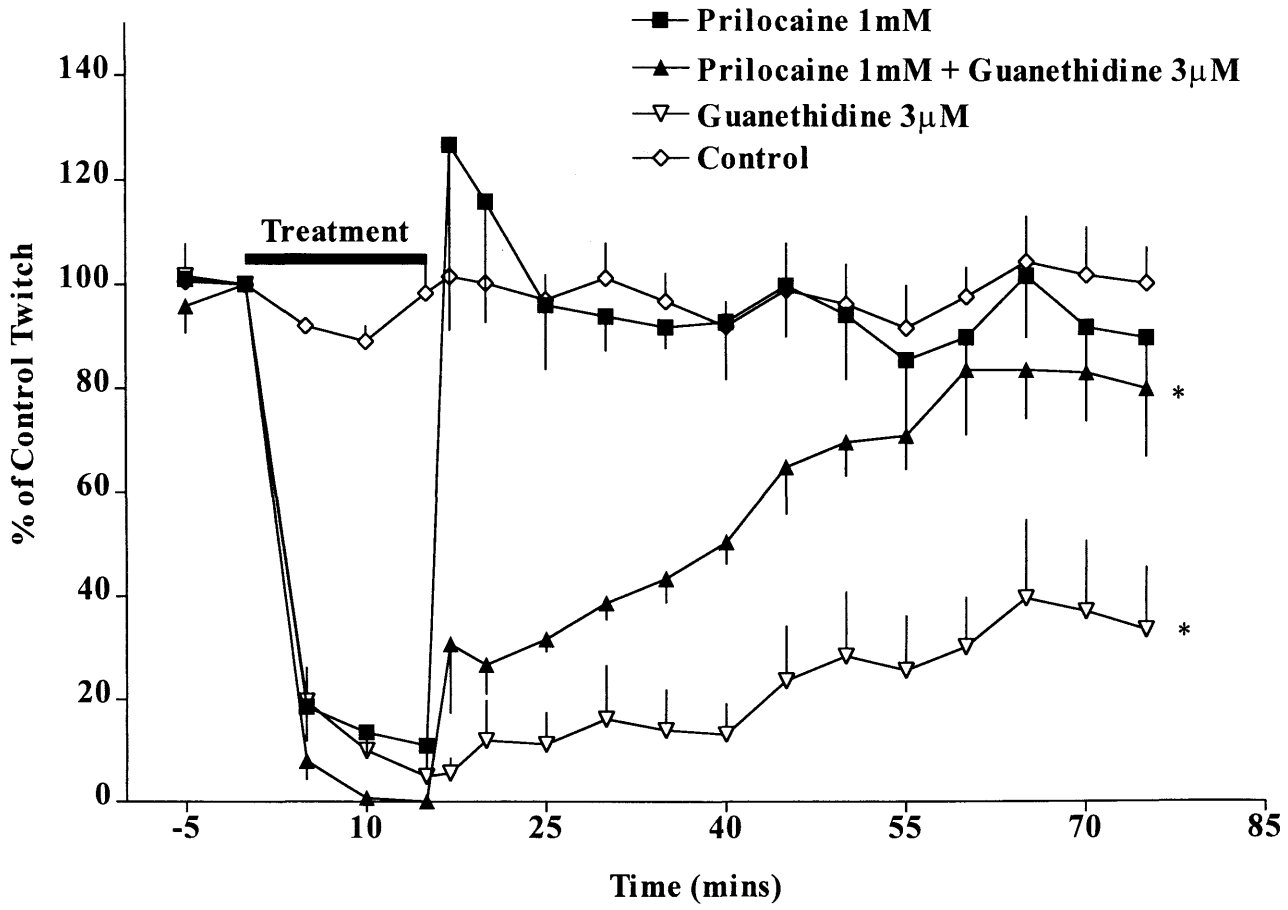


Figure 6.6 Effects of prilocaine (1mM), guanethidine (3μM) and the combination on EFS mouse vas deferens. EFS response in the absence of added agent (control) is also shown. Inclusion of prilocaine (1mM) increased the recovery from guanethidine treatment (10μM) after one hour from 33 to 80%. Curves for guanethidine alone and combination of LA and guanethidine post 15minutes were significantly different ($p<0.05$ indicated by *) by ANOVA, $n\geq4$.

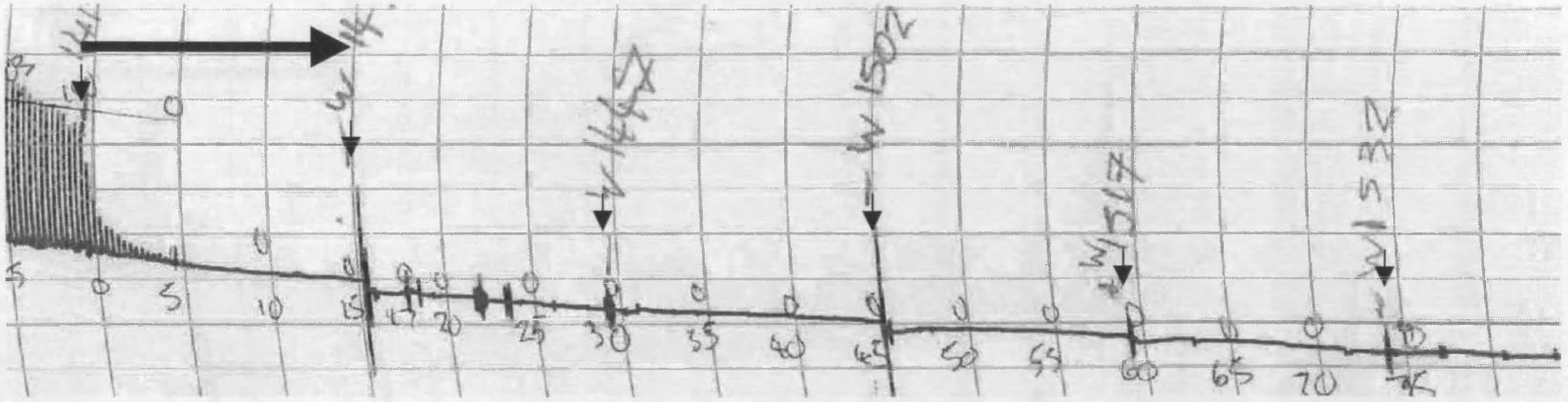


Figure 6.7 Typical trace of vas deferens EFS response and the effect of guanethidine (10μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

