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# The Effect of Soluble Guanylate Cyclase Activators and a Nitric Oxide Releasing PDE 5 Inhibitor on Cavernosal and Anococcygeal Smooth Muscle Function in Conditions of Nitric Oxide Deficiency

A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Medicine (M.D.Res)

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# ABSTRACT

**Introduction:** PDE5 inhibitors improve erections by potentiating nitric oxide (NO)-cyclic guanosine monophosphate system. However, long-term diabetic patients have reduced efficacy secondary to dysfunction of NO system. The aim of this thesis was to investigate *in-vitro* effects of a PDE5 inhibitor (sildenafil), a soluble guanylate cyclase (sGC) activator (BAY41-2272) and an NO-releasing PDE5 inhibitor (NCX-911) on urogenital smooth muscle in conditions of NO deficiency.

**Method:** The effect of these compounds was investigated on tone and electrical field stimulation-induced nitrergic relaxation of cavernosal (human and rabbit) and anococcygeal (rat) smooth muscle and compared to an NO donor (spermine-NONOate) and non-specific sGC activator (YC-1) in the absence/presence of inhibitor of NO synthesis (L-NAME) or inhibitor of sGC (ODQ). In a diabetic rat model, these compounds were assessed in untreated and L-NAME-treated tissues from non-diabetic and diabetic animals.

**Results:** BAY41-2272 was more potent than YC-1 and spermine-NONOate at relaxing rabbit/human cavernosum. ODQ significantly decreased the potency of BAY41-2272 whereas L-NAME did not. BAY41-2272 potentiated nitrergic responses and partially reversed the inhibition of nitrergic responses by L-NAME.

NCX-911 and sildenafil were equally potent at relaxing rabbit and human cavernosum. In presence of L-NAME the potency of sildenafil decreased significantly. Both compounds potentiated nitrergic relaxations equally but failed to induce relaxation in the presence of ODQ.

Nitrergic relaxation was significantly decreased in the diabetic rats but still potentiated by BAY41-2272 but not by sildenafil or NCX-911. The potencies of NCX-911 and BAY41-2272 were unaltered but that of sildenafil was significantly reduced in the diabetic animals.

**Conclusion:** The rank of potency in control tissues was BAY41-2272 > NCX-11 = sildenafil; whereas in NO deficiency BAY 41-2272 > NCX-911 > sildenafil. Endogenous NO derived from nitrergic nerves is significantly decreased in diabetes. NO-releasing PDE5 inhibitors and sGC activators may be effective in management of diabetic erectile dysfunction.

# DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Date……

# ACKNOWLEDGEMENTS

I would like to acknowledge the kind support and dedication of both my laboratory supervisor Dr Selim Cellek and my clinical supervisor Mr David Ralph throughout my period of research at The Wolfson Institute of Biomedical Research and The Institute of Urology. I would also like to acknowledge the assistance of my colleagues in the laboratory Mr Asif Muneer and Mr Rowland Rees for their daily encouragement and comradeship. Most importantly, I would also like to thank my wife Harwinder for her unfounding love, kindness and patience throughout this period of my career.

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# ABBREVIATIONS

| | |
|---|---|
| Ach | Acetylcholine |
| ADP | Adenosine diphosphate |
| AGEs | Advanced glycation end-products |
| AMP | Adenosine monophosphate |
| ATP | Adenosine 5'-triphosphate |
| $Ca^{2+}$ | Calcium |
| BAY 41-2272 | 5- cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b] pyridine-3-yl] pyrimidin-4ylamine |
| cAMP | Cyclic adenosine monophosphate |
| CaM | Calmodulin |
| cGMP | Cyclic guanosine monophosphate |
| DAG | Diacylglycerol |
| EFS | Electrical field stimulation |
| EDHF | Endothelium derived hyperpolarizing factor |
| ET | Endothelin |
| eNOS | Endothelial nitric oxide synthase |
| iNOS | Inducible nitric oxide synthase |
| nNOS | Neuronal nitric oxide synthase |
| g | Gram |
| GAQ | Global assessment question |
| GMP | Guanosine monophosphate |
| GTP | Guanosine triphosphate |
| Hb | Haemoglobin |
| Hz | Hertz |
| $IP_3$ | Inositol 1,4,5-trisphosphate |
| IIEF | International index of erectile function |
| $K^+$ | Potassium |
| L-NAME | N –nitro-L-arginine methyl esther |
| MMAS | The Massachusetts Male Ageing Study |
| MPOA | Medial preoptic area |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| $Na^+$ | Sodium |
| NO | Nitric oxide |
| NANC | Non-adrenergic non-cholinergic |
| ODQ | 1H-[1,2,4]-oxdiazolo[3,4-a]quinoxalin-1-one |
| PDE | Phospodiesterase |
| PKA | cAMP-dependent protein kinase |
| PG | Prostaglandin |
| pGC | Particulate guanylate cyclase |
| $PGI_2$ | Prostacyclin |
| PKG | cGMP-dependent protein kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| PI3 KINASE | Phosphatidylinositol 3 kinase |

| | |
|---|---|
| PVN | Paraventricular nucleus |
| P | Probability |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| SEM | Standard error of the mean |
| SNP | Sodium nitroprusside |
| sGC | Soluble guanylate cyclase |
| STZ | Streptozotocin |
| $TXA_2$ | Thromboxane $A_2$ |
| VIP | Vasoactive intestinal peptide |
| V | Volts |

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# CHAPTER 1.

# INTRODUCTION

# 1. Introduction

## 1.1 Physiology of penile erection

Penile erection is a complex neurovascular event. It is widely accepted that the degree of contraction of the corpus cavernosal smooth muscle is an important determinant of the functional state of penile flaccidity (detumescence) and of erection (tumescence)(Andersson and Wagner, 1995). The balance between the contractile and relaxant factors is known to be controlled by both central and peripheral mechanisms and involves the interaction of three different systems (Andersson, 2001b):

A. The central nervous system

B. The peripheral nervous system

C. Vascular and cavernosal smooth muscle in the penis

### 1.1.1 The central nervous system

The central nervous system (CNS) coordinates incoming sensory information from a variety of sources which may be visual, auditory, cognitive/imaginative, tactile or olfactory. The central pathways integrating these inputs and controlling erectile function are complex and only partially understood. However, there is strong evidence to support the involvement of the paraventricular nucleus (PVN) and the medial pre-optic area (MPOA) within the hypothalamus in the control of erectile function (Giuliano and Rampin, 2000). The MPOA has been postulated to be an integrative centre that collects the input and redistributes to other structures within the CNS such as PVN (Giuliano and Rampin, 2000). The PVN in turn has been suggested to activate selective autonomic pathways that lead to erection (Giuliano and Rampin, 2000). The neurons from the PVN have been reported to project onto the spinal cord either directly (Luiten et al., 1985) or via the median forebrain bundle, pons and medulla (Steers, 1990). The descending pathways from PVN to the spinal cord have been reported to contain a variety of neurotransmitters such as oxytocin, vasopressin, encephalin and dopamine (Giuliano and Rampin, 2000).

Moreover, it has been demonstrated that the direct injection of various agents such as oxytocin, glutamate, nitric oxide (NO) and dopaminergic agonists into the PVN or direct electrical stimulation of PVN elicits episodes of penile erection(assi-Benelli et al., 1979;Giuliano and Rampin, 2000).

### 1.1.2. The peripheral nervous system

Within the spinal cord, there are various specific areas which contain integral components of the erectile system. These are known as the "erection centres" (Figure 1). The thoraco-lumbar erection centre is located between T1 and L2 and gives rise to the sympathetic outflow pathway. This connects to the urogenital tract via the pelvic, cavernosal and pudendal nerves. The sacral erection centre is located between the S2 and S4 segments of the spinal cord and gives rise to parasympathetic outflow pathway. These fibres reach the penis via the pelvic, cavernosal and pudendal nerves. Furthermore, the penis receives dense somatic input from sensory branches of the dorsal nerve, a branch of the pudendal nerve (Andersson and Wagner, 1995).

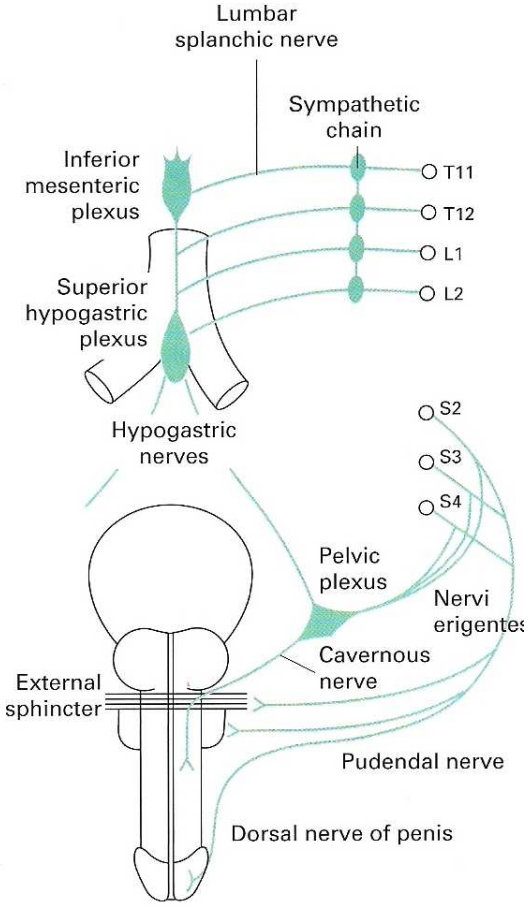


**Figure 1:** The nerve supply of the penis. From (Eardley and Sethia K, 2003)

The individual nerves innervating the penis may contain a number of different transmitters and as a result the nerves are categorized as either being adrenergic or cholinergic according to the predominant transmitter type present. However, non-adrenergic non-cholinergic (NANC) neurotransmitters may be found and indeed be co-localised with either adrenergic or cholinergic nerves. Nitric oxide (NO) is one of the NANC neurotransmitters which has now been widely accepted to be the major mediator eliciting relaxation of the penile smooth muscle (Lundberg, 1996).

During sexual arousal, NO has been reported to be released from parasympathetic nerve terminals (Hedlund et al., 2000b) and these nerves are therefore called nitrergic nerves (Moncada et al., 1997). The released NO causes relaxation of the smooth muscle allowing engorgement of blood into the cavernous space and leading to erection. Noradrenaline released from sympathetic nerves causes contraction of the blood vessels and smooth muscle of the corpus cavernosum, thus leading to detumescence of the penis. Erection of the penis is therefore regulated by a balance between pro- and anti-erectile mediators (Figure 2). Studies with human corpus cavernosum, suggest that when the two systems are simultaneously active, the nitrergic system is dominant over the sympathetic system (Cellek and Moncada, 1997b).

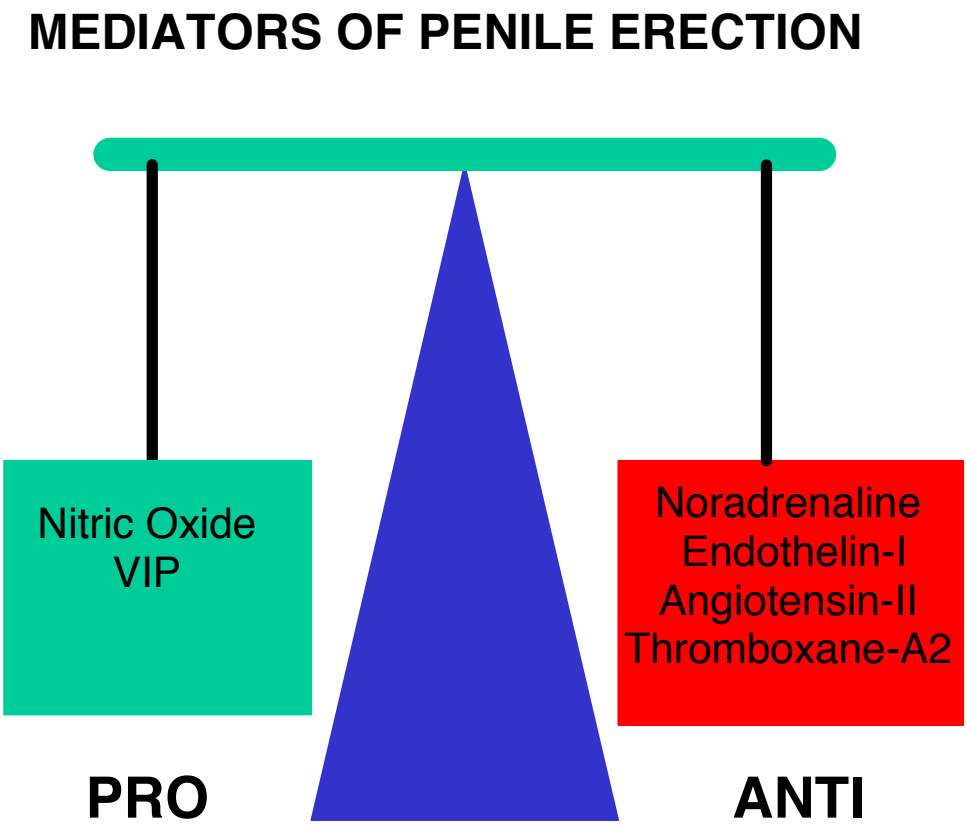


**Figure 2:** Penile erection is regulated by two opposing systems: pro-erectile mediators such as nitric oxide (NO) and vasoactive intestinal peptide (VIP) and anti-erectile mediators such as noradrenaline (NA), endothelin-I, angiotensin II and thromboxane A2.

### 1.1.3. Vascular and cavernosal smooth muscle in the penis

The human penis is composed of paired corpora cavernosa and the single corpus spongiosum (Figure 3). The corpus cavernosum consists of a meshwork of sinusoidal spaces lined with endothelial cells (Andersson and Wagner, 1995;Bossart et al., 1980). In order for an erection to occur, relaxation of penile smooth muscle is required to allow blood to flow into the penile structures. The resulting increase in intra-cavernosal pressure leads to compression of the subtunical venules against the tunica albuginea (the corporeal veno-occlusive mechanism) (Andersson and Wagner, 1995). This process reduces venous drainage from the corpora cavernosa and increases pressure within the corpora, producing an erection. In full rigidity the intracavernous pressure reaches values considerably higher than systemic (systolic) blood pressure with the contribution of the skeletal muscles of the pelvic floor.

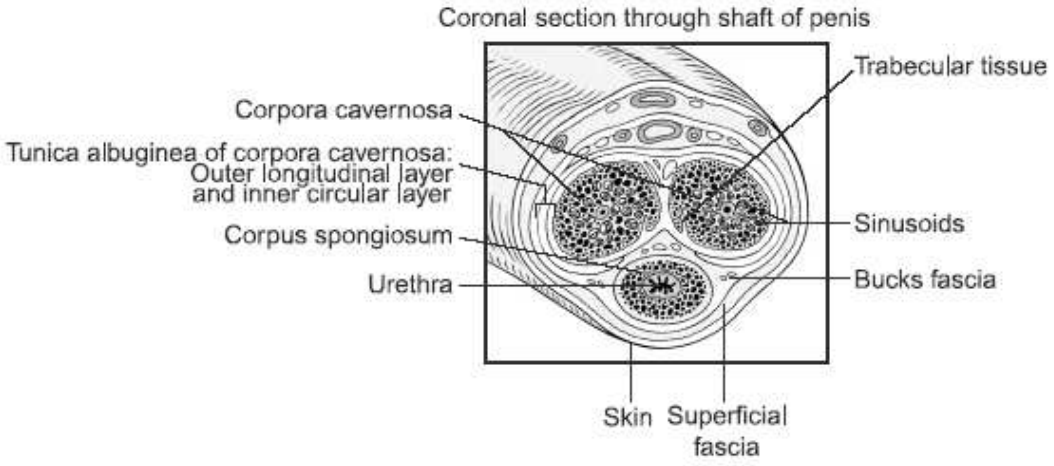


**Figure 3:** Cross section through shaft of penis demonstrating the sinusoids, subtunical venules and tunica albuginea. From (Eardley and Sethia K, 2003)

### 1.1.4. Relaxant factors

#### 1.1.4.1 NO-sGC-cGMP pathway

In the presence of functional adrenergic and cholinergic blockade, electrical field stimulation (EFS) results in relaxation of the corpus cavernosum smooth muscle. In 1990 Ignarro et al (Ignarro et al., 1990) reported that EFS of isolated strips of rabbit corpus cavernosum resulted in the endogenous generation and release of NO, nitrite, and cyclic guanosine monophosphate (cGMP). Further studies demonstrated that the smooth muscle relaxation in response to EFS, in the presence of blockers of noradrenergic and cholinergic pathways (guanethidine and atropine respectively), was abolished by tetrodotoxin and potassium-induced depolarization. Furthermore, the relaxation was markedly inhibited by $N^G$-nitro-L-arginine (L-NNA), oxyhemoglobin, and methylene blue, but was unaffected by indomethacin (Ignarro et al., 1990). These were the first published results to suggest that penile erection is mediated by NO generated in response to nonadrenergic-noncholinergic (NANC) stimulation.

