JUNCTIONAL MODULATION OF SYMPATHETIC TRANSMISSION

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Abstract

Junctional Modulation of Sympathetic Transmission

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This project involved the study of mechanisms which modulate autonomic transmission within the sympathetic nervous system using the mouse vas deferens as a model tissue. Data was collected using contraction studies, electrophysiological techniques with sharp microelectrodes, and fluorescent calcium imaging of both smooth muscle cells and nerve terminal varicosities. An additional series of experiments was conducted using the PC12 cell line, derived from a phaeochromocytoma of the rat adrenal medulla, for flow cytometry experiments using fluorescence-activated cell sorting.

During the course of this project a novel technique for studying the activity of the norepinephrine transporter within a whole organ preparation was developed using the neurotransmitter uptake assay. The uptake of this assay within the nerve terminals of the vas deferens was abolished by desipramine whilst its rate of washout was increased by amphetamine. However, some non-neuronal, peri-nuclear staining which could not be prevented by a range of pharmacological means was also observed. This new technique was then used in other work exploring putative NET regulation by cannabinoids.

The modulatory effects of two pharmacological groups were assessed: testosterone and cannabinoids. Testosterone was found to have a rapid, non-genomic effect inhibiting neurotransmission within the vas deferens. This was a postjunctional effect which appeared to involve modulation of the opening of L-type calcium channels on the smooth muscle cells.

For the studies of cannabinoids, two broad areas of research were conducted. First the effects of Δ^9-tetrahydrocannabinol were investigated with regard to the pre-junctional release of neurotransmitters and the effect of THC on calcium dynamics within individual nerve terminal varicosities. Secondly, a surprising novel effect upon the norepinephrine transporter was identified and examined. This inhibitory effect was revealed initially by contraction experiments demonstrating a decrease in the rate of uptake of noradrenaline from the junction.

This work demonstrates that there are still novel modes of regulation of sympathetic transmission to be uncovered. The ongoing challenge is to establish their role within physiology and pathophysiology.

Abbreviations

2-AG	2-arachidonoylglycerol
4ASP^{+}	4-(4-dimethylaminostyryl)-N-methyl-pyridinium
AM	Acetoxymethyl
ATP	Adenosine-5'-triphosphate
CGRP	Calcitonin gene-related peptide
$\Delta[Ca^{2+}]_t$	Ca^{2+} concentration in the nerve terminals
COMT	Catechol-*O*-methyl transferase
ω–CTX	ω-contotoxin GVIA
DAT	Dopamine active transporter
DMSO	Dimethylsulfoxide
EFS	Electrical field stimulation
EJP	Excitatory junction potential
FAAH	Fatty acid amid hydrolas
FACS	Fluorescence-activated cell sorting
FCM	Flow cytometry
FSC	Forward scatter
5-HT	5-hydroxytryptamine
MAGL	Monoacylglyceride lipase
MAO	Monoamine oxidase
NET	Norepinephrine transporter
NTUA	Neurotransmitter uptake assay
NTV	Nerve terminal varicosity

OG-BAPTA-1-AM	Oregon Green 488 1,2-bis(o-aminophenoxy)ethane-N,N,N=,N=-tetraacetic acid–1acetoxymethyl ester
OG-BAPTA-1-dex	Oregon Green 488 1,2-bis(o-aminophenoxy)ethane-N,N,N=,N=-tetraacetic acid-dextran, potassium salt, 10,000 MW, anionic
PSS	Physiological saline solution
RMP	Resting membrane potential
ROI	Region of interest
SEM	Standard error of the mean
SERT	Serotonin transporter
SSC	Side scatter
THC	Δ^9-tetrahydrocannabinol
VMAT	Vesicular monoamine transporter
WCT	Whole cell Ca^{2+} transient

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Introduction

The Neural Regulation of Smooth Muscle

Smooth and cardiac muscles, distinct from their skeletal cousin, function largely autonomously, needing not the constant reassurance of, or direction from, the central nervous system. Despite this independence, regulation of smooth muscle function is important for homeostasis, metabolic efficiency and the co-ordination of body systems; it allows an alteration of body function in response to changing requirements, either volitional or imposed by the environment. Therefore, understanding how nerves alter smooth muscle function is important, both for the pursuit of knowledge and for more utilitarian reasons – that if we can interfere with this regulation, then we can alter organ function. Furthermore, the body is subject to a range of environmental insults, including exposure to toxins, which can affect smooth muscle and hence bodily functions. Responding to or avoiding such toxicity is also an important part of smooth muscle and autonomic research. The nerves themselves, however, are subject to regulation within the tissues they seek to direct. Such regulation allows finer and local regulation of the tissues they regulate.

In some respects, there is a paucity of knowledge regarding the importance of some regulatory mechanisms at autonomic junctions, and some surprising examples of the presence of receptors where the justification for their existence is far from clear, counterintuitive or even wasteful. However, it seems unlikely that such mechanisms would persist in the context of evolutionary pressure without an ongoing function yielding a competitive advantage. A well known example is the presence of opiate receptors on the sympathetic nerves of the

mouse vas deferens (Hughes *et al.*, 1975a; Hughes *et al.*, 1975b), which powerfully inhibit transmission without evidence for an endogenous role (in the absence of local enkephalinergic innervation or the presence of circulating opiates).

Smooth muscle is inherently diverse in its function and characteristics, due to the many functions it serves throughout the body. It exists as a class because a series of anatomical and functional similarities. For example, most smooth muscle is found surrounding hollow organs where its contraction modifies the intraluminal pressure to effect movement of the contents. However, care must be taken not to overgeneralize, as there are example of smooth muscles which subvert this general rule, such as the anococcygeus (Gillespie & Lullmann-Rauch, 1974; Creed *et al.*, 1975) where smooth muscle forms as an epithelium-free sheet joining bone to viscera. The diversity of smooth muscle exists at many levels: cellular anatomy, electrophysiology, and the relative importance of different intracellular signalling pathways. Smooth muscle is found within a range of bodily systems, including the vascular, lymphatic, gastrointestinal, respiratory and urogenital systems, but also scattered in a range of tissues within which they serve a key role; for example, the piloerector muscles of the skin or the ciliary muscles of the eyes.

The Autonomic Nervous System

The autonomic nervous system has historically been defined by the anatomy of its efferent branch, although many modern authors include the visceral afferent nerves as part of this same system.

Autonomic efferent nerves innervate all body targets other than skeletal muscle and the neurons and glia of the central nervous system. The formal division of the autonomic nervous system is anatomical rather than functional, the three divisions in modern use being the sympathetic, parasympathetic and intrinsic systems. The intrinsic system replaces the 'enteric' nervous system, because of the increasing recognition of the importance of a range of other visceral intrinsic nervous systems such as those of the heart (Jelson *et al.*, 2003) and bladder (Feher *et al.*, 1980; Prieto *et al.*, 1990). The intrinsic neuronal plexuses are contained within the walls of various organs (e.g. the gut) where they form an integrated functional network residing without the central nervous system.

The sympathetic nervous system originates from nerve cells bodies located in the lateral column of the spinal cord from the thoracolumbar regions, ending at approximately L2 (although the specific level can vary depending on the species). The parasympathetic nervous system is described as craniosacral, with efferents that do not travel through the chain of autonomic ganglia that run in paravertebral columns. The cranial nerves containing parasympathetic fibres are the oculomotor (III), the facial (VII), the glossopharyngeal (IX) and the vagus (X), of which the dominant nerve is the vagus. Intrinsic nervous systems, like the enteric, may be innervated by parasympathetic preganglionic or postganglionic

neurons and hence have their intrinsic function modified. This leads to some semantic confusion, as this means that some neurons could be considered as intrinsic enteric neurons or postganglionic autonomic neurons. Fortunately, our semantic confusion does not reflect confused function.

Between the central nervous system and their visceral targets, there are two or more neurons, with the well-known and embryologically explicable exception of the sympathetic innervation of the adrenal medulla. The traditional nomenclature divides these into preganglionic and postganglionic neurons. The synapses between these pre- and post-ganglionic neurons lie in ganglia, which are distributed throughout the body. For the parasympathetic nervous system, the ganglia commonly lie within or close to the target organ; for example, the ciliary ganglion, supplying the parasympathetic innervation to the eye, is located in close appositions (posterior) to the eye. However, in the sympathetic nervous system synapses and nerve cell bodies of postganglionic neurons are located in a paravertebral chain of ganglia, of which the most superior ganglion is the most well studied: the superior cervical ganglion. Despite this general pattern, many exceptions occur including the existence of prevertebral ganglia associated with each of the major vessels arising from the abdominal aorta to the gut, and there are others in which the ganglia are close to their target tissues. The most relevant example of the latter case is the hypogastric ganglion, which provides the sympathetic innervation to the many pelvic urogenital organs including the vas deferens (Langley & Anderson, 1894, 1895, 1896).

These anatomical differences are almost universally reflected in a basic neurochemical code distinguishing the postganglionic sympathetic and parasympathetic neurons. The sympathetic postganglionic neurons release noradrenaline as their primary neurotransmitter, while the parasympathetic postganglionic nerves release acetylcholine. The dominant neurotransmitter from all of the preganglionic neurons is acetylcholine. Other autonomic co-transmitters will be discussed later as relevant to the experimental tissues used in this thesis.

Visceral afferents, long-neglected due to the historical anatomical definition of the autonomic nervous system, are now well-acknowledged as an important associated but independent field of study. The axons of these sensory neurons often run with the efferent autonomic nerves, however they can trigger both somatic and autonomic reflexes.

Vas Deferens

The vas deferens (or ductus deferens, as more technically correct for humans) is a key part of the male reproductive tract that serves the important function of transporting and allowing the final maturation of sperm. Its wall is a thick tube of smooth muscle, composed of inner circular and outer longitudinal smooth muscle cell layers. The vas deferens originates as a continuation of the epididymus, ascending and thickening as it passes towards the prostate, where it empties its contents into the prostatic urethra.

Smooth muscle cells can be of a range of sizes (30-400 μm), with those of the vas deferens tending to be longer (Brading, 1999). There are other cells types within the wall, including the autonomic nerves (discussed below), their associated glia (the Schwann cells), fibroblasts, mast cells, and the enigmatic and controversial Interstitial Cells of Cajal (Burton *et al.*, 2000; Metzger *et al.*, 2008). The lumen of the vas deferens is lined with an epithelium which is functionally important for sperm maturation and can signal to the muscular wall. The thickness of the smooth muscle layers, however, means that the outer longitudinal layer is remote from this biologically active layer. Consequently, the vas deferens provides a simpler system for the isolated study of the autonomic regulation of smooth muscle.

Innervation of the vas deferens

The dense sympathetic innervation of the vas deferens (Sjöstrand, 1965; Furness & Iwayama, 1972) enters from the hypogastric ganglia through a series of short

branches (in the mouse) which approach its surface in its prostatic third. Many of the branches run close to the artery of the vas deferens, spreading mostly towards the epididymal end from this first point of contact. The axons run in large bundles, which meander along the longitudinal axis of the vas deferens, progressively dividing along their course into ever smaller bundles. At some point, these bundles plunge deeper within the wall where they further divide into smaller collections of axon and ultimately single axons. This feature of having single isolated axons running between smooth muscle cells allows the high-resolution study of autonomic transmission at single neuroeffector junctions (Brain *et al.*, 2002). As with all autonomic nerves, they are accompanied by Schwann cells, becoming devoid of this intimate contact only at the points of closest interaction between individual varicosities and the smooth muscle. The varicosities are typically about 1 μm in diameter, ellipsoidal in shape and about 5 μm apart; each varicosity contains around 1000 vesicles (Cottee *et al.*, 1996).

Although the sympathetic nerves provide the main excitatory innervation of the vas deferens, the inner circular smooth muscle layer (Majcen, 1984) (and to a less extent the longitudinal layer) has been shown to have a sparse cholinergic innervation (Jackson & Cunnane, 2002; Cuprian *et al.*, 2005). The close apposition of sympathetic and parasympathetic neurons within the wall of the vas deferens may allow cross-modulation between these two systems (Starke *et al.*, 1989; Fuder & Muscholl, 1995). Such heterotropic interactions are well described in the myenteric plexus and in the heart.

Sensory nerves are also present, and while much has been speculated, little is known of their function. However, a recent study shows that both capsaicin and calcitonin gene-related peptide (CGRP) can modify neurogenic responses in the mouse vas deferens (Sheykhzade *et al.*, 2011a) therefore sensory nerves may play an important fine regulation of responses.

Functionally, the vas deferens, is unusual because it is controlled by a spinal ejaculatory rhythm generator (Stafford *et al.*, 2006), suggesting that endogenously it receives a very low resting tone that is punctuated by high-frequency bursts of activity.

Sympathetic junctional transmission

The classical transmitter from sympathetic nerve terminals is noradrenaline, and in most cases this is the most important functional transmitter. However, it acts not alone but cooperatively with a cocktail of other transmitters and modulators (particularly peptides). These transmitters are co-released (Burnstock, 1976), and probably co-stored. The evidence against co-storage is that the release of each of these transmitter substances can be differentially regulated, and may change under different experimental conditions such as a change in stimulus frequency. However, it may be, as Stjärne argues, that there are different classes of vesicle containing different ratios of neurotransmitters, and that these different classes may mobilise in response to different stimuli (Stjarne, 2001).

Exocytosis of Neurotransmitter Vesicles

Exocytosis is without doubt the dominant, although not exclusive, method of neurotransmitter release from the sympathetic nerves. Classical Na^+-dependent action potentials travel down the postganglionic nerve, traversing the many branches without failure en route to the nerve terminal varicosities. As the nerve terminals depolarize, the predominantly N-type voltage-dependent Ca^{2+} channels (Brock *et al.*, 1989a; Brock *et al.*, 1989b; Brain & Bennett, 1997) in the plasma membrane open, allowing Ca^{2+} to diffuse down its substantial electrochemical gradient. On surprisingly rare occasions (Cunnane, 1984; Macleod *et al.*, 1994; Brain *et al.*, 2002) this Ca^{2+} influx is sufficient to trigger the exocytosis of the contents of a single vesicle from any particular nerve terminal varicosity.

Exocytosis involves several proteins which are collectively known as SNAREs (Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Receptors). The Ca^{2+} binds to the first protein, synaptotagmin, bound to the surface of vesicles which increases its affinity for synaptobrevin and synaptotaxin. The former is also a vesicle bound protein whilst the latter is found on the inner surface of the neurone's plasma membrane. The Ca^{2+}-driven association of these proteins causes the close apposition of the vesicular and plasma membranes resulting in membrane fusion and exocytosis of vesicle contents as has been well reviewed (Calakos & Scheller, 1996; Sudhof, 2004).

In recent years, some authors have argued that total release of vesicular contents may not occur on every occasion. Such "kiss and run" exocytosis occurs as vesicles fuse only transiently with the plasma membrane so that only part of their contents are released (Burgoyne & Morgan, 2003). These discussions challenge the long-held belief that the release of neurotransmitters from autonomic terminals was quantal (Del Castillo & Katz, 1954) and raises the intriguing and controversial possibility that regulated release from synaptic vesicles may contribute to synaptic plasticity (Burgoyne & Barclay, 2002). Indeed it has been shown in calf chromaffin cells that quantal size can vary with stimulation frequency and Ca^{2+} entry (Elhamdani *et al.*, 2001). Work by colleagues in my own lab has also demonstrated that the size of quanta may vary however they attributed this to a range of neurotransmitter packet sizes at sympathetic neuroeffector junctions (Young *et al.*, 2007b, a).

Upon release, the neurotransmitters diffuse to their receptors on the postjunctional smooth muscle where they can act to alter smooth muscle function. Classically, autonomic transmitters act as 'volume' or 'non-directed' transmitters; they accumulate and steadily pool within the extracellular space until they reach a concentration sufficient to activate enough receptors to evoke a response in the smooth muscle cells. However, for some transmitters (particularly ATP) there is strong evidence for directed transmission at close-contact neuroeffector junctions.

Catecholaminergic transmission

The catecholamine noradrenaline is synthesised from its precursors both in the cytoplasm and within the neurotransmitter vesicles themselves. L-tyrosine is converted by tyrosine hydroxylase to L-DOPA (L-3,4-dihydroxyphenylanine), which in turn is subject to the action of DOPA decarboxylase to form the neurotransmitter dopamine. Interestingly, some sympathetic nerves utilize dopamine itself as a transmitter, particularly in the kidney (Elkayam *et al.*, 2008). However, most of the dopamine is transported into the neurotransmitter vesicles by the vesicular monoamine transporter (VMAT) where it is converted by the action of dopamine β-hydroxylase, a membrane-bound enzyme, to noradrenaline. Noradrenaline is stored within the vesicles at a high concentration (0.3–1.0 M) ready for release.

Upon its release by exocytosis noradrenaline pools within the extracellular space from where it can activate receptors on the smooth muscle, or on other cell types

including the nerve from which it has just been released. The receptors for noradrenaline are the α- and β- adrenoceptors. On smooth muscle, the dominant receptors generating contraction are the α_1-adrenoceptors (and to a lesser extent the α_2-), while the β-adrenoceptors (particularly β_2 and β_3) mediate relaxation. The dominant intracellular signalling pathways are mediated by G_q and phospholipase C (for the α_1 receptor) and down- (α_2) or up- (β-) regulation of adenylyl cyclase via G_i and G_s respectively (Summers & McMartin, 1993).

There are some conditions under which the release of noradrenaline may be non-vesicular; this is predominantly through the reverse transport of noradrenaline through NET, which can be induced by the indirectly acting sympathomimetic amines such as tyramine.

Noradrenaline's action is transient, because it diffused out of the junctional cleft into the surrounding extracellular spaces and because it is removed by the action of the two neurotransmitter transporters: the norepinephrine transporter (NET; functionally referred to as uptake-1 in the older literature) and a set of what are mainly octopamine transporters (OCT), the function of which were historically referred to collectively as uptake-2. NET is found predominantly on the sympathetic nerve terminals, allowing the recycling of noradrenaline if it is repackaged by VMAT. Under some conditions, however, the noradrenaline is degraded by a series of enzymes commencing with either monoamine oxidase (MAO) or catechol-*O*-methyl transferase (COMT).

Purinergic transmission

Purines are characterized by the fusion of a pyrimidine and with an imiazole ring. The biologically most important purines are based on adenosine or (to a lesser extent) uracil. From sympathetic nerves, the most important purinergic neurotransmitter is adenosine triphosphate (ATP). The history of the discovery of ATP as a neurotransmitter has been well chronicled by an early, enthusiastic, ebullient, determined, dogmatic and ultimate vindicated pioneer; for reviews, see (Ralevic & Burnstock, 1998; Burnstock, 2006).

ATP is synthesized by mitochondria, to maintain a high cytoplasmic concentration. It is then packaged into vesicles by a mechanism only recently discovered: by the vesicular nucleotide uptake transporter (VNUT), the gene product of SLC17A9 (Sawada *et al.*, 2008). The vesicular concentration may be as high as 100 mM (Stjarne, 2001).

When released by exocytosis, ATP diffuses to its receptors and out of the cleft. The receptors for ATP are of two separate families, the P2X and P2Y families. The P2X receptor are ligand-gated ion channels, while the P2Y family are GPCRs. The P2X receptors are relatively insensitive to ATP, meaning that they respond mainly when the receptors are located close to the release sites. Such tight spatial coupling can be observed in the discrete region of the smooth muscles around each nerve terminal varicosity that responds with an increase in Ca^{2+} concentration in response to the evoked or spontaneous release of packets of ATP in either the vas deferens (Brain *et al.*, 2002), mesenteric artery (Lamont *et al.*, 2003) or bladder (Heppner *et al.*, 2005).

ATP is broken down in the extracellular space by members of a large family of ecto-nucleoside triphosphate diphosphohydrolases (Zimmermann & Braun, 1999), with many pathways eventually leading to adenosine, which can be taken back into nerve terminals using specific transporters (Baldwin *et al.*, 2004).

It is possible that ATP might be released from nerve terminals by routes other than by exocytosis. In other cell types, ATP transport across the membrane can occur through ABC superfamily transporters (of which CFTR is the best known example), or through hemichannels formed from connexin (Stout *et al.*, 2002) or even pannexins (Saez *et al.*, 2005).

Other autonomic transmitters

NPY is an important transmitter in many sympathetic nerve terminals (Neild, 1987; Bradley *et al.*, 2003). Synthesized in the nerve cell bodies, it is transported orthogradely into the nerve terminals, packaged predominantly in large dense cored vesicles with noradrenaline (Lundberg *et al.*, 1983; Lundberg *et al.*, 1990).

While NPY is the most important neurotransmitter peptide released from sympathetic nerves, there are almost certainly other peptides that will be shown to have important effects in modifying junctional transmission at least some sympathetic neuroeffector junctions. For example, several authors have shown that tissue plasminogen activator (tPA) can be released from vascular sympathetic nerves (Peng *et al.*, 1999; Jiang *et al.*, 2002).

Cotransmission in the vas deferens

Stimulation of the sympathetic nerves which innervate the vas deferens, even with quite short trains, evokes a biphasic contraction first observed using rat and guinea-pig tissues (Swedin, 1971b). The first phase is associated with a rapid depolarization of the smooth muscle membrane (Burnstock & Holman, 1961; McGrath, 1978; Stjarne, 1989), a fast excitatory junction potential (EJP), whilst the second often occurs without any change in the smooth muscle cell membrane potential difference.

Both chemical sympathectomy with 6-hydroxydopamine and the use of noradrenergic neurone blocking drugs such as guanethidine and bretylium (Brain & Cunnane, 2008) reduce or abolish the EJPs and both phases of contraction. However, frequently only the second phase of contraction is abolished by α-adrenoceptor antagonists or resperine (Cunnane & Manchanda, 1989a). Thus both phases of contraction are caused by transmitters released from noradrenergic terminals but the responses are associated with different transmitters with evidence from work using exogenously applied ATP and ATP analogues and the use of purinoceptor antagonists strongly supporting the case of ATP as a co-transmitter with noradrenaline in this system. However, whilst the guinea-pig vas deferens frequently displays a clear pharmacological separation of the two phases of neurogenic responses which can be attributable to ATP and noradrenaline (Fedan *et al.*, 1981), the distinction seems less clear-cut in the mouse vas deferens (Stjarne & Astrand, 1985).

Junctional Regulation

Autoregulation

When neurotransmitters or neuromodulators are released from the nerve terminals, they commonly act on prejunctional receptors to modify the subsequent activity of the nerve terminal. Most commonly, this is negative feedback, which is useful to prevent an excess of neurotransmitter release and without causing an uncontrolled cycle of positive feedback.

One example of positive feedback is through a prejunctional action of adrenaline acting on β2-adrenoceptors (Tarizzo *et al.*, 1994), and there is also the possibility of ATP-dependent positive feedback through prejunctional P2X receptors (von Kugelgen *et al.*, 1997; Boehm, 1999).

The most well-studied, and most important, feedback mechanism at sympathetic junctions is the effect of noradrenaline on prejunctional α2-adrenoceptors (Starke, 2001; Trendelenburg *et al.*, 2003; Burnstock, 2006). As with α2-adrenoceptors elsewhere, the main second messenger pathway thought to be involved is G_i, leading to an inhibition of cAMP levels in the nerve terminals. This leads to an inhibition of Ca^{2+} influx, probably through an inhibition of N-type VGCCs, in the sympathetic terminals of the mouse vas deferens (Brain & Bennett, 1997).

ATP, broken down to adenosine, acts on prejunctional adenosine receptors to also inhibit transmitter release (Smith & Lu, 1991; Mendoza-Fernandez *et al.*,

2000; Powell *et al.*, 2000), although it has been argued that such an effect is not mediated through an inhibition of Ca2+ influx (O'Connor *et al.*, 1999). In this way, both of the two main neurotransmitters released from sympathetic nerve terminals can cause negative feedback to brake excessive neurotransmitter release.

Other prejunctional receptors

Most organs are innervated by both noradrenergic (sympathetic) and cholinergic (mostly parasympathetic) nerve terminals, and so the sympathetic nerves are commonly exposed to acetylcholine. The sympathetic nerves terminals contain inhibitory muscarinic receptors and excitatory prejunctional nicotinic receptors. The inhibitory muscarinic receptors are probably the more important ones endogenously, allowing for a peripheral cross-inhibition between sympathetic and parasympathetic nerves. The existence of prejunctional nicotinic receptors on sympathetic nerves led to a historically interesting 'dead-end': the Burn and Rand hypothesis (Burn & Rand, 1959).

The excitatory effects mediated by pre-junctional nicotinic receptors on sympathetic neurotransmitter release have been well documented in many tissues (Starke *et al.*, 1991; Vonkugelgen & Starke, 1991; Carneiro *et al.*, 1993). Other work has demonstrated nicotine's ability to evoke small, random increases of intra-varicosity calcium possibly involving a ryanodine-sensitive calcium store (Brain *et al.*, 2001). No effect was observed on action potential-evoked Ca^{2+} transients although force of contraction and EJP amplitude were increased

suggesting that the small fluctuations were enough to prime the varicosities increasing the probability of both spontaneous and action potential-evoked resale of neurotransmitter.

However, muscarinic receptors have been observed to mediate an inhibition of noradrenaline release in many tissues including the corpus cavernosum of the penis (Saenz de Tejada *et al.*, 1989), various vascular preparations (Vanhoutte *et al.*, 1973; Vanhoutte, 1974; Toda *et al.*, 1990) and the rabbit vas deferens (Eltze, 1988; Dörje *et al.*, 1991; Bradley *et al.*, 2001). In the canine mesenteric artery this effect appears to be mediated by M2 receptors (Zhang *et al.*, 1997) whilst in the rabbit vas deferens M1 activation is involved.

In addition to cholinergic modulation of sympathetic neurotransmitter release, a complete list of endogenous compounds which have a modulatory role include opioids (Henderson *et al.*, 1972; Henderson & Hughes, 1976), neuropeptide Y (Lundberg *et al.*, 1984), 5-hydroxytryptamine (5-HT) (Kapur & Mottram, 1979) and adenosine (Clanachan *et al.*, 1977).

In the mouse vas deferens, functional μ, δ and κ receptors have been demonstrated and also the ORL1 receptor (Trendelenburg *et al.*, 2000) and morphine has been demonstrated to inhibit neurogenic contractions in this tissue (Hughes *et al.*, 1975a) with similar inhibition of purinergic and noradrenergic contractions (Driessen *et al.*, 1993). This observation is pertinent because some drugs acting at presynaptic receptors seem to modulate the release of noradrenaline and ATP in a differential or even opposite manner, for

instance angiotensin peptides (Trachte, 1988; Ellis & Burnstock, 1989a; Ziogas & Cunnane, 1991), calcitonin gene-related peptide (Ellis & Burnstock, 1989b) and prostaglandin E2 (Ellis & Burnstock, 1990).

Interestingly, morphine has been shown to increase the hump phase of bi-phasic contractions (Forsyth & Pollock, 1988) although others have seen no such effect on contractile responses to high frequency stimulation (Driessen *et al.*, 1993). There is some evidence that this dichotomy could be explained by strain differences (Henderson & Hughes, 1976). On cholinergic transmission opioids have been exclusively shown to inhibit transmission in many tissues including human airway (Belvisi *et al.*, 1992), guinea pig airways (Belvisi *et al.*, 1990) and ileum (Paton, 1957; Hughes, 1975) and rabbit heart (Kosterlitz & Taylor, 1959). Whilst a final interesting observation has been made on sympathetic nerves in the heart where morphine and methadone potentiated the cardiac response to sympathetic stimulation which was described an unspecific effect involving modulation of neuronal uptake of noradrenaline (Ledda *et al.*, 1984).

5-HT also appears to have range of effects with regard to its ability of modulate junctional transmission in sympathetically innervated organs. In the rat vas deferens, an inhibition of neurogenic contractions has been observed mediated by the 5-HT1B binding site (Smith & Bennett, 1990), the same autoreceptor as found in the rat brain cortex (Engel *et al.*, 1986). In the mouse vas deferens pre-junctional inhibitory and potentiating responses for 5-HT were observed on electrically induced twitch responses whilst a post-junctional potentiating response was also observed using exogenously applied ATP (Seong *et al.*, 1990).

Interestingly, 5-HT1A antagonists have also been seen to cause post-junctional inhibition of contraction in the mouse deferens (Arkle *et al.*, 2005), however in the guinea-pig vas deferens 5-HT at high concentrations caused post-junctional facilitation of contractile responses (Yoshida & Kuga, 1986).

This gives an idea of the rich plethora of chemicals, neurotransmitters and hormones which can modulate autonomic and specifically sympathetic transmission. Cannabinoids are another chemical group which have been shown to regulate junctional transmission and are a key focus of this work.

Cannabinoids

The significant psychoactive effects of the cannabis plant, *Cannabis sativa*, have been known for centuries through its medicinal and recreational use (Butrica, 2002), however it was not until 1964 that the main psychoactive compound, Δ^9-tetrahydrocannabinol (THC), was isolated (Gaoni & Mechoulam, 1964b). In more recent years, cannabinoids have also been shown to have effects on the sympathetic, parasympathetic (Buckley *et al.*, 1998) and enteric (Pertwee, 2001) branches of the autonomic nervous system. Increasing evidence also points to the involvement of cannabinoid-like receptors in some systems distinct from the classical CB_1 and CB_2 receptors already identified. Endogenous cannabinoid compounds have also been identified which seem to be involved in retrograde signalling for negative feedback. These endogenous compounds are cell membrane-derived molecules released from nerves, blood cells and endothelial, however elucidation of their physiological role has been confounded by the ability of main of these compounds, notably anandamide and N-arachidonoyl-dopamine, to also activate vanilloid VR_1 receptors.

Cannabinoid receptors

A pharmacologically distinct, saturable, high affinity binding site for a tritiated cannabinoid was first demonstrated in membranes from homogenized rat brain in 1988 (Devane *et al.*, 1988). This was rapidly followed by the cloning of the first cannabinoid receptor, CB_1, and its classification as a G-protein coupled receptor (Matsuda *et al.*, 1990). CB2 was first cloned in 1993 and found to have a 44% identity with CB1 at an amino acid level. Both CB_1 and CB_2 are G-protein

coupled receptors with CB_1 mostly localized in the central nervous system and some peripheral tissues whilst CB_2 is found outside of the central nervous system predominately in immune tissues and cells (Pertwee, 1993; Pertwee, 1997, 1999).

The cannabinoid receptors, especially CB_1, display many unique properties. The important role of endocannabinoids in physiology is underlined by the preservation of receptor homology throughout evolution: CB_1 receptors from humans, rats and mice share 97–99% of their amino acid sequence identity. CB_1 receptors are also highly expressed in the brain being the most abundant G-protein coupled receptor with densities 10-50 times greater than more classical receptors such as for dopamine or opioids (Howlett *et al.*, 1990; Herkenham *et al.*, 1991). Another interesting comparison with opioid receptors is made from the comparatively low efficiency of CB_1 receptor coupling to its transduction mechanism with a 7-fold lower ability to couple G-proteins than opioid receptors (Breivogel & Childers, 1998; Felder & Glass, 1998; Manzanares *et al.*, 1999).

Both CB_1 and CB_2 receptors are coupled through $G_{i/o}$ proteins resulting in the inhibition of cAMP and a decrease in phosphorylation activity by protein kinase A. (Devane *et al.*, 1988; Howlett *et al.*, 1990). It has also been established that, with the involvement of the Golf protein, cannabinoid receptor activation also leads to inhibition of Ca^{2+} influx into cells through N (Mackie & Hille, 1992), P/Q (Twitchell *et al.*, 1997), and L (Gebremedhin *et al.*, 1999) type calcium channels, and to the activation of inwardly rectifying potassium conductance (Mackie *et al.*, 1995) and A currents (Childers & Deadwyler, 1996). These observations in the

central nervous system provided the early evidence that cannabinoids could modulate neurotransmitter release and even have a role in short-term plasticity (Schlicker & Kathmann, 2001; Wilson & Nicoll, 2001).

Pharmacological data also suggests the existence of cannabinoid-like receptors which have yet to be identified at a molecular level. Many tissues including mesenteric arteries (Jarai *et al.*, 1999; Wagner *et al.*, 1999; Zygmunt *et al.*, 2002), coronary arteries (White *et al.*, 2001) and the CNS (Kunos & Batkai, 2001) have shown non-CB_1/CB_2 activity. One candidate is GPR55, an orphan receptor first identified in yeast by Glaxo Smith Kline, part of the purinergic subfamily and closely related to GPR35, GPR23 and $P2Y_5$ (Brown, 2007). This receptor is linked to G_{13} resulting in activation of rhoA, cdc42 and rac1 (Ryberg *et al.*, 2007).