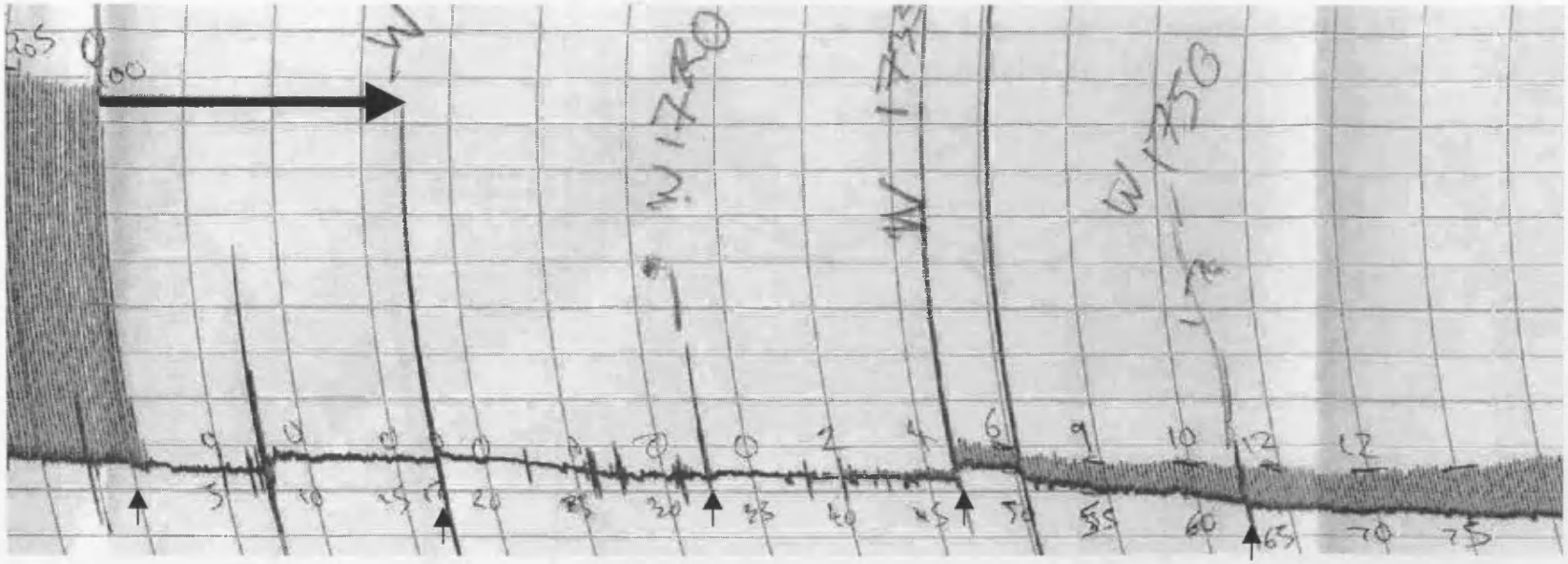


Figure 6.8 Typical trace of vas deferens EFS response and the effect of prilocaine (1mM) with guanethidine (10μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

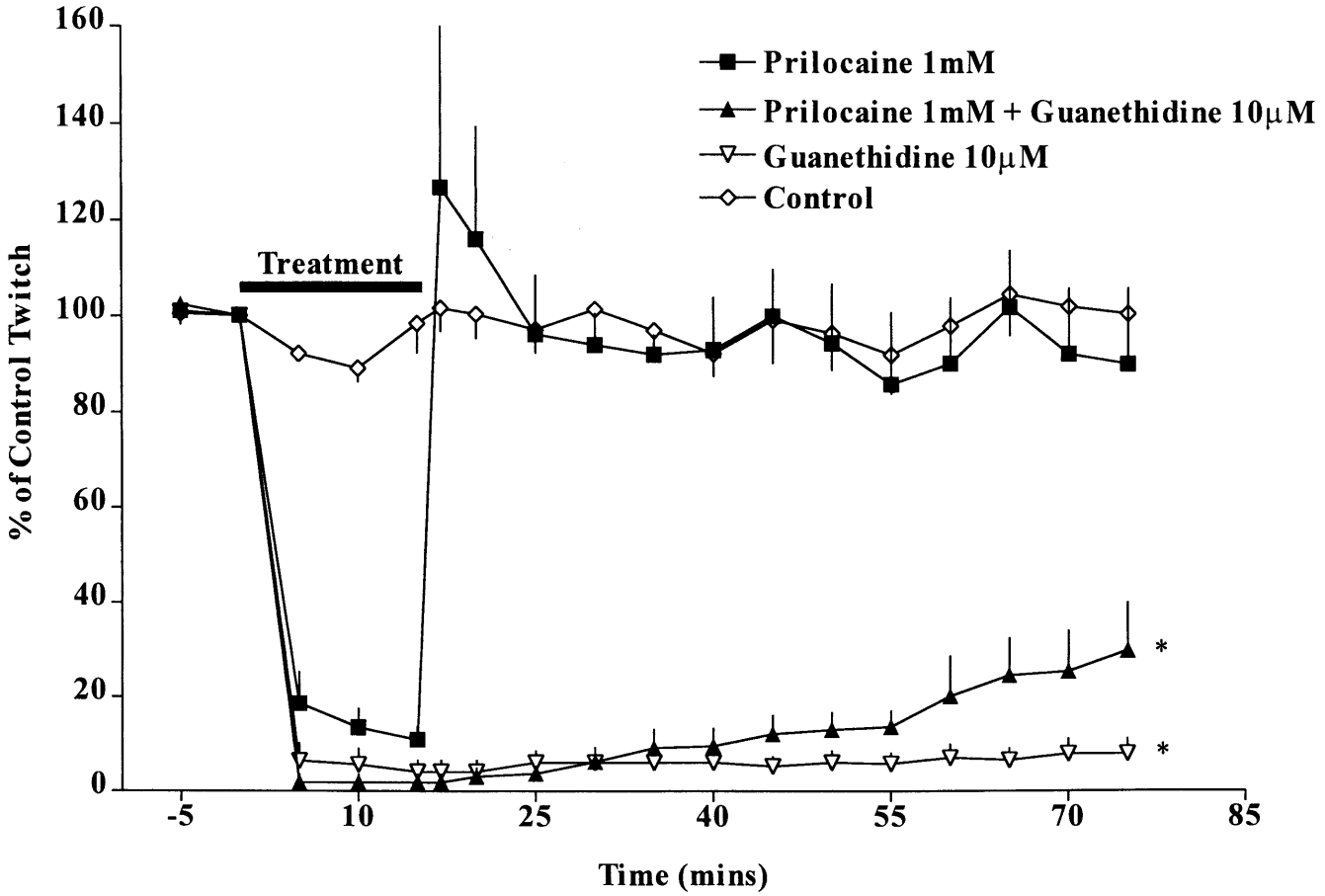


Figure 6.9 Effects of prilocaine (1mM), guanethidine (10μM) and the combination on EFS mouse vas deferens. EFS response in the absence of added agent (control) is also shown. Inclusion of prilocaine (1mM) increased the recovery from guanethidine treatment (10μM) after one hour from 8 to 30%. Curves for guanethidine alone and combination of LA and guanethidine post 15minutes were significantly different ($p<0.05$ indicated by *) by ANOVA, $n\geq4$.