Using immuno-histochemical techniques the enzyme neuronal nitric oxide synthase (nNOS) was demonstrated to be present in the nerve fibres of the pelvic plexus, corpus cavernosum and around blood vessels (Alm et al., 1993;Burnett et al., 1993). Using an antibody against nNOS, labelling studies in the rat have demonstrated that nitrergic nerves which innervate the penis are parasympathetic post-ganglionic nerves (Vizzard et al., 1994) and that nNOS-positive nerves may be found in close proximity to the lumbo-sacral spinal cord (Anderson et al., 1993).

It is now known that in the corpus cavernosum NO may be released from both the endothelium via eNOS and the nitrergic nerves via nNOS. Both nNOS and eNOS are activated by calcium entry into cells and binding to calmodulin associated with the enzyme. It has been suggested that nitrergic-derived NO is functionally more important, as nitrergic relaxation of corpus cavernosum has been reported not to require a functional endothelium after removal by either physical (Kim et al., 1991) or chemical means (Okamura et al., 1999).

However, more recent evidence suggests that eNOS is critically involved in penile erection. It is considered to work in an integrative fashion with nNOS to produce NO required for penile erection. It has therefore been proposed that nNOS may initiate the

erectile process, whereas eNOS is involved in the maintenance of NO-dependent signalling, sustained NO production and maximal erection. It has also been suggested that eNOS may be activated by the process of viscous or shear stress in blood vessels to produce NO continuously (Burnett, 2004). Moreover, the phosphatidylinositol 3 kinase (PI3 kinase) pathway which activates the serine-threonine protein kinase Akt (PKB) has been shown to cause direct phosphorylation of eNOS (Dimmeler et al., 1999;Fulton et al., 1999;Michell et al., 1999). Through this process there appears to be a reduction in the calcium requirement for eNOS which may in turn result in the increased production of NO and erection.

Upon release from nitrergic nerves and the endothelium, NO exerts its action on smooth muscle cells by activating the enzyme soluble guanylate cyclase (sGC). The activation of sGC results in an increase in intracellular cGMP concentration (Figure 4) (Schmidt et al., 1993).

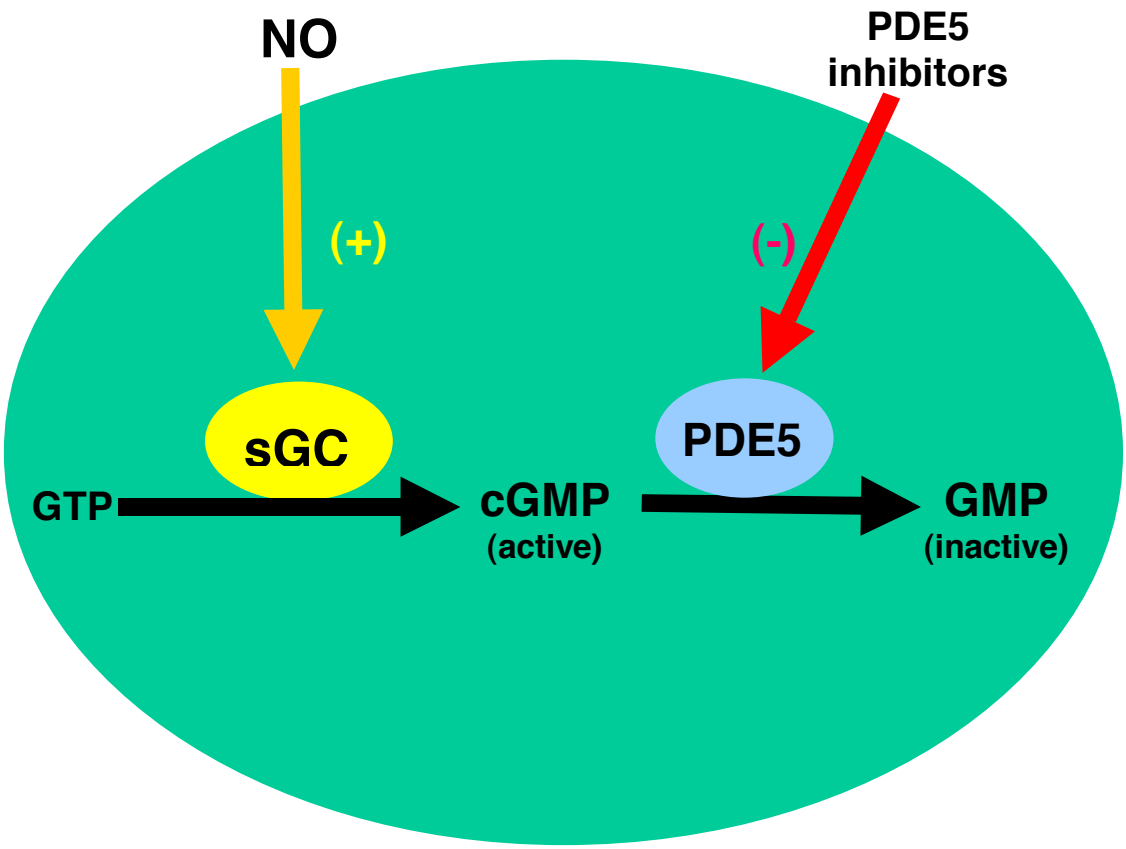


**Figure 4:** Nitric oxide (NO) released from nitrergic nerves and endothelium stimulates smooth muscle relaxation by activation of soluble guanylate cyclase (sGC), which in turn catalyses the conversion of GTP to the active intracellular second messenger cGMP. cGMP is then metabolized by PDE5 to inactive GMP.

Guanylate cyclase activity has been identified in both the soluble and particulate fractions in rats (Hardman and Sutherland, 1969) and other mammals (White and Aurbach, 1969). The activities of these enzymes were characterised (Garbers and Gray, 1974), (Chrisman et al., 1975) and found to be due to different proteins.

The purification of guanylate cyclase from the cytosolic compartment has revealed the soluble isoform to be a heterodimer composed of α- and β-subunits. The β-subunit has a molecular mass of 70 kDa, whereas the α-subunit was reported to be 73 to 82 kDa (Gerzer et al., 1981b;Kamisaki et al., 1986). It has been found to be expressed in the cytoplasm of most mammalian cells and is known to be involved in many important physiological functions, such as platelet aggregation inhibition, smooth muscle relaxation, neuronal transduction and immune modulation (Collier and Vallance, 1989).

The further analysis of sGC has demonstrated the presence of multiple isotypes with different subunit compositions. The most abundant subunits isolated were α1 and β1, which are found in many tissues (Garbers, 1979). It was found that the expression of α1 and β1 individually yielded a protein without catalytic activity, whereas the co-expression of these subunits yielded an enzyme which could be activated by NO (Buechler et al., 1991). Other subunits identified include β2 (Yuen et al., 1990), α2 (Harteneck et al., 1991), α3 and β3 (Giuili et al., 1992). A variant of the α2-subunit, α2i, has an additional 31 amino acids in the catalytic domain (Behrends et al., 1995). It has been suggested that this may increase the ability of this enyme isoform to utilize ATP as a substrate and produce cAMP (Behrends et al., 1995;Koesling et al., 1988;Nakane et al., 1990).

It is now well established that each subunit of sGC can be divided into three functional domains: haem-binding, dimerization, and catalytic (Figure 5).

The haem-binding domain is located at the N terminus of each subunit. The haem prosthetic group is required for activation of sGC by NO (Craven and DeRubertis, 1978), (Ignarro et al., 1982).

Haem is a five-member nitrogen-containing ring in which four nitrogen atoms are co-ordinated with a central iron that can be either $Fe^{2+}$ (ferrous or the reduced form) or $Fe^{3+}$ (ferric or the oxidized form) (Figure 5). Studies have demonstrated that the fifth member of the ring in sGC is an imidazole axial ligand which is coordinated by the β1-

subunit at His105(Stone and Marletta, 1994). Moreover, the mutation of this histidine, which is located near the N terminus of the β1-subunit is associated with an acquired inability of sGC enzyme to bind haem and an enzyme that was unresponsive to NO (Wedel et al., 1994). As discussed previously both α1- and β1-subunits are required to express basal catalytic activity and activation of sGC by NO (Buechler et al., 1991). These results suggest that both subunits play a role in the association of the haem with the enzyme.

However, the presence of Cys78 and Cys124 in the β-subunit was found to be important for coordinating the haem group of sGC (Foerster et al., 1996). Cysteine residues are also known to be important in other haem-containing proteins such as cytochrome C, in which the haem is covalently bound to the protein through thio-ether bonds to two Cys residues.

In contrast to other haem containing proteins, the haem in sGC has a very high affinity for NO (Gerzer et al., 1981b). Even in an aerobic environment, sGC prefers to bind NO rather than oxygen. If sGC binds oxygen, it forms a ferrous-oxy species that must exchange oxygen with NO to form a ferrous nitrosyl complex (Gerzer et al., 1981a;Gerzer et al., 1981b;Gerzer et al., 1981c). Oxidation of the haem group to the ferric state results in the loss of enzyme activity and often a complete loss of the haem moiety from the protein. Thus, reducing agents such as thiols, ascorbate, or dithiothreitol enhance enzyme activation, presumably by maintaining the iron of the metalloporphyrin in the ferrous state that is sensitive to NO. Conversely, oxidising agents such as methylene blue inhibit enzyme activation(Ignarro et al., 1981).

The catalytic domain of each subunit has been demonstrated to be present at the C-terminus. There is reported to be a high degree of homology between the C-terminal regions of all subunits and with the catalytic domain of particulate GC and the C1/C2 catalytic domains of adenylate cyclase(Thorpe and Garbers, 1989). It is well established that both subunits are required to be expressed in order for cyclase activity despite the fact that both α and β subunits contain catalytic domains . Interestingly, the co-expression of the C-terminal portions of α1 and β1 subunits may result in a basally active protein only. It has therefore been suggested that although two catalytic domains are

present, each sub-unit contributes specific residues to a single substrate binding and catalytic site(Liu et al., 1997).

Between the catalytic and haem-binding domains there is an area which is thought to be important in mediating subunit association to form hetero-dimers. This dimerization domain is homologous to the dimerization domain of pGC and is located proximal to the catalytic domains of α and β subunits.

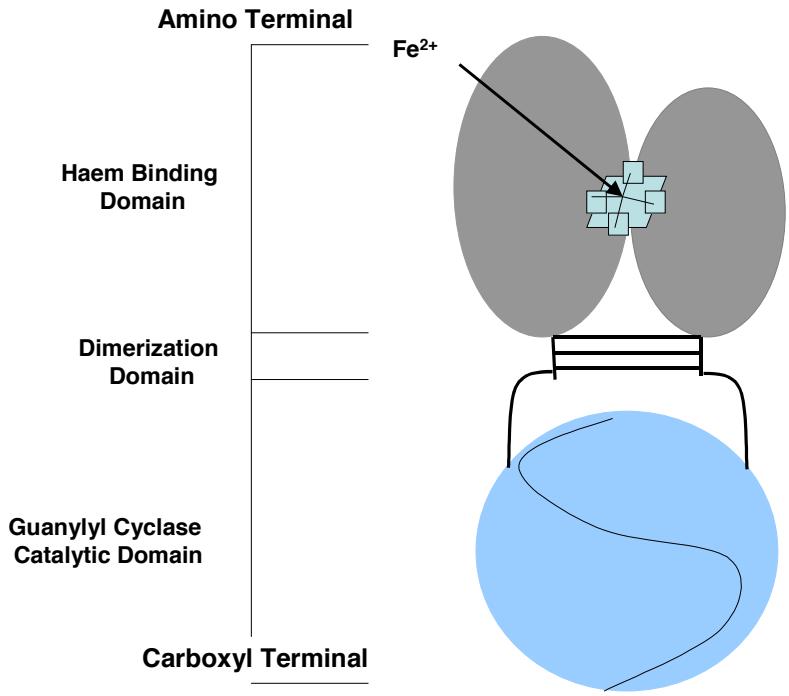


**Figure 5:** The soluble guanylate cyclase enzyme. It is divided in to three domains: Haem binding, dimerization and catalytic.

The free radical NO has been shown to activate sGC by directly binding to form a ferrous-nitrosyl-haem complex. The half-life of this complex is reported to be between 4 minutes and 3 hours in *vitro* studies at 20°C (Hille et al., 1979),(Sharma et al., 1978). NO is reported to bind to the haem ring at the $6^{th}$ position thereby breaking a bond between an axial histidine and iron and forming a bond with the iron. Removal of the haem results in loss of NO responsiveness, therefore indicating that the mechanism is haem-dependent.

Nitrergic nerve stimulation and the administration of NO donors results in an increase in intracellular cGMP concentrations in human and rabbit corpus cavernosum (Bush et al., 1992;Dahiya et al., 1993;Rajfer et al., 1992). Furthermore, analogues of cGMP elicit electrophysiological responses similar to nitrergic nerve stimulation or NO donors (Bowman and Drummond, 1984).

**Mechanism of action of cGMP**

The mechanism by which an increased cGMP concentration results in smooth muscle relaxation is not completely understood. The proposed mechanisms include (Figure 6):

1. cGMP may directly reduce $Ca^{2+}$ entry into cells by inhibiting L-type $Ca^{2+}$ channels (Lincoln, 1989).
2. cGMP may activate a cGMP-dependent protein kinase (PKG) (Lincoln and Cornwell, 1993). However, the effects are unlikely to be mediated by a cAMP-dependent protein kinase (PKA) as in cGK-deficient mice, cGMP induced relaxation was completely abolished whereas cAMP-dependent relaxation was unaffected (Hofmann et al., 2000).

The PKG may:

a. directly inhibit voltage-gated $Ca^{2+}$ channels (Clapp and Gurney, 1991).
b. activate $Ca^{2+}$-sensitive maxi $K^+$ channels and hyperpolarize the membrane (Archer et al., 1994).
c. activate the $Na^+/Ca^{2+}$ exchanger (Furukawa et al., 1991).
d. activate the $Ca^{2+}$-ATPase on the plasma membrane (Yoshida et al., 1991).
e. inhibit $IP_3$ receptor on the endoplasmic reticulum membrane either directly (Komalavilas and Lincoln, 1994) or indirectly by phosphorylating $IP_3$ receptor-related regulatory proteins like phospholamban (Cornwell et al., 1991) or IRAG (Schlossmann et al., 2000).
f. inhibit agonist-induced production of $IP_3$ by binding to $PIP_2$ (Hirata et al., 1990).

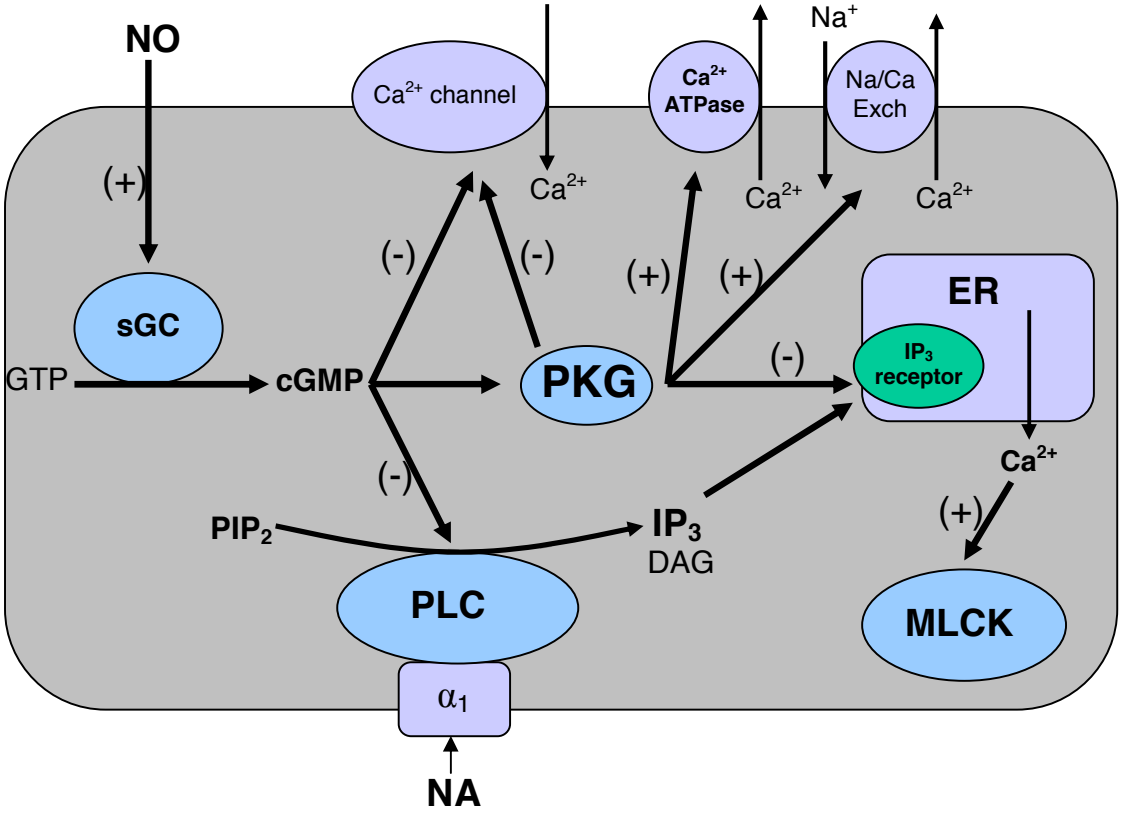


**Figure 6:** Schematic representation of proposed mechanisms involved in regulation of penile smooth muscle tone. NO stimulates sGC which catalyses formation of cGMP. Increased cGMP concentrations lead to activation of PKG. cGMP or PKG reduce intracellular $Ca^{2+}$ concentrations by various mechanisms leading to relaxation. Noradrenaline (NA) on the other hand stimulates $\alpha_1$-adrenoceptors which leads to production of $IP_3$ via PLC. Increased $IP_3$ concentrations lead to contraction by increasing intracellular $Ca^{2+}$ concentrations. PKG, cGMP-dependent protein kinase; ER, endoplasmic reticulum; MLCK, myosin light chain kinase; Na/Ca Exch, sodium calcium exchanger; NO, nitric oxide; NA, noradrenaline, PLC, phospholipase C; sGC, soluble guanylate cyclase. (+) denotes activation and (-) denotes inhibition.