Endocannabinoids

Cannabinoid agonists are divided into classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids by the International Union of Pharmacology (Howlett *et al.*, 2002). Classical cannabinoids either occur naturally in *Cannabis sativa* (of which there are over 60 active compounds) or are synthetic analogues with the common chemical structure of a tricyclic dibenzopyran ring. Non-classical cannabinoids lack the dibenzopyran ring whilst aminoalkylindoles are non-cannabinoid chemicals which show cannabimimetic activity (Pacheco *et al.*, 1991). The final class, eicosanoids, are the chemical group which are found as endocannabinoids and like prostaglandins and leukotrienes are derivatives of arachidonic acid. This chemical origin is then

conjugated with either an ethanolamine or glycerol. The two most common examples in this class are anandamide (also known as *N*-arachidonoyl ethanolamine (AEA)) and 2-arachidonoylglycerol (2-AG). Both are synthesized on demand like other eicosanoids and are released by neurons in a Ca^{2+}-dependent manner.

Endocannabinoids are removed from synaptic and junctional clefts by an energy-independent transporter (Beltramo *et al.*, 1997) which rapidly stops the signalling effects of these molecules. They are then degraded by either fatty acid amide hydrolase (FAAH) if they are an acylethanolamide such as anadamide (Cravatt *et al.*, 1996) or monoacylglyceride lipase (MAGL) for monoglycerides such as 2-AG (Dinh *et al.*, 2002). However, it should be noted that 2-AG can also be inactivated by FAAH. Both FAAH and MAGL are serine hydrolase, the former membrane bound, with both showing high concentrations in the liver and the brain as does the transporter protein (Giuffrida *et al.*, 2001; Gulyas *et al.*, 2004).

Cannabinoids and the Autonomic Nervous System

Four effects of cannabinoids are commonly observed in *in vivo* bioassays: analgesia, hypothermia, immobility and catalepsy. These cannabimimetic effects were so frequently observed in mice that they become known as the "mouse tetrad". However, the mouse is also a well established and frequently used tool for the *in vitro* bioassay of cannabinoid effects and in particular the mouse vas deferens in the assessment of cannabinoid modulation of sympathetic transmission (Thomas & Pertwee, 2006).

The effects of cannabinoids in the mouse vas deferens is mostly considered mediated by CB_1 receptors, the strongest early evidence for this arising from the observation that nonselective cannabinoid receptor agonists WIN 55,212-2 and CP 55,940 potently inhibited the electrically evoked overflow of ^{3}H-noradrenaline (EC50 values 2.7 and 0.27 nM, respectively) and this action was blocked by nanomolar concentrations of SR141716A, a selective cannabinoid CB1 receptor antagonist (Trendelenburg *et al.*, 2000). However, evidence for CB receptors in the mouse vas deferens goes back further to the induction of tolerance to THC with no effect on responses to clonidine, an α_2-agonist, or opioids (Pertwee & Griffin, 1995). Thus, the prejunctional effects of cannabinoids on CB_1 receptors are well established as they are in several other tissues including rat vas deferens (Ishac *et al.*, 1996a), isolated heart (Molderings *et al.*, 1999; Szabo *et al.*, 2001), blood vessels (Deutsch *et al.*, 1997; Ralevic & Kendall, 2002b) and cultured sympathetic neurones (Gobel *et al.*, 2000). Occasional observations also point to CB_2 mediated effects in the mouse vas deferens, such as nanomolar concentrations of the cannabinoid CB2 receptor-selective agonists JWH-015 and JWH-051 blocking electrically evoked sympathetic neurogenic contractions via a mechanism insensitive to SR141716A (Griffin *et al.*, 1997). However, these effects are normally attributed to non-cannabinoid effects and the CB_2 receptor is generally not considered to be expressed in the mouse vas deferens.

Early work into the effects of cannabinoids on the cardiovascular system suggested that cannabinoids lowered blood pressure through a central

sympatho-inhibitory mechanism (Vollmer *et al.*, 1974). However, the *in vivo* effects of cannabinoids on the cardiovascular system seem more complex with both increases and decreases in blood pressure being reported (Stark & Dews, 1980; Dewey, 1986). However, a review of the literature on the cardiovascular effects shows that generally systemically administered cannabinoids cause hypotension and bradycardia by peripheral prejunctional inhibition of sympathetic outflow and increased vagal activity, respectively (Randall *et al.*, 2002).

Such *in vivo* observations and conclusions are supported by *in vitro* studies which demonstrate cannabinoid mediated inhibition of neurotransmitter release in many cardiovascular tissues including rat isolated atria (Ishac *et al.*, 1996b) and mesenteric arteries (Ralevic & Kendall, 2002b). However, others have demonstrated no effect in rat and mouse atria and rat mesenteric arteries for anandamide and synthetic cannabinoids (Lay *et al.*, 2000). The effects of anandamide are particularly varied with relaxation observed in mesenteric arteries (White & Hiley, 1997) and coronary vasculature (Randall & Kendall, 1997) but not rat carotid arteries (Holland *et al.*, 1999) or rat aorta. In addition, in cases of observed anandamide-induced relaxation some studies demonstrate insensitivity to cyclo-oxygenase inhibitors and endothelial denudation (Randall *et al.*, 1996; White & Hiley, 1997) whilst others show sensitivity to cyclo-oxygenase inhibitors (Fleming *et al.*, 1999). Combined, these data show tissue selectivity for the effects of anandamide on blood vessels and for the involvement of prostanoids in mediating its effects. However, it does appear that

peripheral prejunctional inhibition of sympathetic neurotransmission is involved in the hypotensive effects of systemically administered cannabinoids.

An interesting question remains when considering the effects of cannabinoids on the autonomic nervous system: what is the normal physiological role of endocannabinoids? In the central nervous system, retrograde signalling is a well documented and substantiated mechanism of action. However, as previously discussed, endocannabinoids are rapidly taken up by transporters and hydrolysed by enzymes resulting in a short extracellular half life. This problem is exacerbated in the periphery since junctional clefts vary more greatly in size than found centrally and are often wide. Another possibility is that they may directly modify the organization of neuronal membranes due to their high lipophilicity, but this also seems unlikely due to their concentration-dependent, stereoselective, antagonist-sensitive actions on peripheral tissues which points towards receptor mediated signalling pathways. The most likely activity, which is seen in the central nervous system, is that they are released and act prejunctionally, with CB_1 receptors acting as autoreceptors, in a comparable manner to NA, ATP and NPY. This would correlate with the short life expectancy requiring release near to nerves and would still allow for other nerves, blood cells and endothelial cells to release endocannabinoids which modulated autonomic activity. Whatever their exact role, it is clear that cannabinoids have the ability to regulate the activity of autonomic nerves and the responses they evoke in effector organs in the periphery.

Testosterone

Traditionally, the action of steroids has been considered in terms of the hormones binding to specific intracellular receptors, which act as transcription factors either increasing or decreasing the expression of specific genes. The receptors are composed of a ligand-domain, a DNA-binding domain and several transactivation domains which are distributed along the molecule. Such effects are referred to as genomic and are characterized by a time lag between drug application and the response, and by a sensitivity to drugs which inhibit translation (e.g. cycloheximide) or transcription (e.g. actinomycin D). However, in 1942, Hans Selye observed a rapid anaesthetic effect in response to intraperitoneal application of progesterone (Selye, 1942). From this first observation, subsequent evidence has been gathered which demonstrates that in addition to genomic actions, steroids can have rapid effects which must be nongenomic due to their short time lag (often seconds) before a response is seen and their insensitivity to transcription and translation inhibitors.

Many mechanisms have been proposed to explain the gathering reports of nongenomic steroid activities. For instance, differences in the pharmacological properties (e.g. antagonist sensitivity) of the receptors which mediate nongenomic responses from those which mediate genomic effects does not provide convincing evidence that there are separate receptor proteins for mediating nongenomic actions. Consequently, in 1998, a classification of rapid steroid effects was discussed and proposed at the “First International Meeting on Rapid Responses to Steroid Hormones”, held in Mannheim, Germany. The

Mannheim classification can now be used to describe the potential mechanisms for any nongenomic steroid effect (Falkenstein *et al.*, 2000a). It is divided into two broad groups: A (direct steroid action – the steroid is the only agonist acting alone) and B (indirect steroid action – the steroid needs a partner agonist to generate the rapid response). Both groups are then subdivided into I (nonspecific – no receptor involved) and II (specific – steroid receptor with ligand specificity involved). Specific mechanisms are further divided into those mediated by classical steroid nuclear receptors (a) and those mediated by nonclassical steroid receptors (b). Examples have been found for all these groups except BI and BIIa.

The importance of considering genomic and nongenomic steroid effects in the central nervous system mediated by steroids coming from the peripheral glands or originating directly in the nervous system, neurosteroids, has been well reviewed (Melcangi & Panzica, 2006; Melcangi & Panzica, 2009). This area has been widely reviewed in the past for specific groups of steroids such as glucocorticoids which have been demonstrated to have rapid effects including changes in neuronal firing frequency, inhibition of vasopressin release and effects upon behaviour (Makara & Haller, 2001).

In addition, a wide range of rapid steroid effects have been observed in the periphery including inhibition of K_{ATP} channel activity in pancreatic β-cells isolated from mice by 17β-oestradiol (Nadal *et al.*, 1998), dose-dependent increases of mean intracellular Na^+ and K^+ levels in human mononuclear leukocytes caused by aldosterone (Wehling *et al.*, 1987) and oscillations of Ca^{2+}

content in endometrial cells caused by 17b-oestradiol (Pietras & Szego, 1975). Several studies into the effects of steroids on smooth muscle contractility have also been carried out: dexamethasone has been demonstrated to rapidly depress electrically-evoked contractions of longitudinal smooth muscle myenteric plexus preparations from male guinea pigs in a dose-dependent fashion (Persico *et al.*, 1991) and reduced the amplitude of electrically-evoked contractions in isolated ring segments of rabbit ear arteries (Miyahara *et al.*, 1993).

Testosterone's rapid effects on smooth muscle contractility have also been investigated. Experiments using rat detrusor muscle preparations, contracted by electrical field stimulation, showed a concentration-dependent inhibition of responses by testosterone (Hall *et al.*, 2002). This effect could not be blocked by the genomic testosterone receptor antagonist, flutamide (50 μM). A similar effect by testosterone has been found in experiments using myometrial samples from pregnant women (Perusquia *et al.*, 2005). This was found under spontaneous contractile activity and for contraction mediated by high K^+. Testosterone has also been shown to cause an endothelium-independent relaxation in isolated rabbit coronary artery and aorta preparations (Yue *et al.*, 1995).

Materials and Methods

Physiological Salines

Unless otherwise specified, all preparations of the mouse vas deferens were maintained in a physiological saline (PSS) containing (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.1 (PSS). The solutions were bubbled by continuously bubbling the solution reservoir with 95% O_2/5% CO_2. The Henderson-Hasselbach equation was used to check this, with pK=6.1 and a CO_2 solubility of 1.1 g/L at 36˚C and a pressure of 1 atm:

$$pH = pK + \log\left(\frac{\left[HCO_3^-\right]}{\left[CO_2\right]}\right)$$

This yields an equilibrium pH of 7.4 (to two significant figures).

Preparation of the Vas Deferens

All experiments on animals were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 86/09/EEC.

Male Balb/C mice (8-12 week old; Harlan, UK) were killed using cranial concussion followed by cervical dislocation in order to confirm death; this is a Schedule 1 procedure. No regulated procedures (as defined by the Animals (Scientific Procedures) Act) were carried out in experimental work for this thesis.

A midline abdominal incision was performed to penetrate the skin then the linea alba exposing the abdominal and pelvic cavities. Each testicle was identified low in the pelvis and exteriorized, grasping only peri-testicular fat. By tilting each testicle laterally, the epididymus (which runs dorsal to the testicle and in close apposition to it) and vas deferens (which arises as a continuation of the epididymis, after turning sharply at the gubernaculum testis or scrotal ligament) could be identified. The vas deferens runs from the testis to the prostate, which is located around the urethra at the base of the bladder Each vas deferens was excized by initially using blunt dissection to remove excess tissue, including the removal of a large blood vessel (presumably the testicular artery), and by holding the distal epididymis and cutting it from the testicle. Finally, the vas deferens was isolated by cutting off the most prostatic 20% of the vas deferens. At this stage, the epididymis was left intact as a marker for orienting the tissue and in order to secure the tissues in a shall Silgard-lined Petri dish using small pins. This dish contained PSS which was changed at regular intervals in order to maintain the O_2 concentration and pH.

Once in the petri dish, the tissue was examined with a binocular dissecting microscope and was cleared of any remaining connective tissue with forceps and then iris scissors.

Contraction Studies

For contraction studies, the vasa deferentia were further prepared by tying a small loop of suture thread around the prostatic end. Another longer length of thread was tied around the epididymal end of the tissue with the other end left loose for later tying. The epididymis was then cut off.

Each tissue was then transferred to the 5 mL chamber of either a 4-channel or 8-channel organ bath (Letica Scientific Instruments, Spain). The tissue was supported by a rigid metal rod, with a hook at the lower end under which the small loop was placed. The tissue was aligned with the rod, passing through two platinum ring electrodes (custom made in the department by Mr P. Flaxman), aligned so that most of the prostatic end was contained between the electrodes. The electrodes were approximately 5 mm apart. The epididymal end was secured by tying the loose thread end to an isometric force transducer (Letica Scientific Instruments, Spain) held in place above the chamber on a holder that allowed fine manipulation of the transducer position and hence the tension applied to the tissue.

Each 5 mL chamber was contained within a common water bath which acted as a heat reservoir (37˚C). A recirculating pump operates continuously in order to ensure efficient heat exchange by convection. The chambers could be drained from their base (through a valve that was under electronic control) or by overflow out of the top of the chamber as solution enters from below. Both methods of drainage were connected to a Venturi drain or vacuum pump. The bottom of the chambers also contained a small air-stone, allowing continuous

bubbling with the CO_2/O_2 mixture. Unless otherwise stated, the PSS in the chambers was changed every 20 minutes by flushing the chambers 5 times in quick succession. This protocol did not cause a fall in the recorded contractile forces and ensured better exchange of the solution than overflow because it avoids mixing of old and new solution in the chambers. Care was taken to ensure that electrical field stimulation (EFS) was not triggered while any chambers were devoid of fluid; this prevents the problem of a reduced conducting pathway through the tissue leading to an excessively high electric field density, which would result in damaging direct electrical stimulation of the smooth muscle.

Once all tissues were positioned in their chambers, a resting tension of 9.8 mN (equivalent to the force exerted by a 1 g mass) was applied to each of them. The preparations were then allowed to equilibrate for one hour, exposed to EFS every 10 minutes with increasing voltage from 50 V to 90 V in 10 V increments, increasing every 10 minutes (10 pulses at 10 Hz; 0.5 ms width). This method was developed to allow a more rapid equilibration to produce stable, repeatable contractions at a supra-maximal voltage from one hour after developing tension.

Digital recording: sampling and filtering

The isometric force transducers were connected to a bridge amplifier, which was itself connected to a Powerlab (ADInstruments, Oxford, UK) analogue to digital converter. The analogue to digital converter was then connected to a Mac mini (Apple, UK) running Chart versions 4.0-5.5.6 (also from ADInstruments). The sampling frequency was at least 100 Hz (and in many cases 1 kHz); the

contraction is sufficiently slow that no information is lost by sampling at 100 Hz, and this has many advantages including small file sizes, inherent low-pass filtering and the avoidance of spurious precision.

Before tensioning the tissues, the equipment was calibrated on each experimental day by zeroing each 'bridge-amp' and using a 1 g mass to appropriately set the units-conversion parameters. History has dictated the rather anachronistic practice of recording in grams and reporting in millinewtons, with conversion off-line; continuing this practice need not be encouraged!

The Chart software also recorded a TTL signal from a custom-made electrical pattern generator (Martyn Preston, Oxford), via the analogue to digital converter. The pattern generator also sent control signals to the custom-made 4-channel stimulator (or stimulators in the case of the 8-channel baths), which allowed sufficient current to flow to the electrode in order to generate EFS.

Drug exposure

Tissues in the 5 mL chambers were exposed to drugs by exogenous application into the organ baths using a Gilson pipette. Stock solutions of drugs were regulated on the day to ensure that the minimum volume introduced into the baths was 5 µL using a P20 pipette. Drugs requiring a long incubation period or the presence of which needed to persist throughout the course of the experiment were replaced as soon as possible after any washing to change the PSS in the

chamber. For such drugs (e.g. neurotransmitter antagonists used in isolating the purinergic and noradrenergic components of contraction) care was taken to use separate pipettes for introducing these drugs into control and exposure chambers with reference to the drug under investigation (e.g. THC) in order to avoid cross-contamination.

Exogenously applied agonists often evoked responses which varied greatly in the speed with which they evoked a maximum response. Unlike a response to EFS, they also did not return to baseline within a moderately short time course. For instance, in some pilot experiments an intermediate concentration of phenylephrine (1 μM) might evoke a contraction which would persist after 10 mins. Therefore in order to avoid problems of desensitization, tissues would be washed a given time after agonist application or after a particular contractile profile had been observed depending on the protocol of the experiment. These are detailed in the results section.

Contraction analysis

Analysis of contraction data was performed offline using Chart 5.5.6 and its Peak Parameters extension 1.1. Acquisition of data from the traces was automated by recording macros which either searched for EFS markers to find events or which could be run after manual placing of the cursor in order to quickly generate a repeatable selection window following specific parameters.

Baselines were normally calculated from the mean tension during the 10 secs preceding contraction initiation for both EFS- and exogenous agonist-induced contractions. As the mouse vas deferens shows no spontaneous activity under normal conditions in the absence of stimulation, the trace is very stable allowing an accurate recording from this relatively short baseline measurement. Peak amplitude was calculated using as an average of 1 ms window around the maximum point of a trace during EFS or following agonist application; the baseline value before the contraction was then subtracted from this value to give the peak amplitude.

Where appropriate peak area was calculated as the area under the curve for the trace of a contractile response. In cases of agonist-evoked contractions, a specific time frame, dependent on the experimental protocol, was used for selection using the contraction onset as a reference point in order to standardize the data selection windows across an experiment where it was not possible to wait for the trace to return to baseline as previously discussed.

Data were exported to Microsoft Excel for further manipulation and analysis, including normalization. Normalization of contraction data was achieved by one of two methods according to the experimental protocol. The first method involved dividing parameter readings by a "normalizing reading" to a fixed stimulus (10 pulses, 10 Hz, 0.5 ms width, supramaximal voltage) taken at the end of the equilibration period or in the case of experiments using the neurotransmitter inhibitors to isolate the purinergic and noradrenergic components of contraction at the end of the incubation period with the inhibitor.

The second method was by using relative changes calculated by taking the ratio of the given parameter in the presence of the drug or vehicle control and dividing it by the equivalent reading taken before incubation with the drug or matching vehicle of interest.

Intracellular Electrophysiology Studies

Vasa were prepared for electrophysiological recordings by using iris scissors remove the epididymis and then each vasa was cut longitudinally along one side. The tissue could then be turned serosal-side up and pinned flat to the Sylgard-lined base of a custom-made perspex dish (made by Mr P. Flaxman). This arrangement was advantageous as it resulted in a flatter tissue sample and also allowed the tissue to be stretched slightly more thereby reducing movement problems. The physical change to the whole tissue has been previously shown to have no significant effect on the recordings obtained (Young *et al.*, 2007a).

The dish was constructed to allow constant perfusion of chamber with fresh PSS with inflow and outflow driven by a peristaltic pump. The outlet could be altered to regulate the level of PSS within the chamber. The PSS was held in a beaker at room temperature, continuously bubbled with the O_2/CO_2 mixture and flowed through a heater unit which was regulated by a thermostat with a temperature probe in the chamber which automatically maintained the temperature of the PSS in the chamber at 34˚C.

Electrical stimulation was provided by a pair of a pair of custom-made platinum electrodes (made by Mr P. Flaxman) placed around the epididymal end of the tissue. The electrodes were connected to a custom-made stimulator unit for regulating voltage and polarity connected to a custom-made electrical pattern generator (both made by Mr M. Preston). After the electrodes had been put in place, the tissue was allowed to equilibrate for one hour before data collection was undertaken.

The electrodes were made using capillary tubing which was cut into 5 cm lengths and placed into an electrode puller. The threshold for using electrodes was a tip resistance of at least 50 MΩ with a preference for electrodes of 80-90 MΩ. The electrodes were filled with 0.5 M KCl solution and bridge balancing and neutralization of electrode tip capacitance was performed. Membrane potential changes were recorded using a high input Axoclamp-2B impedance amplifier (Axon Instruments, Inc., Sunnyvale, CA, USA) and displayed on a DSO 1602 digital oscilloscope (Gould, Ilford, Essex, UK). The recordings were also digitized with a PowerLab/4SP (AD Instruments) connected to a Power Macintosh G3 (Blue & White) running Chart 3.6 (AD Instruments).

Recordings were taken from each preparation as follows. After impaling a cell with a threshold of a resting membrane potential (RMP) of at least -60 mV, it was allowed to rest for 5 mins in order to ensure a stable recording. The tissue was then stimulated with a train of 5 pulses (5 Hz, supramaximal voltage) at 30 sec intervals. At least five such trains were recorded from each cell. At least four (preferably five) control cells were recorded from each tissue and then the tissue

was incubated with the drug of interest of vehicle as appropriate and a further four or five cells recorded following the same protocol. At the end of each cell recording, the electrode was withdrawn and any offset from zero of the residual potential was recorded to assess any drift in RMP during the recording period.

For experiments conducted with testosterone, a different combination of digitizing hardware and recording software was used. The smooth muscle membrane potentials were recorded using a sharp microelectrode connected to the input headstage of an Axoclamp 2B. However, the data were digitized using a Micro 1401 Mk II (CED, Cambridge) and recorded on a PC (Dell, UK) with Spike 2 software (CED, Cambridge).

Electrophysiology analysis

Analysis of electrophysiology data was performed offline using Spike 2. Data acquired using Chart was imported into Spike 2. A custom-written macro was used to automate the acquisition of data from the traces with the only manual procedure being the placement of the cursor before a train of EJPs. Data were exported to Microsoft Excel for further manipulation and analysis.

Fluorescence Ca^{2+} imaging

As Ca^{2+} is an important regulator of both neurotransmitter release and smooth muscle contraction, understanding the regulation of intracellular and intraterminal Ca^{2+} is important when considering the regulation of neuroeffector coupling. Introducing Ca^{2+} indicators into smooth muscle cells is relatively

straightforward, involving the use of the widely-available membrane-permeable acetoxymethyl (AM) esters . However, the small size of autonomic nerve terminals precludes direct injection and these terminals seem resistant to uptake of the AM-indicators, so a more specialist approach is needed. Such an approach has been described and pioneered in our laboratory (Brain & Bennett, 1997; Brain *et al.*, 2001; Brain & Cunnane, 2008).

Fluorophores used in this study

Throughout, the Ca^{2+} indicators used were based upon Oregon Green 488 BAPTA-1 (OG-BAPTA-1). This is a non-ratioable, synthetic Ca^{2+} indicator, which has the advantage of an excitation peak wavelength that is very close to the narrow 488 nm laser line of the Argon ion laser found in the Leica SP2 confocal microscope (Leica, Milton Keynes, UK). This is a non-ratioble indicator, which implies that it is difficult to monitor slow changes in the resting Ca^{2+} concentration, and is sensitive to movement of image plane. However, the ratioable Ca2+ indicators available at the start of this work were either incompatible with available laser excitation wavelengths (e.g. the excitation ratioable Ca^{2+} indicator Fura-2, usually excited at 340 nm and 380 nm) or had a significantly lower quantum efficiency with more photobleaching and phototoxicity (e.g. the emission ratioable Indo-1). Very recently, a longer-wavelength (488 nm) emission ratioable indicator called Asante Calcium Red has been described, which may be of interest in the future. The signal to noise ratio of this indicator may still be problematic.

Dye delivery – membrane-permeable dye

An AM conjugate of OG-BAPTA-1 (OG-BAPTA-1-AM) was used in experiments monitoring intracellular Ca^{2+} in smooth muscle cells. The indicator was prepared from the 50 µg aliquots supplied by the manufacturer (Invitrogen) by adding 40 µL of a previously prepared Pluronic acid F-127 (Sigma-Aldrich) in dimethylsulfoxide (DMSO) to create a 1 mM stock solution. This was further diluted by the addition of 460 µL of PSS, with 30 s of gentle shaking for mixing, giving a stock solution of approximately 80 µM OG-BAPTA-1-AM in 10% DMSO/2% Pluronic acid F-127, which was divided into four 125 µL aliquots. Care was taken to minimize exposure to light; for example, all Eppendorf tubes containing the indicator solutions were wrapped in aluminium foil. The Pluronic acid solution was prepared by dissolving 0.2g of pluronic acid F-127 powder in 0.8 mL DMSO (approximately, although not precisely, 20% w/v). It should be noted that the powder did not dissolve easily, requiring gentle heating and vigorous shaking.

On the day of use, each vas deferens (prepared as described earlier), was placed in a test tube containing one of the 125 µL aliquot and 875 µL of PSS, making a final concentration of 10 µM OG-BAPTA-1-AM. This was incubated at 33-35˚C for 120 minutes and was continuously bubbled with the 95% O_2/ 5% CO_2 mixture at a very low rate.

After indicator loading the tissue was rinsed with fresh PSS, cut along the longitudinal axis along one side, and pinned serosal-side up to the Silgard-lined

base of a custom-made Perspex chamber (made by Mr P. Flaxman) ready for transfer to the confocal microscope.

Dye delivery – membrane-impermeable dyes

Dextran-conjuated fluorescent Ca^{2+} indicators or fluorescent dyes were orthogradely loaded into the nerve terminals using an adaptation of a technique developed by our laboratory (Brain & Bennett, 1997; Brain *et al.*, 2001).

OG-BAPTA-1 10 kDa dextran (OG-BAPTA-1-dex; Invitrogen) was supplied in 5 mg aliquots. The OG-BAPTA-1-dex is a solid with an appearance similar to that of a soft putty allowing it to be roughly split using a scalpel blade into approximately 1 mg sections which were placed into small eppindorf tubes. These could then be frozen until required. Each 1 mg of dye was then dissolved in 4 µL of PSS which was used to create a concentrated loading solution which could be used for loading approximate 10 tissues.

Each vas deferens was prepared as previously described, with the exception that the prostatic end was cut off on the epididymal side of the point at which the blood vessel first approaches the vas deferens and bifurcates in either direction along the longitudinal axis. This is because the autonomic innervation travels to most visceral organs, including the vas deferens, in parallel with the arterial blood supply. Therefore, it was necessary to section the nerves in such a manner that the loading of the dye (as described below) proceeded in an orthograde manner towards the epididymis.

Each prepared vas deferens was gently sucked (using a syringe and a small length of flexible tubing) into the smoothed tip of a capillary tube, to give a tight fit whilst minimizing the length of vas enclosed within the tube. The capillary tubes were manufactured from filament-containing microelectrode glass; one end of this glass was gently heated in order to create a smooth-edged narrow-diameter orifice. Minimizing the length of tissue in the capillary tube should minimize the extent of tissue hypoxia. Each capillary tube tip was positioned in a Perspex perfusion chamber which was continuously perfused (with recycling for most of the loading period) with PSS from a reservoir that was constantly bubbled with the O_2/CO_2 mixture. The vasa were pinned to the base of the chamber through the epididymal stump to prevent the flow of PSS from displacing the vasa during the loading period. Any PSS in the capillary tubes was then removed using a microelectode filling pipette, which has a very fine bore and could be passed along the entire length of the loading tubes. These filling pipettes were then carefully dried and used to introduce approximately 0.4 μL of the indicator solution on the exposed prostatic tip of each vas deferens. Given its small volume, the amount of indicator applied was not precisely measured, but rather was drawn from the stock solution by capillary action by back-filling the micropipette.

The preparation was left, usually overnight, for an initial period of 5 hours, at which time an automated, peristaltic pump-driven air supply ejected each vas deferens from their respective loading tubes into the bath, so that they could wash for a further 5 hours. Although the indicator solution was also blown into

the chamber, the dilution in the large (approximately 300 mL) recirculating PSS volume rendered its concentration insignificant. The long wash period was necessary in order to remove the rather sticky dye solution from the extracellular space, where it appears to bind strongly to collagen.

In some experiments, Alexa 594 10 kDa dextran (Invitrogen, Renfrew, UK) was used. This was supplied as 5 mg vials and was prepared in a similar manner. The loading protocol with Alexa 594 was the same as for OG-BAPTA-1-dex except that the preparations were left in contact with the dye for 9 hours, manually removed from the capillary tubes and allowed to was in PSS for 3 hours to remove extracellular non-specific dye loading.

The vasa were finally prepared for imaging by longitudinal sectioning through the lumen of the vas deferens and pinned to the Sylgard-lined base of the custom-made chamber as previously described.

Imaging protocol

Tissues were imaged using a Leica SP2 upright confocal microscope, pinned to the base of the custom-made bath previously described using pins made from short sections (1–2 mm) of silver wire (50 μm diameter). The chamber of the bath was continuously perfused using a Minipuls 3 peristalitic pump (Gilson). The perfusion system included a heater which regulated the temperature of the PSS in the chamber under the control of a thermostat which monitored the temperature of PSS in the bath using an electronic thermometer.

Where required, electrical field stimulation was provided using a parallel pair of platinum or silver stimulating electrodes which were positioned around either side of the tissue. For the vas deferens the electrodes would lie at the prostatic end of the tissue. The electrodes were connected to an optically isolated constant voltage stimulator which was controlled by a digital stimulus trigger unit (Applegarth Instruments, Oxford). Electrical events could be synchronized with the frames recorded by the microscope software using a custom-made electronic device (made by Mr M. Preston) which received the transistor-transistor logic frame signals produced by the confocal microscope.

The Leica SP2 microscope allowed the use of three long working distance water immersion objectives (20×, 40× and 63×). The area of tissue imaged varied between experiments as it was possible to control this from within the software in order to create the best images of the structures under observation. Similarly frame rate varied between experiments (specified as required in the results) depending on the area being recorded. In many cases the pinhole was set wider than appropriate for optimal axial resolution in order to increase the emitted light signal received by the photomultiplier tube(s). However, this allowed the laser power and dwell-time to be reduced helping to prevent bleaching and allowing faster scanning.

Image analysis

For experiments using OG-BAPTA-1-AM to label smooth muscle cells, ImageJ 1.38 (http://rsbweb.nih.gov/ij/) running the Java plugin, Leica_SP2_Stacker was used to import the images generated by the confocal imaging software and convert the stacks of individual TIFFs into a single file. A set of macros, custom written in the ImageJ macro language, IJSet, (written by Dr R. Amos), was used to automate some aspects of image loading, measurement and data output.

The macros facilitated the recording of fluorescence intensity over time within individual smooth muscle cells. A region of interest (ROI) was drawn around an individual smooth muscle cell visible within the confocal plane. The average fluorescence intensity was then measured for each frame in a stack. The macros included a simple algorithm which corrected for movement by moving the ROI in each frame to find the best fit for local intensity maxima.

Measurements of Ca^{2+} indicator fluorescence intensity, F, were normally corrected for background noise and converted into ΔF using the equation:

$$\Delta F_t = \frac{(F_t - F_0)}{F_0}$$

which can be simplified to:

$$\Delta F_t = \frac{F_t}{F_0} - 1$$

where F_0 is the 'initial' fluorescence calculated either from the mean of a number of control frames before stimulation or the first frame in the series, and F_t is the fluorescence measured at time t.

For experiments using Alexa 594 10 kDa dextran and OG-BAPTA-1-dex to label NTVs, images were stacked using the Leica_SP2_Stacker for ImageJ. The stacks were then opened in ImageSXM 1.88 (www.liv.ac.uk/~sdb/ImageSXM/) for fluorescent signal analysis using a set of custom-written (written by Dr K. Brain). A ROI was drawn around an individual varicosity and the mean intensity from that ROI recorded for every frame in a series. The size and geometry of the ROI was maintained throughout, however the position of the ROI would alter to correct for any movement using an automated procedure using a best fit algorithm finding the strongest local fluorescence signal. Background fluorescence was measured from a large region with no specific loading of the dye on the first frame of a series. This value was subtracted from all fluorescence readings correcting for both background and noise.