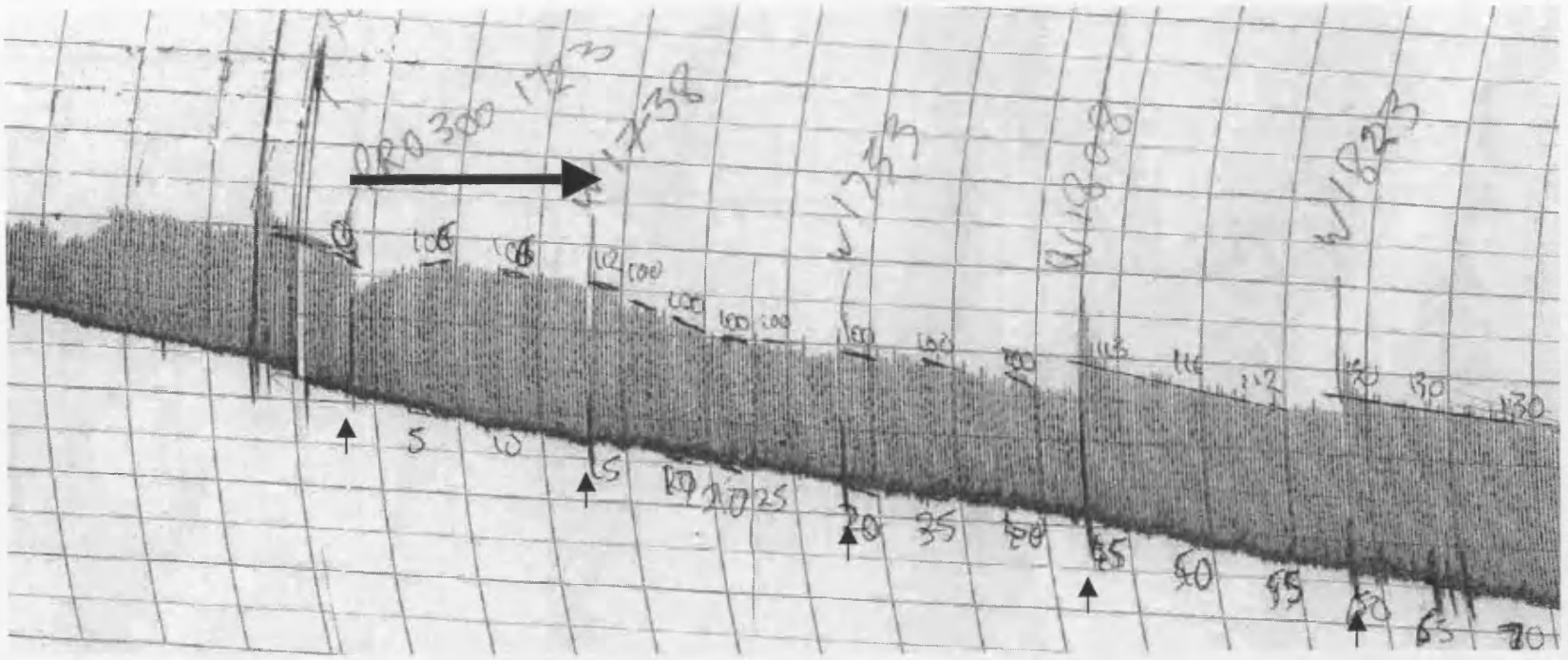


Figure 6.10 Typical trace of vas deferens EFS response and the effect of procaine (300μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

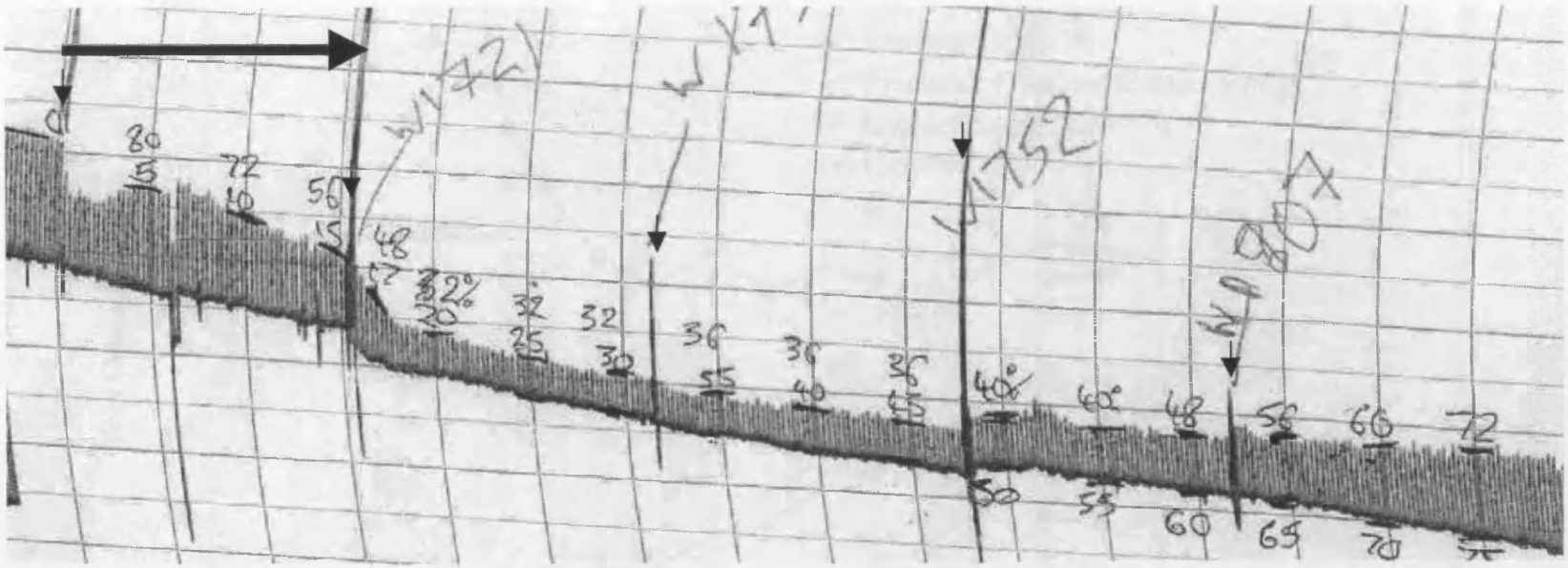


Figure 6.11 Typical trace of vas deferens EFS response and the effect of procaine (300μM) with guanethidine (3μM). The solid horizontal arrow indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