It is known that the concentration of intracellular $Ca^{2+}$ is critical for the regulation of penile vascular and cavernosal smooth muscle tone. A change in the concentration may be achieved by controlling the movements of $Ca^{2+}$ into and out of the smooth muscle cells or the endoplasmic reticulum (Berridge, 1993). An increase in intracellular $Ca^{2+}$ results in an increase in the activity of myosin light chain kinase (MLCK) and in turn the intracellular content of phosphorylated myosin light chain, which allows the smooth muscle cell to contract effectively (Berridge, 1993).

### 1.1.4.2 Endothelium-dependent hyperpolarizing factor (EDHF)

As discussed earlier, NO is widely accepted to be the major mediator of endothelium dependent relaxation in the corpus cavernosum and penile arteries. However, in penile arteries there is another mechanism which remains despite blockade of NO and prostaglandins (Angulo et al., 2003). The hyperpolarisation of the cell membrane has been found to result in the closure of voltage-gated calcium channels. This in turn results in a reduced entry of calcium into both the extra-cellular and intra-cellular compartments and relaxation of the smooth muscle. This process of relaxation is prevented by maxi-K ($K_{ca}$) channel blockade (Spektor et al., 2002) or by not allowing hyperpolarisation with a high $K^+$ concentration (Angulo et al., 2003). Many authors have attributed this activity to an unknown endothelium-dependent hyperpolarizing factor (EDHF). It has been reported that putative candidates for EDHF include derivatives of arachidonic acid, hydrogen peroxide, anadamide and C-type natriuretic peptide. More recently, it has also been demonstrated that endothelium-dependent relaxation is impaired in both human penile corpus cavernosum and penile resistance vessels from diabetic patients (Angulo et al., 2006). However, treatment with dobesilate was found to enhance endothelium-dependent relaxation of penile resistance arteries which is attributed to EDHF. In contrast, it had no effect on endothelium-dependent relaxation of the corpus cavernosum (Angulo et al., 2003). In a diabetic rat model, the combination of a PDE-5 inhibitor and dobesilate was found to significantly improve erectile function greater than each individual agent alone. These results suggest a role for both NO-cGMP and EDHF pathways in erectile function (Angulo et al., 2005).

#### 1.1.4.3 Vasoactive intestinal peptide (VIP)

It has been reported that the penis has a rich supply of nerves containing VIP which are commonly co-localised with nNOS (Domoto and Tsumori, 1994;Ehmke et al., 1995). The effects of VIP are mediated via two VIP receptors (types 1 and 2). Stimulation of the receptors results in an increase in cAMP and activation of cAMP-dependent kinase activity (Miller et al., 1995). Experimental studies suggest that VIP has a role in NANC-mediated corpus cavernosal relaxation but its effect is dependent on the presence of prostaglandins and the generation of NO (Kim et al., 1995). Furthermore, it has been demonstrated that diabetes mellitus is associated with a reduction in the VIP immunoreactivity in nerves associated with cavernosal smooth muscle (Crowe et al., 1983).

#### 1.1.4.4 Prostaglandins

The corpus cavernosum has the ability to produce a wide variety of prostaglandins depending on the local oxygen tension (Andersson and Wagner, 1995;Daley et al., 1996). There at least five active prostaglandin metabolites that have been identified in penile tissues which may act on five major groups of receptors (Andersson and Wagner, 1995). The receptors in turn have been demonstrated to be G-protein coupled and are associated with different second messenger systems (Coleman et al., 1994). The precise role of the prostaglandins and their receptors in the penis has not yet been fully established. However, $PGE_1$ and $PGE_2$ may be involved in relaxation of smooth muscle via stimulating EP receptors whereas $PGF_{2\alpha}$ and $TX_{A2}$ are likely to result in contraction by stimulating TP and FP receptors respectively (Angulo et al., 2002;Hedlund et al., 1989).

### 1.1.5 Contractile factors

It is generally accepted that the most important determinant of the whether the penis is in a state of tumescence or detumescence is the degree of contraction of the corpus cavernosum smooth muscle (Andersson and Wagner, 1995). The activation of the sympathetic nervous system has consistently been demonstrated to result in the inhibition of erection. Furthermore, the tonic inhibitory activity of these nerves has been reported to result in the penis being kept in a flaccid state under normal circumstances (Andersson, 2001b). There are many putative transmitters that may contribute to penile smooth muscle contraction, however noradrenaline and endothelins are currently believed to be the most important (Mills et al., 2001).

#### 1.1.5.1 Noradrenaline

The penile vasculature and smooth muscle has been demonstrated to receive a dense adrenergic innervation. Noradrenaline acts upon $\alpha_1$-adrenoceptors, which are located on the smooth muscle cells of the corpus cavernosum, spongiosum and penile blood vessels (Andersson and Wagner, 1995). The post-receptor mechanisms after activation of adrenergic receptors include the release of membrane-bound phospholipase C, which induces cleavage of phosphoinositol diphosphate ($PIP_2$) to inositol triphosphate ($IP_3$) and diacylglycerol (DAG) (Figure 7). Both $IP_3$ and DAG result in an increase in intracellular $Ca^{2+}$, which in turn activates contractile pathways as described previously (Figure 6) (Berridge, 1993).

However *in vitro* experiments have demonstrated that $\alpha$ adrenoceptor agonists induce a higher force/$Ca^{2+}$ ratio than a direct depolarization-induced increase in intracellular $Ca^{2+}$ (i.e. potassium chloride). These results suggest that there may in addition be a $Ca^{2+}$-sensitizing effect associated with these agonists. One mechanism by which this may occur is through guanosine triphosphate (GTP)- binding proteins recruiting other messenger systems (Karaki et al., 1997;Kuriyama et al., 1998;Somlyo and Somlyo, 2000).

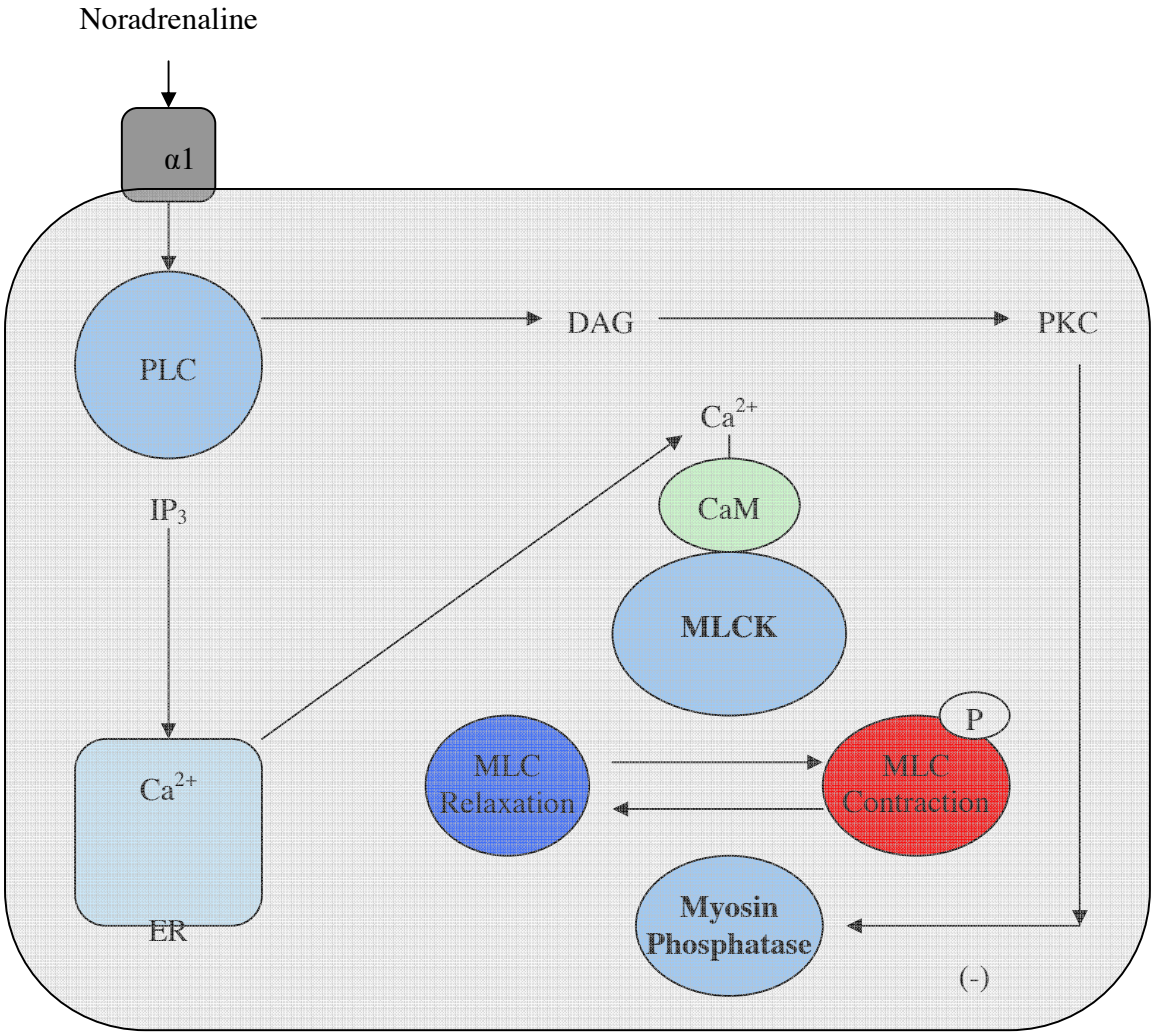


**Figure 7:** The mechanisms by which activation of adrenoceptors results in smooth muscle contraction via calcium dependent and independent mechanisms. Activation of myosin light-chain kinase (MLCK) by $Ca^{2+}$ binding to calmodulin (CaM) leads to phosphorylation of myosin. Whereas, phosphokinase C (PKC) through phosphorylation of CPI-17 results in direct inhibition of myosin phosphatase. The ratio of kinase to phosphatase activities determines the level of phosphorylation and the extent of activation of myosin.

In addition, other vasoactive mediators released from sinusoidal structures, such as angiotensin-II (Comiter et al., 1997), and thromboxane-A2 (Angulo et al., 2006), which possess smooth muscle contractile properties, can also interfere with smooth muscle relaxation and prevent erection. However, their roles have not yet been fully established (Andersson and Wagner, 1995).

### 1.1.5.2 Endothelins

Endothelin-1 (ET-1) is synthesised by the lacunar endothelium and has been reported to potently induce slowly developing, long-lasting contractions in the corpus cavernosum and penile vasculature. This may contribute to the maintenance of corpus cavernosal smooth muscle tone (Becker et al., 2001a). Smooth muscle contraction has also been reported to be produced by ET-2 and ET-3, although with a lower potency than ET-1 (Saenz, I et al., 1991a).

Two ET receptors have been identified (A and B). Whilst ET-B is the most predominant in mammalians, it is ET-A which mediates the vasoconstrictor actions in the penis (Mumtaz et al., 2006). The contractions induced by ET-1 are dependent on trans-membrane calcium flux (through voltage dependent and/or receptor operated calcium channels) and mobilization of $IP_3$-sensitive intracellular calcium stores and calcium sensitization of the contractile machinery (Holmquist et al., 1990;Holmquist et al., 1992). Pathological states such as hypoxia have been found to be associated with an increase in the expression of ET-B receptors. This is a putative mechanism by which blood flow is increased secondary to smooth muscle relaxation (Filippi et al., 2003b;Mumtaz et al., 2006).

### 1.1.5.3 Calcium sensitising pathways

As described previously, the critical step in smooth muscle contraction is an increase in the intra-cellular free calcium [$(Ca^{2+})i$]. The increase in $Ca^{2+}$ is transient whereas the vasoconstrictor induced smooth muscle constriction may be prolonged. One way that this may be accomplished is by increasing calcium sensitivity such that smooth muscle contraction prevails despite the return of $Ca^{2+}$ to near basal levels (Somlyo and Somlyo, 2003).

Calcium sensitization may be initiated by agonist activation of hetero-trimeric G protein coupled receptors. This activation results in the exchange of GTP for GDP on the small GTPase RhoA, and its dissociation from its partner Rho-guanine nucleotide dissociation inhibitor. GTP RhoA is then hypothesized to activate Rho kinase and inhibit MLC phosphatase, thus increasing MLC phosphorylation by basal level activity of MLC kinase. Using this pathway, the myosin phosphorylation and smooth muscle contraction may occur without a change in the concentration of $Ca^{2+}$ from the sarcoplasmic (Figure 8).

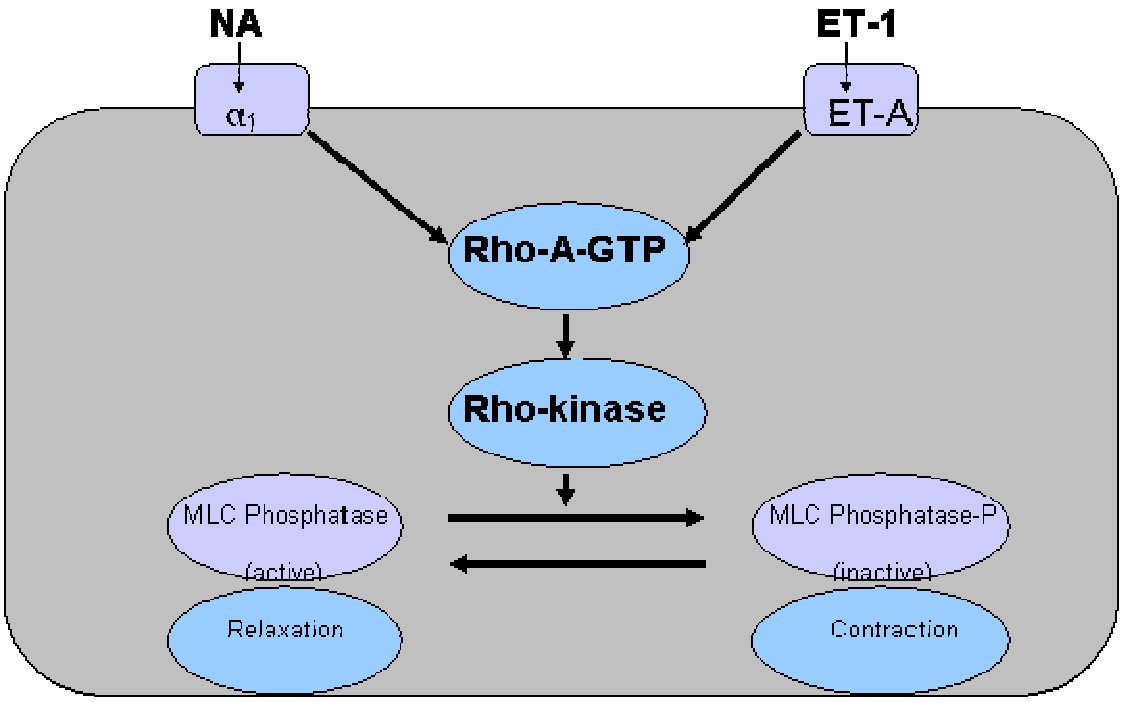


**Figure 8:** Rho/Rho-kinase pathway.

Pathological states may abnormally increase the activation of myosin by Rho-kinase using this mechanism. There is evidence that this may be the case in certain types of ED (Somlyo and Somlyo, 2000). Rees et al demonstrated by immunofluorescence and Western blotting the presence of Rho kinase in the primary culture of smooth muscle cells from human and rabbit corpus cavernosum (Rees et al., 2002). The same group also showed that a Rho-kinase inhibitor (Y-27632) relaxes human and rabbit cavernosal smooth muscle in an NO-independent manner (Rees et al., 2001). These results were further confirmed where Y-27632 caused a significant increase in corpus cavernosal pressure and erection independent of NO, without affecting systemic arterial pressure in rats (Chitaley and Webb, 2001).

## 1.2. Erectile dysfunction

Erectile dysfunction (ED) is defined as the inability to achieve and maintain a penile erection adequate for satisfactory sexual intercourse (1993). It effects up to 150 million men worldwide and this figure is projected to double by the year 2025 (Ayta et al., 1999).

Many studies have attempted to characterise the true prevalence of ED. In a Danish study Ventegodt reported that 5.4% of all patients had a decreased ability to achieve an erection (Ventegodt, 1998). The prevalence was reported to be highest (18%) in those aged greater than 58 years.

More recently, a Dutch study reported 13% of men had difficulties getting an erection and the prevalence increased with age. In the age groups of 40–49, 50–59, 60–69 and 70–79 years, the incidence was found to be 6, 9, 22 and 38% respectively (Meuleman et al., 2001).