In all cases, data was exported to Microsoft Excel for further manipulation and analysis, prior to statistical interpretation.

Monitoring NET Activity with a Fluorescent Substrate

During the preparation of this thesis, work was also being carried out to investigate experimental methods which would make it possible to assess NET rate independent of noradrenaline exocytosis. This was independent of the investigations into the effects of cannabinoids but fortuitously the technique proved useful in testing the hypotheses which required testing following the early cannabinoid investigations using contraction studies.

The Neurotransmitter Uptake Assay (NTUA) Kit (MDS Analytical Technologies, Wokingham, Berkshire, UK) was discovered in a search for products which might permit the measuring of NET rate at the level of an individual nerve terminal. On the manufacturer's website it was described as "a fast, simple, and reliable fluorescence-based assay for the detection of dopamine, noradrenaline, and serotonin transporter (DAT, NET and SERT, respectively) activity" (http://www.moleculardevices.com/Products/Assay-Kits/Transporters/Neurotransmitter.html) and had previously been validated to monitor NET rate in cell culture systems (Jørgensen *et al.*, 2008). The assay contains a proprietary fluorescent indicator dye which mimics serotonin, noradrenaline and dopamine being transported into cells via the neurotransmitter transporters. The assay also contains a masking dye technology which the manufacturer has exclusively licensed from Bayer AG (US Patent Nos. 6,420,183, 7,063,952, 7,138,280 and European Patent No. 0,906,572) which quenches extracellular fluorescence. As it was not possible to be completely certain which compound caused any fluorescence detected, any signal is described as "NTUA fluorescence".

Tissue preparation

Following initial preparation as described above, each vas deference was cut longitudinally through the lumen using iris scissors. The tissue was then pinned to the base of the superfusion bath previously described for imaging studies to create a flat sheet. Once transferred to the stage of a Leica SP2 upright confocal microscope, the bath was continuously perfused with warmed (32–34˚C) PSS at

a rate measured to be 1.65 mL/min. The PSS was stored in a beaker at room temperature and bubbled constantly with the CO_2/O_2 mixture. Bath volume was considered to be approximately 1.5 mL in these experiments. The slightly lower than normal temperature should be noted. It was anecdotally observed that slightly lower temperatures than normal resulted in a better quality of loading.

NTUA preparation

NTUA is supplied in vials lyophilized fluorescent dye and masking compound mix which was stored at -20˚C. The masking compound binds to and quenches the fluophore while it remains in the extracellular space, which implies that fluorescence should only be seen when the fluophore is separated from the masking compound by uptake into cells.

Stocks solutions were prepared by defrosting a vial and adding 10 mL of PSS; the dye mix as encouraged to dissolve by shaking using a sonic mixer. The solution was then divided into ten 1 mL aliquots which were frozen again until the day of use. It was noted that some aliquots did not appear to freeze to solid but there was no correlation with any unusual experimental results or methodological failures when these aliquots were used.

On the day of use, two further dilutions were prepared: a 1:100 dilution by mixing a 1 mL aliquot of NTUA with 99 mL of PSS and a 1:10 dilution by mixing two 1 mL aliquots of NTUA with 18 mL of PSS. Exposure to the tissue was achieved by switching the inflow of the perfusion system to the flask containing

the diluted dye switching back to the normal PSS once the dye flask was empty. Following the imaging protocol below and with the perfusion rate listed above, the 1/100 dilution should be sufficient for two preparations and the 1/10 dilution for one preparation with 12 – 15 mins of perfusion.

NTUA assay imaging

For NTUA assay imaging, the Leica SP2 microscope previously described was configured with an excitation wavelength of 458 nm and an emission band of 490 – 570 nm. A 40× 0.8 NA dipping objective was used to acquire images and the imaging software configured to take 1024 × 1024 px images in 10-slice stacks using the *z*-scanning facility with approximately 1 µm between slices. As no rapid events were under observation, four line averaging was used to improve the quality of images acquired with bi-directional scanning and phase correction applied. The 10-slice stacks allowed for the focal plane drift which was observed over the course of experiments.

In pilot experiments, a field was blindly chosen and the focal plane established using white light. The field was then observed under the confocal microscope while the superfusion was switched to the 1:10 NTUA solution. Due to the necessary random selection of the imaging site, this method sometimes resulted in few or no terminals being observed as the focal plane was too deep or due to a low density of innervation in that region. Nevertheless, many experiments revealed fluorescent structures recognized as nerve terminal varicosities.

This problem was resolved by initially using a 1:100 NTUA solution to perfuse the preparation for 30 mins followed by a further 30 mins wash period. It was then possible to identify a suitable imaging region from the dim images which could be recorded under the confocal microscope. Three stacks of control images were then taken at 2 min intervals. Immediately following the third control stack, the perfusion would be switched to the 1:10 NTUA solution. It had been measured that it took approximately 2 min 15 s for a solution to reach the chamber from its storage beaker or flask therefore continuing to take image stacks at 2 min intervals the next recorded stack was taken at approximately time 0 min in the presence of the higher concentration of NTUA. Image stacks were taken at 2 min intervals to a total of 30 mins. The perfusion was returned to NTUA-free PSS when the 1:10 solution was all used. After a further 30 mins, a final three stacks of images were recorded at 2 min intervals. Where the preparation was to be exposed to a drug or drugs on interest, the initial three control images were taken, the preparation was then perfused with PSS containing the drug(s) for their incubation period and then a further three stacks would be taken before switching to the 1:10 NTUA solution. In these experiments, both 1:10 NTUA solution and NTUA-free PSS would contain the drug(s) of interest.

NTUA image analysis

ImageJ 1.38 (http://rsbweb.nih.gov/ij/) running the Java plugin, Leica_SP2_Stacker was used to import the images generated by the confocal imaging software and convert the stacks of individual TIFFs into a single file. The

stacked TIFFs were automatically saved and opened in ImageSXM 1.88 (www.liv.ac.uk/~sdb/ImageSXM/) for fluorescent signal analysis using a set of custom-written (written by Dr K. Brain). A minimum of four terminals were selected for each experiment using the 1:10 images in order to avoid regions with high non-specific dye loading (see Results and Discussion). A region of interest (ROI) was drawn around a short terminal segment (15–25 μm; 4–6 varicosities). Moving between image stacks, it was possible to restore the ROI once the slice on which the terminal section was in focus had been manually identified. The ROI was automatically fitted to the terminal (using an algorithm based on highest local fluorescent signal) and the mean fluorescent signal of the ROI measured. For photobleaching experiments, the ROI was drawn around single varicosities.

Data were exported to Microsoft Excel and Graphpad Prism for further manipulation and statistical analysis. The protocol used with NTUA allowed changes in transported rate to be monitored. Relative transporter rate was calculated by measuring the change in fluorescent signal over time ($\Delta F/\Delta t$) in the presence of 1:10 NTUA. Data was normalized to the control period (1:100) or the post drug-incubation recordings, as appropriate, so that it was possible to measure a change in the pump rate between the control or post-drug incubation period and the test period with the 1:10 solution (i.e. absolute rate).

EGFP-ChAT mice

In some experiments, ex-breeder mice which express enhanced green fluorescence protein (EGFP) under a choline acetyltransferase (ChAT) promoter (i.e. in cholinergic neurons) were used in order to demonstrate that NTUA was taken into noradrenergic but not cholinergic terminals. These mice were generously donated by P. Bolam (MRC Anatomical Neuropharmacology Unit, Oxford, UK) and were originally supplied The Jackson Laboratory (Bar Harbor, ME, USA), strain B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J, stock number 007902.

Imaging in these experiments was performed as described above for NTUA alone except EGFP was excited with a wavelength of 496 nm and an emission detection band of 510–590 nm. It would have been preferable to use a 488 nm excitation wavelength, however this would also excite NTUA resulting in cross-talk, therefore the suboptimal excitation wavelength was employed. Line-by-line switching of excitation wavelength using the Leica SP2's acousto-optical tunable filter also reduced cross-talk negating the requirement of any spectral deconvolution.

Flow Cytometry

Flow cytometry (FCM) is a technique for counting and examining multiple physical and chemical parameters at a microscopic level. By suspending particles (e.g. cells) in a stream of fluid, electronic detection equipment can analyse thousands of particles per second. A beam of single-wavelength light is focused on to the hydrodynamically-focused stream of liquid and the suspended particles

in the liquid scatter the light whilst fluorescent elements are excited to emit light at a different wavelength. The scattered light is analysed by a detector in line with the light beam to measure forward scatter (FSC) and by detectors which are perpendicular to the light beam to measure side scatter (SSC). Fluorescent detectors measure fluorescent emissions. From these recordings, it is possible to establish various properties of the particles such as cell volume, nuclear shape and membrane roughness.

Cell culture

PC12 cells (donated by Dr P. Sidaway, University of Birmingham) were cultured in RPMI 1640 media with gentamycin (50 µg/mL), horse serum (10% by volume) and foetal calf serum (5% by volume) in an incubator at 37°C and 5% CO_2. Every 3–4 days, cell culture flasks were removed from the incubator and placed in a cell culture hood in order transfer the cells to fresh media and thin their numbers using sterile cell culture techniques in order to maintain suspensions of at least 200,000 cells per mL and encourage rapid regeneration. Cells were resuspended using a plastic Pasteur pipette and a sample of the cell suspension placed in a haemocytometer in order to calculate cell density. Live cells were identified using phase brightness. A calculated volume of the remaining cell suspension was then transferred to a falcon tube using a sterile cell culture pipette and centrifuged (800 rpm) for 5 mins. The cells formed a pellet at the bottom of the tube and the old cell culture media was removed and the cells resuspended with fresh pre-warmed media with the required volume to create a suspension with a density of at least 200,000 cells per mL.

Fluorescence measurements

For FCM experiments, 800,000 cells were transferred to a Falcon tube and suspended in FACS PSS (NaCl 130 mM, glucose 5 mM, HEPES 20 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.3 mM, EDTA 1 mM, 1%BSA) at pH 7.4. The cells were then pre-incubated with any drugs before being spun down again and resuspended with NTUA solution containing the drug(s) under investigation and transferred to a FACS tube. NTUA was prepared as previously described with the exception of using FACS PSS as described above. Flow through the FACS machine was started at "medium" rate (60 µL/min) and dropped to "low" rate (10 µL/min) once cells were detected recording a little under 50 events per second on average. The change of flow rate was to prevent the flow from blocking. The FACS machine was setup with a 405nm laser for excitation fluorescence and a 502 nm long pass dichroic mirror followed by a 510/50 photomultiplier tube (PMT) AmCyan detector. Reflected light was gathered by the Pacific blue detector (450/50 PMT). Recordings were taken for 44 mins. On each day, a negative control tube was run containing PC12 cells in FACS PSS. Using forward- and side- scatter readings, and a cell viability dye in early experiments, a population of cells was selected in each experiment which were used for analysis. The selection criteria were based upon scattering parameters typical for cells of about 10 µm in size (Dr A. Speak, personal communication).

Data analysis

Gated data was saved as an FCS file using the FACSDiva software. The FCS files were analysed using an adaption of a custom written script by Dr Keith Brain in R software (www.r-project.org). The macro calculated linear regression for the data over the first 20 minutes and plotted a graph of the full 44 minutes of data. The regression values for all data were collated in a spreadsheet (Microsoft Excel) and the AmCyan and Pacific Blue data from individual FACS tubes averaged using geometric means. Geometric means were used because the regression values from the two channels showed notably different means and standard deviations. However, the relative errors from the two channels were similar, suggesting that each contained useful information. Geometric means prevented skewing the combined data towards the channel with greater values.

Statistical Analysis

A "goodness of fit" was conducted with a modified Kolmogorov-Smirnov test in Prism 4 (GraphPad Software, Inc., San Diego, CA, USA) to test data for normality before other statistical analysis was conducted. A Student's two-tailed unpaired t-test was then used for all comparisons to test the null hypothesis where data was normally distributed. Where the data was shown to be non-parametric, either a Wilcoxon signed rank test or a Mann-Whitney U test was used and is specified as appropriate in the results. Depending on the experimental protocol used, statistical analyses compared either pre- and post-treatment results or drug of interest and matching vehicle results. The null hypothesis was rejected if $P < 0.05$.

Data are generally presented as mean ± standard error of the mean (SEM). The term *n* normally refers to the number of preparations used. Rarely an equal number of test and control preparations was not used and this is clearly stated. In some cases, the number of terminals, varicosities or cells examined is of relevance and in these cases they are referred to as n_t, n_v and n_c respectively.

Drugs

All drugs and chemicals were purchased from Tocris Bioscience (Bristol, UK) apart from THC and DMSO (Sigma, Poole, Dorset, UK). In most cases, stock solutions were prepared in water and frozen (-20°C) until required. Drugs were subjected to no more than one freeze-thaw cycle. On the day of use, drugs were further diluted in PSS as required.

Preparation of Δ^9-tetrahydrocannabinol

Given the scope of this thesis, it is felt that the preparation of THC is a special case worthy of more detailed description. THC requires time consuming preparation on each experimental day unlike other drugs which could normally be prepared in advance, frozen and defrosted on the day of use requiring only dilution as necessary. Two methods were used for preparing THC. In all cases, THC was supplied in ethanol (28 mg/mL) from which stock solutions were prepared by further dilution with ethanol to 10 mM, aliquoted and frozen for future use. Whenever possible light exposure was minimized as this can degrade THC.

In early experiments, a preparation of THC in the surfactant Tween80 (Sigma) and PSS was used as previously described (Pertwee *et al.*, 1992). An aliquot of THC was defrosted on the day of use, mixed with two parts of Tween80 by mass and the ethanol evaporated by blowing the mixture with a stream of nitrogen gas. Finally, PSS was added in a series of aliquots (50 µl × 2, 100 µl × 2, 200 µl and 500 µl) to create an homogenous 1 mL dispersion. Serial dilutions were then made to the required concentrations.

The method above was found to be very time consuming (given a matching uncontaminated vehicle control would also have to be prepared) and did not always ensure a properly mixed dispersion of THC in PSS and Tween80. The presence of the surfactant also resulted in significant bubble formation at the top of the organ bath chambers in contraction studies. Whilst this did not seem to affect the quality of results obtained, it was not considered a "normal" or favourable observation in organ bath studies. Therefore an alternative preparation method was employed based upon another description from the same laboratory (Thomas & Pertwee, 2006).

On the day of use, aliquots were defrosted and warmed to room temperature. 10 µL of the 10 mM solution was placed in a fresh Eppendorf tube and the ethanol then evaporated with the assistance of a stream of nitrogen gas. THC was then resuspended in 1 mL of DMSO to a concentration of 100 µM. The resulting solution could then be mixed by 1 in 1000 dilution to give a final concentration of 100 nM with a maximum vehicle concentration of 0.1%. DMSO vehicle controls

were prepared in a similar manner to THC by taking 10 μL of pure ethanol, evaporating with nitrogen and addition of 1 mL of DMSO to the tube.

Results

NTUA: Developing a new tool to explore NET function

Transmission at the sympathetic junction is partly dependent upon the presence of noradrenaline within the extracellular space. The concentration of noradrenaline is in a dynamic state based upon its prejunctional release and its rate of clearance from the junction. The norepinephrine transporter (NET) is one of two proteins responsible for noradrenaline clearance, the other being the non-neural uptake 2.

NTUA labelling

Following pilot experiments previously described (see Methods), the standard protocol when using NTUA commenced by incubating the tissue with NTUA (1:100) for 30 mins followed by a further 30 mins wash in normal PSS. It was then possible to visualize structures similar in appearance to varicose nerve terminals as seen using other techniques (Fig. 1A; n = 3, n_t = 30). The fluorescent signal within the terminals linearly increased during incubation with NTUA (1:10), however additional labelling, perinuclear in appearance, also became apparent during this period (Fig. 1B). When the tissue was allowed to wash in normal PSS for a further 40 mins after the reservoir of 1:10 solution was exhausted, the fluorescent signal within the terminals would decrease marginally whereas the perinuclear staining would decrease markedly (Figs. 1A).

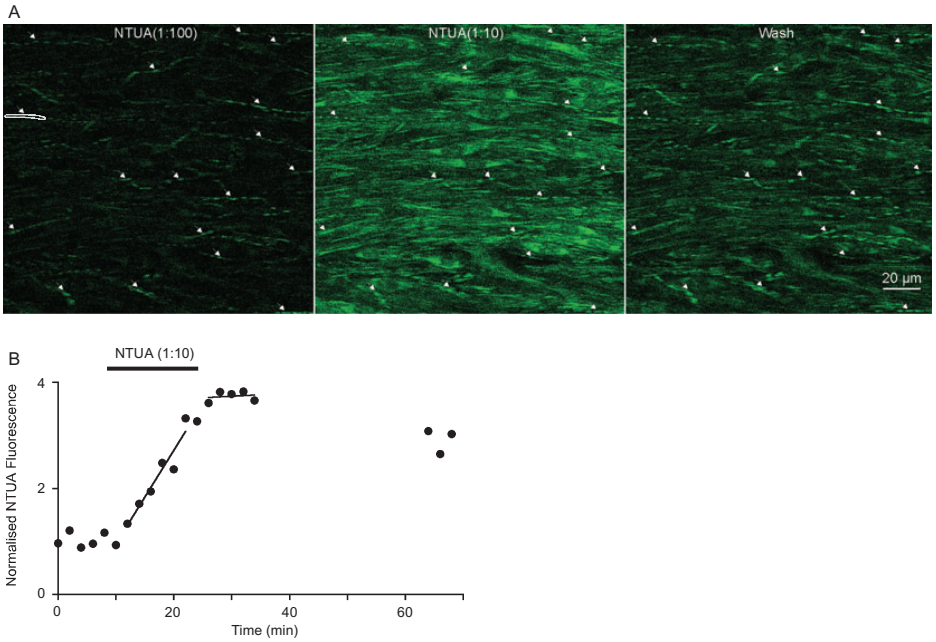


Figure 1
A series of images of the same portion of the mouse vas deferens during neurotransmitter transporter uptake assay (NTUA) exposure is shown. Following exposure to low-concentration NTUA (1:100), varicosities are faintly visible. At the end of NTUA (1:10) exposure, these varicosities were brighter, but there was also significant perinuclear staining in another cells type (probably smooth muscle cells). After returning the preparations to physiological saline (wash) for 30–40 min, the nerve terminal labelling remained, but the perinuclear staining was greatly reduced or abolished. Many nerve terminals (arrows) could be monitored throughout the recording period, although some were too close to non-terminal labelling and were not included in the analysis. The change in fluorescence in the nerve terminal outlined in A, normalized with respect to the mean signal in the control [NTUA (1:100)] period, increased linearly during NTUA (1:10) exposure (B), falling only slowly upon return to physiological saline.

Figure reporoduced from Parker et al (2010).

Given the changes in fluorescence within the terminals was linear during incubation with NTUA (1:10), the technique seemed ideal for elegantly assessing changes in NET activity. The marginal decrease in fluorescence after washing also raised the possibility that it might be possible to use NTUA to measure reverse transport activity of NET within sympathetic nerve terminals.

Specificity for NET

In order to confirm that the fluorescence seen within the varicose terminal structures was due to NTUA being transported into the terminals by NET, experiments were carried out using the NET inhibitor, desipramine. The sample tissues were incubated with desipramine (1 μM) for 6 mins prior to switching to NTUA (1:10) also containing desipramine. In these experiments, no change in florescence was observed within the terminals and when quantified the rate of accumulation during the 1:10 solution exposure period was not significantly different from zero with respect to the control period during incubation with desipramine (Fig. 2A and 2B; $n = 3$, $n_t = 16$).

The reverse transport for NTUA through NET during the wash period was also investigated using amphetamine. Amphetamine is known to increase the removal of free noradrenaline from within terminals (Ross & Gosztonyi, 1975), therefore it was hypothesized that it may also enhance the clearance of NTUA observed during the washout period following exhaustion of the 1:10 solution reservoir. For these experiments, the tissues were treated following the normal protocol until the 1:10 solution was exhausted at which point the perfusion

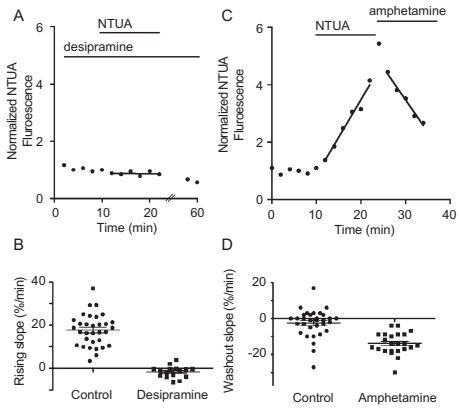


Figure 2
Pretreatment with desipramine (1 mM) prevents fluorescence accumulating in nerve terminals (A). The results of all experiments are summarized in B, for these experiments measuring the relative change in fluorescence at the end of the loading protocol compared with the control period [neurotransmitter transporter uptake assay (NTUA 1:100)].

Adding amphetamine after the NTUA (1:10) exposure causes a rapid loss of fluorescence in the nerve terminal (C), suggesting removal by reversal of norepinephrine transporter. The rate of loss of NTUA fluorescence in the nerve terminal after removal of extracellular NTUA (1:10), both in the presence and absence of amphetamine, is shown in D; each dot represents a different nerve terminal. The exposure protocol is identical to that shown in C.

Figure reproduced from Parker et al (2010).

system was switched to a reservoir of PSS containing amphetamine (10 μM). Amphetamine increased the rate of decrease in fluorescence within the terminals compared to controls by about five times (Fig. 2C and 2D; $P < 0.001$). In some preparations, the fluorescence disappeared altogether before the end of the washout period.

These findings confirm that the changes in intra-terminal fluorescence observed during incubation with NTUA (1:10) and during the further wash period in normal PSS reflect uptake and clearance of NTUA through NET. The actions of desipramine indicate that NTUA fluorescence changes exclusively reflects NET activity within the nerve terminals whilst the results with amphetamine suggest that it may also be possible to use NTUA to assess reverse transporter activity of NET.

Investigating additional cellular labelling

The perinuclear staining previously described upon addition of the 1:10 solution was frequently a cause for problems as it would often prevent the analysis of several varicose terminals which were in a favourable focal plane. The raised "non-specific" fluorescence near such terminals would make it impossible to identify them as separate from the background when the perinuclear staining arose during incubation with the 1:10 solution. Therefore several attempts were made to identify the source of the perinuclear staining and establish a simple pharmacological method of blocking it when required.

The possible role of uptake-2 as the cause of the non-neuronal fluorescence was assessed with two drugs. One effect of hydrocortisone is to inhibit uptake-2 (Moura *et al.*, 1990) and so preparations were incubated with hydrocortisone (30 μM) before the addition of NTUA (1:10). Subjectively, there was no change in the additional cellular labelling observed or in its perinuclear appearance. Hydrocortisone had no effect on the rate of increase of fluorescence within the nerve terminals (control = 23.9 ± 2.6%/min, hydrocortisone = 17.6 ± 1.8%/min, $P > 0.05$, $n = 3$, $n_t = 16$).

The O-methylated noradrenaline metabolite, normetanephrine, is also a potent inhibitor of uptake-2 (Rahman *et al.*, 2008). Incubation with normetanephrine (10 μM) for 30 mins before switching to NTUA (1:10) also did not appear to alter the non-neuronal labelling (Fig. 3A).

Finally, the possibility that SERT might be responsible for the additional cellular labelling was investigated. Citalopram was used at both 100 nM (Fig. 3B) and 1 μM (Fig. 3C) in separate preparations with an incubation period of 30 mins. Neither concentration appeared to alter the non-neuronal labelling observed.

Unfortunately, it was not possible to establish the source of the non-neuronal fluorescence therefore care has to be taken with this technique when analysing nerve terminals to select terminals away from regions of significant additional cellular labelling.

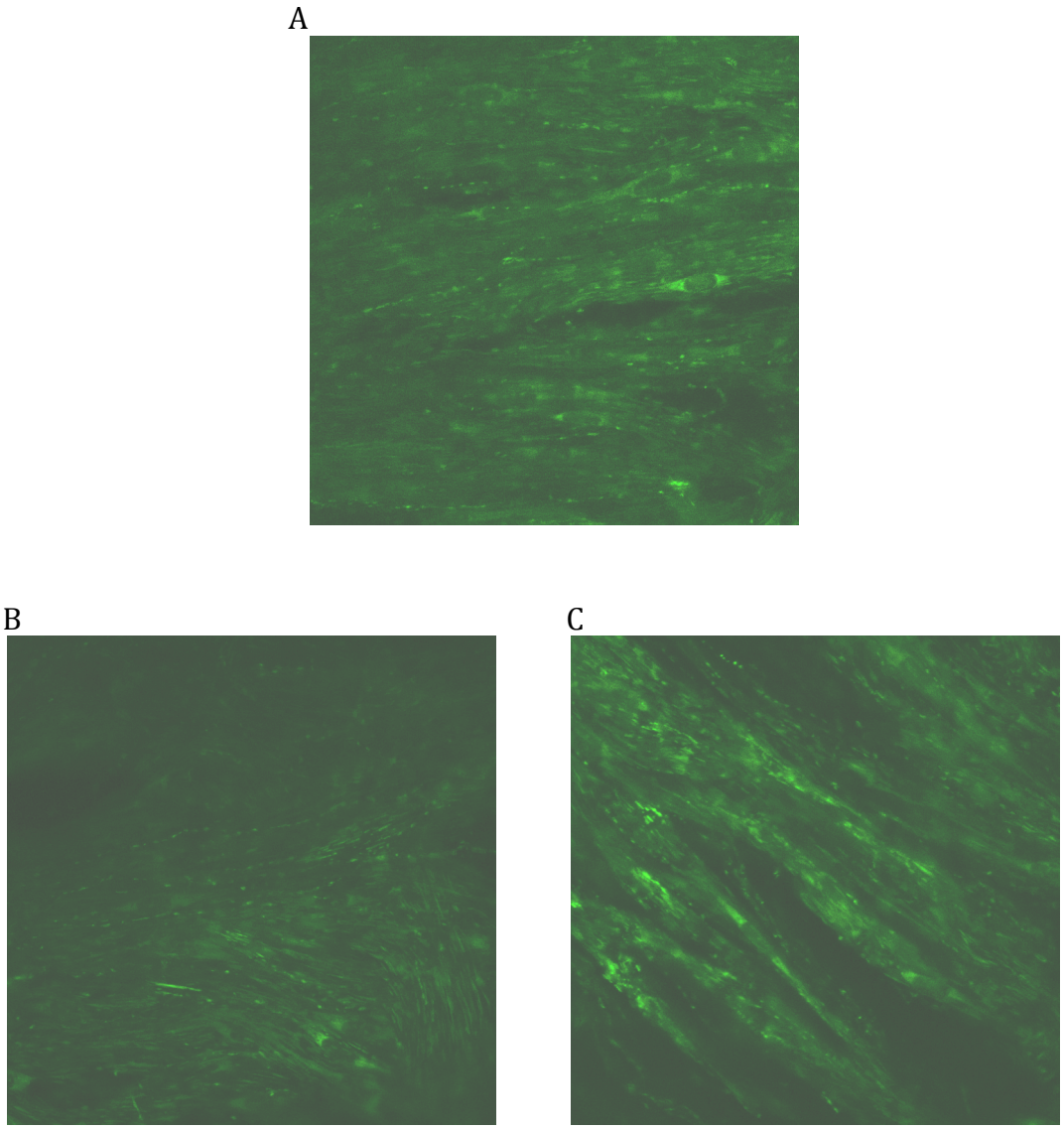


Figure 3
Attempts were made to reduce the perinuclear staining that increased the background "noise" on images using inhibitors of uptake 2. and SERT Pre-incubation with 10 μM normetanephrine (A) did not appear to alter the non-neuronal labelling at the end of the NTUA (1:10) exposure period. The SERT inhibitor, citalopram, did not appear to alter the non-neuronal labelling using either 100 nM (B) or 1 μM (C) concentrations. Therefore, it is unlikely that the non-neuronal uptake and perinuclear staining observed can be attributed to transport of NTUA into smooth muscle cells by uptake 2 or SERT.

Relationship between noradrenergic and cholinergic terminals

If NTUA is a substrate for NET, it should be taken into noradrenergic but not cholinergic terminals. However, given the presence of non-neuronal cellular labelling, the possibility that NTUA might be taken into terminals other than noradrenergic, specifically cholinergic, was investigated. Mice expressing EGFP under a ChAT promotor were used to investigate the relationship between this labelling of cholinergic neurons and NTUA.

EGFP labelling was identified in preparations (n = 3) before incubation with NTUA. EGFP fluorescence detected was mostly found in axon bundles running over the serosal surface of the tissue. Labelling was also observed in smaller, smooth bundles and in a few cases varicose terminals were also identified. After incubation with NTUA, fluorescent labelling with NTUA and EGFP was clearly distinguished as separate with no NTUA signal from EGFP-labelled structured (Fig. 4A). However, in most cases, only NTUA fluorescence could be viewed within the field be imaged due to the scarcity of cholinergic innervation.

It was interesting to observe that in some instances isolated cholinergic terminals labelled with EGFP were in close association with NTUA-labelled noradrenergic terminals. A similar anatomical observation was also made for nerve terminals bundles identified as cholinergic and noradrenergic by their respective fluorescent markers. Noradrenergic terminals were found to be present at a density of at least an order of magnitude greater than the cholinergic terminals.

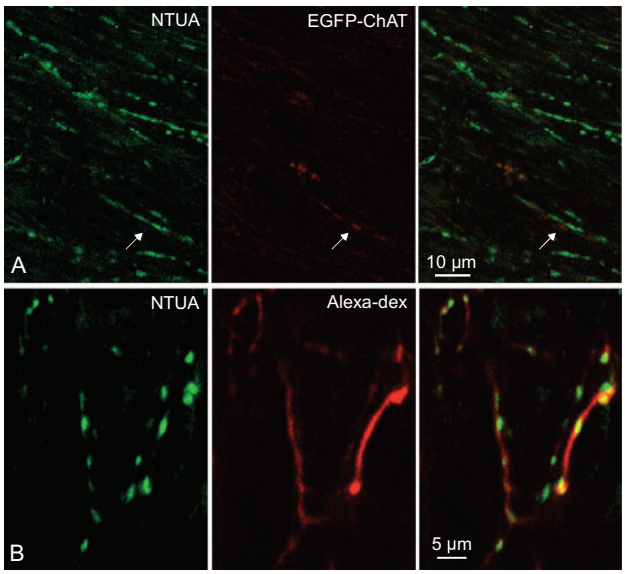


Figure 4
Neurotransmitter transporter uptake assay (NTUA) (1:10) labelling (green) in nerve terminals of the mouse vas deferens in transgenic mice expressing enhanced green fluorescence protein (EGFP) under the choline acetyltransferase (ChAT) promoter (A). Cholinergic terminals (EGFP positive; pseudo-coloured red) were very sparse (the only one present in this field is marked with an arrow), but commonly ran close to NTUA-labelled terminals. No NTUA labelling was found in EGFP-positive terminals. This suggests that the NTUA specifically labels sympathetic noradrenergic terminals. There is some cross-talk between the channels; no attempt at cross-correction has been made. In normal Balb/C mice, loading a proportion of the nerve terminals with Alexa 594 dextran (red) prior to NTUA (1:10) exposure shows that NTUA labelling (green) is confined to the varicosities and does not spread to the intervaricose nerve terminal segments (B).

Figure reproduced from Parker et al (2010).

These observations confirm that neuronal NTUA labelling can be confidently attributed to its localization within noradrenergic terminals. The anatomical observations also demonstrate the possibilities for cross-talk between noradrenergic and cholinergic pathways due to their close association. However, the question remained of the exact intraterminal location of NTUA.

Intraterminal location of NTUA labelling

Although, the neuronal labelling observed with NTUA appeared punctate, it was not clear whether the fluorescent signal was anatomically restricted to the varicosities or if some labelling occurred within intervaricose axonal segments. Identification of this would help to identify whether NTUA was freely diffusing within the cytoplasm of noradrenergic terminals or if it was taken up into an organelle which would restrict its movement and therefore isolate its position within the neuron.

A 10kDa dextran conjugate of Alexa 594 was orthogradely loaded into the sympathetic nerve terminals of the mouse vas deferens overnight in order to fluorescently label late endosomes and lysosomes within the nerves (Hortsch *et al.*, 2010). The Alexa 594 could be visualized throughout the cytoplasm in both the varicosities and the inter-varicose axonal regions of the terminals. These terminals were co-loaded with NTUA allowing the distribution of the two indicators to be compared with the resulting observation the NTUA was only to be found in the varicosities of the sympathetic terminals (Fig. 4B, $n = 2$, $n_t = 21$).