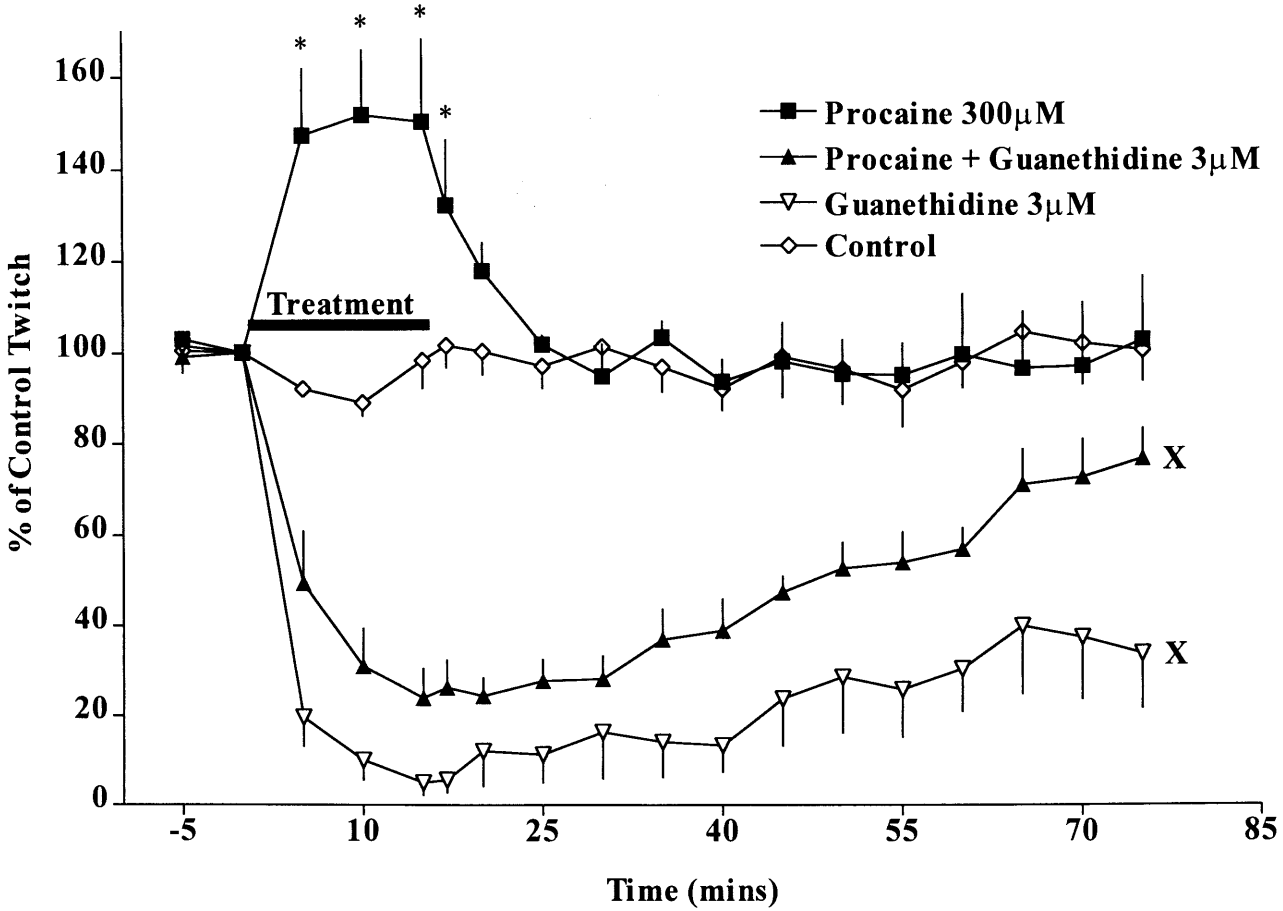


Figure 6.12 Effects of procaine (300μM), guanethidine (3μM) and the combination on EFS mouse vas deferens. EFS response in the absence of added agent (control) is also shown. Inclusion of procaine (300μM) increased recovery from guanethidine treatment (3μM) after one hour from 33 to 77%. Curves at points indicated by (*) were significantly different from the corresponding control ($p<0.05$ by t-test following ANOVA of 0-25min data) $n\geq4$. Guanethidine and guanethidine in combination with procaine curves post 15 minutes were significantly different indicated by (x) by ANOVA ($p<0.05$), $n\geq4$.

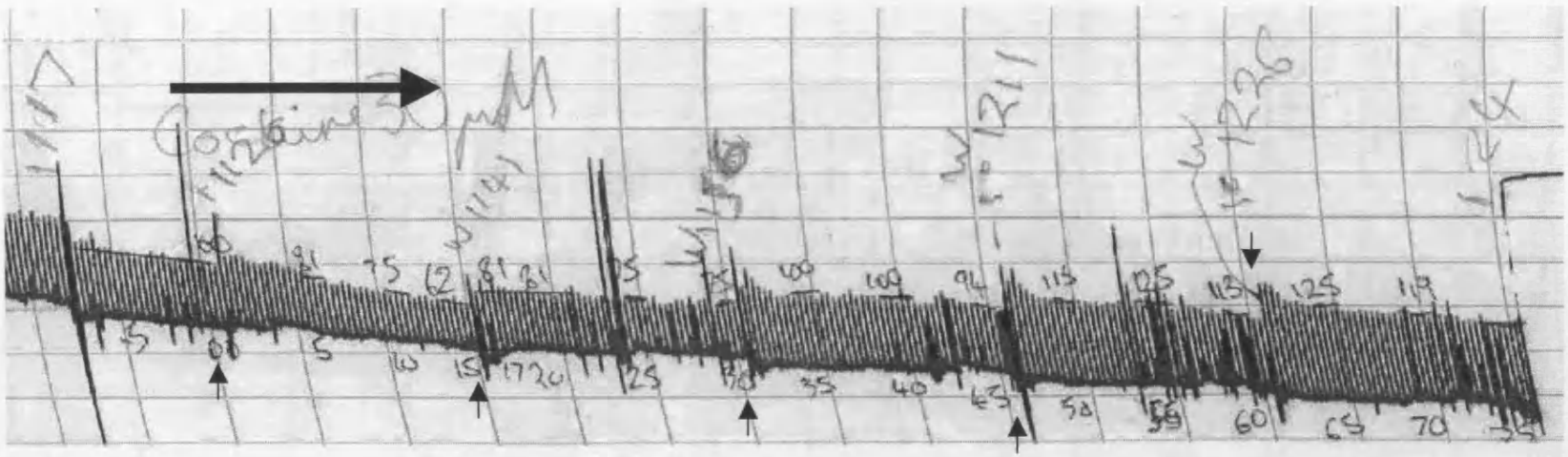


Figure 6.13 Typical trace of vas deferens EFS response and the effect of cocaine (30μM). The solid horizontal bar indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

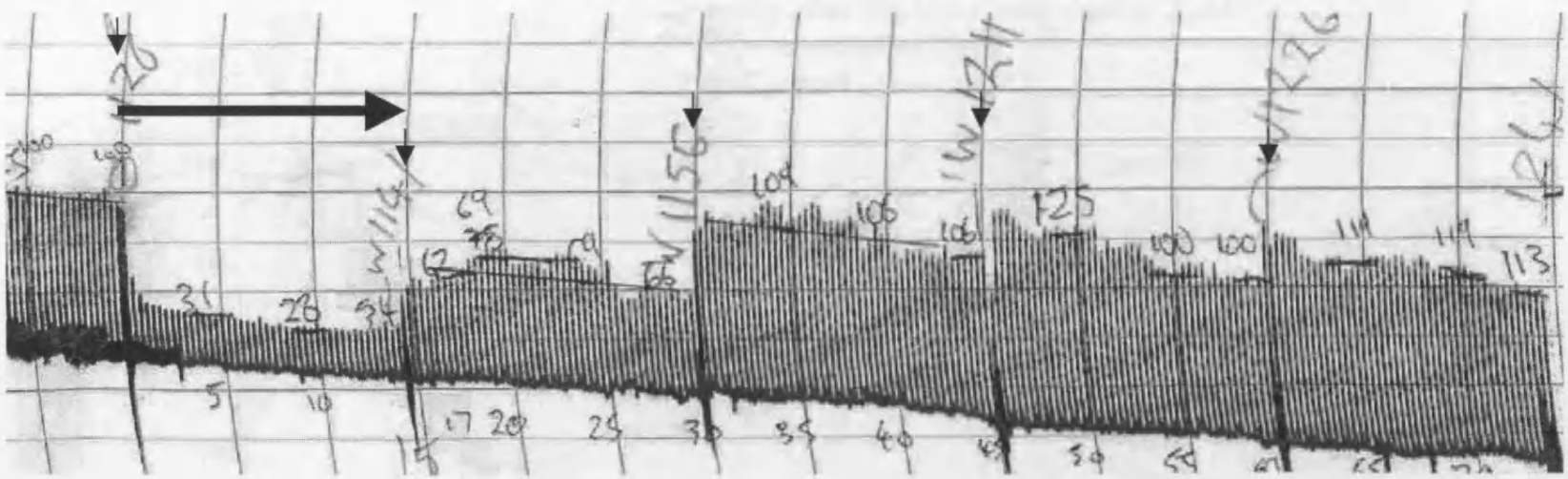


Figure 6.14 Typical trace of vas deferens EFS response and the effect of cocaine (30μM) with guanethidine (3μM). The solid horizontal bar indicates the duration of drug addition and the arrows indicate tissue washes. Changes of response amplitude were measured against the basal response (that immediately prior to drug addition at time 0).