The Massachusetts Male Aging Study (MMAS) is the most well known study to assess the prevalence of ED (Feldman et al., 1994). This study reported the results of a regional survey of 1709 men aged 40–69 years. Of the 1709 men surveyed, 1290 men completed a paper questionnaire. Of these men 52% reported ED, with 10% having complete ED. Moreover, the results suggest that the probability of complete ED at age 70 was threefold that at age 40, the probability of moderate ED was two-fold, while that of minimal ED remained constant across all age groups. While the MMAS is regarded as representative of the population in Massachusetts, it has been criticised for its validity in terms of sociocultural diversity as 96% of the study population were Caucasian in origin.

In summary, studies of the prevalence of ED most commonly suggest that the incidence of ED increases sharply with age (Feldman et al., 1994). Furthermore, ED is a common cross-cultural condition, affecting 19% to 67% of men aged 40 to 80 years, in both developing and industrialized countries.

However, it is now well understood that epidemiological studies may underestimate the true incidence of ED because of the perceived embarrassment that is associated with the condition (Chun and Carson, III, 2001). In an Australian study conducted in primary care, questionnaires were distributed to consecutive adult male attendees at 62 general medical practices. Of the 1240 completed questionnaires 39.4% of

men reported ED, however only 11.6% of these men had sought treatment for the disease (Chew et al., 2000).

## 1.3. Pathophysiology of erectile dysfunction

ED can be caused by either psychogenic or organic causes, however in many patients the disorder is of mixed aetiology with both factors present. The psychogenic component of ED is reported to be especially important in younger men (aged less than 35 years) (Melman and Gingell, 1999) and in elderly men who start a relationship with a new partner. Diseases which become more prevalent with age such as diabetes and vascular disease, are now known to be an integral in the pathogenesis of ED in the ageing male. It has been reported that in patients older than 50, up to 50% of these patients may have ED secondary to vascular disease (Kaiser, 1999). The presence of ED of any cause is itself associated with psychological distress. This of course may in turn reduce the probability of achieving satisfactory erectile function (Feldman et al., 1994).

ED occurs when the normal function of one or more of the neuronal or muscular systems is interrupted and this is discussed below.

### 1.3.1 Central nervous system

The number of oxytocinergic neurons in the rat PVN and supraoptic nucleus decreases substantially with aging (Bazhanova et al., 1998). This age-related decrease may impair sexual function, since penile oxytocinergic neurons are postulated to have an important role in erection and ejaculation (Andersson and Wagner, 1995).

The selective neuronal loss in the brain has been proposed to be related to an increase in the rate of programmed cell death by oxidative and nitrosative stress in these neurons (Torreilles et al., 1999). Peroxynitrite (produced by the reaction between NO and superoxide) is a strong oxidizing and nitrating agent. Increased levels of peroxynitrite have been demonstrated to occur with ageing. In the rat hypothalamic MPOA, the increased levels are postulated to be secondary to inducible nitric oxide synthase (iNOS) (Vernet et al., 1998). Moreover, an age-related increase in iNOS expression in the hypothalamus of the male rat has been observed. This region is involved in the control, the synthesis and neuronal secretion of Gn-RH and oxytocin and other factors which regulate penile erection (Ferrini et al., 2001). It is therefore possible that iNOS may have a pathological role in the reduction of Gn-RH and oxytocin. This in turn may result in

sexual dysfunction, which may manifest itself as a lowered serum testosterone level and reduced erectile function in ageing males.

### 1.3.2 Spinal cord

In spinal cord injury, patients with lesions above the sacral parasympathetic centre tend to have maintenance of reflex erections. These patients usually have short lasting erections which can be induced with minimal stimulation. This in contrast to lesions below the sacral centre, where there are no reflex erections and subsequently severe ED (Yarkony, 1990).

### 1.3.3 Peripheral nervous system

ED may occur when there is disruption to the afferent nerves which bring sensory information to the CNS or when there is a lesion in the autonomic nerves mediating arterial and trabecular smooth muscle relaxation. This may result secondary to conditions such as alcoholism, chronic renal failure and in cases of viral infections (e.g. HIV).

### 1.3.4 Vascular disease

Arterial disease can result in ED by restricting the inflow of blood into the penis. The most common cause of this is atherosclerosis. Furthermore, ED and cardiovascular disease share many common risk factors such as diabetes mellitus, hypertension, hypercholesterolemia and smoking. This has led to the hypothesis that ED may be an early manifestation of vascular disease i.e. endothelial dysfunction (Sullivan et al., 1999). The mechanisms involved in producing ED in vascular disease are complex and include arterial re-modelling (Saenz, I et al., 1991b), increased vasoconstriction (Schubert and Mulvany, 1999) and impaired neurogenic and endothelium dependent vasodilatation (Simonsen et al., 2002).

### 1.3.5 Diabetes mellitus

Many epidemiologic studies have reported an increased risk of ED in diabetic men (Aytac et al., 2000). The prevalence of ED has been reported to affect between 35-50% of diabetic patients (Akkus et al., 2002;Kolodny et al., 1974;McCulloch et al., 1980). In an Italian study the authors reported that ED was more common in type 1 than type 2 diabetics. Furthermore, a positive relationship was demonstrated between ED, poor metabolic control and age (Fedele et al., 2001). These results indicate that diabetes is a very significant risk factor for the development of ED.

In diabetes, the severity of ED has been demonstrated to be related to both the severity (Metro and Broderick, 1999), and duration of diabetes (McCulloch et al., 1980). However, it is likely that the aetiology of ED in diabetes is multifactorial. It is now well established that there is a higher incidence of peripheral neuropathy (Kolodny et al., 1974), autonomic neuropathy (Bruna and Navarro, 2005), microangiopathy (McCulloch et al., 1980) and arterial insufficiency (Metro and Broderick, 1999) in diabetic patients with ED than in potent diabetic patients.

The proposed mechanisms of ED in diabetic patients will now be discussed.

**a. Nitric oxide**

Studies have shown a reduction in eNOS- and nNOS- mediated cavernosal smooth muscle relaxation in diabetic animals (Azadzoi and Saenz, I, 1992;Cartledge et al., 2001b). Using immunhistochemistry, it was demonstrated in streptozotocin (STZ)-induced diabetic rats, that nitrergic nerves undergo selective degeneration whereas anti-erectile noradrenergic nerves remain intact. As a result, the nitrergic relaxation responses *in vitro* and erectile responses to cavernous nerve stimulation *in vivo* were found to be attenuated in diabetic animals, whereas the noradrenergic responses were enhanced (Cellek et al., 1999).

In a rat model of type I (BB/WORdp diabetic prone) and type II (BBZ/WOR) diabetes, the amount of the nNOS protein has been shown to be reduced in diabetic rats (Vernet et al., 1995). Moreover, both diabetic models showed marked decreases of penile NOS activity and penile nNOS content compared to non-diabetic controls. By

administering an inhibitor of NO synthase, (L-NAME), to the drinking water of rats following the establishment of diabetes with STZ, it was demonstrated that L-NAME is able to protect the nitrergic nerves from morphological and functional impairment. These results suggest that selective nitrergic degeneration in diabetes is NO-dependent and that inhibition of NO synthase may be neuroprotective in this condition (Cellek et al., 1999).

*In vitro* studies using corpus cavernosum from patients undergoing implantation of a penile prosthesis demonstrated that EFS-induced relaxation was less pronounced in the smooth muscle from diabetic men than in the smooth muscle from non-diabetic men. Furthermore, the degree of impairment was found to increase with the duration of diabetes(Saenz, I et al., 1989). Moreover, it has been found that NOS -containing (nitrergic) neurons innervating the penis of streptozotocin-induced diabetic rats undergo a selective degenerative process in two phases. In the first phase, nitrergic nerve fibres have been demonstrated to partially lose their NOS content and function, whereas in the second phase, nitrergic degeneration is considered to take place in the cell bodies in the ganglia. This results in an eventual complete loss of nitrergic function and nNOS content. Not surprisingly the changes in the first phase were found to be reversible with insulin replacement but the neurodegeneration in the second phase was not (Cellek et al., 2003). Thus these findings are in keeping with previous observations that the degree of erectile impairment increases with the duration of diabetes. These were further confirmed in human penile tissue from patients with diabetic ED (Tuncayengin et al., 2003).

Other authors have suggested that the diabetes induced reduction in cavernosal NO could be due to a lack of essential co-factors for NOS enzyme (Escrig et al., 2002). It is also hypothesized that diabetes may impair the activity of guanylate cyclase, thereby decreasing the production of cGMP (Seftel et al., 1997). Thus decreased NO and its effector molecule, cGMP, may participate significantly in the development of diabetes induced ED.

**b. Advanced glycation end products (AGEs) and superoxide production**

AGEs are thought to develop in diabetic patients secondary to hyperglycaemia and are the products of non-enzymatic reactions between glucose and lipids, proteins or nucleic acids (Cartledge et al., 2001a). It is now known that glucose reacts with the amino group of amino acids, resulting in the production of Schiff bases. These bases undergo a reversible reaction to form more stable Amadori products, however some of these glycosylation products undergo further chemical modifications and ultimately become irreversible glycosylation end-products, termed AGEs (Brownlee et al., 1988;Cartledge et al., 2001a). AGEs are able to form covalent bonds with vascular collagen, which may result in vascular thickening, reduced elasticity, endothelial dysfunction and atherosclerosis (Bucala et al., 1991);Singh, 2001 703 /id}. AGEs have also been found to accumulate in ageing and diabetic tissues, and may form at an accelerated rate when glucose levels are pathologically elevated (Bucala et al., 1991;Seftel et al., 1997). Furthermore, high-performance liquid chromatographic analysis has demonstrated a significant elevation of the AGEs in the penile tissue but not in the serum of diabetic patients compared with that of non-diabetic patients (Seftel et al., 1997).

*In vitro* both haemoglobin (Hb) and HbA1c, an isoform of glycosylated haemoglobin have been found to significantly impair the relaxation of rat corpus cavernosum to acetylcholine in a dose-dependent manner. L-arginine addition has been found to reverse the impairment caused by Hb, but not glycosylated haemoglobin. The addition of pyrogallol (a donor of superoxide anions) resulted in a similar impairment in acetylcholine-induced relaxation in control tissues. However, the addition of SOD completely reversed the HbA1c-induced impaired relaxation. These results suggest that there is impaired endothelium-dependent smooth muscle relaxation in the corpus cavernosum from diabetic rats secondary to the presence of AGEs (Cartledge et al., 2001a). Furthermore, AGEs have been shown to decrease compliance in the corpus cavernosum and interfere with smooth muscle relaxation by generating free radicals or reactive oxygen species (ROS) that react with NO (Cartledge et al., 2001a). Further studies demonstrate that a specific iNOS inhibitor (PNU- 1945 1) significantly potentiates the relaxation of pre-contracted human cavernosal tissue. These results suggest there may be a pathological mechanism for AGE-mediated ED via up-regulation

of iNOS and down regulation of eNOS (Seftel et al., 1997). ROS and superoxide anions have been shown to be elevated in diabetic rat penises. The product of the reaction between ROS and NO (peroxynitrite) does not itself elicit smooth muscle relaxation but may have a role in peroxide-induced cell damage and death (Cartledge et al., 2001a). AGEs can contribute to diabetic ED by generating oxygen free radicals, which induce oxidative cell damage and quench NO, culminating in decreased cGMP and impaired cavernosal smooth muscle relaxation (Bivalacqua et al., 2005;Cartledge et al., 2001a;Khan et al., 2001).

As discussed previously, in diabetes nitrergic neurones have been suggested to undergo a selective nitrergic degenerative process in two phases. More recent evidence suggests that AGEs increase the ROS and caspase-3-dependent neuronal apoptosis in a synergistic fashion with endogenous NO (Cellek et al., 2004). It has therefore been postulated that the apoptosis may be prevented with treatment by an NOS inhibitor, a pan-caspase inhibitor, a soluble receptor of AGEs s well as anti-oxidants. These results suggest that NO and AGEs act synergistically to produce irreversible nitrergic degeneration in diabetes.

**c. Endothelial dysfunction**

ED can be considered to be an early manifestation of systemic endothelial dysfunction (Billups, 2005). It is now well established that ED often precedes and predisposes subsequent atherosclerosis. Moreover, endothelial dysfunction is a reflection of the loss of NO activity or biosynthesis at the endothelial level. Despite this, the precise mechanisms involved in diabetes- associated endothelial dysfunction are incompletely understood. The known factors associated with endothelial dysfunction in diabetes include defects in endothelial NO synthase (eNOS) expression and activity; increased oxidative stress; decreased or impaired NO-independent relaxing factors; changes in the production or action of hormones, growth factors and cytokines; and the increased generation and activity of opposing vasoconstrictors (RhoA/Rho-kinase). The best-characterized of these mechanisms are the result of hyperglycaemia. Elevated free fatty acids which are seen in patients with insulin resistance, may induce endothelial dysfunction through the activation of protein kinase C (PKC), the increased production of

reactive oxygen species (ROS), elevation in triglyceride and LDL, and decrease in HDL levels (Creager et al., 2003). More recent evidence suggests that the effects of hyperglycaemia and insulin resistance on endothelial cells are additive, since defects in both glucose and lipid metabolism produce similar effects with the resultant decrease in endothelial NO availability (Du et al., 2006;Du et al., 2003). The association between metabolic syndrome, insulin resistance and obesity and ED in men are now well characterised and understood (Esposito et al., 2005).

**d. ET-B receptor and ultrastructural changes**

ED in diabetic patients may be the result of an imbalance toward increased penile vasoconstriction possibly secondary to endothelin (ET) receptor signalling and ultrastructural changes in the endothelium. ET-1 produced by the vascular endothelium is a potent vasoconstrictor in the penis (Mills et al., 2001). ET-1 has been shown to be elevated in the plasma of diabetic patients (Takahashi et al., 1990). It has been hypothesized that ET-A receptors in cavernosal tissue have a vasoconstricting role. Thus, the elevation of the ET-A receptor and its ligand may cause the penile vasculature to have an imbalance toward vasoconstriction (Mumtaz et al., 2006). Furthermore, the ET-B receptor has been associated with pro-mitogenic effects which have been suggested to account for early ultrastructural changes of atherosclerotic-like lesions in diabetic patients (Khan et al., 2001;Sullivan et al., 1997). In a hypercholesterolemia rabbit model there was reported to be a reduction in the number of ETB receptors which may account for the reduced relaxation responses seen (Sullivan et al., 1998). Additionally, it has been shown that the tunica albuginea from diabetic rats is diminished and irregularly arranged. The structural alteration in the tunica albuginea is hypothesized to contribute to diabetic ED by impairing the veno-occlusive mechanism (Lu et al., 2004).

e. **Rho kinase**

As described previously, calcium sensitization may be initiated by agonist activation of hetero-trimeric G protein coupled receptors. Abnormally increased activation of myosin by Rho-kinase using this mechanism may have a role in ED (Somlyo and Somlyo, 2000).

In a diabetic rabbit model it has been demonstrated that the corpus cavernosum exhibited increased sensitivity to ET-1 compared to controls (Chang et al., 2003). This increased sensitivity was associated with a 2-3 fold up-regulation of the ET-A receptor and could be blocked by the Rho-kinase inhibitor Y-27632. These results suggest that the increased ET-1-induced contraction is largely dependent upon Rho-kinase (Chang et al., 2003). This mechanism suggests a possible role involving the transduction pathways for the ET receptors in the pathogenesis of diabetic ED. Moreover, Rho-kinase expression has now also been found to be increased in the diabetic vasculature and implicated in diabetes-induced nephropathy (Gojo et al., 2007;Xie et al., 2006).

**f. Cyclic GMP dependent kinase (PKG)**

It has been reported that the second messenger cGMP elicits cavernosal smooth muscle relaxation primarily through the action of PKG (Lincoln and Cornwell, 1993). The PKG acts by altering intracellular calcium levels and opens calcium-dependent potassium channels leading to hyperpolarisation of smooth muscle cells(Chang et al., 2004). Studies have demonstrated that PKG-1 knock-out mice have impaired cavernosal smooth muscle relaxation in response to neuronal and endothelial NO (Hedlund et al., 2000a). Furthermore, *in vitro* studies have shown that both isoforms of PKG-1 (a and b) are able to protect cGMP from hydrolysis, with the a sub-type conferring a higher degree of protection (Kotera et al., 2003). Moreover, in diabetic rabbit corpus cavernosum, both isoforms of PKG-1 were found to be significantly reduced (PKG-1a was decreased more than PKG-1b) as well as a decrease in PKG-1 activity in the corpus cavernosum of diabetic rabbits as compared with normal rabbits (Chang et al., 2004). It is therefore possible that a decrease in both the quality and quantity in PKG-1 may be a mechanism by which to promote diabetic ED by reduced activity of the cGMP second messenger pathway.

### 1.4. Current pharmacological treatments for erectile dysfunction

There are currently a broad range of options available for the management of ED. They include oral agents, intracavernosal injection (papaverine, phentolamine, prostaglandin E1, VIP), transurethral vasoactive agents (prostaglandin E1), vacuum erection devices, vascular surgery and penile prostheses.