The localization of NTUA to the varicosities raised the hypothesis that the fluorescent substrate was transported by VMAT into varicosities after transport into the cytoplasm by NET. Incubation with reserpine (1 μM), a VMAT inhibitor, for 90 min prior to the addition of NTUA (1:10) did not alter the rate of uptake of NTUA (Fig. 5B; reserpine = 33 ± 4%/min, control = 24 ± 3%/min, $n = 2$, $n_t = 28$, one-way *t*-test $P = 0.97$). However, the rate of decreased of NTUA fluorescence during the washout period was significantly increased (Fig. 5C; reserpine -10.1 ± 1.2%/min, control -2.6 ± 1.4%/min, $n = 2$, $n_t = 28$, $P < 0.05$). Interestingly, after the washout period, NTUA fluorescence was observed within the inter-varicose regions of the terminals in the preparations incubated with reserpine (Fig. 5A).

Consequently, it was possible to identify the intra-terminal location of NTUA after uptake by NET as within the varicosities. The results with reserpine would also suggest that its accumulation within vesicles following transport by VMAT normally protects the fluorescent substrate from being removed from the terminals by the reverse transport action of NET.

NTUA as a tool to track varicosities

Fluorescence recovery after photobleaching (FRAP) was used to investigate the possibility of NTUA movement within nerve terminals. A mixture of high intensity 458 nm and 476 nm light was focused for 2 mins on a small, square area of tissue covering one or two varicosities. Compared to a stack of images acquired before photobleaching, NTUA labelling was almost totally abolished in the varicosities of interest (Fig. 6A). A fall in the intensity of fluorescence of

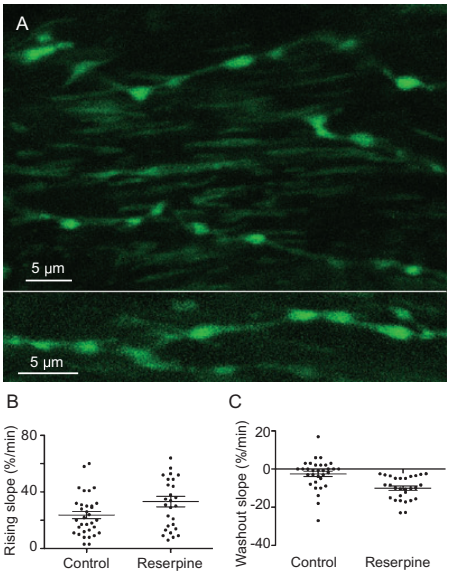


Figure 5

High magnification confocal images of nerve terminals filled with neurotransmitter transporter uptake assay (NTUA) in the presence of reserpine (1 mM) are shown (A). Two representative images from well-separated fields in the same preparation have been included and show that the intervaricose axonal segments fluoresce. After loading the terminals with NTUA (1:100), reserpine (1 mM) was applied; after 90 min, NTUA (1:10) was applied, as in Figure 2, for the measurement of uptake (B) and washout (C) rates.

Figure reproduced from Parker et al (2010).

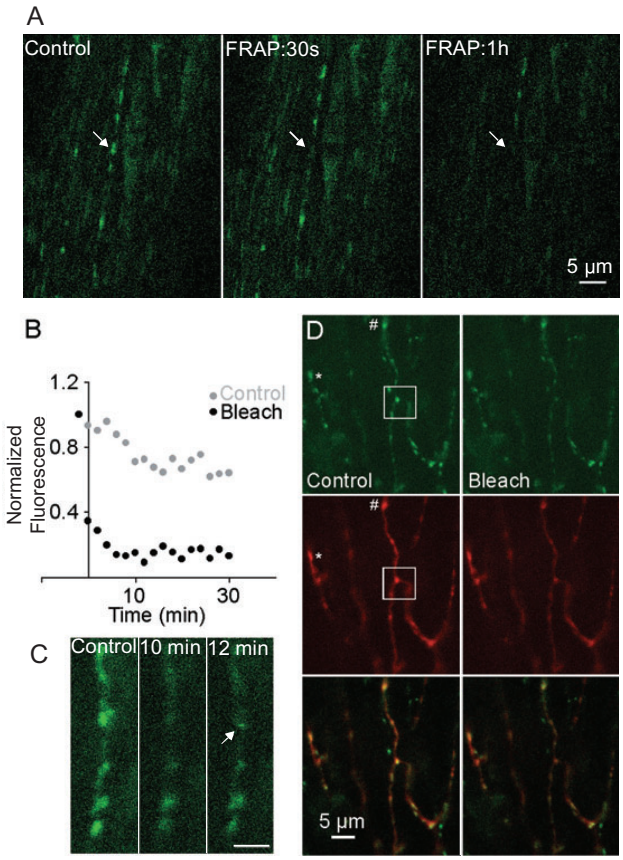


Figure 6
Fluorescence recovery after photobleaching (FRAP) the neurotransmitter transporter uptake assay (NTUA)-labelled terminal at the arrow caused a persistent loss of fluorescence (A) which only slowly recovered with respect to control varicosities on the same terminals (B). In C, three frames from another such experiment are shown, with photobleaching at t = 0 min. Between the recordings at t = 10 and 12 min punctate, fluorescence returned in a diffraction-limited spot within the target varicosity (arrow); this fluorescence persisted throughout the rest of the experiment. Such FRAP experiments were also carried out in nerve terminals filled with Alexa 594 dextran (D). In such cases, the varicosity in the photobleached region (boxed) lost its NTUA labelling, but the Alexa 594 labelling was lost throughout the entire terminal that crossed this target region. For example, note that the varicosity indicated with a # has a significant fall in Alexa 594 (red) labelling but not NTUA labelling; this varicosity is on the nerve terminal that crosses the target region. However, the varicosity at * does not lose labelling on either channel. These findings suggest that the FRAP protocol does not disrupt the nerve terminal (as Alexa 594 can diffuse to the target region through the nerve terminals to be bleached) and confirms that even a 10 kDa cytoplasmically located molecule can readily move between varicosities.

Figure reproduced from Parker et al (2010).

neighbouring varicosities, sometimes on different nerve terminal branches, was observed. It was considered that this phenomenon arose from the scattering of the photobleaching light beam due to the thick nature of the vas deferens as an experimental tissue.

Measuring the time course of recovery of fluorescence within the photobleached varicosities showed a very slow recovery time (time to recover to 1/e) of 120 mins (media of n = 6, range 107 – 250 mins; Fig. 6B). Nearby varicosities were used as controls to assess and correct for any decrease in NTUA labelling over time. Occasionally, a gradual stepwise recovery of fluorescence occurred (Fig. 6C) as small (diffraction limited) spots of fluorescence reappeared within a photobleached varicosity.

In one experiment, a tissue loaded with Alexa 594 dextran was used and high intensity 594 nm light was added to the photobleaching procedure. Consistent with the belief that Alexa 594 dextran is freely diffusible within the cytoplasm, the fluorescence intensity of this fluorophore fell proportionally across all varicosities of the terminal (Fig. 6D). NTUA fluorescence was still only decreased local to the region of photobleaching.

These observation are consistent with little cytoplasmic exchange of NTUA. Assuming that NTUA is freely diffusible within the cytoplasm, this would further suggest that due to the actions of VMAT NTUA fluorescence arises solely from within varicosities under normal conditions. This assumption also allows the conclusion that there is only rare vesicular movement between varicosities and

that NTUA allows the observation of such movement under certain experimental conditions.

PC12 cells as an assay system to assess NTUA

The neuroendocrine PC12 cell line expressed both NET and DAT, and so provides a simple cellular system in which the function of the these transporters can be assessed. While the use of transporter over-expressing cell lines with the use of a plate reader has been demonstrated (Jørgensen *et al.*, 2008), it has not been determined whether or not PC12 cells express the transporter at a sufficiently high density to allow this method to be used, or whether flow cytometry can be used with this kit. Being able to use flow cytometry may be a useful tool because it allows a cell-by-cell comparison of the co-localization of more than one fluorescent probe.

When PC12 cells were mixed in a NTUA (1:100) solution, no significant uptake could be detected using flow cytometry. However, the same exposure protocol did allow PC12 cells, which had settled on a microscope slide, to be viewed with a confocal microscope (results not shown). This suggests that the detection threshold for flow cytometry is higher than that for confocal imaging (i.e. that this approach is less sensitive).

Exposing PC12 cells to an increased concentration of NTUA (1:10), led to a linear increase in the fluorescent signal from the cells over the first 20 minutes of

exposure to the compound (Fig. 7A and B). The slope of this linear relationship was used to determine the rate at which the compound was taken into the cells.

Without NTUA (DMSO-only control) there was no significant change in the fluorescent signal (0.03 ± 0.92 ΔF/min; n = 6 experiments, 50 cells/s, so about 60,000 cells per experiment). As some of the slope values in these control experiment were negative, the mean rather than the geometric mean (as described in the methods) of the two emission channel signals was used.

In the presence of NTUA (1:10), using the same protocol, there was a linear increase in the fluorescent signal (259±34 ΔF/min; n=9; Fig C). To determine whether uptake by DAT was important in these undifferentiated PC12 cells, the DAT inhibitor GBR12909 (10 nM) was added to the cells prior to (for 30 minutes) adding the NTUA and commencing flow cytometry. Under these conditions the fluorescent signal increased at a rate of 180 ± 20 ΔF/min (n = 5), which was not significantly different from NTUA alone (P = 0.13). This suggests that DAT uptake is not important in these undifferentiated PC12 cells, although the statistical power may be too low to detect a small but important effect.

Following preincubation with the NET inhibitor desipramine (100 nM), the rate of NTUA uptake measured using the same protocol was 144±25 ΔF/min (n = 6), which was significantly slower than in the control (P < 0.05).

Citalopram (100 nM) was used to determine whether or not there was any evidence for SERT transporting the NTUA into these cells. Under these

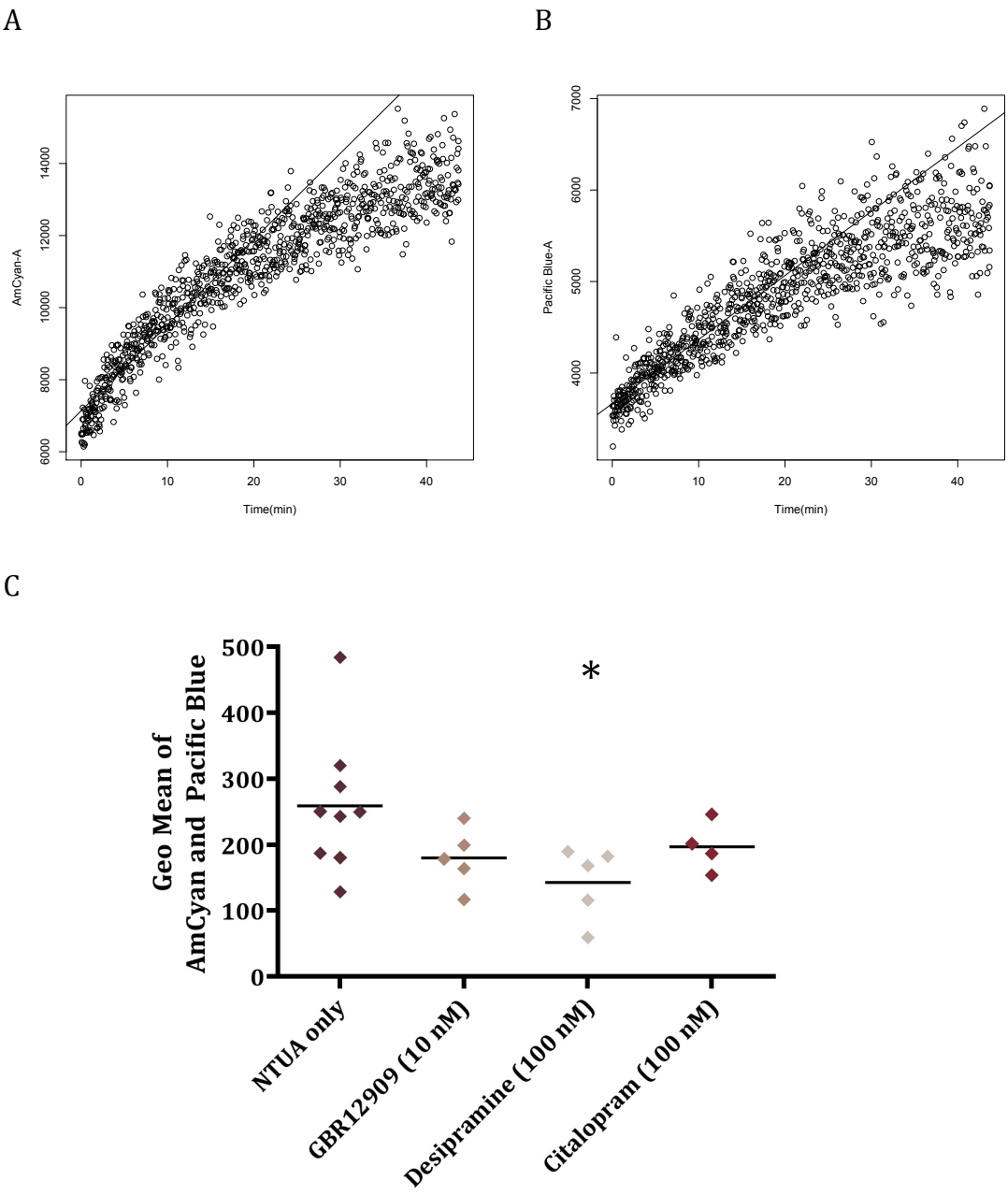


Figure 7
PC12 cells exposed to NTUA (1:10) showed a linear increase in the fluorescent signal detected from the cells using both AmCyan (A) and Pacific Blue (B) filters. Linear regression could then be used to calculate a rate of uptake within the first 20 minutes of exposure to NTUA as shown on the graphs. The uptake of NTUA was not significantly altered by pre-incubation with either the SERT inhibitor, citalopram, or the DAT inhibitor GBR12909 (C). However, pre-incubation with the NET inhibitor, desipramine, caused a significant decrease in the rate of NTUA uptake with respect to controls (* $P < 0.05$).

conditions, the rate of uptake was 197 ± 19 ΔF/min (n = 4), which was not significantly different from NTUA alone (P = 0.27).

These findings show that NTUA can be used during flow cytometry to monitor NET-dependent uptake, although a contribution from other transporters or non-specific uptake cannot be ruled out.

Effects of Testosterone on Neurotransmitter Release

A novel finding for testosterone

Reports of rapid, nongenomic effects of testosterone in other smooth muscle preparations have become widespread in recent years (Costarella *et al.*, 1996; Crews & Khalil, 1999a; Hall *et al.*, 2002). Therefore, investigations were carried out to assess the possibility of similar effects on the mouse vas deferens given its close anatomical location and association to the source of testosterone production, the testis.

Preliminary vehicle testing

Due to the solubility of testosterone in ethanol, there was concern that relatively high concentrations of ethanol might be present in perfusing PSS solutions at certain higher concentration of testosterone. Therefore, vehicle controls for experiments using testosterone were conducted and demonstrated that 1% ethanol had no significant effect on the mean normalized amplitude of contraction in mouse vas deferens (data not shown).

Testosterone inhibits neurogenic contractions

An initial protocol was conducted using a concentration of testosterone (100 µM) previously shown in other tissues (Hall *et al.*, 2002) to cause nongenomic effects. The effect of this concentration of testosterone was assessed over time. A mild stimulation protocol of trains of five pulses (0.5 ms pulse

width, supramaximal voltage, 0.1 s interval) was used to evoke contractions in the tissue.

Testosterone reduced the neurogenic contraction of mouse vas deferens within the first 20 mins after application of the drug (mean normalized amplitude in the presence of testosterone 0.42 ± 0.03, n = 2; mean normalized amplitude of controls 1.13 ± 0.06, n = 2, $P < 0.05$). This effect persisted until the end of recording at 50 minutes (Fig. 8). This prompted experiments to investigate the concentration-response relationship for testosterone using concentrations of 3, 10, 30, 100 and 300 μM (n = 6) and an incubation time of 20 mins. Testosterone caused a concentration-dependent inhibition of contractile responses to electrical field stimulation (Fig. 9), with significant inhibition for 100 and 300 μM concentrations only ($P < 0.05$). The calculated EC_{50} value was 118.9 μM.

Location and transduction of testosterone's effect

The initial findings were a novel effect for testosterone within the mouse vas deferens and was considered worthy of further investigation. The next steps taken were to identify whether this effect was pre- or post-junctional in origin and whether it involved the classical androgen receptor.

It is possible to use α,β-metATP as an agonist to evoke purinergic contractions which are reproducible provided the tissue is thoroughly washed with fresh PSS immediately after the contractile response has peaked and that a sufficient rest period is allowed between stimulations. α,β-metATP (1 μM) was used to

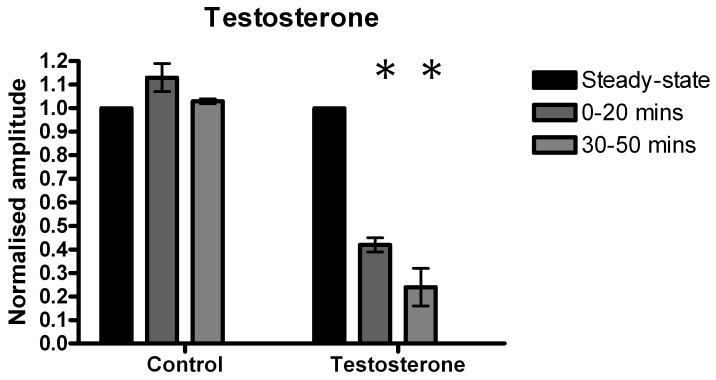
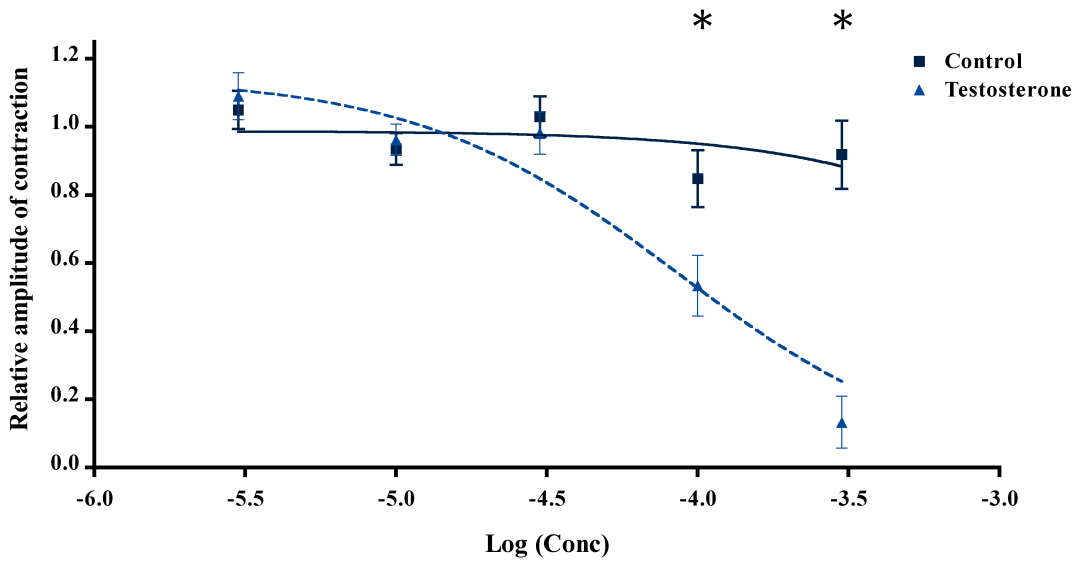


Figure 8
Testosterone (100 μM) caused a significant inhibition of neurogenic contractions within the mouse vas deferens that reached a plateau after 20 minutes of incubation with respect to time-matched ethanol (0.1%) vehicle controls (* $P < 0.05$). There was no further significant increase in the inhibitory effect observed beyond 20 minutes.

A



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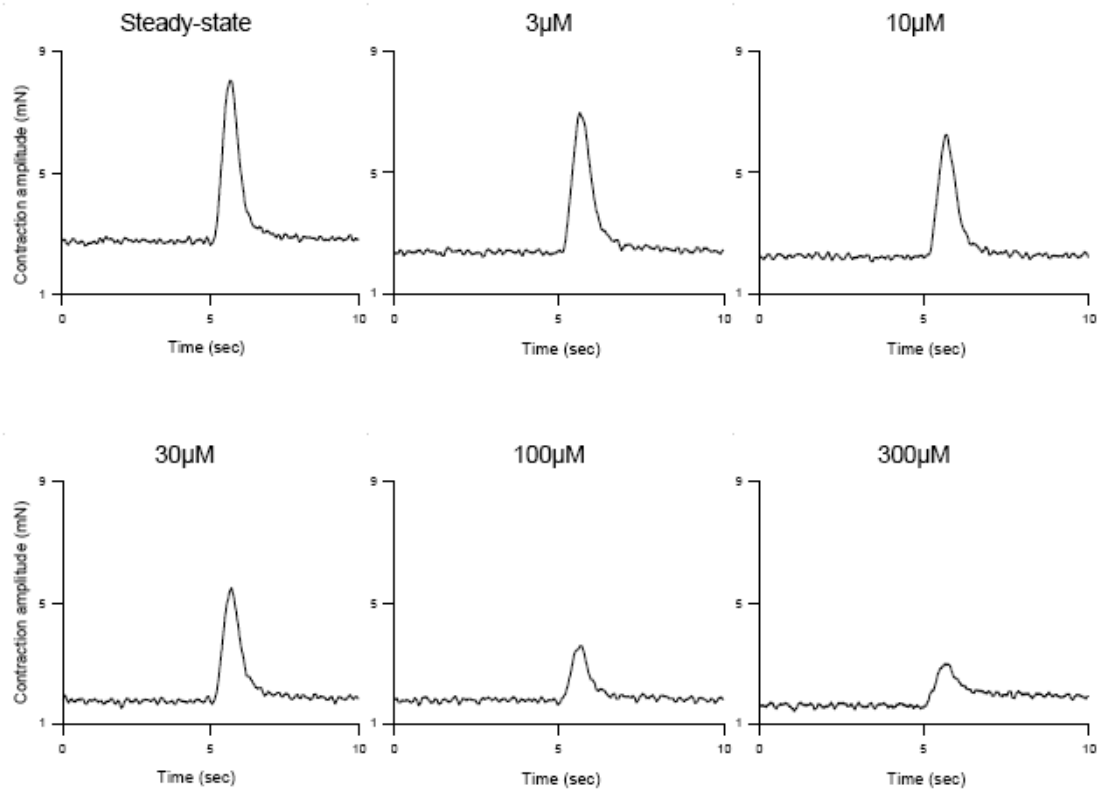


Figure 9
Testosterone caused a concentration-dependent inhibition of neurogenic contractions within the mouse vas deferens (A), an effect which was significant with respect to ethanol vehicle controls at 100 μM and 300 μM (* $P < 0.05$). (B) shows sample traces of neurogenic contractions evoked after 20 minutes incubation with a range of concentration of testosterone and before the addition of either testosterone or ethanol (steady-state).

stimulate the tissue instead of EFS with a rest period of 30 minutes between doses of the agonist. Testosterone (100 μM) caused a significant inhibition of agonist-evoked contractions (Fig. 10) with the ratio of normalized peak amplitudes of contraction before and after addition of drug being 0.99 ± 0.12 for vehicle controls and 0.56 ± 0.05 for testosterone ($P < 0.05$, $n = 6$). This suggested that testosterone was inhibiting contractions through a post-junctional effect.

The androgen receptor antagonist, flutamide, was used to investigate whether this novel effect for testosterone involved the androgen receptor. EFS was once again used to evoke contractions given its more physiological effects. Tissues were incubated with flutamide (50 μM) for 15 minutes and control responses recorded. The tissues were then incubated with either testosterone (100 μM) or ethanol (0.1%) as a vehicle control. Despite the presence of flutamide, testosterone still caused a significant inhibition of electrically evoked contractions compared to the vehicle (Fig. 11; $P < 0.05$, $n = 6$). The normalized peak amplitudes for the contraction peaks were 0.27 ± 0.1 after the addition of testosterone compared to 0.61 ± 0.05 with flutamide alone and 0.56 ± 0.07 for ethanol vehicle controls in the presence of flutamide.

Assessing the nongenomic nature of testosterone's effect

Given the rapid onset of testosterone inhibitory effect and the lack of involvement of the androgen receptor, it seemed possible that this effect may be mediated by a nongenomic pathway previously not elucidated for testosterone within the mouse vas deferens. Therefore investigations were carried out to see

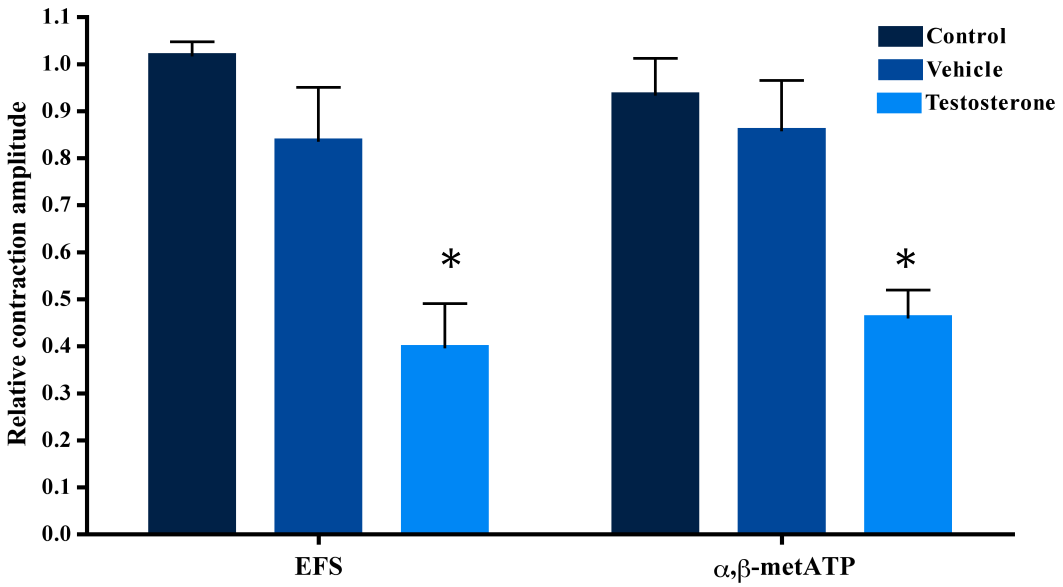


Figure 10
In the same series of experiments, tissues were stimulated with both electrical field stimulation to elicit neurogenic contractions and with the exogenous application α,β-metATP (1 μM) to elicit post-junctional purinergic contractions. Testosterone (100 μM) caused a similar magnitude of inhibition of contractions elicited by both means. For both EFS- and α,β-metATP-evoked contractions, the inhibitory effect of testosterone was significantly different from the contraction evoked in the presence of ethanol (0.1%) vehicle controls. (* $P < 0.05$).

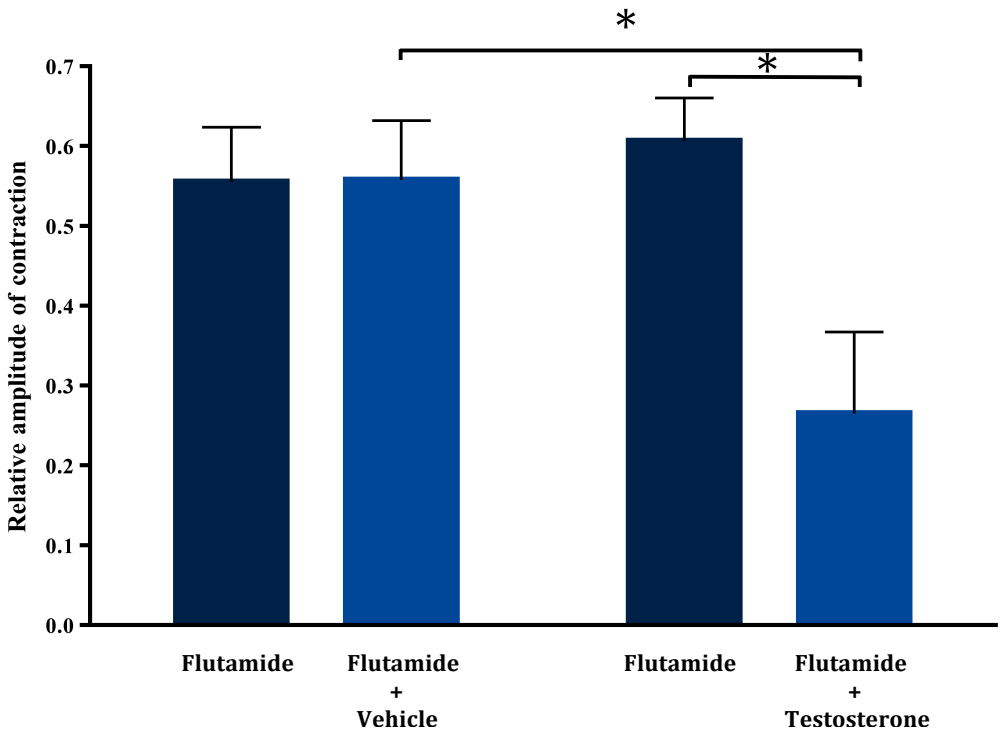


Figure 11
Tissuse were incubated with the androgen receptor antagonist, flutamide (50 μM), for 15 minutes. Flutamide alone did not cause a significant change in the amplitude of contractions (data not shown). Despite the presence of flutamide, testosterone (100 μM) still caused a significant inhibition of neurogenic contractions both with respect to matched tissues in the presence of flutamide alone and when compared to ethanol vehicle controls (* $P < 0.05$).

if testosterone's inhibitory effect persisted when genomic pathways were blocked.

In these experiments, half the tissues were exposed to the translation inhibitor, cycloheximide for 3 hours before control recordings were taken followed by the addition of either testosterone or ethanol vehicle control. Those tissues not exposed to cycloheximide were incubate with the matching vehicle, DMSO (0.1%). Testosterone's inhibitory effect persisted in the presence of cycloheximide (300 μM) with average normalized peak amplitudes of 0.85 ± 0.02 for controls and 0.54 ± 0.08 after incubation with testosterone (Fig. 12; $P < 0.05$, $n = 6$).

Interestingly, control readings in the presence of cycloheximide were significantly greater in amplitude than matching DMSO controls (normalized peak amplitudes: DMSO = 0.55 ± 0.05, cycloheximide = 0.85 ± 0.02; $P < 0.05$, $n = 6$). The ability of cycloheximide to increase contraction amplitudes appeared additive with testosterone's ability to reduce contraction amplitudes.