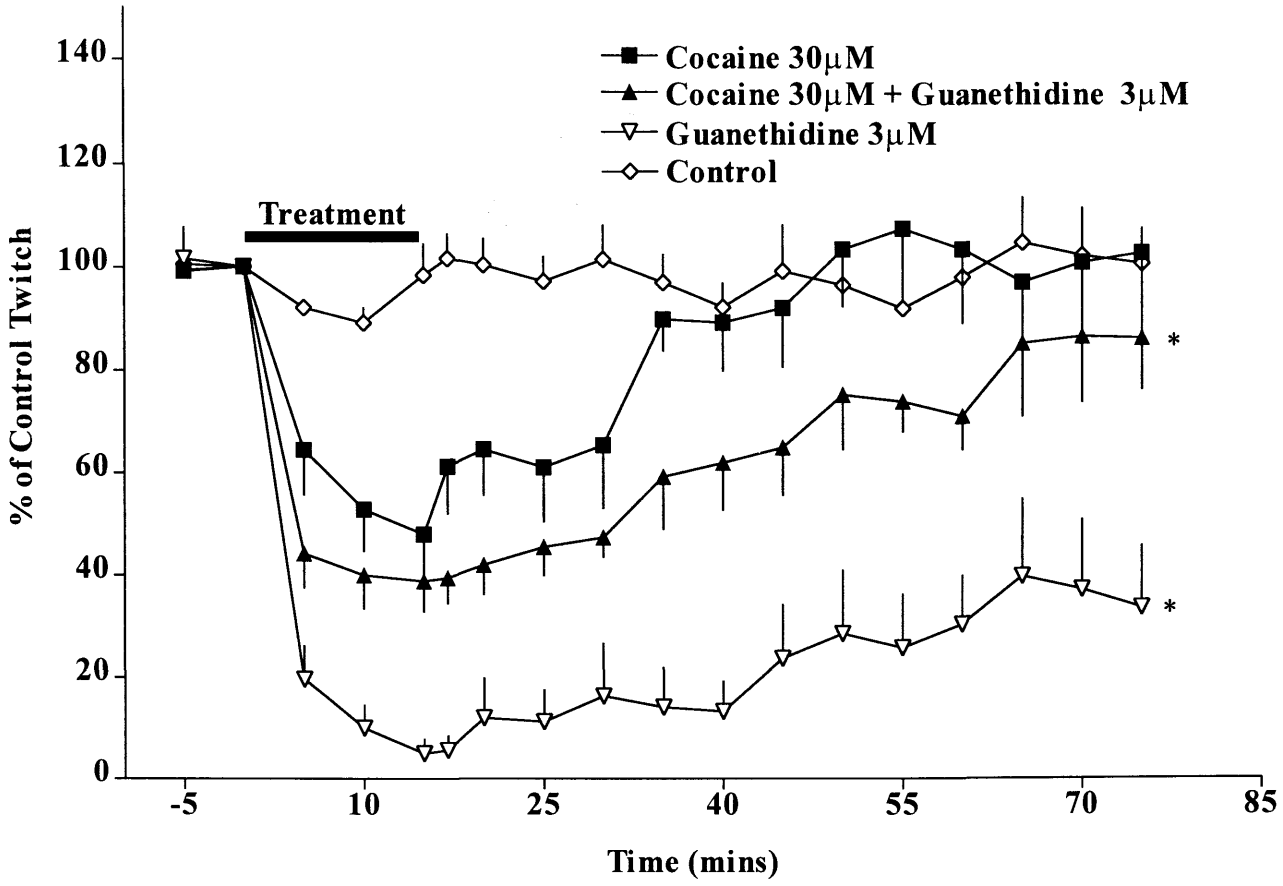


Figure 6.15 Effects of cocaine (30μM), guanethidine (3μM) and the combination on EFS mouse vas deferens. EFS response in the absence of added agent (control) is also shown. Inclusion of cocaine (30μM) increased the recovery from guanethidine treatment (3μM) after one hour from 33 to 86%. Curves for guanethidine alone and combination of LA and guanethidine post 15minutes were significantly different ($p<0.05$ indicated by *) by ANOVA, $n\geq4$

6.5 Discussion

The results presented in this chapter clearly demonstrate an interaction with both amide and ester type local anaesthetic agents, on the actions of the NET substrate, guanethidine. The concentrations used here were chosen as a result of uptake studies performed in cultured cells (see chapter 3), where the *in vitro* functional potency of the local anaesthetic agents for NE uptake inhibition was determined.

The inhibition by prilocaine of guanethidine action and therefore its uptake was dependent upon the concentration of guanethidine (substrate) and was competitive in nature. When the concentration of guanethidine was raised ~3 fold from 3μM to 10μM the reduction in efficacy due to prilocaine (1mM) was reduced from 80% to 25% (figure 6.16). This difference was significant ($p<0.05$). At these concentrations of guanethidine (3-10μM) it would be expected that further increases in concentration would tend to overcome the competitive action of prilocaine (1mM). It is estimated from a simple bench study of arm volume, that in IVRGBB, the concentration of prilocaine is around 1mM and guanethidine reaches around 70μM, significantly higher than the top concentration used here. However, there can be great differences of efficacy between tissues and indeed species and as this study used a preparation of mouse vas deferens, further investigation of the interaction is warranted preferably in the form of a clinical study. In addition, absolute "effector site" concentrations in man cannot be predicted.

The ester, procaine (300μM), also reduced the efficacy of guanethidine (3μM) by 66% when co-incubated, in common with the prilocaine (figure 6.16). Procaine was used in order to examine the effect of an ester type local anaesthetic in addition to the amide prilocaine. The mechanism of action of procaine, which caused an increase in twitch amplitude, is not known. However, the concentration of procaine used (300μM) was insufficient for local anaesthetic action, i.e. nerve block. It is thought that the increase in twitch amplitude may be due to inhibition of GTPase activity within the postsynaptic cells. Hageluken *et al.,* (1994) reported that procaine, at the concentration used here, inhibited GTP hydrolysis in cAMP differentiated HL-60 cells. This effect could potentiate the effects of G_q type G protein coupled receptors like α_1 adrenoceptors by prolonging the active phase, determined by duration of GTP hydrolysis. Tetracaine, bupivacaine, and lidocaine were reported to increase GTP hydrolysis while procaine had the opposite effect.

It is postulated that the local anaesthetics have differential actions on G-proteins and nucleoside diphosphate kinase (NDPK) which may, in part, explain the differential effects of procaine and prilocaine on vas deferens contraction (Hageluken *et al.*, 1994).

Kitamura *et al.,* (1986) demonstrated that procaine depolarises smooth muscle membranes by inhibition of K^+ conductance, although their experiments used a higher concentration of procaine (1mM). It is also known that dual effects of local anaesthetics can be seen where contractions in smooth muscle are induced at lower concentrations, due to actions on cellular sources of Ca^{2+} (Feinstein & Paimre, 1969).