Oral agents are the least invasive option and are the most accepted form of treatment accepted by the majority of patients as a first line. In the following section, I present the current evidence for PDE5 inhibitors and briefly describe the role of other agents (Table 1).

| **Oral Treatments** | **Mechanism of action** |
|---|---|
| Sildenafil citrate (Viagra™) | PDE5 inhibitor |
| Tadalafil (Cialis™) | PDE5 inhibitor |
| Vardenafil hydrochloride (Levitra™) | PDE5 inhibitor |
| Phentolamine (Vasomax™) | α-adrenoceptor antagonist |
| Doxazosin (Cardura™) | α-adrenoceptor antagonist |
| Prazosin (Minipress™) | α-adrenoceptor antagonist |
| Yohimbine | α-adrenoceptor antagonist |
| Terazosin (Hytrin™) | α-adrenoceptor antagonist |
| Trazodone (Desyrel™) | α-adrenoceptor antagonist |
| Apomorphine (Uprima™, Ixense™ and Taluvian™) | Dopamine receptor agonist |

**Table 1:** The current oral agents for the treatment of male erectile dysfunction.

### 1.4.1 PDE5 inhibitors:

The second messengers cGMP and cAMP are metabolized to GMP and AMP respectively by a superfamily of enzymes called phophodiesterases (PDEs) (Francis et al., 2001). Among all the PDEs PDE5, 6 and 9 are specific for cGMP and PDE5 is the predominant PDE in the corpus cavernosum (Francis et al., 2001;Michell et al., 1999).

**PDE5: cGMP-specific phosphodiesterase**

The physiological significance of PDE5 in the regulation of penile smooth muscle tone was confirmed by the first successful clinical use of a specific inhibitor of PDE5, sildenafil in the treatment of ED (Boolell et al., 1996). By inhibiting PDE5 hydrolytic activity sildenafil use has been found to be associated with a higher rate of accumulation of cGMP within the penile vasculature and cavernosal smooth muscle in response to NO. This increase in cGMP in turn enhances the erectile response (Ballard et al., 1998). Three different isoforms of PDE5 have been reported in human tissue, PDE5A1, A2 and A3 (Lin et al., 2000). In human corpus cavernosum PDE5A2 has been found to be the most abundantly expressed isoform (Lin et al., 2000).

**PDE5 inhibitors**

Sildenafil (Viagra$^{TM}$), vardenafil (Levitra$^{TM}$) and tadalafil (Cialis$^{TM}$) are the currently available PDE-5 inhibitors. Sildenafil and vardenafil differ only minimally in terms of their structure, while tadalafil differs markedly from both sildenafil and vardenafil in terms of its molecular structure. The PDE5 inhibitors have the same mechanism of action. However, they differ in their efficacy for the inhibition of the enzyme, in their selectivity for PDE5 over other isoenzymes such as PDE6 and in their pharmacological properties.

**Potency and selectivity**

The potency of the PDE5 inhibitors can be measured *in-vitro* by assessing the $IC_{50}$ value (concentration at which the enzyme activity is 50% inhibited) (Table 2). Using these values vardenafil exhibits a PDE5 inhibitory potential approximately five times higher than that of sildenafil (Kim et al., 2001). PDE6 plays an important role in the conversion of light impulses into nerve impulses in the retina. For PDE6 sildenafil and vardenafil show a lower selectivity than tadalafil. With respect to PDE11 tadalafil shows only 5 times greater selectivity with respect to PDE5 (Baxendale et al. 2001). PDE11 has been detected in a variety of human tissues, e.g. in the heart, pituitary gland, brain and testes. The physiological significance of PDE11 and the possible consequences of its inhibition have not yet been fully established.

**Pharmacokinetics**

All three drugs are rapidly absorbed from the gastrointestinal tract, with peak plasma levels being attained within 1 hour in the case of sildenafil (Milligan et al., 2002) and vardenafil (Rajagopalan et al., 2003) and after 2 hours in the case of tadalafil (Corbin and Francis, 2002). Food intake causes no delay or reduction in tadalafil absorption (Sussman, 2004), whereas it is known to reduce and delay sildenafil (Nichols et al., 2002;Rajagopalan et al., 2003). The mean half-lives ($t_{1/2}$) of sildenafil and vardenafil are 3 - 4 hours whereas that of tadalafil is approximately 18 hours (Corbin and Francis, 2002) (Table 2). The elimination of sildenafil, vardenafil and tadalafil takes place overwhelmingly via the liver, mostly via the cytochrome enzyme P450 (CYP3A4) (Corbin and Francis, 2002).

**Clinical efficacy**

Results from clinical trials suggest that all three PDE-5 inhibitors are effective in a wide range of patient groups (Brock et al., 2002;Eardley et al., 2001;Hellstrom et al., 2003). Treatment with vardenafil at a dose of 20 mg produced an improvement in the ability to achieve an erection in 80% of ED patients (Porst et al., 2001). In a comparable study of sildenafil (100 mg dose) by Goldstein, 84% of ED patients were successfully treated (Goldstein et al., 1998). Treatment with tadalafil 20 mg produced an improvement

in the ability to achieve an erection in 81% of ED patients (Padma-Nathan, 2003). More recently comparative studies between vardenafil and sildenafil suggest that patients do not have a significant preference between these two PDE5 inhibitors (vardenafil 38.9%; sildenafil 34.5%; and no preference 26.6%) (Rubio-Aurioles et al., 2006).

**Adverse effects**

The most common side effects seen with sildenafil include headache, flushing, dyspepsia, and rhinitis (Fagelman et al., 2001). The adverse effects with tadalafil and vardenafil are similar to sildenafil, however tadalafil is associated with a higher incidence of back pain (4-9%) and myalgia (1-7%).

| | **Sildenafil** | **Vardenafil** | **Tadalafil** |
|---|---|---|---|
| Time to onset | 30-60 min | 25-40 min | 45 min |
| Duration of action | 4-8 hours | Up to 6 hours | 24-36 hours |
| $IC_{50}$ for PDE5 (nM) | 3.5-3.7 | 0.1-0.7 | 0.9-1.8 |
| *PDE1 | 80 | 500 | >4450 |
| *PDE2 | >8570 | 44290 | >14800 |
| *PDE3 | 4630 | >7140 | >14800 |
| *PDE4 | 2190 | 43570 | >14800 |
| *PDE5 | 1 | 1 | 1 |
| *PDE6 | 10 | 16 | 190 |
| *PDE7 | 6100 | >214000 | >14800 |
| *PDE8 | 8500 | >214000 | >14800 |
| *PDE9 | 750 | 4150 | >14800 |
| *PDE10 | 2800 | 21200 | >14800 |
| *PDE11 | 780 | 1160 | 5 |

**Table 2**: Pharmacological properties of three PDE5 inhibitors sildenafil, tadalafil and vardenafil are shown as "time to onset" and "duration of action" obtained from clinical studies. $IC_{50}$ values are from *in vitro* enzyme studies. * denotes the ratio of $IC_{50}$ for that PDE enzyme over $IC_{50}$ for PDE5 (Corbin and Francis, 2002).

### 1.4.2 Alpha-adrenoceptor antagonists

The sympathetic nervous system via stimulation of α-adrenoceptors is considered to be the major determinant of cavernosal smooth muscle contraction and detumescence. Currently available antagonists include phentolamine, yohimbine, prazosin, doxazosin and terazosin. However none of these agents are commonly used for the treatment of ED due to poor efficacy and high incidence of adverse effects (Brock, 2000;Guay et al., 2002;Kirby et al., 2005).

### 1.4.3 Dopamine receptor agonists - apomorphine

Apomorphine has been used in the treatment of a variety of medical conditions, including Parkinson's disease. More recently, apomorphine has been shown to be effective in eliciting penile erection in both animal models and in humans (Lal, 1988).

Apomorphine has been found to activate oxytocinergic neurons in the paraventricular nucleus (PVN) in the hypothalamus. This in turn projects to other key areas of the brain which regulate erectile function (Chen et al., 1999). Penile erection in rats, which increased in response to intraperitoneal injection of apomorphine, was reported to be inhibited by haloperidol, a central dopamine receptor antagonist but unaffected by domperidone, a peripheral dopamine antagonist (assi-Benelli et al., 1979). In rats, apomorphine was reported to directly activate the sacral pro-erectile nucleus. After stimulating the PVN, the sacral parasympathetic pro-erectile outflow may be activated, which results in a release of NO. There is then subsequent smooth muscle relaxation in the penis, corporal engorgement and erection (Giuliano et al., 2001).

A sublingual route was used after it was discovered that subcutaneous formulations also stimulate an erectile response in men whilst minimizing side effects (Heaton et al., 1995). Sublingual apomorphine (Uprima™, Ixense™ and Taluvian™) is a centrally acting agent licensed for the treatment of ED in most European and South American countries, but not currently in the United States.

Double-blind, placebo controlled crossover trials have shown that 2 and 3 mg of apomorphine were statistically superior to placebo in inducing an erection sufficient for intercourse in 38-53 % of patients depending on the underlying ED aetiology such as coronary artery disease, benign prostatic hypertrophy, hypertension and diabetes (Dula et

al., 2001;Dula et al., 2000). However, a recent post marketing observational cohort study of general practice prescribing for Uprima in the UK has reported a low level of effectiveness and a high incidence of discontinuation as a result of lack of efficacy (Maclennan et al., 2006). This is in keeping with the personal experience of most urologists and it is therefore now seldom prescribed.

### 1.5. Where do PDE5 inhibitors fail?

PDE5 inhibitors prevent the hydrolysis of cGMP by PDE5, promoting its accumulation which ultimately augments penile smooth muscle relaxation. It is thought that a certain amount of NO is required for PDE5 inhibitors to be efficacious. Disease states which exhibit deficiencies in NO-cGMP pathway (due to decreased expression and activity of neuronal and endothelial nitric oxide synthases, impaired NO release or diminished NO bioavailability) will limit the efficacy of PDE5 inhibitors. If the pathophysiology is severe, PDE5 inhibitors will not be able to compensate the reduction in the NO-cGMP pathway. This will result in a failure of erectile dysfunction treatment. Highest rates of failure have been seen in long-term diabetes and after treatment for prostate cancer as summarised below:

#### 1.5.1 Diabetes mellitus

**Sildenafil**

The sildenafil Diabetes Study Group reported that 56% of men with ED and diabetes who received sildenafil (25-100mg) for 12 weeks reported improved erections (GAQ). This is contrast to placebo where only 10% reported better erections (Rendell et al., 1999). In a more recent double-blind, placebo-controlled, flexible-dose study patients were randomized to receive sildenafil (25-100 mg) or placebo for 12 weeks. The erectile function domain of the IIEF (See Appendix) showed only a 6-point increase in the mean score over placebo (Figure 9). However, men with mild/moderate ED achieved a higher overall score compared with men with severe ED (Rendell et al., 1999;Stuckey et al., 2003). The percentage of improved erections (GEQ, 66.6 vs. 28.6%) and successful intercourse attempts (63 vs. 33%) was significantly higher with sildenafil compared with placebo. Moreover, similar responses were also reported in Type II diabetic patients (Boulton et al., 2001). In this study the majority of needed to take the maximum permitted dose in order to achieve an effect (100mg) (Kalinichenko et al., 1999).

**Vardenafil**

In a multicentre double-blind placebo-controlled fixed-dose trial, patients with diabetes (type 1 or type 2) and ED were randomized to take 10 or 20 mg vardenafil or placebo as needed for 12 weeks. With respect to the erectile function domain, the dose-dependent final scores for the 10- and 20-mg dose were 17.1 and 19.0 compared with 12.6 for placebo ($P < 0.0001$). These two doses significantly enhanced the rates of successful penetration ($P < 0.0001$) and successful intercourse ($P < 0.0001$) compared with placebo. The results suggest that vardenafil was effective in increasing intercourse success rates irrespective of baseline ED severity, HbA1c level and for both type 1 and 2 diabetes. However, the results did not show an improvement in erectile function domain to normal ranges (Goldstein et al., 2003). Similar results have been reported in a prospective, randomized study in PDE5 inhibitor-naive patients with type 1 diabetes. Vardenafil treatment significantly improved the erectile function domain score ($P < 0.0001$) of the IIEF (From 13 to 20) (Ziegler et al., 2006) (Figure 9). These results are encouraging but are again inferior to results seen in other groups.

**Tadalafil**

In a study in both type 1 and type 2 diabetics, tadalafil was given at a dose of 10-20 mg taken up to once daily for 12 weeks. At doses of 10mg or 20mg, the erectile function domain score was improved by 6.4 and 7.3 respectively regardless of baseline HbA1c level (Saenz, I et al., 2002) (Figure 9). More recently a retrospective review of placebo-controlled trials reported that at baseline, patients with diabetes had more severe ED than patients without diabetes, with mean IIEF erectile function domain scores of 12.6 and 15.0 respectively. In patients with diabetes receiving tadalafil at 20 mg, there was reported to be a mean improvement of 7.4 in their erectile function domain score against baseline versus 0.9 for placebo ($p<0.001$). Furthermore, the baseline erectile function domain scores were found to correlate inversely with baseline HbA1c levels. However, as for other PDE5 inhibitors, the response to tadalafil was lower in men with diabetes than in men without diabetes (Fonseca et al., 2004).

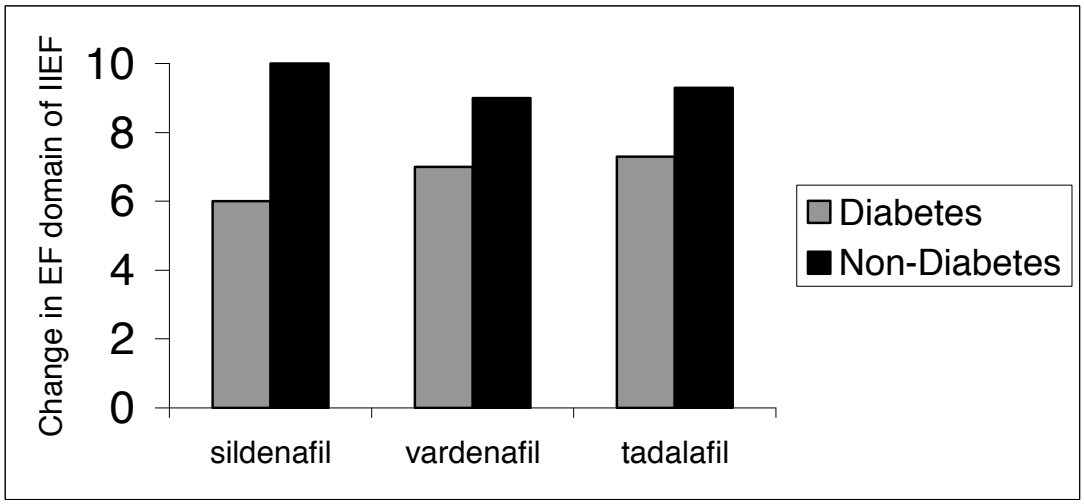


**Figure 9:** The change in erectile function (EF) domain of the IIEF in non-diabetic patients compared with diabetic patients after treatment with sildenafil 100mg (Carson et al., 2002;Stuckey et al., 2003), vardenafil 20mg (Hellstrom et al., 2002;Ziegler et al., 2006) and tadalafil 20mg (Saenz, I et al., 2002;Seftel et al., 2004).

### 1.5.2 Prostate cancer

An intact neurogenic innervation of the penis is required for physiological erectile responses. As a consequence patients with prostate cancer after either radical surgery or radiotherapy, have a high risk of post-operative ED.

**Sildenafil**

In an open label study of sildenafil with ED after radical prostatectomy (RP), 53% of men receiving sildenafil (50-100mg) reported improved erections, whereas 40% reported an enhanced ability to achieve and maintain erections (Lowentritt et al., 1999). In this study the mean IIEF erectile function domain score increased from 9 to 14 ($p < 0.001$) (Figure 10). Moreover, it was demonstrated that degree of ED was directly related to the degree of nerve sparing, a higher pathological stage and age.

In a retrospective review of seven studies, the reported response rate to sildenafil was 14%-53% for an end point of erection sufficient for vaginal intercourse. This study also demonstrated evidence for a lower response rate after non-nerve-sparing (0%-15%) versus nerve-sparing surgery (35%-75%; but not after unilateral (10%-80%) versus bilateral nerve-sparing surgery (46%-72%). It was suggested that the odds of responding improved 12-fold with preservation of at least one neurovascular bundle (Feng et al., 2000;Montorsi and McCullough, 2005).

The efficacy of sildenafil in patients with ED following radiation therapy for prostate cancer was assessed after three-dimensional conformal external beam irradiation or brachytherapy without androgen deprivation. Results were assessed at 3 time points. These were less than 12, 13-24 and 25-36 months following the completion of radiation therapy. The corresponding mean IIEF erectile function domain scores for these three time points after brachytherapy or external beam irradiation was 26/23, 22/19 and 17/15, respectively. Moreover, the percentage of patients who achieved normalization of the IIEF erectile function domain at the three time points in the brachytherapy or external beam irradiation groups was 60%/50%, 48%/42% and 26%/19%, respectively. The results of this study suggest that sildenafil can modestly improve erectile function following radiation therapy for prostate cancer. However, there appears to be a clear time dependence for the response to this therapy to be successful (Ohebshalom et al., 2005).

Moreover, sildenafil appears to improve erectile function to a greater degree following brachytherapy rather than external beam radiotherapy (Ohebshalom et al., 2005;Shemtov et al., 2004).