Whole cell Ca^{2+} transients and testosterone

A possible mechanism by which testosterone might be able to reduce the amplitude of contractions, both those evoked by EFS and by exogenously applied α,β-metATP, was hypothesized as the modification of Ca^{2+} dynamics within smooth muscle cells. The most obvious mechanism would be via the direct or indirect inhibition of L-type voltage-gated Ca^{2+} channels. As these channels have

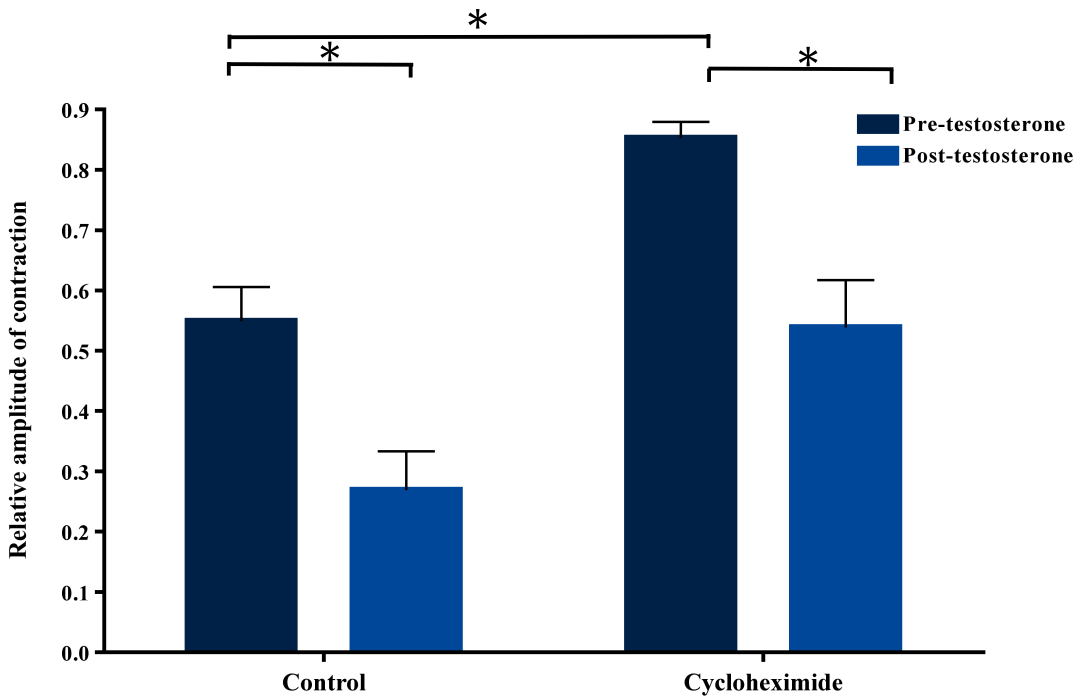


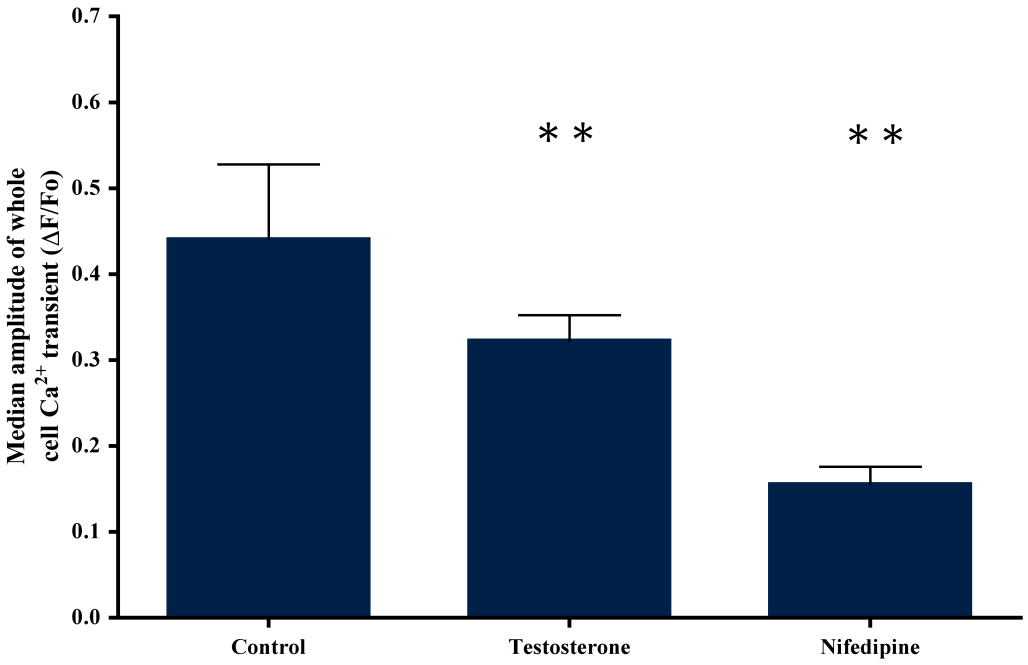
Figure 12
The inhibitory effect of testosterone on neurogenic contractions persisted in the presence of the translation inhibitor, cycloheximide (300 μM), and with its matched vehicle control, DMSO (0.1%). However, cycloheximide also cause a significant increase in the amplitude of recorded neurogenic contractions compared to DMSO before the addition of testosterone (* $P < 0.05$). Although the effect of cyloheximide appeared to be additive with the inhibitory effect of testosterone, there was no significant difference for the contractile amplitudes in the presence of testosterone between the cycloheximide and DMSO tissues.

been previously been shown to be principally responsible for the whole cell Ca^{2+} transients (WCTs) observed in smooth muscles cells, the effect of testosterone on WCTs was investigated.

Vasa deferentia loaded with the Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM were observed under a Leica SP2 confocal microscope. Series of 100 frames were captured at 5 Hz over regions with a minimum of 5 whole smooth muscle cells in the imaging field. WCTs were initiated by EFS (pulse width 0.5 ms, 10 pulses in train, interval 0.05 s, supramaximal voltage). This protocol evoked WCTs intermittently within individual smooth muscle cells but WCTs were qualitatively assessed as being of repeatable intensity when evoked. For quantitative analysis, WCTs were defined as those fluorescence readings upon stimulation more than two standard deviations above the mode for each cell.

Testosterone (100 μM) caused a significant decrease in the median amplitude of WCTs compared to matching ethanol vehicle controls (Fig. 13A; Wilcoxon matched pairs test $P < 0.05$, $n = 4$, $n_c = 22$). Testosterone also significantly reduced the number of whole cell events per series (Fig. 13B; Wilcoxon matched pairs test $P < 0.05$, $n = 4$, $n_c = 22$) compared to vehicle controls prior to the addition of testosterone which may be interpreted as reducing probability of inducing WCTs in the smooth muscle cells. Subsequent incubation with nifedipine (1 μM) for 15 mins caused a further decrease in the amplitude and probability of WCTs which was significantly different from both control and testosterone readings (Wilcoxon matched pairs test $P < 0.05$, $n = 4$, $n_c = 22$).

A



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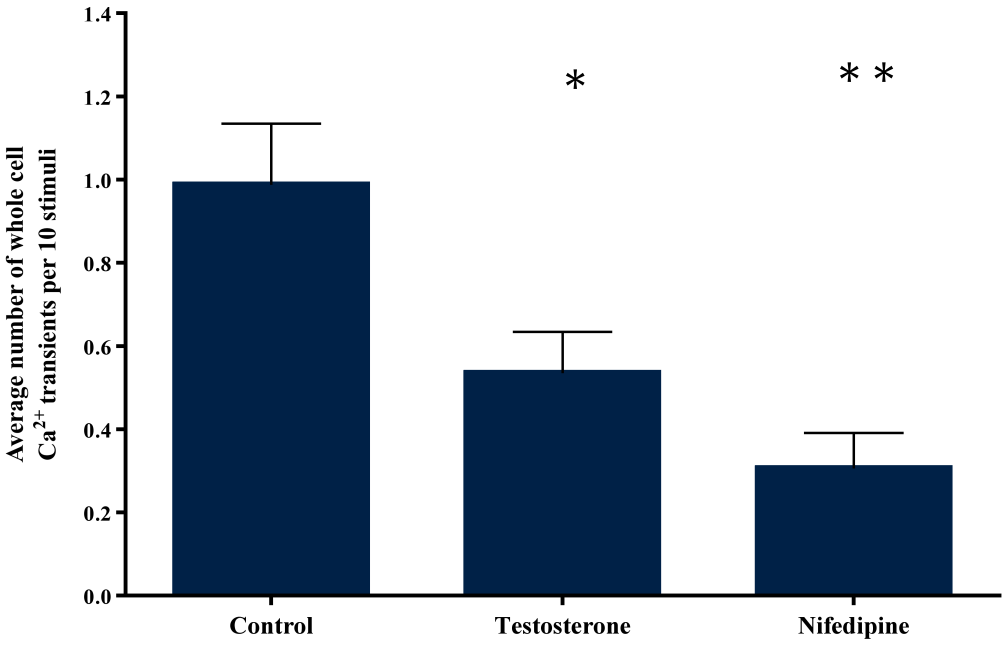


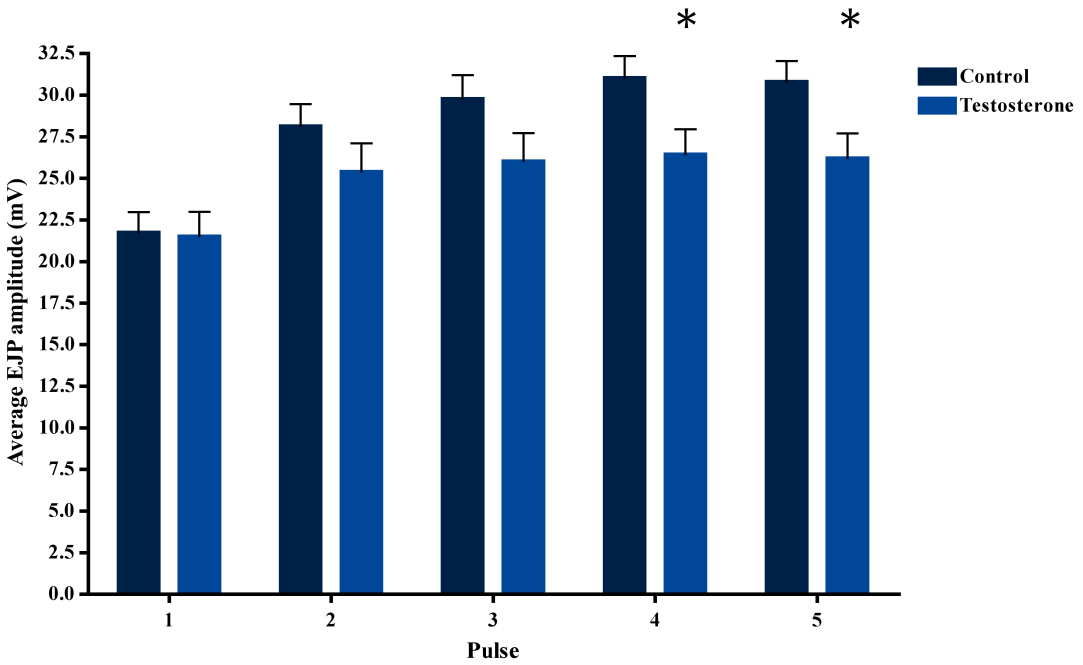
Figure 13
Testosterone (100 µM) caused a significant decrease in the median amplitude of whole cell calcium transients evoked by electrical field stimulation compared to ethanol (0.1%) vehicle controls (A). A significant decrease in the frequency of WCTs was also discovered measured as the average number of WCTs per train of electrical stimuli (B). Both the amplitude and the frequency of WCTs could be further reduced by incubation with nifedipine (1 µM). * $P < 0.01$, ** $P < 0.05$.

An additional pre-junctional effect for testosterone

Intracellular electrophysiological recordings of smooth muscle cells were also made to investigate the effect of testosterone on neurogenic purinergic transmission. As the previous experiments had indicated that testosterone's effects on contraction were post-junctional it was not expected to find any effect on excitatory junction potentials (EJPs) recorded although an effect on P2X receptor opening cannot be totally ruled out.

EJPs were evoked by electrical stimulation (pulse width 0.1 ms, 5 pulses in train, interval 0.2 s, supramaximal voltage). Responses to trains of five stimulations were compared before and after addition of testosterone (100 μM) with a minimum incubation time of 20 mins. No significant difference was found on the first pulse between the testosterone (n = 6, n_c = 36) and control (n = 6, n_c = 37 cells) EJPs (Fig. 14A; P = 0.91). However, the ratios of later pulses to the first pulse were lower after incubation with testosterone (P < 0.05). For the fifth to first pulses, the ratios were 1.50 ± 0.06 for controls compared to 1.27 ± 0.03 for testosterone (P < 0.05). This result is indicative of reduced facilitation which is attributable to a pre-junctional action of testosterone and is a second novel effect discovered in the vas deferens for testosterone. A final interesting observation from sample traces (Fig. 14B) is that in the presence of testosterone broadening on the EJPs appears to occur compared to control recordings.

A



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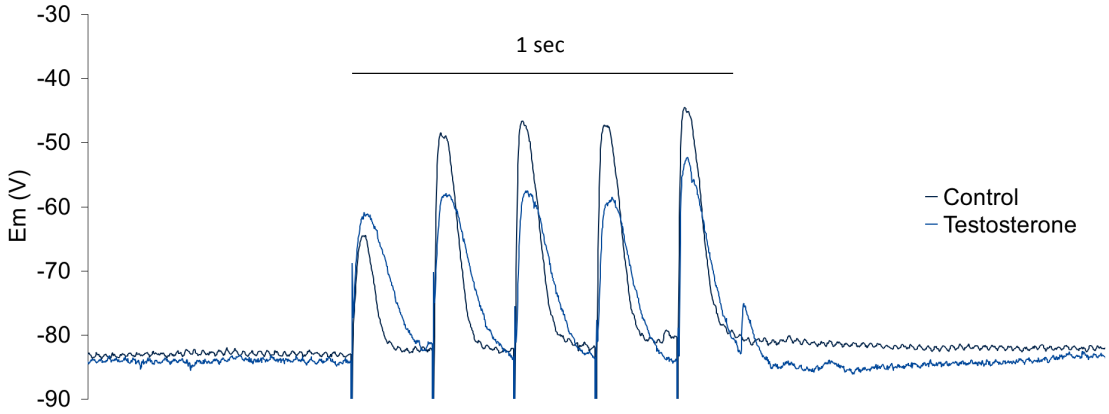


Figure 14
Testosterone (100 μM) caused a significant inhibition of the fourth and fifth EJPs evoked by a train of pulses of electrical stimulation (A, * $P < 0.05$) compared to ethanol vehicle controls. Sample traces (B) suggest that testosterone may cause a broadening of the EJP compared to ethanol vehicle controls (not statistically analysed).

Effects of THC on Neurotransmitter Release

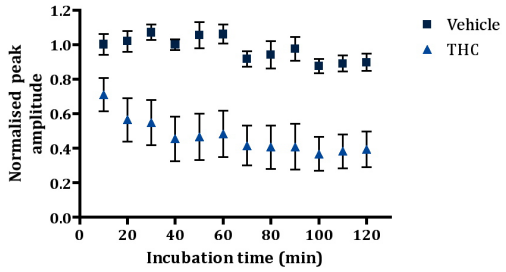
The effect of THC (100 nM) over time

A basic assessment of THC was initially carried out in order to confirm whether it was possible to establish similar effective concentrations and incubation periods to those previously established by other research groups. Therefore, experiments were first conducted to establish if any changes in the magnitude of responses to THC on the contractile properties of the mouse vas deferens, evoked through EFS, could be observed with increasing incubation time. For these initial experiments, a previously established effective concentration (100 nM) of THC was employed (Thomas & Pertwee, 2006).

THC (100 nM) caused a significant inhibition of neurogenic (10 pulses, 10 Hz, 0.5 ms, 90 V) contractions over a 120 min time period compared to vehicle (0.1 % DMSO) controls from 20 mins onwards (Fig. 15a) ($P < 0.05$, $n = 4$). Inhibition showed a plateau at approximately 40 mins. Peak amplitudes of contraction after 40 mins incubation were an average 45 ± 0.13 % of those evoked before the addition of THC compared to 100 ± 0.03 % for vehicle controls.

The effect of THC (100 nM) on neurogenic contractions was further investigated whilst pharmacologically isolating the noradrenergic and purinergic components of contraction. In the presence of α,β-metATP (1 μM), the isolated noradrenergic component of contraction was inhibited with significant inhibition from 10 mins after the start of incubation with THC ($P < 0.05$, $n = 4$). Maximal inhibition was

A



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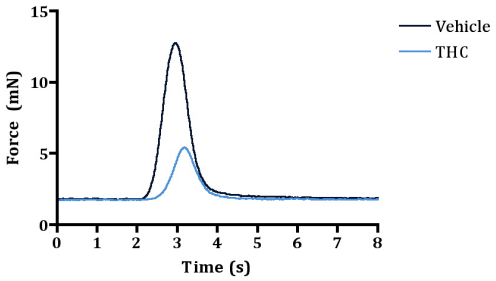

Figure 15

A. A graph showing how the average amplitude of neurogenic contractions (10 pulses, 10 Hz, 0.5 ms, 90 V) are reduced after application of THC (100 nM) versus vehicle control alone (0.1% DMSO). The inhibitory effect reaches maximal from 40 minutes after the start of incubation.

B. Example traces of neurogenic contractions after 40 minutes of incubation with THC (100 nM) and vehicle control (0.1% DMSO). The traces commence 2 seconds prior to the onset of electrical stimulation.

reached at approximately 30 mins showing a plateau from 40 mins (Fig 16a). Peak amplitudes of contraction after 30 mins incubation were an average 27 ± 13 % of those evoked before the addition of THC compared to 95 ± 3.8 % for vehicle controls.

The purinergic component of contraction was isolated with the α_1 antagonist, prazosin (100 nM). Inhibition of the purinergic component of contraction was much smaller in magnitude than of the noradrenergic component. Maximal inhibition of neurogenic contractions was observed at 30 mins with peak amplitudes an average 79 ± 1.9 % of those evoked before the addition of THC compared to 102 ± 8.7 % for vehicle control (Fig. 16c; $P < 0.05$, $n = 5$). This was the only time point at which a significant inhibition of contraction compared to vehicle was observed. A significantly greater inhibition of noradrenergic contractions was found compared to purinergic contractions (Fig. 16e; $P < 0.05$).

From these data, all future experiments used a 30–40 mins incubation period with THC before recording results in order to ensure maximal effect was achieved before data were collected.

Concentration-dependent effects of THC

The effect of THC applied over a concentration range (1–300 nM) on the pharmacologically isolated noradrenergic and purinergic components of contraction elicited by EFS (10 pulses, 10 Hz, 0.5 ms, 90 V) was investigated using a 30 mins incubation period for each concentration. THC caused a

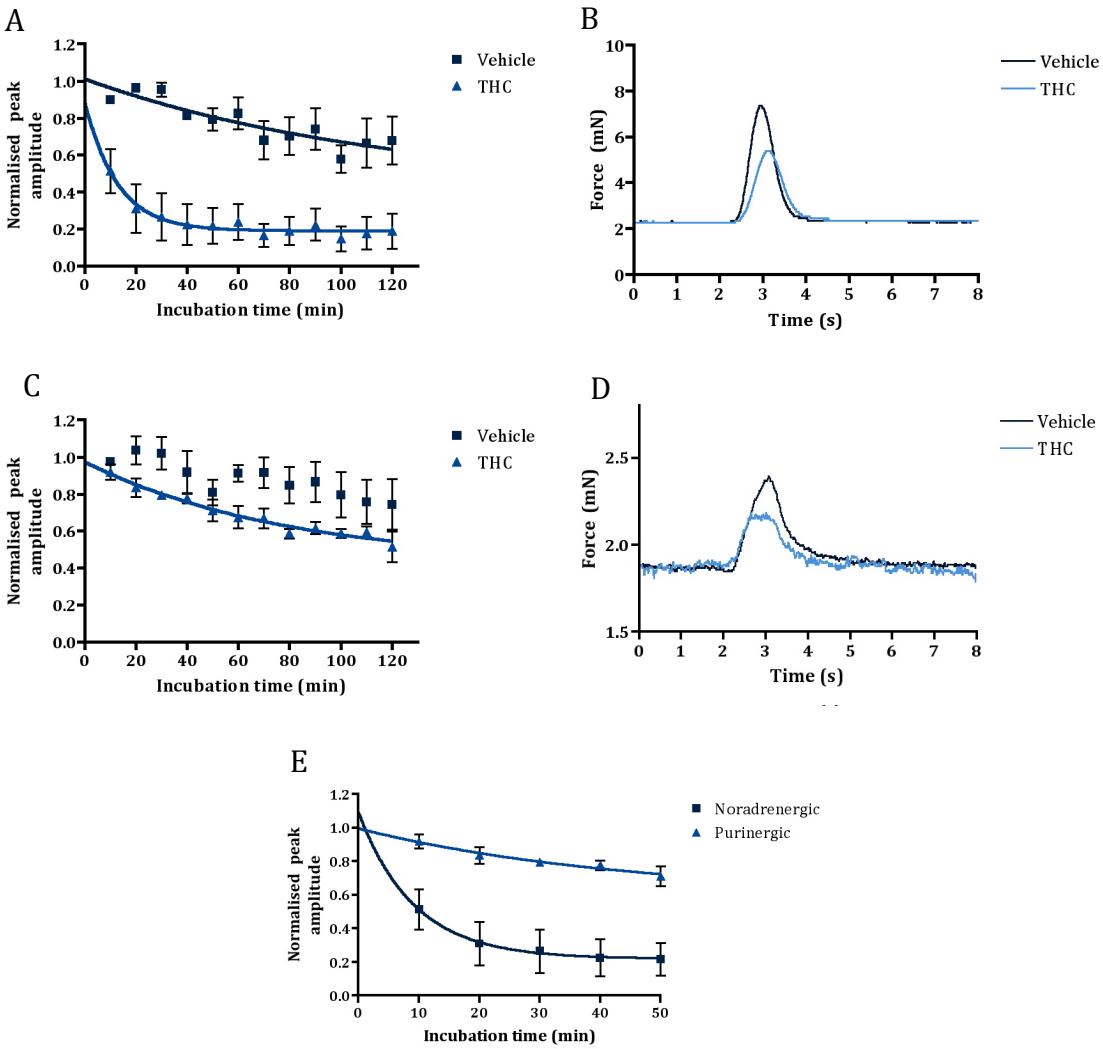

Figure 16

A. A graph showing how the average amplitude of pharmacologically isolated noradrenergic neurogenic contractions (10 pulses, 10 Hz, 0.5 ms, 90 V) are reduced after application of THC (100 nM) versus vehicle control alone (0.1% DMSO). The inhibitory effect reaches maximal from 30 minutes after the start of incubation.

B. Example traces of noradrenergic neurogenic contractions after 30 minutes of incubation with THC (100 nM) and vehicle control (0.1% DMSO). The traces commence 2 seconds prior to the onset of electrical stimulation.

C. A graph showing how the average amplitude of pharmacologically isolated purinergic neurogenic contractions (10 pulses, 10 Hz, 0.5 ms, 90 V) are reduced after application of THC (100 nM) versus vehicle control alone (0.1% DMSO). The inhibitory effect reaches maximal from 30 minutes after the start of incubation.

D. Example traces of neurogenic contractions after 40 minutes of incubation with THC (100 nM) and vehicle control (0.1% DMSO). The traces commence 2 seconds prior to the onset of electrical stimulation.

E. A graph comparing the effects of THC (100 nM) on noradrenergic and purinergic components of contraction over 50 mins.

concentration-dependent inhibition of peak amplitude to both noradrenergic (Fig. 17a) and purinergic (Fig. 17b) neurogenic contractions compared to vehicle controls. This effect was significant at only 100 and 300 nM on the noradrenergic component of contraction ($P < 0.05$, $n = 4–6$, T-test) and at only 30 nM on the purinergic component ($P < 0.05$, $n = 5–6$, T-test). The pEC_{50} for THC was -8.7 ± 0.13 nM (equivalent EC_{50} of 1.8 nM) for the noradrenergic contractions and -8.3 ± 0.39 nM (4.3 nM) for the purinergic contractions. Unfortunately, noticeable run-down is observed on the vehicle data for both of these sets of experiments. Linear regression was used to calculate a run-down trend from the vehicle data and a correction factor was applied to each THC data point. The resulting graph (Fig. 4c) compares the concentration-dependent effect of THC on the noradrenergic and purinergic components of contraction. THC caused a greater inhibition of noradrenergic compared to purinergic contractions with a significant difference at 100 and 300 nM ($P < 0.05$, $n = 6$). From these data, pEC_{50}s of -8.5 ± 0.17 nM (equivalent of 3.0 nM) and -8.8 ± 0.33 (1.7 nM) were calculated from the effects on the noradrenergic and purinergic components respectively.

Given the run-down problems in the original data, this experiment was repeated. Unfortunately similar problems with run-down were again observed (Fig. 17d and 17e). Please see the discussion for comment on the cause and effects of run-down in this experiment.

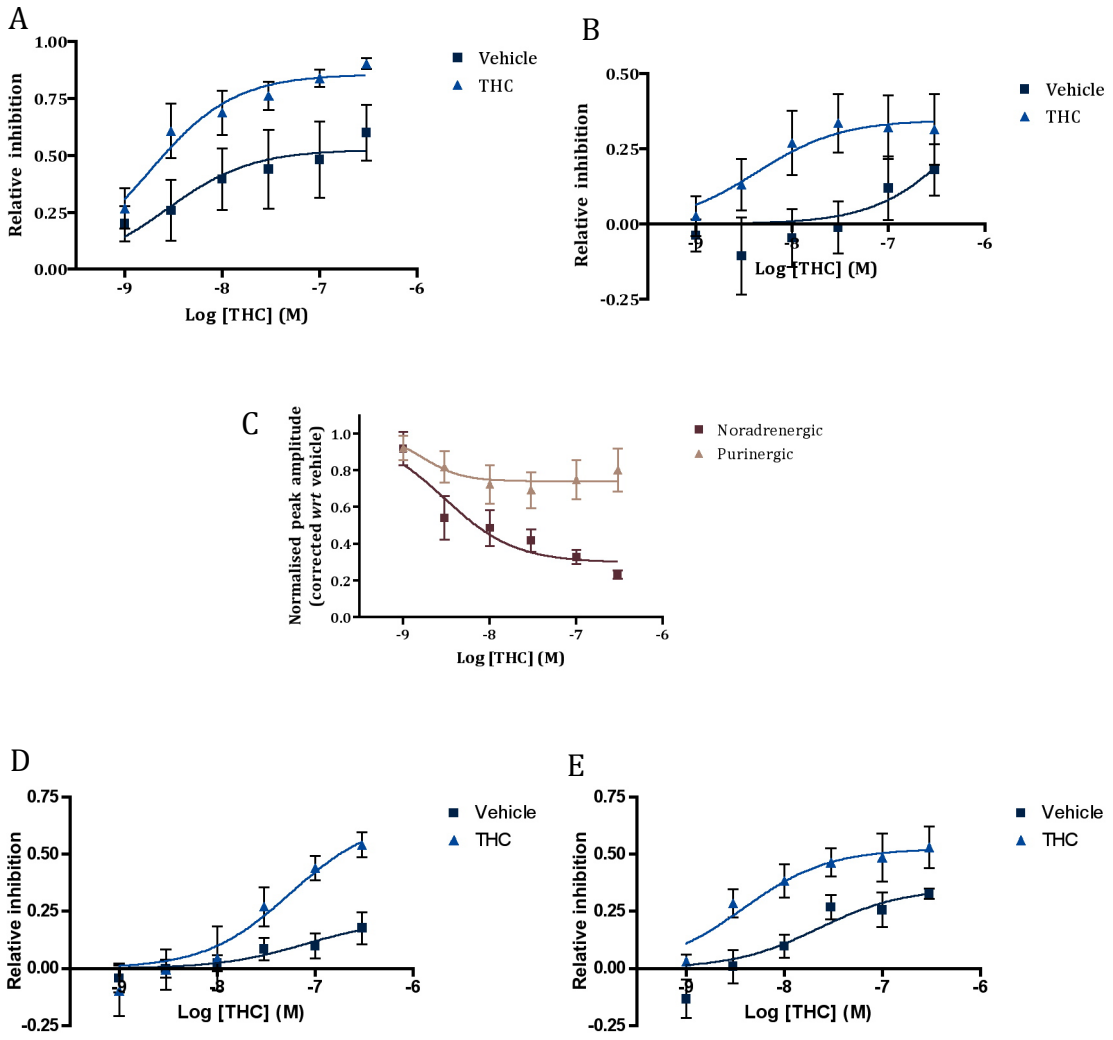

Figure 17

A. A graph showing the concentration-response curves for the inhibitory effect of THC (1–300 nM) versus matching vehicle controls on the peak amplitude of noradrenergic neurogenic contractions (10 pulses, 10 Hz, 0.5 ms, 90 V) isolated in the presence of α,β-metATP (1 μM).

B. A graph showing the concentration-response curves for the inhibitory effect of THC (1–300 nM) versus matching vehicle controls on the peak amplitude of noradrenergic purinergic contractions (10 pulses, 10 Hz, 0.5 ms, 90 V) isolated in the presence of prazosin (100 nM).

C. A graph comparing the concentration-response effects of THC (1–300 nM) on pharmacologically isolated noradrenergic and purinergic contractions, peak amplitudes corrected with respect to (wrt) matching vehicle controls.

D. A graph showing the concentration-response curves from a repeat series of experiments examining the effects of THC (1–300 nM) on noradrenergic contractions.

E. A graph showing the concentration-response curves from a repeat series of experiments examining the effects of THC (1–300 nM) on purinergic contractions.

Effect of THC on contractions evoked by exogenously applied agonists

The site of action of THC was investigated using exogenously applied agonist, phenylephrine and α,β-metATP, to evoke noradrenergic and purinergic contractions respectively. The agonists were applied to the tissue in the chambers of the organ bath from lowest to highest concentration at 20 minute intervals. The tissues were washed immediately after a maximal response was observed in order to try to minimize desensitization to the agonists. For each set of tissue, half the tissues were exposed to THC (100 nM) whilst the other half were controls with only the vehicle, DMSO (0.1%) added to the chamber.

Tissues stimulated with phenylephrine were exposed to six concentrations from 0.1 μM to 30 μM. No significant effect was observed on incubation with THC compared to vehicle controls (Fig. 18a). This suggests that the inhibitory effect observed during concentration-response experiments with THC on the noradrenergic component of contraction was mediated by a pre-junctional effect.

α,β-metATP was used to stimulate the tissue with concentrations from 10 nM to 30 μM. As with most of the contraction data presented in this thesis, normalization of contractions was calculated relative to a standard stimulus (10 pulses, 10 Hz, 0.5 ms, 90 V) at the end of the equilibration period. However, in one experimental run, the response of the tissue to EFS was very small so that the normalized responses to agonist were larger than the distribution of responses from the other samples. Consequently it was decided that these data points were outliers and discounted from the statistical analysis. Therefore in this set of experiments there were six preparations exposed to THC (100 nM) but

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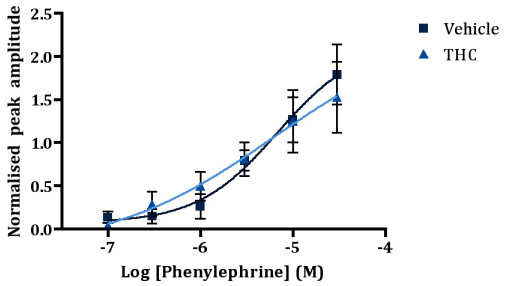

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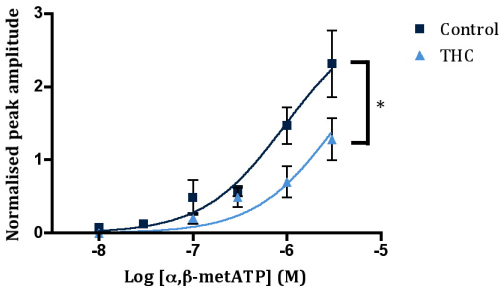

Figure 18

A. A graph showing the effect of THC (100 nM) versus DMSO (0.1%) vehicle control on contractions evoked by the exogenous application of the adrenergic agonist phenylephrine (0.1–30 μM).

B. A graph showing the effect of THC (100 nM) versus DMSO (0.1%) vehicle control on contractions evoked by the exogenous application of the purinergic agonist α,β-metATP (10 nM–3 μM) (* $P < 0.05$).

only four exposed to DMSO (0.1%) vehicle as a control used for analysis. THC caused a significant inhibition to the peak amplitude of contractions elicited by α,β-metATP (Fig. 18b; $P < 0.05$, $n = 6$, ANOVA). This suggests that the inhibitory effect observed during concentration-response experiments with THC on the purinergic component of contraction was mediated, at least in part, by a post-junctional effect.

Effect of THC on a range of stimulus frequencies

As it is known that the ratio of NA and ATP released by sympathetic nerve terminals varies with frequency, it was considered interesting to see if the effects of THC varied in some manner with stimulation at a range of frequencies of the pharmacologically isolated components of contraction. In order to compare the different frequencies, 30 secs of stimulation were used at all frequencies. Repeatable responses to stimulation were achieved using a range of rest times between trains dependent on the frequency (0.5 Hz – 5 mins; 1 Hz – 10 mins; 2 Hz – 10 mins; 5 Hz – 15 mins; 10 Hz – 20 mins). The order in which frequencies were used was randomized for each experimental set in order to avoid any cumulative desensitization by always using lowest to highest frequencies. Normalization was carried out by comparing the responses to each frequency before and after incubation with THC (100 nM) or DMSO vehicle control thereby creating a ratio for each frequency.