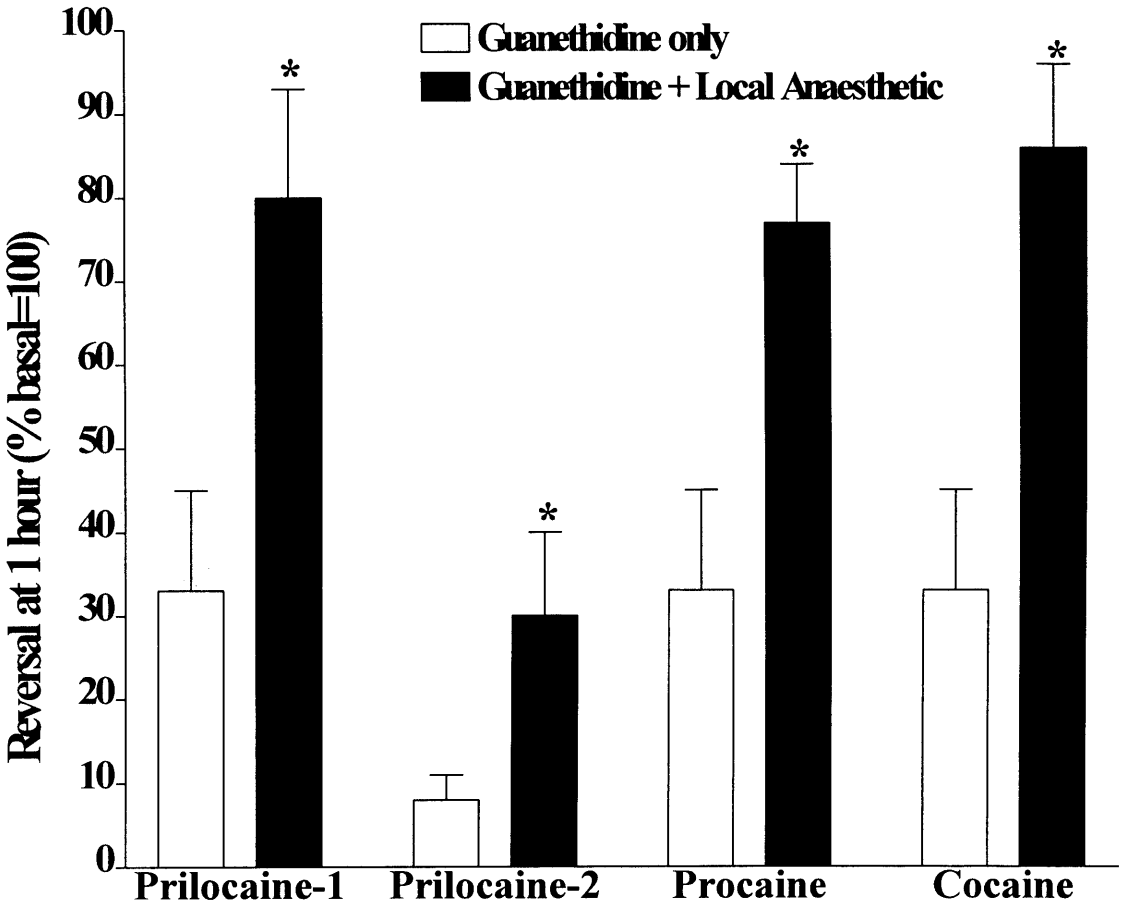


Figure 6.16 Graph showing the reversal after one hour of EFS twitch response. Open bars show reversal of guanethidine alone and filled bars show reversal when coadministered with local anaesthetic. Prilocaine-1 indicates reversal of 3μM guanethidine and prilocaine-2 indicates reversal of 10μM guanethidine. Asterisks indicate significant differences ($p<0.05$, n=4-7).

It is worthy of note that the degree of twitch depression produced by 30μM cocaine alone was only ~50%. This is in marked contrast to the inhibition produced by prilocaine of >70%. However it is unlikely that the initial reduction in contraction in response to local anaesthetic application is due to NET inhibition. It is more likely that this results from interference with membrane or nerve, voltage sensitive channels (e.g. Na^+). Cocaine is used only at 30μM, too low a concentration to have full effect on sodium channels but enough to have an effect on NET.

In summary, it has been clearly demonstrated that local anaesthetic agents antagonise the inhibitory effect of guanethidine on EFS mouse vas deferens tissue, an action likely to be mediated via an interaction with the NET protein. This supposition is based on the results of Chapters 3 and 4 and the observation that cocaine which is known to interact with NET (Van Dyke, 1982) also reverses the effects of guanethidine in this preparation (figure 6.16).

CHAPTER 7

General Discussion.

7.1 General summary of findings

The hypothesis of this thesis was that the questionable efficacy of IVRGBB (Kingery, 1997) may be due to an inhibition of guanethidine uptake by local anaesthetics. The rationale for this is that cocaine, a local anaesthetic, is known to inhibit NET.

Both cell types examined in this thesis (namely SH-SY5Y and 293-hNET) were found to take up [^{3}H]norepinephrine in a time dependent and saturable manner which was inhibited by the known hNET inhibitor, imipramine. This confirmed active uptake by the NET. Furthermore, these cells when prepared as membranes, bound the highly selective NET antagonist [^{3}H]nisoxetine in a concentration, time dependent and saturable manner. Each cell type did however express varying degrees of non-specific uptake of [^{3}H]norepinephrine and binding of [^{3}H]nisoxetine (see chapters 3 & 4). Most of the local anaesthetic agents examined, lidocaine, prilocaine, bupivacaine, dibucaine (amides), procaine, tetracaine and cocaine (esters) inhibited both the specific uptake of [^{3}H]norepinephrine and specific binding of [^{3}H]nisoxetine in a concentration dependent manner to a maximum of 100% with the exception of cocaine which inhibited nisoxetine binding to maximum plateau of 80% (see chapters 3 & 4). These findings support the hypothesis of local anaesthetic inhibition of NET uptake rate. Binding experiments identified a large difference between expression levels of hNET in SH-SY5Y and 293-hNET cell membranes. This discrepancy was confirmed by performing Western blotting and immunochemiluminescence on both cell types, suggesting a ~160 fold expression level difference between cell types (see chapter 5). The 293-hNET cells appeared to express the greater amount of hNET immunoreactive protein yet did not reflect this in their uptake rate and capacity (see chapter 3). Expressed, but non-functional protein in 293-hNET cells is presumably responsible for this discrepancy, as the expression system is not regulated as it should be in an endogenous, neuronal system. As local anaesthetic agents were found to inhibit the uptake and binding of [^{3}H]norepinephrine and [^{3}H]nisoxetine, the interaction with NET was examined in a sympathetically innervated, *ex vivo*, electrically stimulated tissue. Local anaesthetics were found to competitively inhibit the blocking effect of guanethidine on the EFS twitch response in mouse vas deferens, supporting the original hypothesis and questioning the coadministration of these two drugs in the Bier's block.

7.2 Differences between hNET expression and norepinephrine uptake between cell types

There were clearly differences between the two cell lines examined, particularly in respect of the binding of [^{3}H]nisoxetine. As [^{3}H]nisoxetine is a highly selective ligand for the NET it was used to measure the expression levels of the transporter. Isotopic dilution experiments yielded values of pK_d and B_{max} in 293-hNET cells of 8.41±0.05 and 5934±475 fmol mg protein^{-1} respectively (see chapter 4). SH-SY5Y cells however, expressed hNET protein in much lower amounts. This resulted in problems of obtaining accurate results from the isotopic dilution protocol. Low specific binding (~800dpm) was masked by high non-specific binding compounding "isotope dilution" correction factors.

The high concentrations of [^{3}H]nisoxetine that would be required to perform direct saturation experiments in SH-SY5Y cells would have been unfeasible due to the cost of radioligand. In order to determine the relative expression levels of hNET protein in the two cell types, PAGE and immunochemiluminescence was performed (see chapter 5). Using differing concentrations of solubilised SH-SY5Y and 293-hNET cell protein in the same gels, resulting band density was measured using a transmission scanner. Taking into account the protein concentrations, 293-hNET cells were estimated to possess a ~160 times greater density of the active 80kDa form of immunoreactive hNET protein. Working from the B_{max} calculated for 293-hNET cells, a 160 fold lower value would be around 30fmol mg protein^{-1} . This value would be at the limits of detection using isotopic dilution (see chapter 4). It must be noted that binding experiments were performed on cell membrane preparations whereas western blotting was performed from whole cell solubilised protein. Western blotting experiments therefore may include hNET protein which is intracellular as opposed to membrane bound and may overestimate density. The greater expression levels of hNET in 293-hNET cells is a result of the transfection process where the gene for hNET was inserted using the vaccinia virus T7 expression system (Fuerst *et al.*, 1986) and Lipofectin. In contrast SH-SY5Y cells express hNET protein at much lower levels as an endogenous protein. The greatly different expression levels are not however, reflected in uptake capacity and rate. The capacity of [^{3}H]NE (~25nM) uptake for 293-hNET and SH-SY5Y cells were 1647±44 and 1129±167 fmol mg protein^{-1} respectively, showing slightly increased capacity in 293-hNET cells (data from uptake time course experiments, chapter 3). The rates of [^{3}H]NE (~25nM) uptake were 95.3±5.2 and 194.6±19.7 fmol mg protein^{-1}

min^{-1} in SH-SY5Y and 293-hNET cells respectively showing a 2 fold increase in initial uptake rate not reflecting the much larger (160 fold) expression level in 293-hNET cells. These findings may suggest that much of the NET protein expressed in 293-hNET cells is non-functional. Galli *et al.,* (1996) estimated B_{max} of 293-hNET cells as 13.1pmol mg $protein^{-1}$, using the high affinity cocaine analogue [^{125}I]3β-[4-iodophenyl]tropan-2β-carboxylic acid methyl ester as a ligand. Their experiments indicated a single population of noninteracting transporter sites with an average of $9x10^5$ transporters $cell^{-1}$ or $\sim10^{-6}$ as an approximation. The uptake of [^{3}H]norepinephrine was found to follow Michaelis-Menten kinetics (Galli *et al.*, 1995).