**Vardenafil**

In a double-blind study after bilateral nerve sparing retropubic radical prostatectomy, patients were randomized to take placebo or 10 or 20 mg of vardenafil. After 12 weeks both vardenafil doses were reported to be significantly superior to placebo ($p < 0.0001$) for all efficacy variables assessed. The mean erectile function domain score improved from 9.2 to 15.3 after treatment with the higher dose of Vardenafil (Figure 10). When the average intercourse success rate per patient receiving 20 mg vardenafil was assessed, it was reported that this variable was successful in 74% in men with mild to moderate ED and 28% in men with severe ED (compared to 49% and 4% for placebo, respectively). The results of this study therefore suggest that vardenafil is able to significantly improve some of the key elements of erectile function in men with mild ED after nerve sparing RP but is less effective in cases of severe ED (Brock et al., 2003;Ziegler et al., 2006). Similar results have now been reported in another randomized, placebo controlled, double-blind trial in the USA and Canada (Nehra et al., 2005).

**Tadalafil**

In a double-blind randomized controlled study involving men with ED after bilateral nerve sparing radical prostatectomy, patients were randomized to either tadalafil or placebo. Of the patients treated with tadalafil 62% reported improved erections at the completion of the study vs. 23% of controls. Furthermore, 54% of intercourse attempts were reported to be successful in penetrative intercourse compared to 32% of controls. However, the mean erectile function domain score showed only a small increase for patients receiving tadalafil (5.9) (Figure 10) (Montorsi et al., 2004).

Following three-dimensional conformal external beam irradiation for prostatic carcinoma, the efficacy of tadalafil was assessed in a double-blind, placebo-controlled, cross-over study over a 12 week period. The patients received 20 mg of tadalafil or placebo for 6 weeks. There was reported to be a significant increase in the mean erectile

function domain score from baseline with tadalafil (8.4 to 17.7), but not with placebo (9.5). Furthermore, sixty-seven percent of the patients reported an improvement in their erectile function with tadalafil (placebo 20%), with 48% reporting the successful ability to have intercourse with tadalafil (placebo 9%) ($p<0.0001$) (Incrocci et al., 2006).

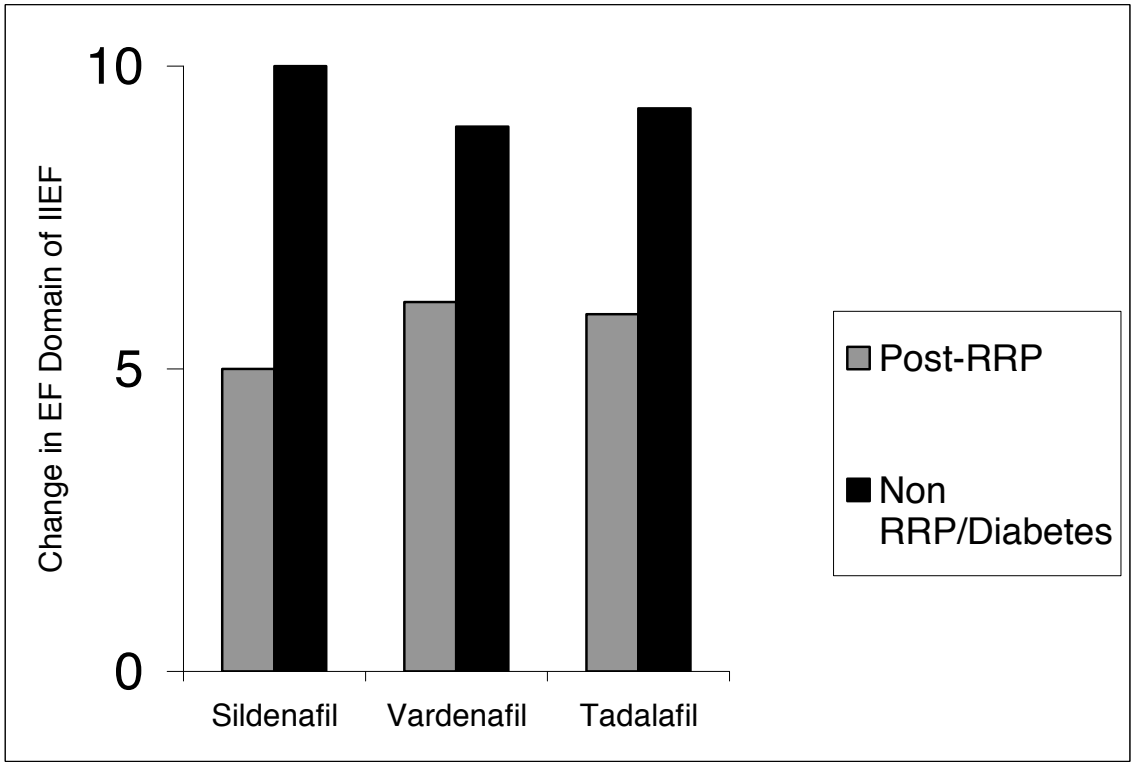


**Figure 10:** The changes in the erectile function (EF) domain of the IIEF in patients with no diabetes/radical prostatectomy after treatment with sildenafil (100mg) (Lowentritt et al., 1999), vardenafil (20mg) (Brock et al., 2003) and tadalafil (20mg) (Montorsi et al., 2004) versus post radical prostatectomy patients.

### 1.6 Soluble guanylate cyclase activators

In the previous section ( see 1.5) I have discussed areas where PDE5 inhibitors fail. In order to achieve improved efficacy in these groups of patients, newer therapeutic strategies are required. One novel approach is to target the NO-independent activation of the enzyme sGC. The benzylindazole derivative, YC-1 is an activator of sGC (Friebe and Koesling, 1998). YC-1 has been shown to relax vascular (Wegener et al., 1997) and cavernosal (Mizusawa et al., 2002;Nakane et al., 2002) smooth muscle. YC-1 has also been shown to evoke erectile responses when given intracavernously and enhance erections induced by cavernous nerve stimulation and apomorphine when given systemically (Mizusawa et al., 2002). However it has been reported to have phosphodiesterase (PDE) inhibitory activity (Galle et al., 1999) and stimulate the synthesis and release of NO (Wohlfart et al., 1999).

More recently, the pyrazolopyridine derivative BAY41-2272 (5- cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b] pyridine-3-yl]pyrimidin-4ylamine) has been synthesized and shown to stimulate soluble guanylate cyclase in a NO-independent manner without an effect on cGMP breakdown (Becker et al., 2001b;Stasch et al., 2001). In contrast to YC-1, it has been shown to have a distinctly higher potency and no phosphodiesterase inhibitory activity (Stasch et al., 2001). However, it is not yet known whether NO-independent activation of sGC can lead to relaxation of cavernous smooth muscle.

### 1.7 Nitric oxide releasing PDE5 inhibitors

A further potential approach to improving efficacy is the combination of a NO-releasing compound with a PDE5 inhibitor. Recent evidence suggests that the PDE5 inhibitor sildenafil has successfully been combined into a NO releasing formulation (sildenafil nitrate; NCX-911) (Figure 11).

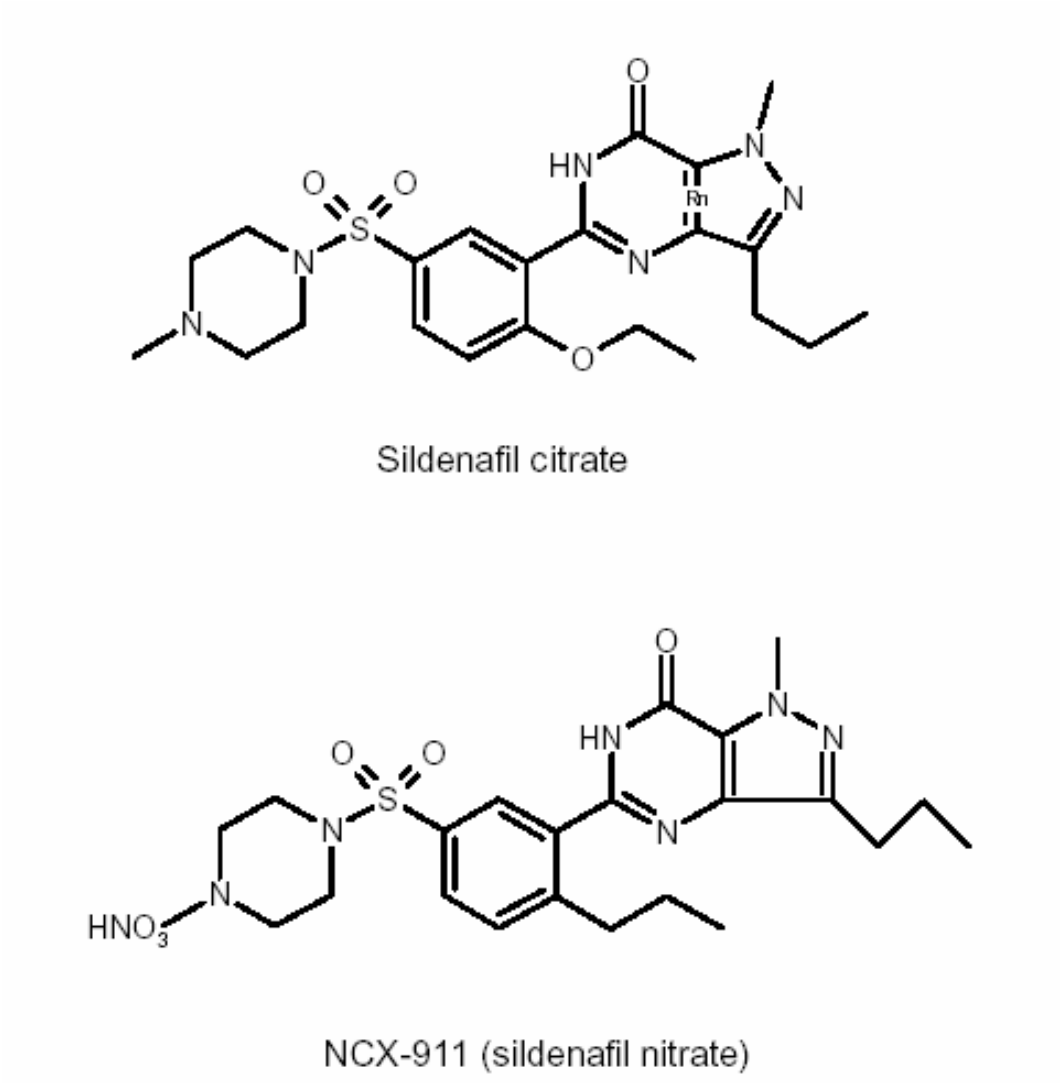


**Figure 11:** The structures of sildenafil and NCX-911

It has been demonstrated that NCX-911 and sildenafil have similar selectivity and potency with respect to inhibiting PDE5 in-vitro (Riffaud JP, 2001).

NCX-911 has been previously shown to release NO spontaneously and increase cGMP concentrations by activating sGC and inhibit PDE5 (Seidler et al., 2002). Furthermore, NCX-911 has been shown to increase cGMP in a concentration dependent manner in the absence of endogenous NO. In comparison, it was found that sildenafil had little effect on cGMP concentrations under the same conditions (Riffaud JP, 2001).

In a hypercholesterolemia rabbit model of erectile dysfunction, NCX-911 was shown to be significantly more potent (5-10 times) than sildenafil at reducing the phenylephrine-induced tone in the corpus cavernosum (Shukla et al., 2005). However it is not yet known whether NO-releasing PDE5 inhibitors have improved efficacy in conditions of NO deficiency.

### 1.8. Hypothesis and Aims of Thesis

Although the current therapeutic options for erectile dysfunction have been shown to be safe and efficacious, there clearly remains significant scope for further improvement. It is now well accepted that certain groups of patients, such as long-term diabetic patients and patients following treatment for prostate cancer respond less well to PDE5 inhibitors. Moreover, in these difficult to treat patient groups there does not appear to be any significant benefit for one PDE5 inhibitor over another. The reduced response to PDE5 inhibitors has been mainly attributed to a lack of NO bio-availability which may be in part due to a lack of endogenous NO production or availability. Therefore, new approaches which may potentially relax cavernosal smooth muscle without the need of endogenous NO are attractive.

I therefore hypothesised that in conditions of NO deficiency in the corpus cavernosum and anococcygeus muscle, NO-independent sGC activators and NO-releasing PDE-5 inhibitors may be more potent than PDE5 inhibitors at reducing smooth muscle tone and in potentiating nitrergic relaxations.

The aim of my thesis was therefore to investigate *in vitro* effects of a PDE-5 inhibitor (sildenafil), a NO-independent soluble guanylate cyclase activator (BAY 41-2272) and an NO-releasing PDE5 inhibitor (NCX-911) on cavernosal (human and rabbit) and anococcygeal (rat) smooth muscle in conditions of NO deficiency. My further aim was to compare their efficacy with a non-selective sGC activator (YC-1) and a NO donor (spermine-NONOate).

# CHAPTER 2.

# MATERIALS AND METHODS

## 2.0 Materials and Methods

The hypothesis was tested *in vitro* using urogenital tissue obtained from male rabbits, rats and patients. All animal experiments were conducted according to the rules outlined by the Home Office, Animals (Scientific Procedures) Act 1986 (project number 70/5161). The patients donating tissue to this study gave fully written informed consent and the study was approved by the Ethics Committee of the Riverside Health Authority and the Ethics Committees of the Mid Sussex National Health Service Hospital (RREC 2911).

### 2.1 *In vitro* experiments using rabbit corpus cavernosum

Male New Zealand White rabbits weighing 3.0–3.5 kg (Harlan, UK) were housed in the Biological Services Unit, Wolfson Institute for Biomedical Research, University College London in conditions conforming to Home Office regulations. The rabbits were allowed to accommodate to their environment for at least one week before being used. The rabbits were killed with an intravenous overdose of phenobarbitone (200 mg/kg) (Euthesate, Willows Francis Veterinary, UK) injected into the marginal vein of the ear (Figure 12).

**Figure 12**:Phenobarbitone being injected into the marginal vein of a rabbit

After confirmation of death, a lower midline incision was made above the symphysis pubis. The penile bulb was exposed by de-gloving the penis of overlying skin and fur. Once each crus was identified, care was taken to detach the penis as proximal as possible to its attachment to the pubic bone. The surrounding ischiocavernous and bulbospongiosus muscles were dissected off the penis and the whole organ placed in a container containing cold Krebs' solution, which was continuously bubbled with 95% oxygen and 5% carbon dioxide.

The penis was then pinned in a dish containing cold Krebs' solution and the remaining overlying connective tissue and urethra were dissected free (Figure 13). A ventral corporotomy was made on each side of the penis and using fine dissecting instruments the erectile tissue was carefully dissected from the surrounding tunica albuginea (Figure 14). The corpus cavernosum was excised and cut longitudinally to obtain four identical strips (3 × 8 mm). Both ends of the cavernosal strips were ligated

using ties (which were used to mount strips) (Figure 15). The cavernosal strips were then mounted between ring electrodes in horizontal superfusion chambers.

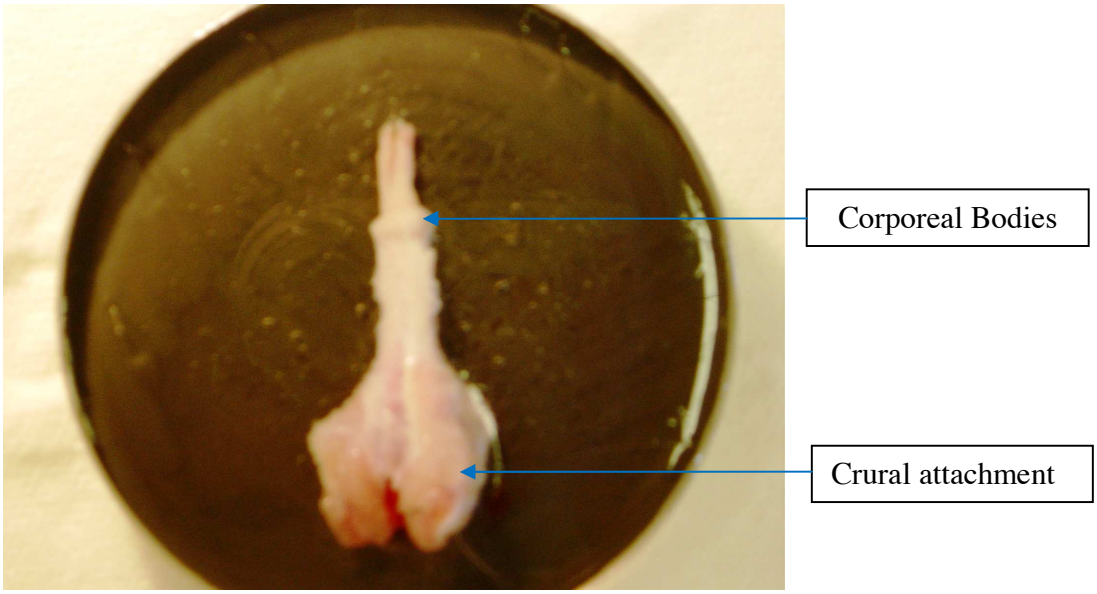


**Figure 13:** The rabbit penis is placed in a dish containing Krebs' solution. The specimen is held down gently with pins whilst the corpus cavernosum is dissected out.