As suspected, a Kolmogorov-Smirnov test for normality demonstrated that the ratio data had a non-gaussian distribution therefore non-parametric analysis of

the data was done using a Mann-Whitney U test. Initial analysis of the data measuring peak area, as for the experiments changing the number of pulses in a train of EFS, revealed no significant difference between THC and Vehicle responses at any frequency on either the purinergic (P = NS, n = 6; Fig. 19a) or the noradrenergic (P = NS, n = 5 Fig. 19b) components of contraction. It was surprising that no effect of THC was revealed in this experiment and particularly that THC did not increase the peak area at high frequencies in a similar manner to that seen with longer trains of stimulation on the noradrenergic component of contraction. It was considered possible that a changing ratio of transmitters between frequencies might be masking any changes, therefore it was decided to use a method of analysis which would more sensitively identify changes in the contractile response. Therefore, as a 30 sec train of stimulation evokes the classic twitch and hump response even at low frequencies, it was decided to analyse the peak amplitude for the twitch and the hump separately. Sadly, unlike the guinea-pig vas deferens that of the mouse does not show an elegant split between the pharmacological origins of the twitch and hump. The twitch responses were analysed looking for the amplitude of the largest peak during the first 10 secs of stimulation whilst the hump was measured as a mean of the hump amplitude compared to baseline before stimulation during the last 10 secs of EFS (Fig. 19g).

THC (100 nM) caused a significant decrease in the peak amplitude of the twitch response to the noradrenergic component of contraction at 1, 2, 5 and 10 Hz EFS (0.5 ms, supramaximal voltage) compared to vehicle controls (Fig. 19E; $P < 0.05$, n = 5, T-test). At 1 Hz, the ratio of peak amplitude before and after 40 mins

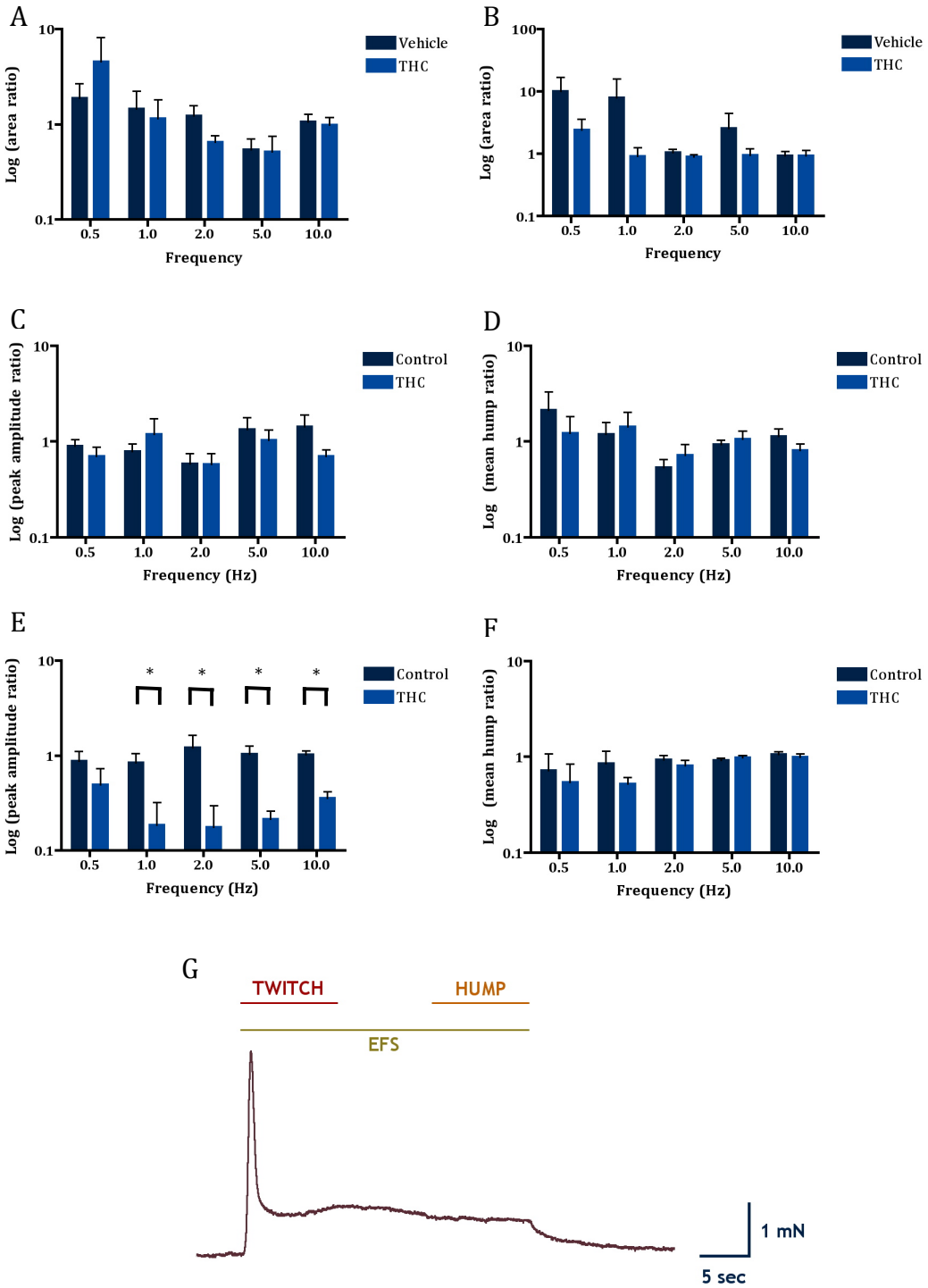

Figure 19

A. A graph showing the peak area as a ratio of pre and post exposure after incubation with either THC (100 nM) or DMSO (0.1 %) vehicle control on the purinergic component of contraction. EFS was applied for 30 seconds at range of frequencies (0.5–10 Hz).

B. A graph showing the peak area as a ratio of pre and post exposure after incubation with either THC (100 nM) or DMSO (0.1 %) vehicle control on the noradrenergic component of contraction.

C. A graph showing the effects of THC (100 nM) on the twitch component of the purinergic response over a range of frequencies.

D. A graph showing the effects of THC (100 nM) on the hump component of the purinergic response over a range of frequencies.

E. A graph showing the effects of THC (100 nM) on the twitch component of the adrenergic response over a range of frequencies (* $P < 0.05$).

F. A graph showing the effects of THC (100 nM) on the hump component of the adrenergic response over a range of frequencies.

G. A representative trace of a noradrenergic contraction evoked by 30 seconds of EFS at 10 Hz to demonstrate the collection of data for the above graphs. The twitch response was measured by finding the peak amplitude measurement within the first 10 seconds of EFS. The hump response was measured by averaging the contractile amplitude within the last 10 seconds of EFS.

incubation with THC was 0.18 ± 0.14 compared to 0.84 ± 0.22 with vehicle alone. At 10 Hz the ratio was 0.36 ± 0.06 for THC compared to 1.02 ± 0.11 for vehicle controls. However, THC did not cause a significant change in the mean amplitude for the hump during the last 10 secs of stimulation at any frequency of stimulation (Fig. 19F; *P* = NS, *n* = 5, T-test). At 10 Hz, the ratio of mean amplitude before and after incubation with THC was 0.99 ± 0.08 compared to 1.05 ± 0.07 for the vehicle alone.

On the pharmacologically isolated purinergic component of contraction, THC (100 nM) did not cause a significant difference in the amplitude of the twitch peak (Fig. 19C) or the mean amplitude of the hump during the last 10 secs of EFS (Fig. 6D at any frequency (*P* = NS, *n* = 6, T-test). At 10 Hz, the ratio of the twitch amplitude before and after incubation was 0.70 ± 0.12 with THC and 1.43 ± 0.47 with vehicle alone. For the mean hump amplitude the average ratio was 0.80 ± 0.14 with THC and 1.13 ± 0.22 with vehicle alone.

Electrophysiology studies

In order to further explore the effect of THC on both evoked and spontaneous ATP release from the nerve terminals, voltage-following electrophysiology using sharp intracellular electrodes was performed. Vasa deferentia were prepared for recording as described in the Methods section. All experiments were performed in the presence of prazosin (100 nM) and nifedipine (1 μM) in order to reduce contraction during the stimulus trains and hence maintain the recordings. In brief, from each vas deferens a set of at least four cells were recorded from using

a protocol of 5 stimuli at 5 Hz delivered every 30 s for a cycle of five trains as controls. For each cell, the microelectrode was withdrawn at the end of the stimulus protocol in order to assess the resting membrane potential. The superfusion reservoir was switched to one containing THC (100 nM) or DMSO (0.1%) vehicle, the tissue was left to equilibrate for 40 minutes, and then the recording protocol was repeated. The amplitudes of matching EJPs were averaged across the five cycles of EFS trains to give five average EJP amplitudes for each cell sampled.

THC (100 nM) caused a significant reduction in the amplitude of all EJPs evoked by a train of 5 EFS pulses versus EJPs evoked before addition of the drug (Fig. 20b; $P < 0.01$, n_{cells} = 18–19, n_{preps} = 4, ANOVA). In a separate set of control experiments, no significant effect was observed after the addition of vehicle (0.1 % DMSO) alone (Fig. 20a; $P > 0.05$, n_{cells} = 17–18, n_{preps} = 4, ANOVA). Comparing the cells sampled within a preparation, THC reduced the amplitude of the first EJP to an average 57.0 ± 8.8 % of controls compared to 95 ± 12 % for vehicle. There was a significant difference between the amplitude of the first EJP evoked in the presence of THC versus DMSO (0.1%) vehicle (Fig. 20c; $P < 0.05$, Mann-Whitney test). THC had no significant effect on resting membrane potential of the preparations versus both control readings and DMSO vehicle, (Fig. 20d; $P > 0.05$, T test), suggesting that there was no postjunctional effect of the THC sufficient to affect the resting conductances determining the resting membrane potential.

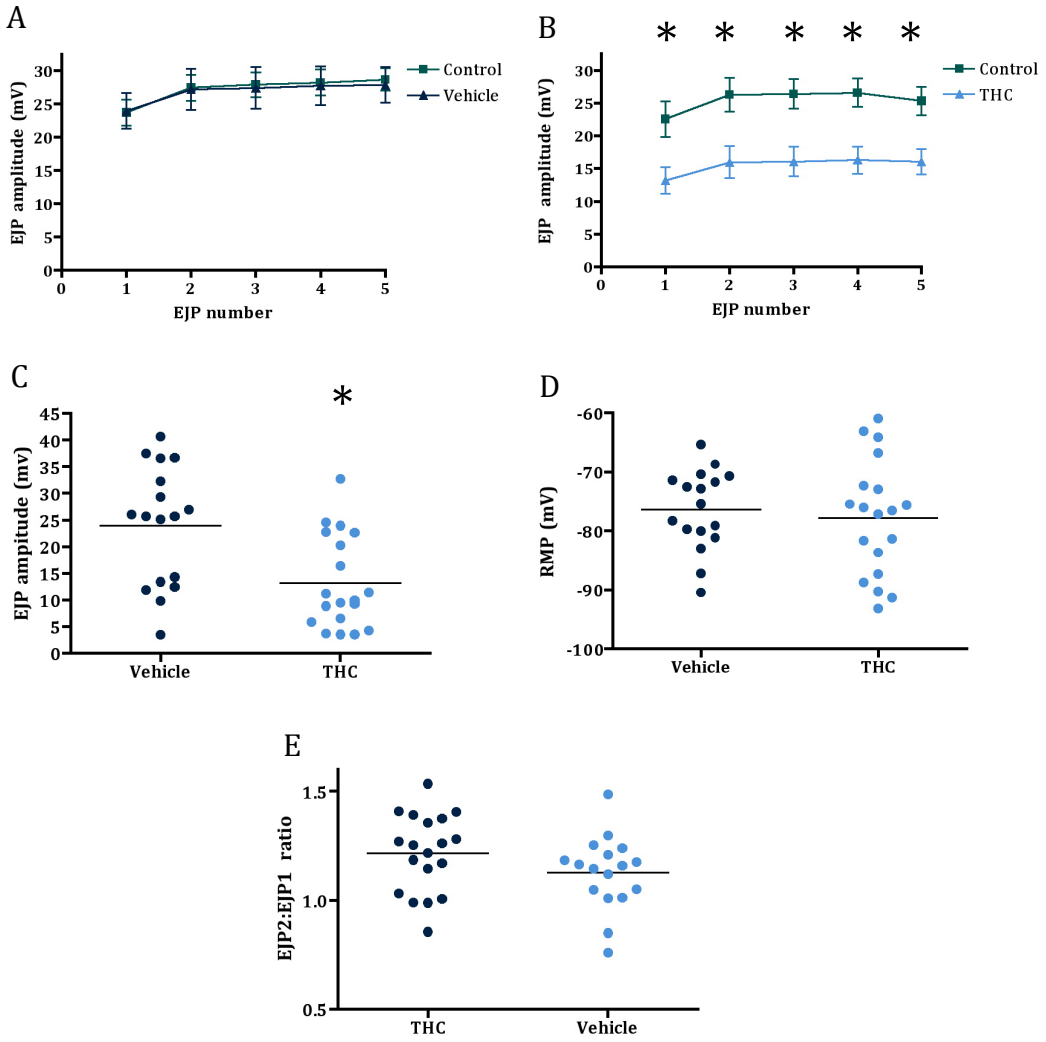

Figure 20

A. A graph showing the average amplitude of a train of EJPs (5 pulses, 5 Hz) evoked before and after incubation with 0.1% DMSO vehicle.

B. A graph showing the average ampltude of a trains of EJPs (5 pulses, 5Hz) evoked before and after incubation with THC (100 nM). * $P < 0.05$

C. A graph showing the distribution of amplitudes for the first EJP in a train of 5 in the presence of either THC (100 nM) or DMSO (0.1%) vehicle. * $P < 0.05$

D. A graph showing the distribution of resting membrane potentials in the presence of either THC (100 nM) or DMSO (0.1%) vehicle.

E. A graph showing the distribution of ratios between the first and second EJPs of a train of 5 in the presence of either THC (100 nM) or DMSO (0.1%) vehicle.

To determine whether THC might affect the prejunctional mechanism of frequency facilitation, its effect on the ratio of the second to the first EJP amplitude was investigated. The time course of the dominant component of frequency facilitation, referred to as F2-facilitation, is 0.4 s (Bennett, 1973; Blakeley *et al.*, 1984a), which implies that the stimulus frequency of 5 Hz should show significant facilitation. The ratio of the second and first EJPs (Fig. 20e; $P > 0.05$) was not significantly affected. To further investigate whether there were postjunctional effects of THC, either on the kinetics of the P2X receptor or on the membrane time resistance or capacitance, the rise and fall times of the EJPs were investigated. Neither the rise time of the first EJP (data not shown; $P > 0.05$) nor the fall time of the fifth EJP (data not shown; $P > 0.05$) were significantly affected by THC.

There were some concerns that 5 stimuli at 5 Hz might have, in addition to frequency facilitation, some component of temporal summation. In the mouse vas deferens the time constant for repolarization of the EJP is 31±7 ms (Cunnane & Manchanda, 1989b), implying that over 200 ms the membrane potential will still be elevated by e^(-200/31)=0.0016, or 0.16% of the initial amplitude. While this is small, the resting potential appeared to remain at a membrane potential somewhat more positive than that suggested by the simple assumption of an exponential recovery. This sustained component of depolarization is currently being investigated by Dr R. Haddock. To avoid this slow component of repolarization and any residual summation, the stimulus frequency was extended in some experimental sets ($n = 5$) from 5 Hz to 2.5 Hz. Qualitatively,

there was still some residual summation and the effects of THC were similar to those described for the 5 Hz stimulation protocol.

Effects of THC on Nerve Terminal Ca^{2+} Regulation

Having shown that THC affects both noradrenergic and purinergic transmission, it seemed possible that THC could affect a common pathway involved in the release of both of these cotransmitters. Given that several prejunctional receptor systems affect nerve terminal Ca^{2+} influx and that THC is known from some other systems to inhibit Ca^{2+} influx in nerve cell bodies, investigations were carried out to investigated the effects of THC on nerve terminal Ca^{2+} regulation.

Nerve terminals were orthogradely loaded with OG-BAPTA-1-dex, as described in the Methods section, and viewed with a Leica SP2 upright confocal microscope. Field stimuli were applied using a pulse width 0.5 ms, matching the contraction experiment protocol; for each nerve terminal, a stimulus voltage threshold was determined, which was usually in the range of 30 – 60 V. Such as stimulus protocol led to an all-or-none $\Delta[Ca^{2+}]_t$ response, which has previously been shown to be tetrodotoxin-sensitive (Brain & Cunnane, 2008).

Effects of THC (100 nM in Tween 80) on NTV transients

Tissue samples orthogradely loaded with OG-BAPTA-1-dex were used to study the effects of THC (100 nM in 0.015% Tween-80) NTC calcium transients. For each nerve terminals, 6 sets of 5 impulses at about 5 Hz (more precisely, at a

stimulus interval of 0.216 ms), were delivered, yielding a set of 32 images with the stimulus triggered at the start of the 7th frame, synchronized using the microscope's TTL signal (see Methods). This allows the residual Ca^{2+} concentration to be measured soon after each action potential in the train, as the recording frequency matches the stimulus frequency. Six control series were acquired at 2 minute intervals, after which the superfusing solution was switched to one containing THC (100 nM) and allowed to equilibrate for 20 minutes. A further 6 sets were acquired. Analysis of the ratio of $\Delta F/F_0$ before and after the addition of either THC or vehicle (Tween 80) revealed a significant decrease in Ca^{2+} transient amplitude in the presence of THC compared to vehicle for the first EFS pulse (Fig. 21; $P < 0.05$; sample images at Fig. 22). On average, THC reduced $\Delta F/F_0$ amplitude by 20 ± 3% ($P < 0.05$, $n_{varicosities} = 41$, $n_{strings} = 12$, $n_{preps} = 7$), whereas vehicle produced no significant change (+1 ± 3%; $P > 0.05$, $n_v = 39$, $n_s = 14$, $n_p = 6$). There was no obvious consistent change in the kinetics of $\Delta[Ca^{2+}]_t$ recovery (data not shown).

Exploring the relationship between THC and NET

From the above work, it is argued that CB_1 receptors inhibit the release of both the principal neurotransmitters, ATP and noradrenaline, following brief trains of stimuli. Although this has not been verified as the definite receptor pathway through the use of a selective CB_1 antagonist such as SR141716A (Rinaldi-Carmona *et al.*, 1994), CB_1 is the only known cannabinoid receptor expressed on the mouse vas deferens(Thomas & Pertwee, 2006). For completeness, it was

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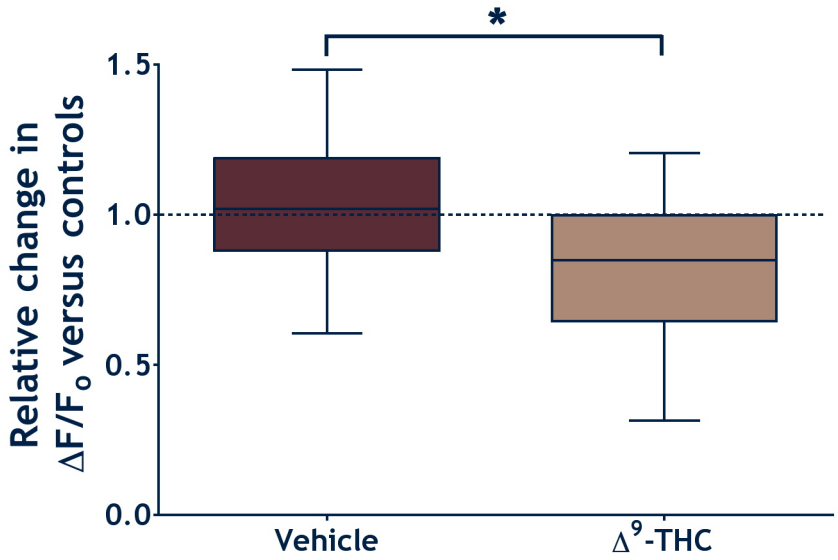

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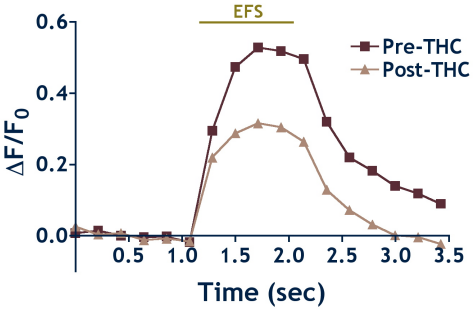

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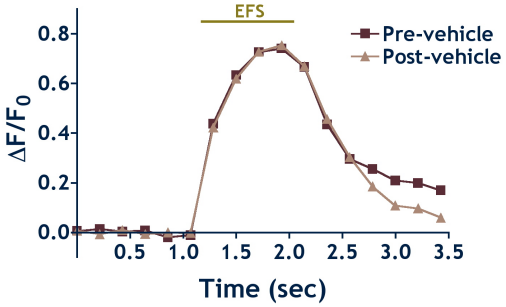


Figure 21

A. THC (100 nM) caused a significant decrease in the amplitude of NTV calcium transients versus vehicle controls (* $P < 0.05$).

B. A sample trace of the fluorescent changes observed within an individual NTV before and after incubation with THC.

C. A sample trace of the fluorescent changes observed within an individual NTV before and after incubation with Tween 80 vehicle controls.

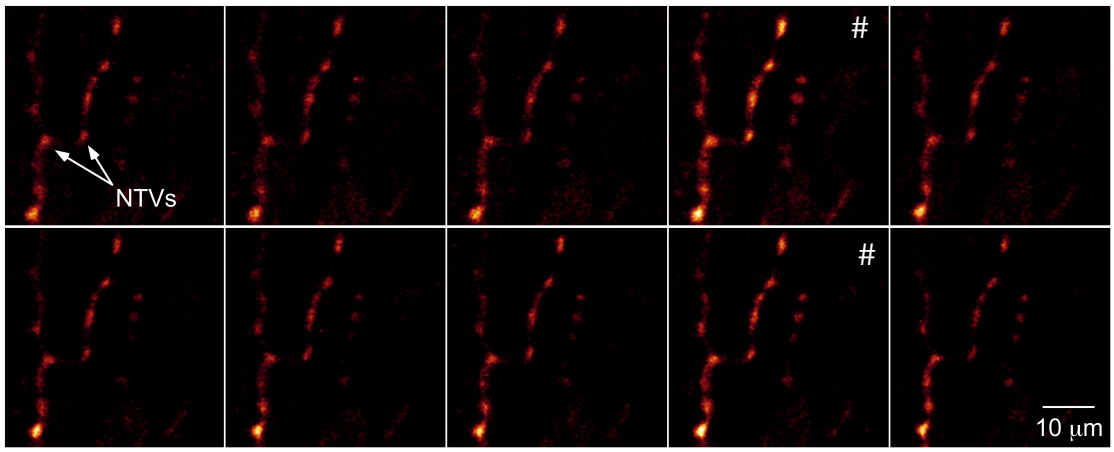


Figure 22
Scans (5 Hz) of sympathetic NTVs acquired before (top) and after (bottom) incubation with 100 nM THC. Scans are (from left to right): 3 pre-stimulation, 1 during stimulation (#) and 1 post-stimulation.

decided to investigate the effects of THC on longer trains of stimuli, which led in a rather surprising direction.

Effect of THC on a range of stimulus train lengths

Further investigations into the potential differential effects of THC on noradrenergic and purinergic transmission, measured using contractile responses, looked at the effects of THC (100 nM) using a range of pulse numbers in trains of EFS (1-200 pulses, 10 Hz, 0.5 ms width, 90 V). This was considered an interesting study as previous studies have demonstrated that the ratio of the principal transmitters released by autonomic terminals may vary with train length. However, since a range of train lengths evokes peaks with different shapes, from a single peak for short EFS train lengths to the classic twitch and hump response for longer trains, it was considered preferable to use peak area as the measurement for this data as opposed to the usual twitch amplitude. This gave a measure of the full magnitude of the contractile response in terms of force and time as opposed to force alone for peak amplitude.

Very surprisingly, THC caused a significant increase in the peak area of contractions evoked using trains of 100 and 200 pulses of stimulation (Fig. 23A; $P < 0.05$, $n = 6$) in the presence of α,β-metATP (1 μM) to isolate noradrenergic responses. At 200 pulses, peak areas of contractions evoked in the presence of THC were an average 1150 ± 230 % of those evoked by a standard 10 pulses (10 Hz) before the addition of THC compared with 490 ± 38 % for vehicle controls.

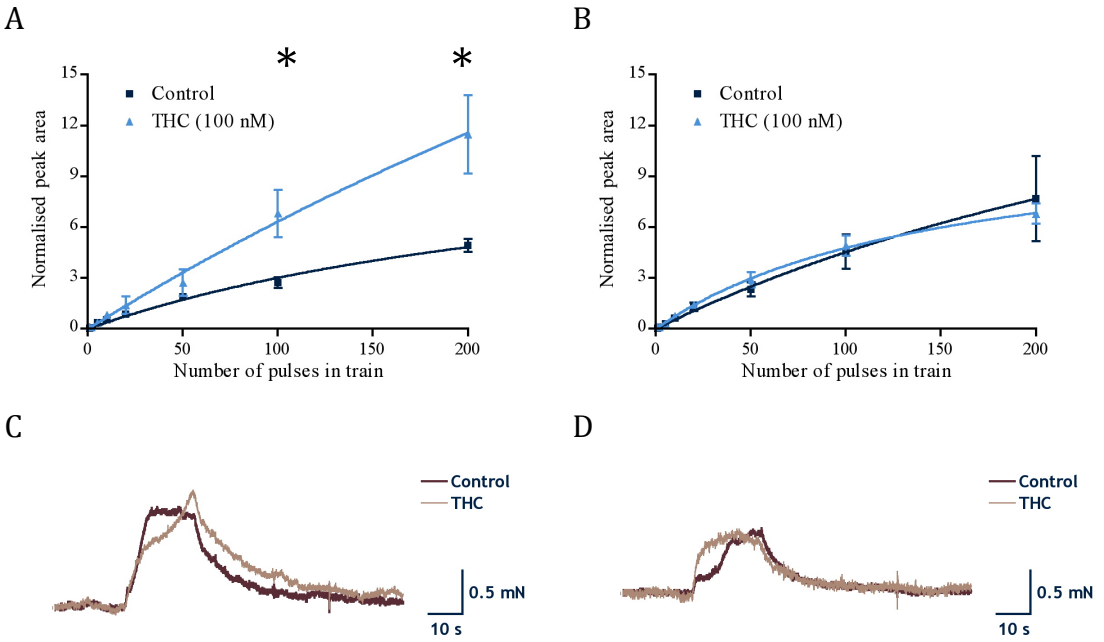


Figure 23

A. THC (100 nM) caused a significant increase in the peak are of noradrenergic neurogenic contractions evoked in the presence of α,β-metATP (1 μM) versus DMSO (0.1%) vehicle controls for trains of 100 and 200 pulses of stimulation (10 Hz). *$P < 0.05$.

B. THC (100 nM) did not cause any change in the peak are of purinergic neurogenic contractions evoked in the presence of prazosin (100 nM).

C. Sample traces of noradrenergic neurogenic contractions evoked by EFS with a 100 pulses train of stimulation in the presence of DMSO (0.1%) vehicle and THC (100 nM).

D. Samples traces of purinergic neurogenic contractions evoked by EFS with a 100 pulses train of stimulation in the presence of DMSO (0.1%) vehicle and THC (100 nM).

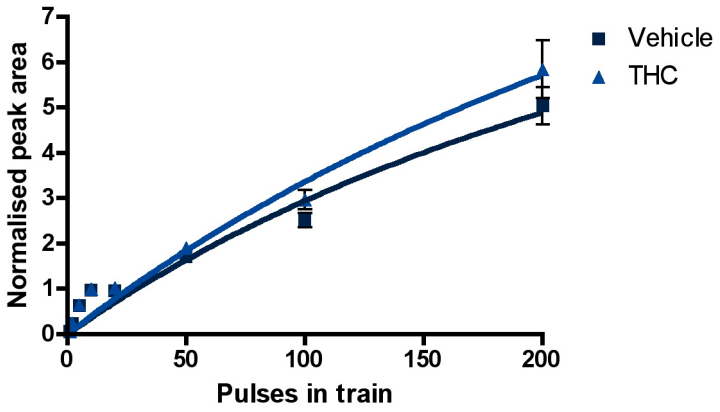
No significant effect was observed on the peak area of purinergic contractions in the presence of THC compared to vehicle controls (Fig. 23B; $P > 0.05$, $n = 6$).

Identifying the receptor subtype(s) involved in modifying the noradrenergic component

In order to confirm that the effect observed on noradrenergic contractions was attributable to THC and a cannabinoid receptor, the experiment was repeated in the presence of the CB1 antagonist, AM251. In the presence of AM251 (1 μM) there was no significant difference in normalized peak area between contractions evoked in the presence of THC (100 nM) and the vehicle, DMSO (maximum 0.1%), alone over the same range of pulses trains (Fig. 24A). AM251 had thus appeared to block the potentiating effects previously observed in the presence of THC with longer trains of pulses suggesting that the original response may be a CB1 mediated response.

Surprisingly, the CB2 antagonist, AM630 (1 μM), also blocked the potentiating effect previously elicited by THC (100 nM) for the noradrenergic component of contraction (Fig. 24B). In the presence of AM630 there was no significant difference between contractions in the presence and absence of THC at any train of pulses. This data would suggest that the original effect may also involve a pathway that is neither CB1 nor CB2 mediated but involves a receptor sensitive to both AM251 and AM630.

A



B

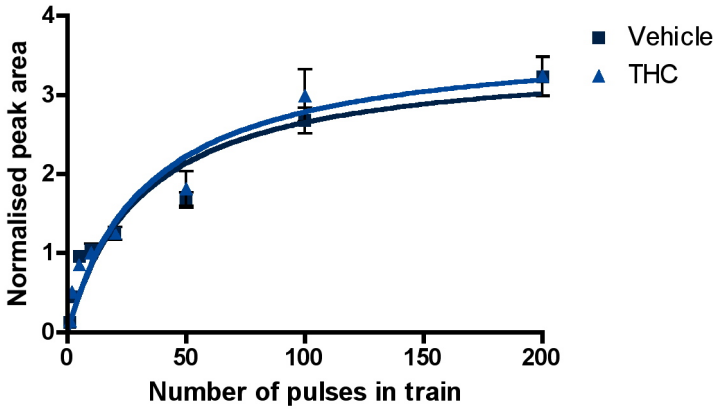


Figure 24

A. The CB1 antagonist, AM251 (1 μM), blocked the potentiating effect of THC (100 nM) on the peak area of long trains of stimulation in tissues pre-incubated with α,β-metATP (1 μM) to isolate the noradrenergic component of contraction.

B. The CB2 antagonist, AM630 (1 μM), blocked the potentiating effect of THC (100 nM) on the peak area of long trains of stimulation in tissues pre-incubated with α,β-metATP (1 μM) to isolate the noradrenergic component of contraction.

Role of NET in modifying the noradrenergic component

The most likely mechanism by which THC might cause an increase in peak area was considered the inhibition of the norepinephrine transporter type-1 (NET). This hypothesis was investigated using the NET inhibitor, desipramine, to test whether the potentiating effects of THC were blocked in its presence. In the presence of desipramine (100 nM) there was no significant difference between contractions evoked in the presence of THC (100 nM) or the vehicle, DMSO, alone (Fig. 25). Taken with the cannabinoid receptor antagonist experiments, this suggested that THC's surprising potentiating effects on peak area for the noradrenergic component of contraction is mediated via an inhibition of NET via a pathway involving a CB1- and CB2-like receptor.