After determination of the amino acid sequence of NETs by Pacholczyk, (1991) it was possible to identify potential N-glycosylation sites. Melikian *et al,*, (1994) characterised an anti-peptide antibody (N430) that recognises hNET in immunoprecipitation experiments. Within 30 minutes of pulse labelling, stably transfected LLC-PK1 cells synthesise and N-glycosylate hNET proteins as revealed by immunoblotting and immunoprecipitation (Melikian *et al.,* 1996). hNET proteins shift to larger M_r forms over 2-4 hours in these cells with the latter form enriched at the cell surface. When N-glycosylation is prevented by the addition of tunicamycin, maturation to the larger (80kDa) form is prevented and whole cell NE transport activity is reduced (Melikian *et al.*, 1994). hNET forms synthesised in the presence of tunicamycin are found to have a greatly reduced half-life compared with controls and loss of antagonist ([^{125}I]RTI-55) binding sites in LLC-PK1 membranes. These data confirm the importance of N-glycosylation sites on functional hNET protein consistent with their extracellular location on the proposed model (see figure 1.6). N-glycosylation appears to be important for the physical maturation and surface targeting of biogenic amine transporters. N-glycosylation itself may play little or no role in direct ligand recognition, but rather sugar addition may increase the probability that transporter proteins fold properly and/or are protected from degradation so that they can be efficiently trafficked to the plasma membrane. In rat brain tissue, only a single 80kDa band was found, suggesting the predominance of fully processed forms of NET in native tissue (antibody specific for both rodent and human forms) (Kippenberger *et al.*, 1999). Interestingly, despite SH-SY5Y expressing the hNET protein endogenously, both 80 and 54kDa bands were present in our immunochemiluminescence studies (see chapter 5). SH-

SY5Y cells may represent an intermediate between native tissue and transfected cells. The N-glycosylation process was clearly preserved in both cell types.

7.3 Differences in local anaesthetic effects on [^{3}H]norepinephrine uptake and [^{3}H]nisoxetine binding between SH-SY5Y and 293-hNET cells

Characteristics shared by both cell types examined were the inhibition of [^{3}H]NE uptake and displacement of [^{3}H]nisoxetine by all local anaesthetic agents. Very recently, Sato *et al.* (2000), have published data in agreement with this for NET expressed in COS cells.

When pK_i values for SH-SY5Y and 293-hNET cells in [^{3}H]nisoxetine binding experiments were compared including all local anaesthetics and imipramine, there was a strong positive correlation with an r^2 value of 0.88 (see figure 4.10). However, when the values for cocaine and procaine are omitted, the remaining pKi values yielded a stronger correlation with an r^2 value of 0.99. This indicates a difference in the displacement of [^{3}H]nisoxetine by cocaine and procaine between cell types, both of which are ester type local anaesthetic agents. The reason for these discrepancies between cell lines are unclear but may be attributable to the differing lipid environment of the two cell membranes, i.e. neuronal and non-neuronal.

The same local anaesthetic agents were capable of inhibiting the uptake of [^{3}H]NE in both 293-hNET and SH-SY5Y cells with a good correlation between the two cell types (r^2=0.998) indicating that the pharmacology of the transporter (with respect to local anaesthetic inhibition of uptake) is the same (see figure 3.7). Ester type local anaesthetic agents were in general more potent than the amide forms.

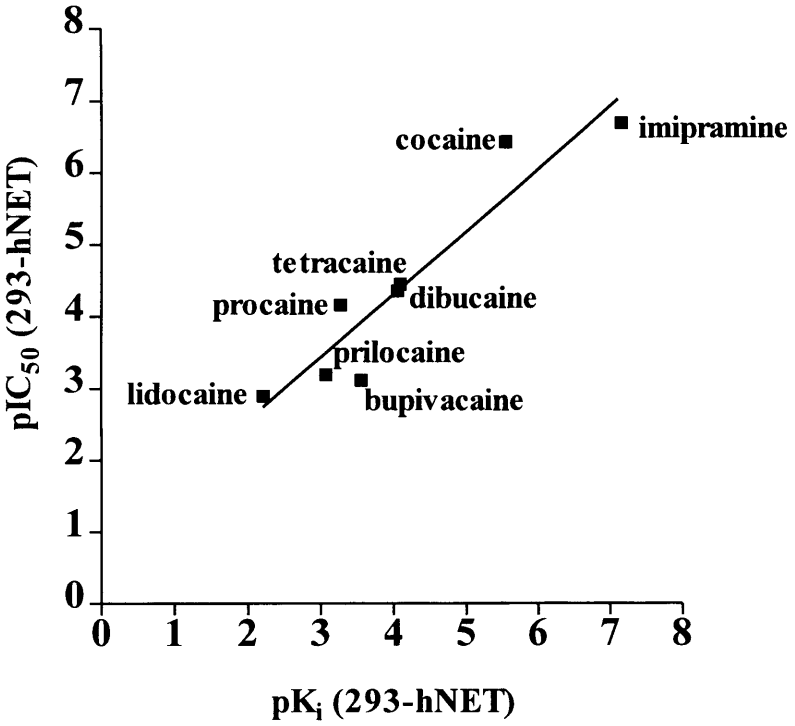


Figure 7.1 Correlation between pK_i and pIC_{50} in 293-hNET cells (r^2= 0.88, $p<0.05$).

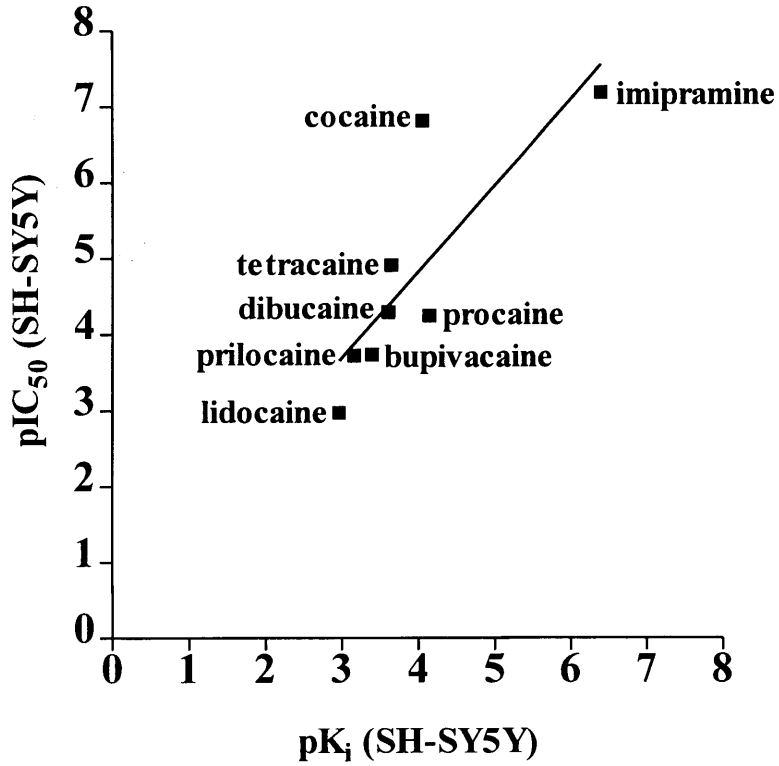


Figure 7.2 Correlation between pK_i and pIC_{50} in SH-SY5Y cells (r^2= 0.66, $p<0.05$)