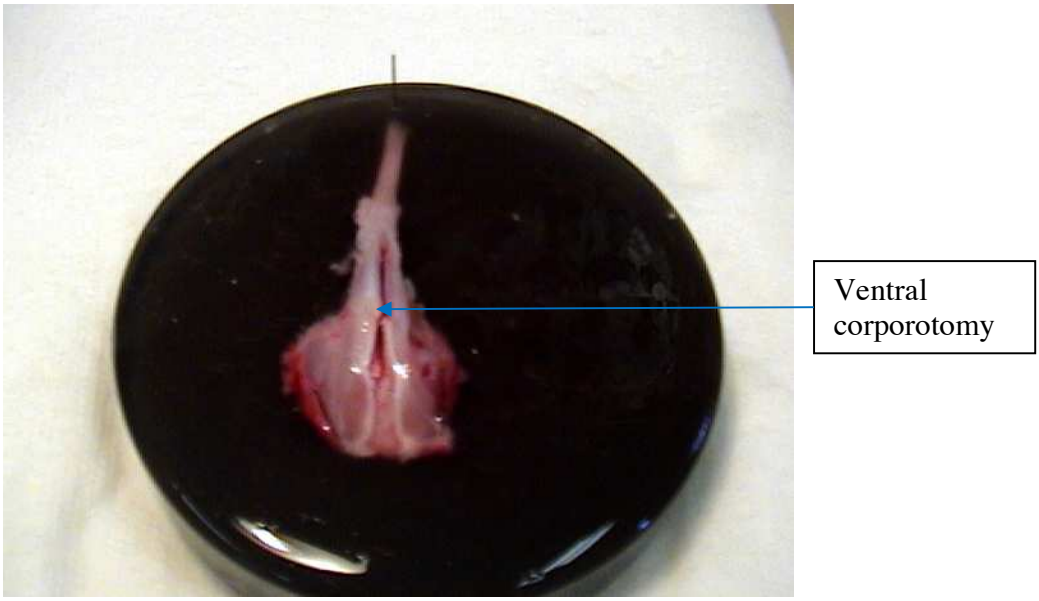


**Figure 14:** The overlying bulbospongiosus and connective tissue have been removed, exposing the ventral surface of the penis. A ventral corporotomy is made to help facilitate dissection of the corpus cavernosum.

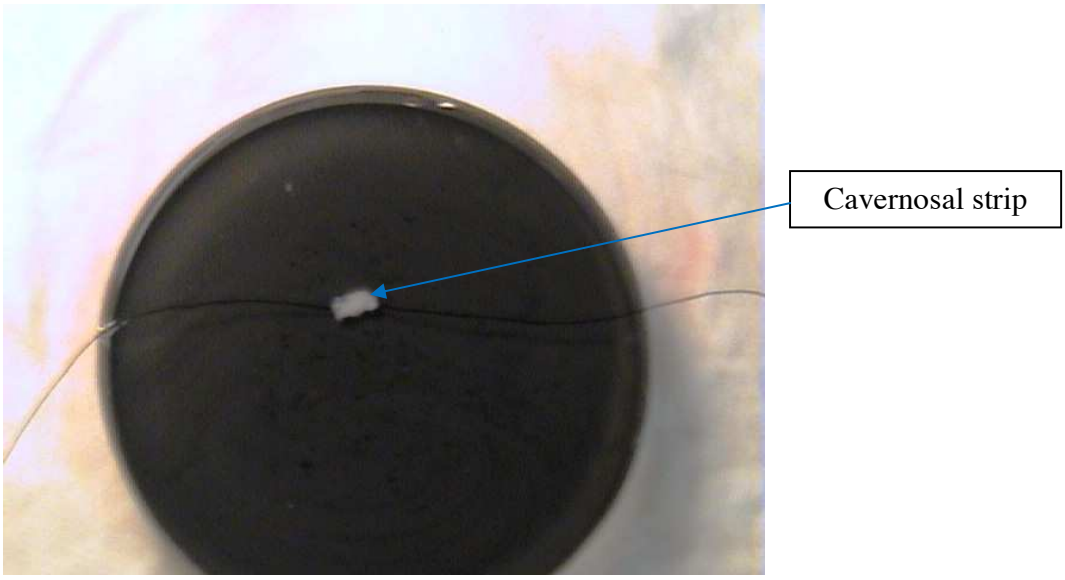


**Figure 15:** Each end of dissected rabbit corpus cavernosum is tied using 4-0 silk sutures. This then enables the strip to be easily mounted in a superfusion chamber.

### 2.2 *In vitro* experiments using human corpus cavernosum

Whole corpus cavernosum was obtained from patients undergoing penectomy for gender reassignment at Charing Cross Hospital, London and Sussex Nuffield Hospital, Brighton, UK. Tissues were transported from hospital to the laboratory in ice-cold Krebs' solution in less than 2 hours. After arrival they were transferred to Krebs' solution at room temperature and cleaned of adherent tissue and blood. Eight strips (3 x 8mm) were cut from middle part of each corpus cavernosum.

### 2.3 *In vitro* experiments using anococcygeus muscle from diabetic and non-diabetic rats

Male Wistar rats (Harlan, UK) weighing 200–250 g were divided into two groups. Diabetes was induced in one group by a single intraperitoneal injection of streptozotocin (STZ; 75 mg/kg) as described previously (Cellek et al., 2003;Cellek et al., 1999). The other group was injected with the vehicle (saline). Sixteen weeks after STZ injection the animals were weighed and killed with cervical dislocation. Blood samples were collected for analysis of serum glucose levels (Reflolux S Glucometer, Boehringer Manheim, Germany) and bilateral anococcygeus muscles were isolated for functional studies as described previously (Cellek et al., 2003).

An abdominal midline incision was made and the genital organs, bladder and urethra cleared, then the pelvis split. The exposed colon was cut at the pelvic brim and the pelvic portion pulled forward to reveal the two underlying muscles surrounded by connective tissue. A maximal length of each muscle was then carefully removed. Each

anococcygeus muscle was cleaned of adherent tissues and mounted horizontally between two ring electrodes in horizontal superfusion chambers.

**2.4 Horizontal superfusion chamber:**

Each preparation (human or rabbit corpus cavernosum or rat anococcygeus muscle) was mounted horizontally between two ring electrodes (4 mm diameter) in superfusion chambers (37°C) as described previously (Cellek and Moncada, 1997b) (Figures 16 and 17). The chambers were perfused with Krebs' solution at a constant flow of 1.0 ml/min by means of peristaltic pumps (Miniplus 2, Gilson, United Kingdom). One end of the preparation was tied to a FT03C (Grass Instruments, Massachusetts, USA) force-displacement transducer connected to a Linearcorder WR 3101 (Graphtec, Tokyo, Japan) and a computer for registration of isometric changes in tension (Figure 17). A schematic diagram of the laboratory set- up is shown in Figure 18.

The preparations were stretched until they reached approximately the *in situ* length (0.4–1 g in rabbit corpus cavernosum, 0.5 g in rat anococcygeus muscle, 0.6 g in human corpus cavernosum) and allowed to equilibrate for 90 min. The preparations were stimulated electrically for 5 s with trains of rectangular pulses of 50 V, 0.3 ms pulse duration and at a range of frequency of 0.5-25 Hz, delivered by Grass S88 stimulators. The recorder was run at a rate of 25 mm/min to record the mechanical responses. The mechanical responses were also recorded on a computer by a specialized data acquisition system (Axon Instruments, USA). The chemicals were applied directly to the reservoir holding the Krebs' solution.

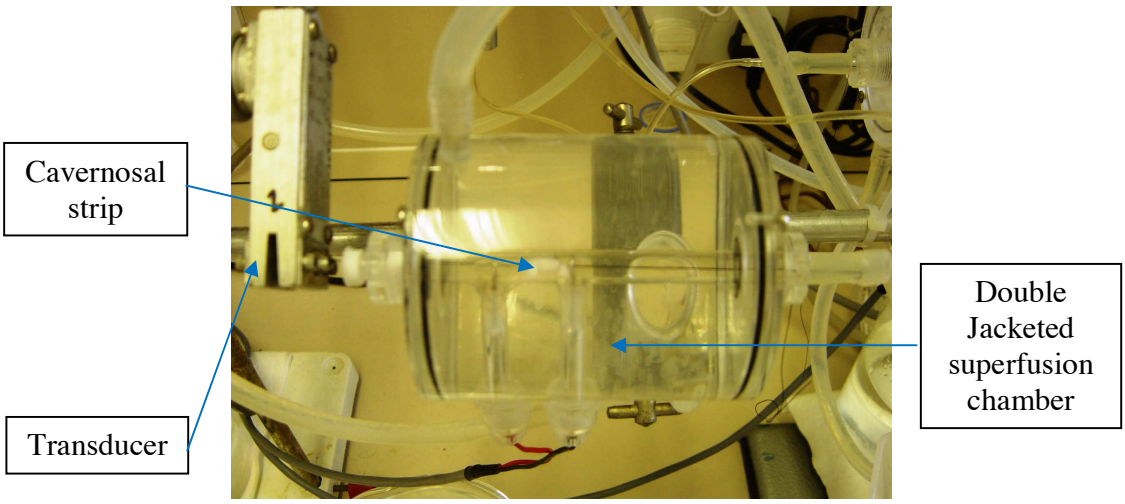


**Figure 16:** Each dissected strip of corpus cavernosum is mounted into a superfusion chamber. One end is attached to a transducer to detect changes in tension and the other end fixed.

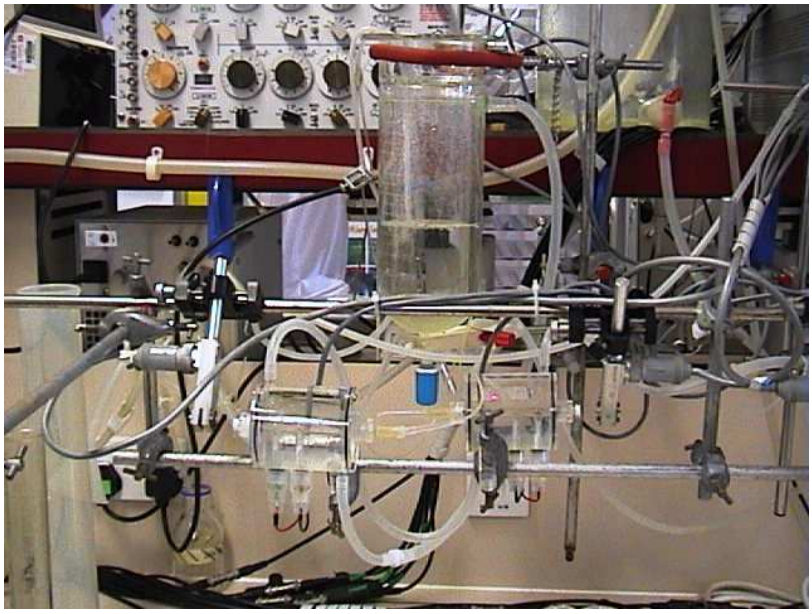

**Figure 17:** Overview of the laboratory set up showing superfusion chambers, organ bath and tubing.

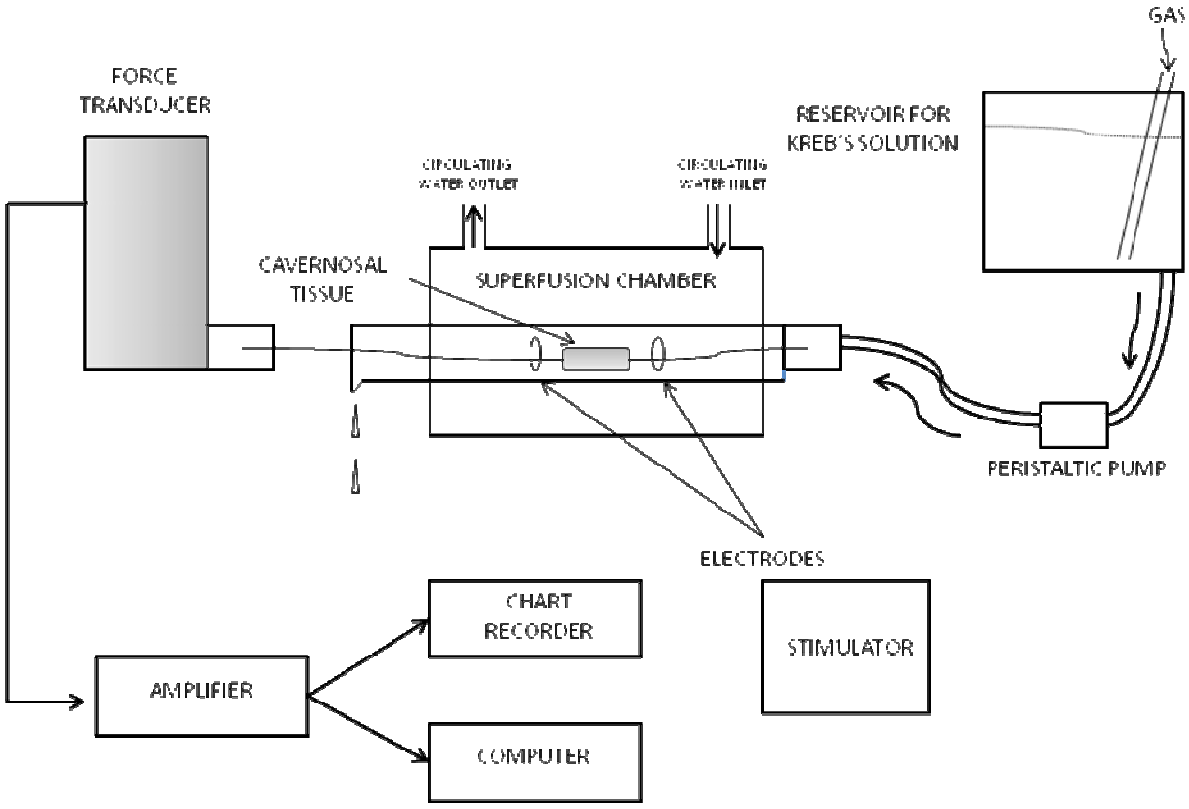


**Figure 18:** Schematic illustration of the experimental set up

**2.5 Krebs' solution and chemicals**

Fresh Krebs' solution was prepared each day of the following ionic composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, $KH_2PO_4$ 0.5, glucose 11.5, indomethacin 0.01, dexamethasone 0.01. The solution was bubbled continuously with 95% oxygen and 5% $CO_2$ and the pH of the solution was maintained at approximately 7.0-7.2.

The inducible form of NOS (iNOS) can be induced by trace amounts of endotoxin in the buffer (Rees et al., 1990) and cause loss of tone (Cellek and Moncada, 1997a). Dexamethasone (0.01mM) was added to the Krebs' solution to prevent induction of iNOS (Rees et al., 1990). The cyclooxygenase inhibitor indomethacin (0.01mM) was added to prevent synthesis of prostaglandins since these can cause non-neurogenic relaxations (Daniel et al., 1979).

NCX-911 was a gift from NicOx, France. Sildenafil citrate was extracted from Viagra® tablets as the free base, analysed by 1H-NMR and LC/MS to confirm identity and purity (>97%) and then converted to its citrate salt by Dr. D. Madge at the Medicinal Chemistry Department, Wolfson Institute for Biomedical Research, University College London. Tetrodotoxin was obtained from Calbiochem, UK. BAY41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl]-pyrimidin-4ylamine) was a gift from Bayer AG, Germany. All other chemicals were obtained from Sigma, UK.

## 2.6 Statistical analysis and presentation of the results

The effect of drugs on phenylephrine-induced contraction was expressed as a percentage reduction in tone. Results are expressed as mean ± standard error of the mean. Statistical analysis was performed using Student's unpaired two-tailed t-test. A probability value (P) less than 0.05 was considered to be statistically significant. The statistical analysis was performed using Graphpad Prism software (version 3.00, GraphPad Software Inc, San Diego, California, USA). *n* denotes either the number of patients or the number of animals. $EC_{50}$ values were calculated using Microcal Origin software (Version 4.0, Microcal Software Inc, USA).

## 2.7 Experimental design

### 2.71 Effect of compounds on elevated tone in the human and rabbit corpus cavernosum and rat anococcygeus muscle

Strips of corpus cavernosum from the rabbit and human and anococcygeus muscle from rat were incubated in Krebs' solution and allowed to equilibrate for 90 minutes. Electrical field stimulation (EFS) was applied to assess the viability of the tissue. Guanethidine (10μM) and scopolamine (10μM) were added to the organ bath to inhibit adrenergic and cholinergic responses respectively after which EFS was stopped. The $EC_{80}$ for phenylephrine for each tissue (rabbit and human corpus cavernosum, rat anococcygeus) was then determined by constructing a concentration response curve with cumulative additions of the compound (10 nM-1 mM). The effects of BAY41-2272, YC-1, spermine NO-NOate, sildenafil or NCX-911 were then investigated on the tone elevated by $EC_{80}$ of phenylephrine in the absence or presence of an inhibitor of sGC, ODQ (30$\mu$M) or an inhibitor of NO synthase, L-NAME (500$\mu$M).

### 2.72 Investigation of the effects of compounds on nitrergic relaxations

Nitrergic relaxations were obtained using EFS in the rabbit and human corpus cavernosum and rat anococcygeus muscle after treatment with scopolamine and guanethidine and elevation of the tone by $EC_{80}$ of phenylephrine. To study the effect of the compounds on nitrergic relaxations 2 sub-threshold concentrations (30 and 50 nM for BAY41-2272; 10 and 30 nM for sildenafil and NCX-911) were used (concentrations which do not reduce phenylephrine-induced tone) in the absence or presence of L-

NAME. The effect on the duration and magnitude of relaxations was assessed by measuring the area under the curve of each relaxation before and after the compound.