Role of Uptake 2 in modifying the noradrenergic component

As the uptake of noradrenaline from the sympathetic junction is dependent upon two proteins, NET and uptake 2, it was considered prudent to investigate whether THC might also modify the activity of uptake 2. Normetanephrine (10 μM) was incubated with the tissues for 20 minutes in order to block uptake 2. Interestingly, this also blocked the potentiating effect of THC on the noradrenergic component of contraction raising the possibility that both NET and uptake 2 are involved in the effect of THC (Fig. 26). It should be noted that in these experiments, a small but insignificant inhibition of contractile peak area can be observed for longer trains of stimulation. It may be considered at this point that the original experimental set which precipitated this work was

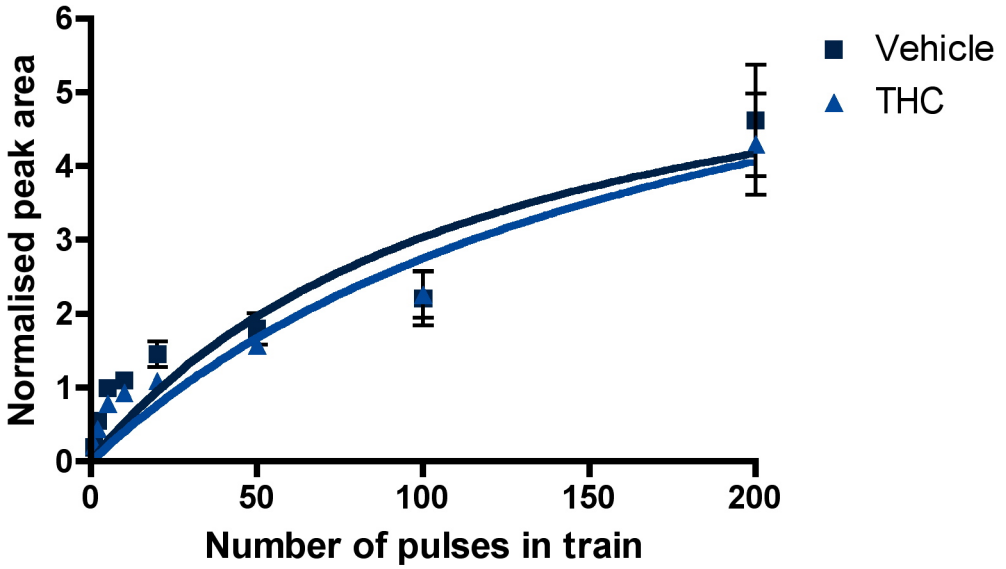


Figure 25
The NET inhibitor, desipramine (100 nM), blocked the potentiating effect of THC (100 nM) on the peak area of long trains of stimulation in tissues pre-incubated with α,β-metATP (1 μM) to isolate the noradrenergic component of contraction.

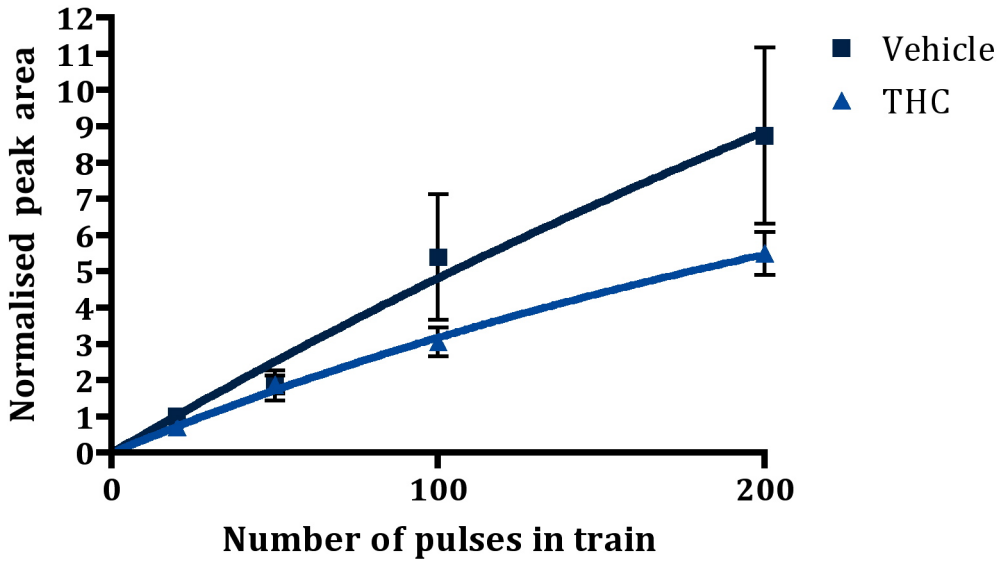


Figure 26
The uptake 2 inhibitor, normetanephrine (10 μM), blocked the potentiating effect of THC (100 nM) on the peak area of long trains of stimulation in tissues pre-incubated with α,β-metATP (1 μM) to isolate the noradrenergic component of contraction. The apparent inhibition caused by THC at 100 and 200 pulse trains was not significantly different from DMSO (0.1%) vehicle controls.

composed of anomalous data were it not for imaging experiments conducted later.

Investigating THC's potential effect on NET using agonists with a range of affinity for NET

It was decided to further investigate the possible effects of THC on NET activity using the application of exogenous agonists. It was hypothesized that by using three agonists (noradrenaline, phenylephrine and methoxamine) with a range of affinities for NET difference in the changes to the contractile responses caused (or not) by THC would be revealed. Theoretically, methoxamine having no affinity for NET would be unaltered by incubation with THC given it had already been demonstrated that THC had no post-junctional effects. Noradrenaline's responses on the other hand were expected to be modified due to its affinity for NET whereas phenylephrine which is know to have an intermediate affinity for NET would be expected to show an intermediate effect of THC. Although peak amplitude was to be analysed as in previous exogenous agonist work, it was considered unlikely that this parameter would reveal the changes in the contraction profile that would be expected by THC inhibiting NET. Therefore, peak rise time and peak area were also analysed. It was also decided in order to standardize analysis of the contraction peaks evoked by these agonists to sample data using a selection window of 30 sec before the initial rise point for a contraction to calculate baseline and a 60 sec selection window after the initial rise point for peak amplitude and area analysis. Preliminary experiments were carried out to establish suitable concentrations of the three agonists which

evoked responses of approximately equal magnitude. A rest time between sequential doses for each concentration used, giving a repeatable response to that test concentration, was also established.

Agonists were introduced into the organ bath chambers by pipette. In early experiments 5 µL doses were used however it was thought this might be causing irregular mixing in the chambers so 50 µL doses were used in later experiments. The control responses (before incubation with THC or DMSO) were compared between these two methods and it was established that there was no significant difference between the variance of the two data sets therefore it was considered acceptable to combine these results for analysis.

The peak amplitude, peak area and rise time for each exogenously-evoked contraction were analysed. THC (100 nM) did not cause a significant change in any of these parameters versus DMSO (0.1%) vehicle controls for either noradrenaline (Fig. 27A–C), phenylephrine (Fig. 27D–F), nor methoxamine (Fig. 27G–I) (n = 8 for each exogenous agonist).

Do endocannabinoids modify the noradrenergic component?

However, it was considered worth investigating whether other cannabinoids might evoke a similar response to that send with THC. The endocannabinoid, anandamide, seemed like a good choice as an endogenous candidate which might be involved in eliciting this response under normal physiological conditions for the purposes of altering junctional activity. At a common *in vitro* concentration

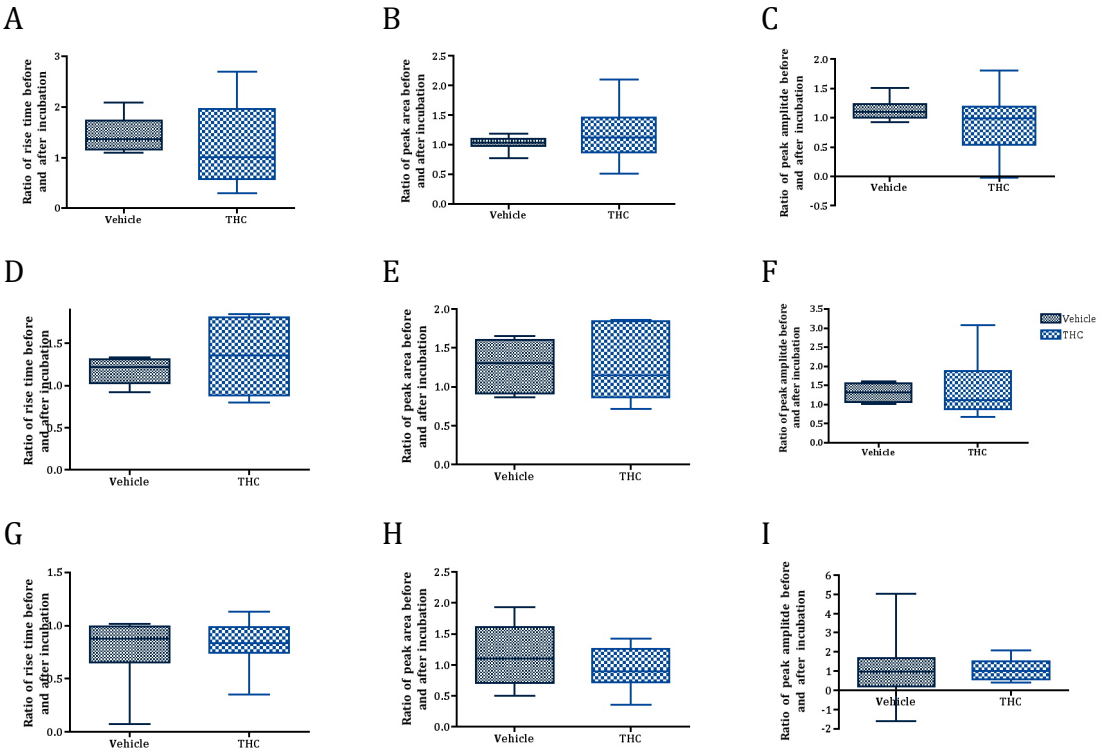


Figure 27

A–C: The ratios of rise time, peak area and peak amplitude (calculated before and after incubation with either THC (100 nM) or DMSO (0.1%) vehicle control) were not significantly different between the two exposure protocols for contractions evoked by the exogenous agonist, noradrenaline (3 μM).

D–F: The ratios of rise time, peak area and peak amplitude were not significantly different between exposure to THC (100 nM) and DMSO (0.1%) vehicle control for contractions evoked by the exogenous agonist, phenylephrine (10 μM).

G–I: The ratios of rise time, peak area and peak amplitude were not significantly different between exposure to THC (100 nM) and DMSO (0.1%) vehicle control for contractions evoked by the exogenous agonist, methoxamine (10 μM).

(1 μM), anadamide caused no significant difference in the peak area of contractions against vehicle controls evoked using the same stimulus protocol (1-200 pulses in a train) as was used for the original THC experiments (Fig. 28). This work was again carried out in the presence of α,β-metATP (1 μM) in order to isolate the noradrenergic component of contraction. Although further investigation is necessary with other cannabinoids, this raises the possibility that the effect observed with THC is specific to that cannabinoid.

Using NTUA to assess effects of THC on NET

The development of the use of NTUA to assess NET function raised the exciting possibility of being able to directly assess the effect of THC on NET activity. Therefore, a series of experiments was carried out to monitor the changes in NET rate analysed with NTUA in the presence of THC in order to further test the hypothesis suggested by the contraction experiments described earlier.

Tissues were prepared for imaging following the standard NTUA protocol developed. After exposure to NTUA (1:100) to find a suitable site for imaging, the preparations were incubated with either THC (100 nM) or vehicle (0.1% DMSO) in PSS for 40 mins. Following the incubation period, tissues were incubated with NTUA (1:10) as normal with either THC or DMSO added to the mixture as appropriate. The presence of THC or DMSO was continued during the washout period.

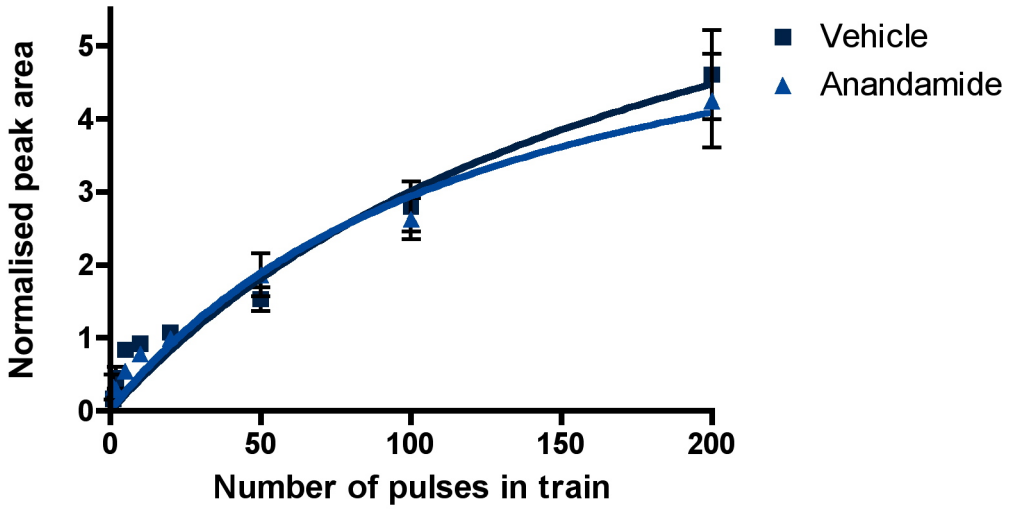


Figure 28
The endocannabinoid anandamide (1 μM) did not cause a significant change in the peak area of noradrenergic neurogenic contractions, pharmacologically isolated with α,β-metATP (1 μM) and evoked by a range of trains of stimulation, versus DMSO (0.1%) vehicle control.

THC (100 nM) caused a significant decrease in the rate of increase of fluorescence during incubation with NTUA (1:10) compared to vehicle controls (Fig. 29A, n = 5, Mann-Whitney Test $P < 0.05$). In the presence of THC fluorescence increased by 44 ± 4.3%/min (n = 5, n_t = 29) compared to vehicle controls of 96 ± 18%/min (n = 5, n_t = 26). THC caused no significant change in the rate of fluorescence decrease during the washout period (Fig. 29B).

In a separate series of experiments, tissues were also incubated with the CB1 antagonist, AM251, in addition to THC or DMSO for 40 mins. There was no significant difference in the rate of increase of fluorescence between tissues exposed to THC and DMSO in the presence of 1μM AM251 (n = 6, data not shown).

NTUA allowed the quantitative analysis of the newly discovered effect of THC on the rate of NET activity. In conjunction with the results from contraction data, these findings suggest that his effect is mediated by the CB1 receptor.

PC12 cells as a tool to test the interaction between THC and NET

PC12 cells were used in order to determine whether or not the interaction between cannabinoid receptors and NET occurred in another cell type. CB1 receptors have been shown to be present on PC12 cells (Juttler *et al.*, 2004), although this finding has been contradicted by other groups who find no evidence for CB1 mRNA in PC12 cells (Molderings *et al.*, 2002) on undifferentiated PC12 cells.

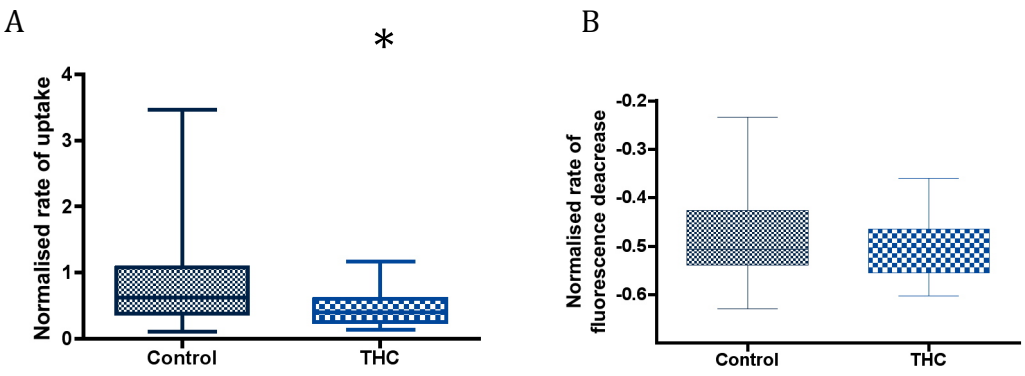


Figure 29

A. THC (100 nM) caused a significant decrease in the rate of uptake of NTUA (1:10) into nerve terminals of the mouse vas deferens versus DMSO (0.1%) vehicle control (*$P < 0.05$).

B. There was no significant difference in the rate of washout of NTUA between tissues exposed to THC (100 nM) and those exposed to DMSO (0.1%) vehicle control.

Following preincubation of the PC12 cells for 40 minutes in 100 nM THC, the rate of uptake of NTUA was 307 ± 35 ΔF/min (Fig. 30), which was not significantly different from a matched control set without THC (296 ± 42 ΔF/min; n = 6 for each; P = 0.46). Hence, THC had no detectable effect on the NET rate in these cells.

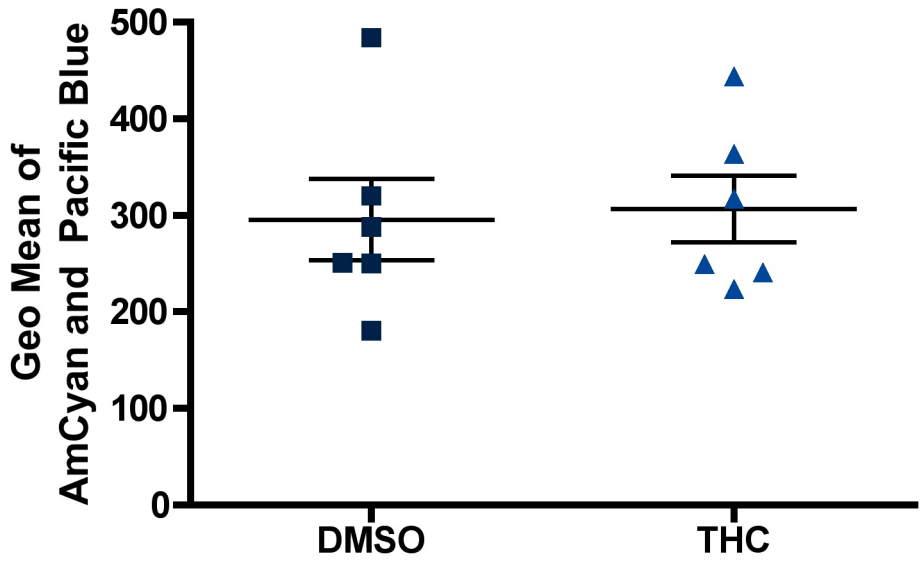


Figure 30
PC12 cells pre-incubated with either DMSO (0.1%) vehicle as control or THC (100 nM) showed no significant difference in the rate of uptake of NTUA (1:10).

Discussion

In this project, the effect of two compounds on sympathetic junctional transmission have been investigated. Namely the androgenic steroid, testosterone, which is usually considered for its role in the stimulation and control of the development and maintenance of male characteristics in vertebrates, and the cannabinoids, those compounds which are chemically active at the cannabinoid receptors and are extracted from or related to those compounds extracted from the Cannabis family of plants.

However, before discussing the effects of cannabinoids and testosterone on junctional transmission, the development of a key new experimental technique, the use of NTUA as a tool for monitoring NET rate and neurotransmitter vesicle tracking, will be discussed. This methodology was developed during the course of this project and provides an exciting opportunity to study dynamically *in vitro* a neurotransmitter transporter within isolated tissue samples.

NTUA as an experimental tool for monitoring NET rate

Autonomic neurotransmitters are, classically, volume transmitters. This implies that it is the sustained concentration of the neurotransmitter in the extracellular milieu that will determine its effect. Therefore, the key feature controlling the concentration (and hence effect) is the balance between neurotransmitter release and its subsequent uptake, removal or degradation. This is certainly true for noradrenaline, where exocytosis provides supply, and the removal is through the noradrenaline transporter (NET).

Drugs that act prejunctionally to modify transmitter release may therefore do so by affecting exocytosis (which might include effects on synthesis or packaging) or noradrenaline uptake. While there are many techniques designed to explore transmitter release, there are fewer approaches available to specifically study uptake.

Intriguingly, NET can affect both uptake and release; the effect on release is indirect, through the provision of recycled noradrenaline. Hence, being able to dynamically study changes in NET rate are experimentally useful for understanding both normal sympathetic physiology and the mechanism by which drugs act.

Existing approaches for monitoring NET

The importance of NET for clearing noradrenaline can be seen in the effect of known NET inhibitors, such as desipramine, on the rate of relaxation of tone

following field-stimulation-induced contraction. This means that the rate of relaxation can be used as an indirect measure of a drug's effect on the transporter. The problem which this approach is it relies on many other tissues factors; for example, any technique that affects the relaxation of the smooth muscle cells themselves (which might include effects on postjunctional K^+ channels or Ca^{2+} pumps) will similarly affect muscle relaxation.

The overflow of noradrenaline, subsequently detected using a variety of approaches such as amperometry or cyclic voltametry, is a widely used approach, but has the problem that it's interpretation depends also on an understanding of the rate of neurotransmitter release. There are also questions of sensitivity, with most approaches requiring an aggressive EFS protocol in order to produce any detectable overflow.

An adaptation of this overflow technique, well known to readers of popular pharmacology textbooks, is the use of radioactively labelled NET substrates such as levo-[ring-2,5,6-3^H]norepinephrine (Raffel *et al.*, 2013). However, these compounds are also substrates for uptake-2, the existence of which confounds the interpretation of the relative importance of NET.

The PET probe I^{123}-MIBG, a substrate for NET, is very useful for in vivo imaging, but it's very poor spatial resolution prevents this approach being used for cellular or subcellular imaging. Consequently, I^{123}-MIBG is a useful technique for macroscopic identification of sympathetic innervation density, particularly in the heart (Miranda *et al.*, 2013; Miranda *et al.*, 2014), or for the identification of NET-

expressing tumours such as phaeochromocytomas and paraganglionomas (Lenders *et al.*, 2014).

There are some existing fluorescent substrates for NET, of which the only compound that has generated significant interest is 4-(4-dimethylaminostyryl)-N-methyl-pyridinium ($4ASP^+$). Similar to the first published use of NTUA (Jørgensen *et al.*, 2008), this compound is extensively used to assay NET activity in cultured cells (Schwartz *et al.*, 2003; Mason *et al.*, 2005). Previous work by former colleagues has attempted to use $4ASP^+$ to identify nerve terminals in a range of isolated organ preparations, including the anococcygeus and vas deferens, however this work was unsuccessful in terms of confidently identifying these structures (Amos, 2007).

NTUA as a tool for monitoring NET

Given the many problems associated with the existing technique for assessing NET activity, any possible new tool would present an exciting new opportunity to explore sympathetic function and neuropharmacology. After commencing this project, Jørgensen *et al*. (2008) reported the use of NTUA for "The measurement of neurotransmitter transporter uptake activity" in cell lines overexpressing the NET, DAT or SERT transporters. This finding in cell lines was seen as an opportunity to explore the use of NTUA in mature nerve terminals expressing native receptors for the identification of sympathetic nerve terminals and as a means of monitoring transporter activity and its modulation.

In the initial experiments, it was found that vasa deferentia exposed to a diluted solution of the NTUA (1:10 dilution compared to the manufacturer's recommend solution of 1 aliquot diluted in 10 mL, here defined as a 1:1 solution) developed notable fluorescent labelling in structures reminiscent of nerve terminals and in other structures, typically with an apparently perinuclear appearance. Upon returning the solution to the usual PSS, the intensity of the fluorescent signal in the terminals-like structures diminished slowly, whilst the apparently non-neuronal signal reduced rapidly and often disappeared.

Unfortunately, imaging using this experimental protocol was exceedingly difficult because of the random selection of an imaging sight prior to any fluorescent signal being present. It was also particularly important to get the correct plane of focus, as if not correctly set this would subsequently confound the ability to accurately assess changes in the fluophore intensity and derive a measure of transporter activity from this. Hence, a modified protocol involving the initial pretreatment with a 1:100 dilution of NTUA was developed and can be seen in the work presented in this thesis. This approach allowed the identification of an area of average innervation density close to the serosal surface. This permitted the consistent gathering of quantifiable data, using the 1:100 dilution to provide a baseline for interpreting subsequent transporter rate during the period of exposure to a higher (1:10) concentration of NTUA. Unfortunately, the undesirable cellular labelling of apparently non-neuronal tissue was rarely apparent with the 1:100 solution and therefore it was still possible that the accurate tracking of terminals would not subsequently be possible due to the appearance of contiguous non-neuronal labelling. Using this

protocol, the data can be assessed by either of two main methods. Firstly, an absolute measure of the transporter rate can be obtained from the slope of the linear (first 12 minutes) portion of the uptake curve following the switch to the 1:10 dilution. This has the advantage of simplicity, but doesn't account for changes in the illumination intensity or photomultiplier gain. Therefore, to allow for pooling of data between experiments, a normalization procedure was used, dividing this slope by an average of the fluorescent signal in the 6 minutes immediately prior to the switch to the 1:10 solution. This allows a measurement of the relative (but not absolute) transporter rates between the control (1:100) and test (1:10) conditions.

Validating NTUA as a tool for monitoring sympathetic terminals

The experiments with the NET inhibitor desipramine (1 μM) showed that the uptake of NTUA into terminal-like structures was abolished, implying that these structures accumulate unmasked NTUA through the action of this transporter. Further, amphetamine, which has previously been demonstrated to reverse NET activity (Ross & Gosztonyi, 1975), increased the rate of removal of NTUA from the terminal-like structures in a manner similar to that seen with other NET substrates such as bretylium. The non-neuronal labelling was unaffected by desipramine, and therefore cannot be attributed to the action of NET.

The experiment with EGFP-ChAT mice indicates that the neuronal structures labelled with NTUA are not cholinergic, with the interesting corollary that there are no cholinergic/noradrenergic terminals in the mature mouse vas deferens.

This confirms previous immunohistochemical conclusions based on the absence of co-expression of vACh transporter and tyrosine hydroxylase (Jen *et al.*, 1999). The co-localization of NTUA in orthogradely labelled terminals, with Alexa 594-dextran, is further evidence that the strings of punctate labelling is, in fact, contained within varicosities within nerve terminals.

Unfortunately, it was not possible to totally exclude the possibility of NTUA uptake into sensory nerve terminals, the third major population of neurones found in the mouse vas deferens. However, this would seem unlikely given the effects of desipramine and amphetamine on blocking the uptake and increasing the removal of NTUA respectively from the terminal-like structures and given the lack of evidence for NET expression on sensory nerves.

NTUA as a tool for studying VMAT and vesicular tracking

The punctate appearance of the NTUA fluorescence immediately suggested that the NTUA was restricted to the varicosities of the nerve terminal. Such localization seemed attributable to the further uptake of NTUA into vesicles by the action of VMAT. This was likely as most substrates for NET are also substrates for VMAT and this hypothesis was supported by reserpine, an inhibitor of vesicular uptake (Iversen, 1967; Iversen *et al.*, 1967), which prevented the punctate appearance of NTUA instead resulting in a fluorescent pattern expected of more diffuse spread throughout the nerve terminals. The resulting pattern of fluorescence was comparable to the Falck-Hallarp type of fluorescence found in the terminals of animals exposed to reserpine and then

acutely re-exposed to catecholamines (Hamberger *et al.*, 1964). In addition the localized bleaching with slow recovery during FRAP experiments suggests that NTUA is tightly restricted to varicosities, most likely by containment within vesicles.

Reserpine, well known as an inhibitor of VMAT-2 (for review, see Varoqui and Erickson (1997)), also increased the rate of NTUA fluorescent decreased during the washout period. This is consistent with a cytoplasmic location of intra-terminal NTUA in the presence of reserpine resulting in an increased susceptibility of loss by reverse transport through NET. Given this observation, it may be considered surprising that NTUA uptake was unaffected by reserpine without the NTUA being sequestered by the normal action of VMAT except noradrenaline uptake by NET has been shown to be unaffected by resperine (Kopin & Gordon, 1962). The assumption that NTUA is normally sequestered by its uptake into vesicles may also be questioned given the results with amphetamine which increased the rate of loss of NTUA fluorescence during the washout period. However, previous work has demonstrated amphetamine's ability to reverse VMAT as well as NET transport (Pifl *et al.*, 1995; Pifl *et al.*, 1999).

The FRAP experiments suggest that it may be possible to use NTUA to monitor the movement of vesicles between neighbouring varicosities given the gradual and occasionally diffraction-limited spots of fluorescence observed. However, to what extent this would be a useful method given the demonstrated low probability of exchange remains questionable and unproven. Nevertheless, this

does suggest that NTUA may be used to assess NET activity at a very fine level, potentially to a single nerve terminal varicosity.

Unfortunately, it also seems unlikely that NTUA may be used to assess VMAT activity in a quantitative manner given reserpine had no affect on NTUA uptake. However, qualitative information may be made regarding VMAT's activity from an assessment of the punctate or diffuse pattern of NTUA fluorescence within the nerve terminal.

Specificity of NTUA

The experimental results with desipramine and amphetamine, as previously discussed, strongly suggest that NTUA uptake into terminals is dependent on NET. Additionally, given rate of uptake was roughly zero in the presence of reserpine, this indicates that uptake into varicosities is exclusively NET dependent. Such a hypothesis is also supported by the evidence that NET is densely expressed on noradrenergic terminals in the mouse vas deferens (Schroeter *et al.*, 2000) and, although desipramine can also act as an inhibitor of DAT, its specificity is roughly 4000 times greater for NET (review by Baldessarini (2001)).

Although it is possible to be reasonably confident that varicose uptake is specific to NET activity, the perinuclear, non-neuronal fluorescence observed does raise questions about the specificity of NTUA for NET. The non-neuronal labelling was unaffected by the use of NET, DAT and SERT inhibitors with the latter two also

not appearing to affect terminal fluorescence. In addition, uptake-2 inhibitors were unable to abolish this fluorescence. This does raise the intriguing question of the route by which NTUA is entering the apparently non-neuronal tissue; direct entry to the non-neuronal tissue by cellular damage would seem unlikely and whilst entry via another transporter is possible the most likely candidates have all been assessed. However, redundancy is present in many physiological systems and it is noted that experiments using multiple inhibitors at the same time were not conducted therefore the possibility does remain that it may be via a combination of these transporters that NTUA causes the non-neuronal labelling. The investigation of SERT activity may be questioned as previous studies have failed to demonstrate functional SERT activity in vas deferens (Yaris *et al.*, 2003) but for completeness this test was carried out given NTUA is known to be a substrate for all three transporters.

Finally, the usefulness of NTUA as a tool to identify sympathetic terminals and monitor NET activity would be diminished if NTUA were to compete with noradrenaline for NET uptake or to alter contractile responses to noradrenaline. However, it has been shown that NTUA (1:10) made no significant effect on the rate of relaxation for contractile responses compared to controls whereas desipramine slowed this response (Parker *et al.*, 2010). This work also demonstrated that NTUA reduced the peak amplitude of neurogenic contractions but not those elicited by exogenously applied noradrenaline. Therefore the use of NTUA to assess NET activity in the mouse vas deferens may be considered a useful technique which does not alter the normal physiological activity of this transporter. However, the presence of NTUA when measuring some contractile

parameters will modify the results obtained most likely due to a pre-junctional effect of NTUA on noradrenergic transmission, most likely by displacement of NTUA from vesicles.

The future use and limitations of NTUA

The development and validation of using NTUA within an isolated organ was conducted during the preparation of this thesis and has been demonstrated as a method for monitoring both NET activity and vesicle movement between varicosities. NTUA now provides an optical method by which NET transport rate can be assessed to the level of an individual varicosity. The only problems associated with the technique arise from the non-neuronal labelling. However, it appears that despite this complication, sufficient data may be acquired from a normal number of tissue preparations.

The use of NTUA to study the effects of drugs on NET activity has already been demonstrated in the literature. Carbachol has been shown to decrease NET activity measured using NTUA via a muscarinic pathway (Parker *et al.*, 2010). Therefore, this technique was used to investigate the potential effects of THC in altering NET activity which were discovered in the experimental work for this thesis.

Testosterone

The androgen receptors are the classical pathway by which testosterone is thought to mediate its affects on cells. As with signalling for all members of the steroid hormone receptor superfamily, this involves entry of testosterone or any other androgen into the target cell by passive diffusion through the plasma membrane. The molecule must also pass through the nuclear membrane where, as with androgen receptors, the receptor is expressed within the nucleus. Having bound to the receptor, the resulting steroid-receptor complex activates by a series of confirmational changes in the tertiary and quaternary structure of the steroid and receptor proteins. Once activated the complex can bind to the chromatin in specific DNA sequence regions known as the steroid response elements where it forms a transcription factor regulating the synthesis of the mRNAs and encoded proteins for the steroid responsive gene lying downstream of the binding site. Changes in the rate and amount of synthesis of these proteins thus becomes the ultimate outcome mediated by androgen signalling.