When comparing pK_i values with pIC_{50} values within cell lines, positive correlations were also observed, more strongly with 293-hNET, (293-hNET r^2=0.88, SH-SY5Y, r^2=0.66, see figures 7.1 & 7.2). It may have been expected that pIC_{50} and pK_i values would be almost identical if compounds are acting in the same way with NET to both inhibit uptake and displace [^{3}H]nisoxetine. In reality, actual values of pIC_{50} and pK_i within cell lines generally differed significantly despite a positive correlation. However in SH-SY5Y cells, pIC_{50} and pK_i values for lidocaine and bupivacaine did not differ significantly, and in 293-hNET cells the same values for lidocaine and prilocaine were not different. Differences in these values may be explained by the use of different buffering systems in the two types of experiment, as reported by other groups in G protein coupled receptor systems (Hashiba *et al.,* 2001). The different protein binding capacities of the local anaesthetic agents coupled with the different protein availability in each experiment may also have influenced the observed pK_i and pIC_{50} values. However, if protein binding was a contributory factor to this phenomenon, one might expect agents with low protein binding capacities to show similar values. Lignocaine, bupivacaine and prilocaine showed similar values within cells but however, are quoted as having 64%, 95% and 55% protein binding (Tucker & Mather, 1980; Stichartz *et al.,* 1990).

It is notable that cocaine, did not displace [^{3}H]nisoxetine by 100% but appeared to reach a maximum of ~80% in SH-SY5Y cells. An explanation cannot be offered for this, but it may suggest isoforms of the transporter for which cocaine is selective leaving a fraction still bound to [^{3}H]nisoxetine. However, such subtypes have not yet been reported and nisoxetine is considered to be a highly selective ligand for NET (Tejani-Butt, 1990).

7.4 Interaction of local anaesthetic agents with the sympathetic blocking effect of guanethidine in *ex vivo* mouse vas-deferens tissue.

In vitro data presented clearly indicates an interaction of local anaesthetics with NET. The next logical step (in the absence of clinical trials) was to examine an *ex vivo* sympathetically innervated tissue. The mouse vas deferens was chosen for these studies. The aim was to demonstrate if the inhibitory effect of local anaesthetics on NET was transferable to an equivalent sympathetically innervated tissue system and to demonstrate

substrate concentration dependence. Dependence upon substrate concentration i.e. guanethidine would support a competitive inhibition of NET by local anaesthetic agents.

Mouse vas deferens is a good example of a sympathetically innervated tissue (Bentley & Sabine, 1963). Other tissues, such as guinea-pig ileum were rejected as interference from parasympathetic innervation may have occurred and sympathetic responses ellicit relaxation not contraction in ileum. Electrical field stimulation (EFS), of mouse vas deferens tissue evokes neurogenic contraction which is reversible by prazosin and tetrodotoxin which block α_1 and Na^+ channels respectively. This proves neurogenic contraction and excludes the possibility of myogenic contraction, making the preparation suitable for studying an interaction with nerve function.

The findings from chapter 6 support the hypothesis, showing a significantly quicker recovery from guanethidine block when local anaesthetics (prilocaine, procaine and cocaine) were coadministered to the preparation. Furthermore, the recovery of twitch response observed was clearly dependent upon substrate (guanethidine) concentration. By definition, if the effect can be overcome by increasing the substrate concentration, the inhibition is said to be competitive.

An interesting phenomenon observed during the mouse vas deferens experiments was the increase in twitch amplitude in the presence of procaine (300μM) (see chapter 6). This concentration was clearly insufficient to induce nerve block (concentration chosen by previous uptake experiments rather than clinical potency). It is thought that the increase in twitch amplitude may be due to inhibition of GTPase activity within the postsynaptic cells. Hageluken *et al.* (1994) reported that procaine, at the concentration used here, inhibited GTP hydrolysis in cAMP differentiated HL-60 cells. This effect could potentiate the effects of G_q type G protein coupled receptors like α_1 adrenoceptors by prolonging the active phase, determined by duration of GTP hydrolysis. Tetracaine, bupivacaine, and lidocaine were reported to increase GTP hydrolysis while procaine had the opposite effect. Kitamura *et al.* (1986) demonstrated that procaine depolarises smooth muscle membranes by inhibition of K^+ conductance, although their experiments used a higher concentration of procaine (1mM). It is also known that dual effects of local anaesthetics can be seen where

contractions in smooth muscle are induced at lower concentrations, due to actions on cellular sources of Ca^{2+} (Feinstein & Paimre, 1969).

7.5 Clinical relevance.

At this point it must be made clear that it is not possible to determine the exact concentration achieved at the effector site in tissues of either local anaesthetic or guanethidine due to various factors that may effect penetration and availability of agent. The agents introduced, in order to reach the effector site, must penetrate tissue which may have varying lipid or protein content. Both factors which can affect pharmacological efficacy. IVRGBB subjects may have varying arm volumes affecting final concentration of intravenous agents. Such factors cannot be simulated in our *in vitro* and *ex vivo* preparations.

It is estimated from a simple bench study of arm volume in 19 volunteers, that in IVRGBB, the concentration of prilocaine is around 1mM and guanethidine reaches around 70μM, significantly higher than the top concentration used here (17 fold dilution in tissue estimated). However, there can be great differences in efficacy between tissues and indeed species. As already stated, it is impossible to know the exact concentration of agents at the site of action in tissue studies. The presence of competitive, non-specific protein, binding to the local anaesthetic agents and guanethidine *in vitro,* may have an effect on the efficacy of IVRGBB. An increase in guanethidine concentration in IVRGBB to overcome the local anaesthetic competition cannot be performed in man as this may cause associated side effects when the cuff is deflated, allowing redistribution systemically producing postural hypotension, sickness, diarrhoea, permanent denervation etc..

Based on the results of this thesis and the above considerations, I would strongly advise that until further clinical research is carried out, guanethidine and local anaesthetic agents should not be coadministered in IVRGBB.

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Publications arising from this thesis

Full papers

1. JOYCE P.I., ATCHESON R., MARCUS R.J., HEFFERNAN A.M., ROWBOTHAM D.J., LAMBERT D.G., (2001) Interaction of local anaesthetic agents with the endogenous norepinephrine transporter in SH-SY5Y human neuroblastoma cells. *Neuroscience Letters* **305**, 161-164.
2. JOYCE P.I., BLAKELY R.D., ROWBOTHAM D.J., LAMBERT D.G., Interaction of local anaesthetic agents with the recombinant human norepinephrine transporter expressed in 293HEK cells. In final stages of preparation for *Anesthesia and Analgesia.*
3. JOYCE P.I., RIZZI D., CALO' G., ROWBOTHAM D.J., LAMBERT D.G., Effect of guanethidine and local anaesthetics on the electrically stimulated mouse vas deferens. In final stages of preparation for *Brit. J. Anaesthesia.*

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