However, several observations with androgens and other steroid hormones in many different animal and cell systems contraindicate the above mechanism as the sole pathway by which steroids mediate their effects. These effects include responses:

- which occur too rapidly (within seconds to minutes) to be attributable to changes in mRNA and protein synthesis;
- which cannot be inhibited by antagonists of the classic, genomic steroid receptors;

- which cannot be blocked by the use of inhibitors of transcription and translation;
- which occur in cells which that do not carry out transcription or translation (e.g. spermatozoa);
- which occur in cultured cells not expressing the classic, genomic steroid receptors;
- which are caused by steroids coupled to high-molecular weight substances that prevent free diffusion of the molecule through the plasma membrane.

In addition, some instances are recorded where specific steroids mediate a response which cannot be mimicked or displays a totally different potency profile using a similar steroid with slightly different chemical structure. Such observations cannot be explained by the classical, genomic receptors with many hypothesizing that they are mediated by a separate group of receptors expressed on the surface of the plasma membrane signalling through typical extranuclear pathways such as G-protein coupled receptors and tyrosine kinase.

However, another mechanism has also been proposed whereby the classic steroid receptors have actions independent of any interaction with DNA instead interacting with signal transduction proteins in the cytoplasm causing changes in many cellular processes such as ion transport.

Effects of testosterone on contraction

In this project, the effects of short term incubation with testosterone on neurogenic contractions in the mouse vas deferens were investigated. The results showed a concentration-dependent inhibition of neurogenic contractions which became statistically significant at concentrations of testosterone greater than or equal to 100 μM with a calculated EC_{50} of 118.9 μM. These findings draw comparison with similar studies in rat isolated urinary bladder where neuromuscular transmission was inhibited in a concentration-dependent manner (Hall *et al.*, 2002). In this work a threshold concentration of 100 μM testosterone was also found to give statistically significant inhibition.

In other studies, lower concentrations of testosterone have been found to reduce the magnitude of smooth muscle contractions. In studies on blood vessels, testosterone and other sex hormones have been shown to reduce the contractility of smooth muscle. This has been show in both endothelium dependent (Chou *et al.*, 1996; Geary *et al.*, 2000; Tep-areenan *et al.*, 2002; Thompson & Khalil, 2003) and endothelium independent preparations (Yue *et al.*, 1995; Perusquia & Villalon, 1999; Quan *et al.*, 1999; Teoh *et al.*, 2000). There was variation in the nature and sensitivity of the response according to the vessel type and species used as an experimental model. However, in the majority of these studies the concentration of testosterone required to elicit a significant response was generally lower than that used in this project and within the normal physiological range (Mayo Medical Laboratories). Similar results have also been shown in myometrial tissue from pregnant humans where testosterone and other androgens caused a reduction of spontaneous

myometrial contractility as well as those induced by KCl (Perusquia *et al.*, 2005). Again, these results were within a normal physiological range for testosterone and therefore it was hypothesized that non-genomic effects of androgens may play a role in maintaining pregnancy.

Testosterone has also been demonstrated to have non-genomic effects on cholinergic contractions in smooth muscle. In one study on rabbit tracheal smooth muscle, testosterone at concentrations of or above 1 nM caused a significant relaxant effect to tissue precontracted with either acetylcholine or cabachol (Kouloumenta *et al.*, 2006). This was established to be a non-genomic effect that involved the epithelium and NO pathways. However, interestingly in this study, testosterone had no effect on tissue precontracted with KCl.

It is also interesting to note that in this project the vehicle for testosterone, ethanol did not appear to have any inhibitory effects of its own on the contractile response of the vas deferens. In previous studies using rat and rabbit bladder models, such an effect has proved a confounding factor at similar concentrations to the 0.1% used in this experimental work (Ohmura *et al.*, 1997; Kim *et al.*, 1999; Hall *et al.*, 2002).

Postjunctional effects of testosterone

In order to identify the location for the inhibitory effect of testosterone, experiments were conducted using α,β-metATP to evoke purinergic contractions without a neuronal component. In these experiments, 100 μM testosterone

significantly inhibited contractions evoked by the exogenous agonist suggesting that the inhibitory effect of testosterone was postjunctional in origin. Previous studies in the rat vas deferens have also shown a postjunctional inhibitory effect of testosterone on exogenously induced contractions elicited by KCl and Ca^{2+} (Lafayette *et al.*, 2008).

The Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM, was used to assess the effect of testosterone on WCTs in the mouse vas deferens. In these experiments, testosterone significantly reduced both the mean amplitude of WCTs and the number of WCTs per experimental series. As WCTs are attributed to the opening of L-type voltage-gated Ca^{2+} channels, it is believed that testosterone's effect is via an ability to modify the opening probability of these channels within the mouse vas deferens. Interestingly, nifedipine, a member of the dihydropyridine family of calcium channel blocker which primarily block L-type Ca^{2+} channels, was able to further reduce the amplitude and frequency of WCTs. This effect was both significantly different from control readings and from reduced amplitude and frequency of WCTs in the presence of testosterone. This would suggest that testosterone's effect was either via a modulatory mechanism or as a partial antagonist with at least a residual component of channel function still active thereby allowing nifedipine to demonstrate its well established actions.

Previous work in other tissues has also led to suggestions that testosterone may modify calcium dynamics within smooth muscle cells. In the rat uterus, testosterone along with other steroids, was demonstrated to relax contractions evoked by KCl (Gutierrez *et al.*, 1994). These contractions are known to depend

on extracellular calcium entry through L-type channels. The relaxing effect was counteracted by exposure to $CaCl_2$, therefore the authors conclude that the effect of testosterone must be occurring at a membrane level. In coronary smooth muscle, testosterone has been shown to cause significant and concentration-dependent relaxation of the sustained contractions evoked by PGF2α and KCl (Crews & Khalil, 1999a, b). However, testosterone and the other steroids under investigation had no effect on the transient contractions evoked by caffeine, an activator of Ca^{2+} release from intracellular stores, in a Ca^{2+}-free extracellular solution. This work was conducted in deendothelialized coronary artery strips isolated from castrated male pigs and deendothelialized aortic strips isolated from male and female Sprague-Dawley rats.

Isolated cells studies have also demonstrated a specific effect for testosterone on L-type Ca^{2+} channels with no apparent effect on T-type Ca^{2+} channels. In work using the model vascular smooth muscle cell line, A7r5, and microflurimetry, testosterone caused a dilatory effect on contractions evoked by KCl (Hall *et al.*, 2006). This effect persisted in the presence of T-type Ca^{2+} blocker, pimozide, but was not apparent in the presence of nifedipine.

There is also evidence from patch-clamp experiments that testosterone binds to L-type Ca^{2+} channels causing a concentration-dependent inhibition of their currents (Scragg *et al.*, 2004; Scragg *et al.*, 2007). This result was found using physiologically concentrations of testosterone (IC_{50} 38 nM) in A7r5 smooth muscle cell line and HEK 293 cells stably expressing either L- or T-type Ca^{2+} channels. Interestingly, a single point mutation trialled in this work almost

completely abolished the L-type channel sensitivity to nifedipine and also rendered the channels insensitive to testosterone.

Evidence for non-genomic and non-androgen receptor mediated effects

The effect of testosterone observed during this project may logically be attributed to a non-genomic mechanism of action as the effects occurred with a short incubation period of 20 minutes. Classical genomic responses, as outlined in more detail above, usually require hours or days to appear due to the signalling pathways involved and their ultimate effect of modifying the transcription of genes into mRNA and its subsequent translation into protein molecules (Falkenstein *et al.*, 2000b).

The use of translation inhibitors such as cycloheximide are well characterized for demonstrating if a steroid response persists in the presence of such a compound and therefore provides more concrete evidence for a non-genomic mechanism of action (Beato & Klug, 2000; Losel *et al.*, 2003). In this project cycloheximide was pre-incubated with the tissue samples for three hours and the inhibitory effect of testosterone persisted in being observed. This increases confidence in the proposal that this project demonstrates a novel non-genomic effect of testosterone within the mouse vas deferens. Although some studies have used a translation inhibitor alone to confirm non-genomic effect (Lafayette *et al.*, 2008), on reflection it would have provided a more robust confirmation to additionally conduct experiments using a transcription inhibitor such as actinomycin D

(Perry & Kelley, 1970) as is the case in other studies of non-genomic steroid effect (Perusquia *et al.*, 2005).

The genomic testosterone receptor antagonist, flutamide, is widely used to investigate if the actions of testosterone may be attributed to this classical signalling pathway (Neri *et al.*, 1972; Peets *et al.*, 1974; Nazareth & Weigel, 1996). In this project, pre-incubation with flutamide did not modify the inhibitory effect of testosterone on neurogenic contractions in the vas deferens.

In summary, the data collected during this project suggests that the inhibitory effect which testosterone elicited on neurogenic contractions in the mouse vas deferens may be attributed to a non-genomic, non-androgen receptor pathway.

Evidence for an additional pre-junctional effect?

The electrophysiological experiments conducted with testosterone revealed a possible second novel effect for this steroid on autonomic and smooth muscle signalling. Namely, a decrease in the facilitation of successive EJPs within a train. However, there was no significant difference in the amplitude of the first EJP for each train versus vehicle controls.

Facilitation is observed in many smooth muscle tissues when the amplitude of successive EJPs evoked by a train of stimuli gradually increase in amplitude until a constant level is reach (Blakeley *et al.*, 1984b). This is normally observed with short, low frequency trains. The rate of facilitation increases with the frequency

of nerve stimulation. It is believed that facilitation contributes to the process of causing sufficient net membrane depolarization to reach the threshold for opening voltage-dependent Ca^{2+} channels (Brock, 1992).

The exact molecular mechanism for facilitation has always been widely debated and remains to be fully elucidated to date. However, one popular mechanism is that of the residual calcium hypothesis (Katz & Miledi, 1965) whereby a residual change in the Ca^{2+} concentration persists within the nerve terminal after each successive stimulation (Brain & Bennett, 1997). It is also known that various Ca^{2+} channels are thought to play a role in the calcium transients observed within the sympathetic terminals of the mouse vas deferens (Wright & Angus, 1996; Waterman, 1997). Meanwhile, there is no evidence for nifedipine affecting junction potentials in the vas deferens (Surprenant *et al.*, 1983; Beattie *et al.*, 1986; Stjarne *et al.*, 1991). This raises the intriguing hypothesis that testosterone may also have a non-genomic effect on these other types of Ca^{2+} channels and thereby reduce the rate of accumulation of residual calcium remaining within the nerve terminal between successive stimulations.

Physiological and pathophysiological effects of androgens on the autonomic nervous system

As can be observed from the various papers cited in the discussion above, non-genomic effects of testosterone have been found at the junctions between the autonomic nervous system and smooth muscle in various species. The range of concentrations at which these effects have been observed has varied widely from

supraphysiological micromolar concentrations, as seen in this project, down to physiological nanomolar concentrations (Heaton, 2003; Losel *et al.*, 2003).

Many would argue that even lower concentrations such as 50 μM testosterone, which has been shown to cause half-maximal relaxation in rat aorta (Costarella *et al.*, 1996) are non-specific and non-physiological, and therefore of limited value in being studied. However, caution should be attached to any such perspective. First, an *in vitro* experimental system should not be considered wholly comparable with an *in vivo* model or non-experimental physiology. *In vivo*, drugs reach receptors by local diffusion from their source of production or release, or by local delivery via the bloodstream. *In vitro* experiments require the drug to diffuse throughout the organ bath, cross multiple tissue layers and accumulate at the target protein sites. Therefore the concentration of drug at the target proteins is probably much lower than in the organ bath (Busatto & Jurkiewicz, 1985).

Secondly, there is benefit to be gained from an understanding of effects of higher than normal concentrations of testosterone. Androgens are widely used in the treatment of many conditions including hypogonadism, myopathy, mood disorders and osteoporosis (Heaton, 2003). It has been demonstrated that intramuscular injections of testosterone can cause supraphysiological concentrations to persist for several days (Dobs *et al.*, 1999).

Interestingly, a range of mechanisms has also been observed or proposed by which testosterone exerts non-genomic effects on the autonomic nervous system

and smooth muscle cells. The effect on Ca^{2+} currents observed in this and other projects (Gutierrez *et al.*, 1994; Crews & Khalil, 1999a) is just one mechanism. For instance in one study using the rat aorta, an increase in efflux of potassium ions causing a hyperpolarization was the proposed mechanism for relaxation (Costarella *et al.*, 1996). Similar transduction pathways for the non-genomic effects of steroids involving K^+ channels have been seen in many smooth muscle preparations including vascular (Yue *et al.*, 1995; Deenadayalu *et al.*, 2001; Tep-areenan *et al.*, 2002; Jones *et al.*, 2004) and duodenum (Diaz *et al.*, 2004). However, other studies showed no role for K^+ channels in the signal transduction pathways (Nakajima *et al.*, 1995; Ogata *et al.*, 1996).

With regard to the role of signalling pathways involving neighbouring tissues to the smooth muscle cells there is a mixture of observations. In the rat vas deferens (Lafayette *et al.*, 2008) an epithelium-independent effect was characterised demonstrating no role for nitric oxide, cGMP or epithelial K^+ channels. Whereas an endothelium-dependent mechanism was observed in human coronary artery (Mugge *et al.*, 1993), rat aorta (Honda *et al.*, 1999), canine coronary artery (Chou *et al.*, 1996) and airway smooth muscle (Kouloumenta *et al.*, 2006). These pathways do seem to involve the release of nitric oxide.

What is clear, is that research into the non-genomic effects of steroids and their physiological and pathophysiological roles on modulating the autonomic nervous system and smooth muscle cells is still in its infancy. Further work will

no doubt shed further light on the specific signalling pathways involved and the current variation observed both between and within experimental models.

Cannabinoids

Cannabinoid research developed from the study of the pharmacological effects of the material from the plant, *Cannabis sativa* (Mechoulam *et al.*, 1976; Razdan, 1986, 1987). It was established that the primary active compound from the plant was a chemical with an ABC-tricyclic ring system possessing a benzopyran moiety, Δ^9-THC (Gaoni & Mechoulam, 1964a). Many other active compounds were also identified from the plant including Δ^8-THC, cannabinol (CBD), cannabidiol (CBD) and cannabichromene. These compounds all became known as the Classical Cannabinoids.

Two G-protein-coupled receptors, CB1 and CB2, have been discovered over the years in mammalian tissue. Δ^9-THC is a partial agonist and binds equally well to both receptors. Studies have revealed variation in the location of expression of the two receptors: CB1 is localized inside and outside the central nervous system whilst CB2 is mainly found in the periphery (Howlett *et al.*, 2002; Pertwee, 2005). There is also evidence for receptor mediated effects of cannabinoids which are not attributable to CB1 or CB2. This has led to the hypothesis that additional cannabinoid receptors exist although their full identification and characterization remains elusive (Jarai *et al.*, 1999; Wiley & Martin, 2002; Begg *et al.*, 2005).

Studies of the cannabinoid receptors have also revealed membrane-phospholipid-derived endogenous ligands , known as endocannabinoids, which with the receptors form an endocannabinoids system (Di Marzo, 1999). The two

most significant endocannabinoids which have received the greatest characterization and use are *N*-archidonoylethanolamine (anandamide, AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995).

Although the exact physiological and pathophysiological functions of the endocannabinoids system have yet to be fully identified, the potential therapeutic opportunities that research suggests may be available are diverse. Modulation of the endocannabinoid system is hoped to be used in the treatment of multiple sclerosis (Baker *et al.*, 2001; Malfitano *et al.*, 2005), obesity (Cota *et al.*, 2003; Pagotto *et al.*, 2005), inflammatory and neuropathic pain (Rice *et al.*, 2002; La Rana *et al.*, 2006), blood pressure regulation and cardiovascular disease(Pacher *et al.*, 2005) and allergic asthma (Lunn *et al.*, 2006), to name but a few conditions. It is hoped that the information gathered during the course of this project adds at least one small brick to the growing wall of knowledge regarding cannabinoids that in the future their role may be more fully understood, and therapeutic benefit may be harnessed for patients by taking advantage of the endocannabinoids system.

Vehicles for dissolving THC

Cannabinoids generally have a very low water solubility. Consequently, a non-aqueous vehicle is required for their storage and delivery to experimental tissues. They are usually supplied as powders or in ethanol solution. Made up into aliquots of an appropriate size for a day's experimental work, they are most

stably stored in ethanol at –20°C. However, if maintained in ethanol for admixture with PSS in an organ bath, the concentration of ethanol would frequently be too high and prove a confounding factor in the data obtained. Therefore one of two vehicles in normally used, either a mixture of Tween-80 and PSS (Pertwee *et al.*, 1992) or DMSO (Thomas & Pertwee, 2006).

As described in the methodology, it was rapidly established during preliminary experimental work that the use of DMSO was easier on two accounts. First, the highly viscous nature of Tween-80 made it challenging to ensure that THC was evenly mixed throughout the combined solution of this vehicle and PSS. It is also noted that given the high lipohilicity of THC (Thomas *et al.*, 1990), it may be sequestered by Tween 80 delaying equilibration time between THC free in solution and that present in the tissue (Roth & Williams, 1979). Secondly, as Tween-80 may be considered a detergent or surfactant, it generated large quantities of foam and bubbles within the organ bath given the ongoing oxygenation of the PSS in the chamber. No such problems were encountered with the use of DMSO although the serial dilution process was time consuming.

THC versus endocannabinoids

Initial interest in cannabinoids arose through the marked psychoactive effects that had been observed throughout centuries with the medicinal and recreational use of the cannabis plant, *Cannabis sativa* (Butrica, 2002). The main psychoactive compound of the cannabis plant, THC (Pertwee, 1988), was isolated by Gaoni and Mechoulam (1964a). It has similar binding properties to

both CB1 and CB2 receptors with reported K_i values of 5.05 – 80.3 nM for CB1 and 3.13 – 75.3 nM for CB2 (Pertwee, 2008). It is also reported to have good binding at the putative cannabinoid receptor GPR55 (for review see Pertwee *et al.* (2010)). The classic endocannabinoids, AEA and 2-AG, show a much greater difference in K_i values between the two receptors without being receptor selective. A similar problem occurs when considering other phytocannabinoids and synthetic cannabinoids. The stability of the agonists must also be considered: all cannabinoids are light sensitive however some, such as AEA, require even more specialist handling due to a high propensity to become oxidized (Thomas & Pertwee, 2006). Additionally, all cannabinoids also appear to have activity at non-CB1 and non-CB2 targets, however this is particularly the case for endocannabinoids. Citing AEA as an example, again, this cannabinoid also has activity at vanilloid TRPV1 receptors (Pertwee, 2005) which are known to be present in the mouse vas deferens (Sheykhzade *et al.*, 2011b).

Consequently, THC may be considered a good representative cannabinoid to use experimentally with the judicious use of receptor-specific antagonists as required. The additional consideration that it is the cannabinoid compound that most humans are likely to come into contact with makes it an appealing choice as well. Finally, along with nabilone, a synthetic analogue of THC, it was one of the first two cannabinoids licenced for medical use.

At this point, it is also interesting to note that it was initially thought that THC exerted its pharmacological properties by perturbation of the phosopholipid constituents of biological membranes. This view was held partly because of the

high lipid solubility and low water solubility of THC (reviewed in Pertwee (1988)). Of course, this view changed in the late 1980s when specific cannabinoid receptors were discovered in mammalian tissues.

Inhibition of contraction

Having established a preference for using DMSO as a vehicle, preliminary studies were conducted using 100 nM THC. In these initial experiments, THC caused a significant inhibition of neurogenic contractions reaching a maximal effect over 30-40 minutes. This result is comparable to previous studies (Pertwee *et al.*, 1992; Pertwee *et al.*, 1993) therefore an appropriate and stable response could be elicited for the purposes of this project.

It is interesting to consider these effects with respect to the plasma concentration of THC to which individuals are acutely exposed when smoking cannabis. In experiments studying inhalation of THC in humans, plasma concentrations of THC on average ranged from 358 nM to 73 nM in the first 30 minutes after inhalation using cigarettes with 3.4% THC content (Abrams *et al.*, 2007). Interestingly, the authors of this paper also reported the observation of self-titration of THC intake. Therefore, a standard concentration of 100 nM THC may be considered appropriate for interpreting effects with regard to persons who expose themselves to the acute effects of cannabis.

Little evidence for a postjunctional effect of THC

Classic pharmacology experiments were conducted using the exogenous agonists phenylephrine and α,β-metATP to evoke adrenergic and purinergic contractions respectively without any nerve stimulation. In these studies, THC had no significant effect on the amplitude of contractions evoked by phenylephrine. Such a result may be interpreted as suggesting that the previous results observed with THC on neurogenic contractions may be attributed to a pre-junctional effect of THC.

On the other hand, THC did cause a significant inhibition of contractions evoked by α,β-metATP suggesting a post-junctional effect on this signalling pathway. However, the electrophysiological studies did not give any indications of a post-junctional effect of THC as resting membrane potentials were unaffected by the presence of THC versus controls. Additionally, the rise and fall times of EJPs were unaffected giving no indication for a direct or indirect effect of THC on the P2X receptors.

These findings are partially consistent with the widely held view that the effects of cannabinoids on sympathetic transmission are pre-junctional. However, it should be noted that one study using a range of cannabinoid agonists has demonstrated a possible post-junctional effect also inhibitory in nature (Ralevic & Kendall, 2002a) in rat mesenteric arteries. With high concentrations of cannabinoids they observed an inhibition of contractile responses to noradrenaline, methoxamine, α,β-methylene ATP, and vasopressin. Additionally they observed inhibition of neurogenic contractions which was not prevented by

the application of CB1 or CB2 antagonists. It is possible that the effect observed with the exogenous application of α,β-metATP in this study may be similar to this.

In summary, it would be hasty and rash to rule out the possibility that post-junctional cannabinoid receptors may exist and we have merely yet to establish the receptors and endogenous agonists involved.

Distinguishing the effects of THC on purinergic and noradrenergic components of contraction

Numerous studies have demonstrated a biphasic pattern to neurogenic contractions within the vas deferens of many non-human species (Ambache & Zar, 1971; Swedin, 1971a; Westfall & Westfall, 2001) composed of an initial transient twitch and a secondary tonic component known as the hump. This observation lead to suggestions that a second neurotransmitter to noradrenaline was involved in eliciting these responses with Burnstock (1972) proposing ATP as the second transmitter. It was later demonstrated that stimulation of the vas deferens causes the release of purines (Westfall *et al.*, 1978) strengthening the hypothesis that ATP acted as a co-transmitter with noradrenaline.

Debate also exists over the nature of storage and release of these co-transmitters with four scenarios proposed (Knight *et al.*, 2003):

1. ATP and NA may be stored and released from the same vesicles;
2. ATP and NA may be stored and released from separate vesicles;

3. ATP and NA might be stored and released from different sets of varicosities;
4. ATP and NA may be stored and released from the same vesicles but in different proportions in different varicosities.

Consequently, it seemed appropriate to investigate whether 100 nM THC might have a differential effect on the purinergic and noradrenergic components of contraction.

In the initial experiments looking at the effects of THC over time, THC caused a proportionally and significantly greater inhibition of pharmacologically isolated noradrenergic neurogenic contractions than purinergic. However, in concentration-response experiments, a similar pEC_{50} of 1.8 nM and 4.3 nM was established for the action of THC on the noradrenergic and purinergic components of contraction respectively. This demonstrated that the different response in the initial experiments could not be attributed to an infra-maximal dose of THC to effect changes on the purinergic component.

Strangely, for the concentration-response experiments on the purinergic component of contraction only the response to 30 nM THC was significantly different from vehicle controls. It is believed that this may be attributed to two factors. The amplitude for the residual purinergic contractions in the presence of prasozin were much smaller in absolute amplitude than the residual noradrenergic contractions. Consequently, the relative error on the normalized purinergic data was greater. Additionally, although run-down was noted for both the noradrenergic and purinergic experiments, this also contributed to the even

smaller absolute contraction amplitudes for the purinergic data. Therefore, it is proposed that the data collected may have been underpowered by sample size to show a significant effect of THC on residual purinergic contractions. This may also explain for the apparently greater proportional inhibition to the noradrenergic component of contraction.

It is interesting to contrast this finding with data from the perfused rat mesenteric bed preparation in which reduction of purinergic responses was greater (Pakdeechote *et al.*, 2007a). However, this finding could be attributed to the use of U46619 to raise tone in the preparation as it has been shown that this compound reveals a purinergic component of neurotransmission (Pakdeechote *et al.*, 2007b) and that the contribution of ATP transmission increases with raised tone (Rummery *et al.*, 2007) despite purinergic responses being much smaller than noradrenergic responses. Although the differential effects observed on the contractile responses to the two neurotransmitters in this project may be interpreted as arising from different pathways of modulation, I believe that they arise from a nonlinear relationship between transmitter release and the contractile response elicited.

Inhibition of nerve terminal Ca^{2+} influx as a unifying mechanism to explain inhibition

The inhibition of neurogenic contractions by THC, both those elicited by purinergic transmission and those elicited by noradrenergic transmission, suggested that a common pathway was involved inhibiting the release of

neurotransmitters from nerve terminal varicosities. The confocal microscopy studies examining calcium dynamics within NTVs demonstrated that THC significantly reduced the amplitude of NTV calcium transients. It is known that calcium transients within the NTV are dependent upon functioning N-type Ca^{2+} channels (Brain & Bennett, 1997) therefore THC must be modifying the activity of these channels. This is the first time that this effect of THC has been demonstrated at the level of an individual NTV.

It is well established that both CB1 and CB2 receptors are G protein-coupled receptors that regulate by inhibition adenylyl cyclase and therefore cellular levels of cAMP (Matsuda, 1997; Rhee *et al.*, 1998). It has been shown, mostly from studies in the central nervous system, that downstream signalling via CB1 receptors causes inhibition of voltage-gated Ca^{2+} channels (L, N, and P/Q type) (Felder *et al.*, 1993; Mackie *et al.*, 1993; Mackie *et al.*, 1995) and activates the inward rectifying K^+ channels (Mackie *et al.*, 1995; McAllister *et al.*, 1999). There is also evidence that cannabinoids causes receptor-independent changes in the functional properties of many voltage-gated ion channels including Ca^{2+} channels, Na^+ channels and various types of K^+ channels (Oz, 2006). Effects on ligand-gated ion channels have also been observed.

Previously, it has also been demonstrated that similar signalling pathways may operate in the peripheral nervous system as well. In the guinea-pig myenteric plexus, low levels of external Ca^{2+} have been shown to enhance cannabinoid-induced inhibition of neurogenic contractions (Coutts & Pertwee, 1998). The inverse effect was observed by elevating the external Ca^{2+} concentration.

Attenuation of the response also occurred when intracellular levels of cAMP were increased through the use of forskolin, 8-bromo-cAMP or a phosphodiesterase inhibitor.

The results from this project and the study described above, are both consistent with a negative coupling between CB1 receptors and voltage-gated Ca^{2+} channels via cAMP.

THC's effects on long trains of stimuli – a surprise!

The effects of THC were further characterized in contraction experiments looking at variable trains of stimulation that might alter the proportions of ATP and NA released. First, experiments were conducted using increasing lengths of trains of stimulation on the pharmacologically isolated noradrenergic and purinergic components of contraction. In these experiments, THC had no significant effect upon the peak area of purinergic contractions versus DMSO controls. However, for the noradrenergic component of contraction, THC actually caused a significant increase in the peak area versus DMSO controls for stimulation with trains of 100 and 200 pulses.

This effect was not seen in the presence of inhibitors of either CB1 or CB2 receptors. Additionally this effect was not observed in the presence of desipramine which inhibits NET. Surprisingly, in the presence of normetanephrine, which is known to inhibit uptake 2, this effect of THC also disappeared. Indeed, qualitatively an inhibitory effect for THC was thought to be

revealed again, however on further analysis this was not found to be statistically significant. Taken together, these findings would suggest that THC's potentiating effect on noradrenergic contractions is mediated via a receptor that is sensitive to both AM251 and AM630 but is therefore distinct from the CB1 and CB2 receptors. This effect would also appear to involv both NET and uptake 2 and therefore involve the regulation of noradrenaline uptake both pre- and post-junctionally.

The experiments using a range of frequencies of stimulation also involved long trains of stimulation. Interestingly, in these experiments, THC cause a significant inhibition of the twitch phase of the noradrenergic component of contraction but not the purinergic component. No significant effect was observed on the hump phase in either the purinergic or the noradrenergic component of contraction. Therefore, this data does not indicate an effect of THC regulating the uptake of NA from the junctional cleft.

NET inhibition: NTUA imaging

The novel technique of using NTUA to assess NET activity within individual nerve terminals and varicosities was used to further investigate the putative effect of THC on NET. In these experiments, THC was shown to decrease the rate of uptake confirming that THC has an inhibitory effect of this uptake transporter. However, it did not appear to cause reverse activity of NET. This effect did not occur in the presence of the CB1 receptor antagonist, AM251. This suggests that the effect on NET is mediated via the CB1 receptor or, as previously

hypothesized from the contraction data collected, on a novel receptor sensitive to both AM251 and the CB2 antagonist, AM630.

At this time, it is only possible to hypothesize upon the nature by which THC may regulate NET. First, it is noted within the literature that there is significant evidence for the regulation of NET activity by cellular protein kinases and phosphatizes (Ramamoorthy *et al.*, 2011). One specific example is the regulation of NET activity via a PKC-mediated pathway (Apparsundaram *et al.*, 1998a; Apparsundaram *et al.*, 1998b). It is also interesting to note that under certain conditions, there is evidence for stimulus trafficking of CB1 receptors causing G_q linkage as opposed to G_i (Lauckner *et al.*, 2005). There is also some evidence for cAMP/PKA-mediated regulation of NET activity, however this does not appear to be a general phenomenon as there is notable variation between species and cells lines used in experimental studies (Mandela & Ordway, 2006b).

Perhaps more intriguing is the work exploring how changes in neurotransmission through the sympathetic junction initiate NET regulation and modulate NET function. Early work showed that in the cat atria electrical nerve stimulation caused increased retention of exogenous noradrenaline (Gillis, 1963; Gillis *et al.*, 1966). It as has also been hypothesized that depolarization-induced mechanisms of NET regulation may exist due to the association between vesicular compartments and NET (Kippenberger *et al.*, 1999), and the co-localization of NET at sites of release (Savchenko *et al.*, 2003). Meanwhile calcium has been shown to increase NET function and trafficking within PC12

cells (Uchida *et al.*, 1998) and KCl stimulation of PC12 cells has caused increases in NET function (Mandela & Ordway, 2006a).

Together, these studies raise many exciting possibilities for how cannabinoids may regulate NET function. Mechanisms may involve a direct intracellular link between cannabinoid receptors and NET. Alternatively, the regulation of NET may occur secondary to changes in noradrenaline release and nerve terminal calcium dynamics.

NET inhibition: PC12 cells

The experiments using NTUA and PC12 cells to investigate the effects on the regulation of NET activity did not show any significant difference between exposure to THC or DMSO vehicle control. As there is disagreement regarding the expression of CB1 receptors on PC12 cells (see results), this may be interpreted in one of two ways. Either CB1 receptors are not expressed on PC12 cells or the effect of THC regulating NET activity is mediated via a non-CB1, non-CB2 receptor which is not expressed on PC12 cells.

Summary

In many ways I feel like the work presented in this thesis makes a very minor contribution to our understanding of the regulation and study of sympathetic junctional transmission. The comparison I often use when talking to friends and relatives is to say that it is like building a house and only laying two or three bricks in the whole building. However, it is also satisfying to reflect on new contributions to the field which have been presented above, namely:

- the development of a new experimental tool in the form of NTUA for dynamically studying NET activity within a whole organ preparation;
- the discovery and exploration of a non-genomic effect of testosterone on junctional transmission within the mouse vas deferens;
- the first use of a technique for imaging nerve terminal calcium dynamics within individual nerve terminal varicosities to study the effects of cannabinoids on sympathetic junctional transmission;
- the discovery and exploration of a novel effect of THC regulating the activity of NET within the mouse vas deferens.

Nevertheless, I hope that these findings may form a stepping stone on which others can further elucidate the functions and regulation of the sympathetic nervous system. There are areas of further research which I would have liked to explore myself but for the pressures of time when completing my experimental

work. Specifically, the use of calcium channel and signalling pathway antagonists to explore in more detail the pathways involved in the inhibitory effect of THC on neurotransmitter release, a greater study of the effects of endocannabinoids within this model system, and exploring whether THC's effect on NET activity could be found in other model systems (e.g. rat mesenteric artery). However, I am sure that as my work has ultimately gone in directions which I had never predicted, so it will be the case for those who follow on from me.

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