# CHAPTER 3.

# RESULTS

# 3.0 Results

## 3.1 Determination of the $EC_{80}$ for phenylephrine

The $EC_{80}$ for phenylephrine was determined by constructing concentration response curves with incremental cumulative additions of the compound ($10^{-8}$ – $10^{-3}$ M) in the absence of electrical field stimulation (EFS) (n=8; Figure 19). The calculated $EC_{80}$ was 3$\mu$M in both rabbit and human corpus cavernosum.

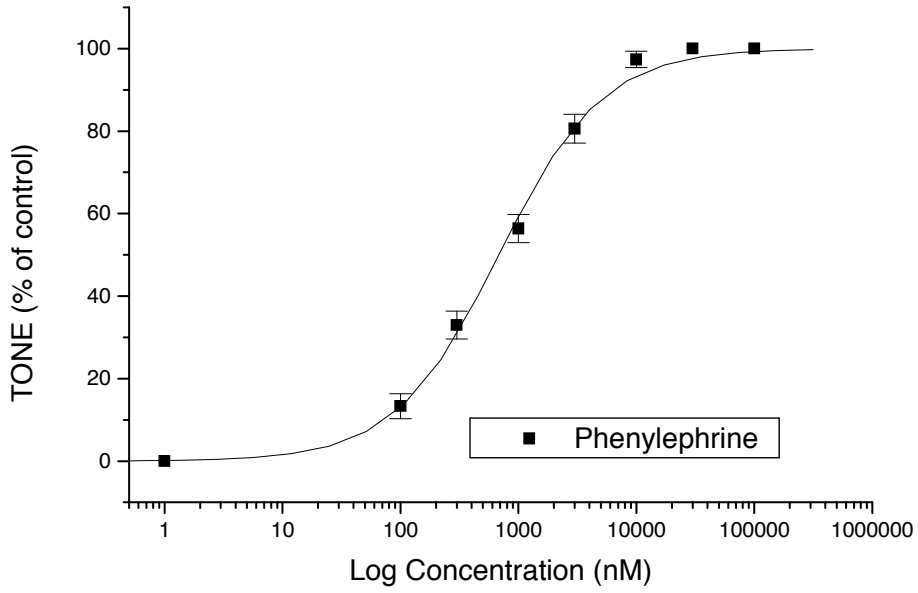


**Figure 19:** Concentration response curve to phenylephrine in strips of human corpus cavernosum. $EC_{80}$ for phenylephrine (3 $\mu$M) was then calculated using this curve. Each data point represents mean of 8 separate experiments, error bars represent the error of the mean.

### 3.2 Eliciting nonadrenergic noncholinergic relaxation responses to EFS in rabbit and human corpus cavernosum

Electrical field stimulation (EFS; 50 V, 0.3ms pulse duration, 0.5-25Hz, for 5s, every 120 s) elicited reproducible contractions of both rabbit and human corpus cavernosum strips. These contractions were inhibited with guanethidine (10$\mu$M). Further addition of scopolamine (10$\mu$M) inhibited any cholinergic component of neuronal stimulation. In the presence of scopolamine and guanethidine, further addition of phenylephrine (3 $\mu$M) caused elevation of the tone. EFS under these conditions elicited reproducible nonadrenergic noncholinergic (NANC) relaxation responses (Figure 20). The optimum frequency (i.e. giving the greatest relaxation response) was found to be 5 Hz for both tissues. These frequency-dependent relaxations were completely inhibited by an inhibitor of sGC (ODQ; 30 $\mu$M), an inhibitor of NO synthase (L-NAME, 500 $\mu$M; Figure 22) or tetrodotoxin (TTX, 1 $\mu$M; not shown) indicating that they were nitrergic in nature and neurogenic in origin. L-NAME also elevated the tone at 23.25% and 28.2% in human corpus cavernosum and in rabbit corpus cavernosum respectively (mean, n=8 for each).

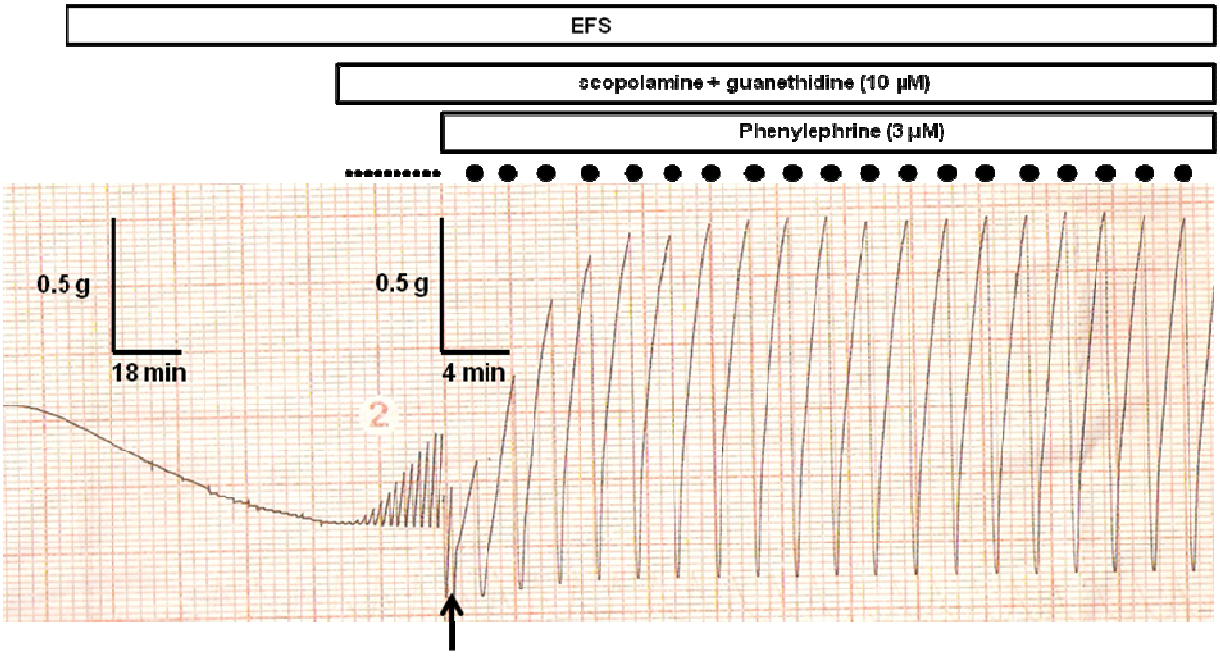


**Figure 20:** Electrical field stimulation (EFS) (50 V, 0.3 ms pulse duration, 5 Hz, for 5 s, every 120 s, denoted with solid dots) was applied to rabbit corpus cavernosum. The addition of scopolamine and guanethidine (both at 10 $\mu$M) and phenylephrine (3 $\mu$M) resulted in elevation of the tone and the appearance of NANC relaxation responses. In this trace the baseline and the speed of the recorder were adjusted (as denoted with an arrow) to allow the full responses to be demonstrated on the trace.

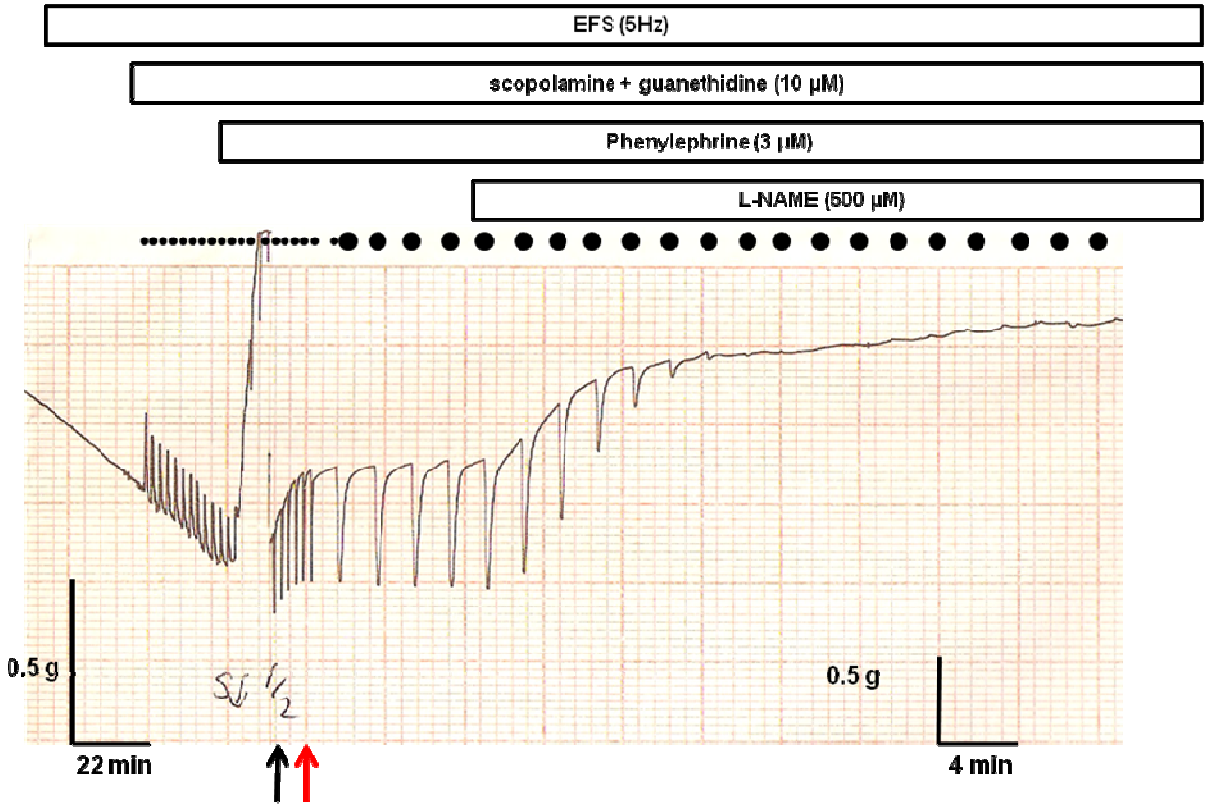


**Figure 21:** EFS (50 V, 0.3ms pulse duration, 5Hz, for 5s, every 120 s, as denoted by solid dots) elicited contractions in rabbit corpus cavernosum. Addition of phenylephrine (3 $\mu$M), scopolamine (10 $\mu$M) and guanethidine (10 $\mu$M) abolished EFS-induced contractions and elevated the tone. Under these conditions same EFS elicited NANC relaxation responses which were abolished by L-NAME (500 $\mu$M). The addition of L-NAME also resulted in further elevation of the tone. On this trace please note that the sensitivity of the recorder was reduced by 50% and the baseline was adjusted (denoted by black arrow) and the speed of the recorder was reduced (denoted by red arrow) to allow demonstration of the full responses on the trace.

### 3.3 Investigation of the effect of sGC activators on rabbit and human corpus cavernosum

#### 3.31 Effect of BAY41-2272 on phenylephrine-induced tone

The addition of cumulative concentrations of BAY41-2272 resulted in a concentration-dependent reduction of phenylephrine-induced tone in both rabbit ($EC_{50}$=407.5±22.7 nM) and human ($EC_{50}$=300.3±33.8 nM) cavernosal strips (n=12 for both) (Figure 22, Tables 3 and 4).

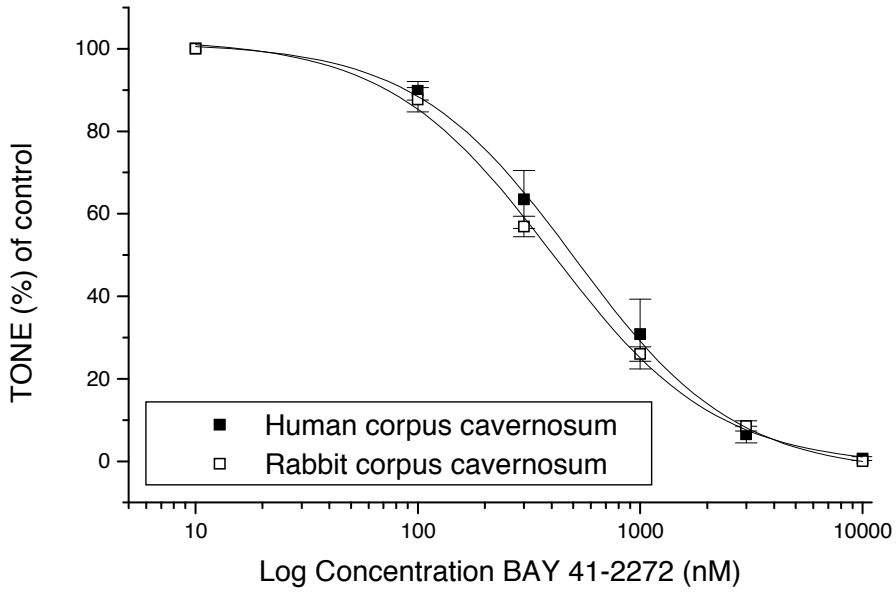


**Figure 22:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in human (closed squares) and rabbit corpus cavernosum (open squares). Each data point represents mean of 12 separate experiments, error bars represent the error of the mean.

### 3.32 Investigation of the effect of BAY41-2272 on phenylephrine-induced tone in the presence and absence of ODQ

The sGC inhibitor, ODQ (10 and 30 $\mu$M) caused a rightward parallel shift in the dose-response curve to BAY41-2272 in both the rabbit and human corporal tissues. In the presence of 30 $\mu$M ODQ, $EC_{50}$ of BAY41-2272 was 1905.3±14.4 nM and 1407.3±158 nM and in the rabbit and human tissues, respectively (n=8; Figures 23 and 24, Tables 3 and 4).

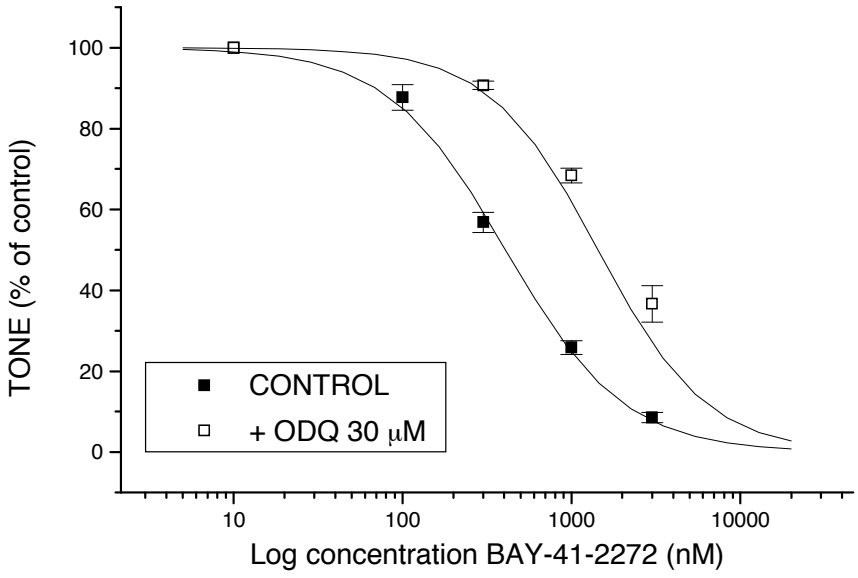


**Figure 23:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 30 $\mu$M ODQ (+ ODQ, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

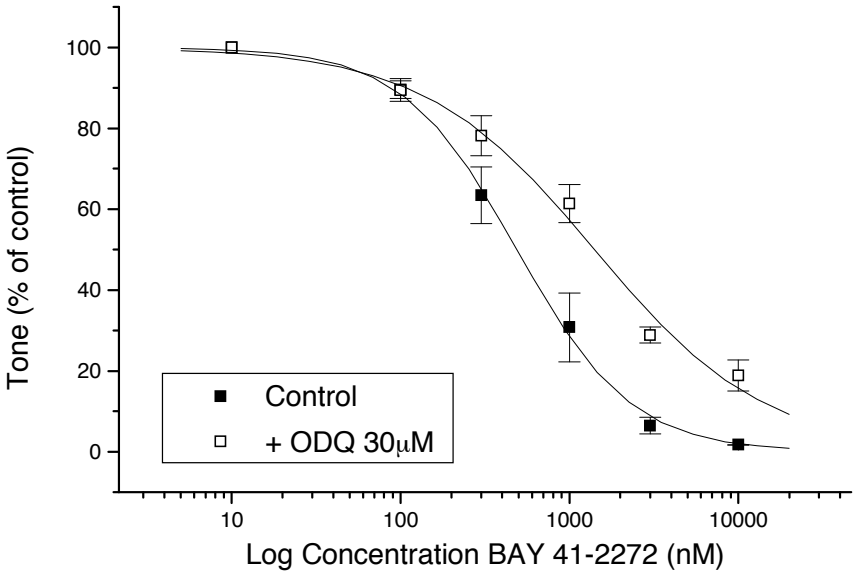


**Figure 24:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (CONTROL, solid squares) or presence of 30 $\mu$M ODQ (+ ODQ, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.33 Effect of BAY41-2272 on phenylephrine-induced tone in the presence and absence of L-NAME

The addition of the NO synthase inhibitor L-NAME at 500 $\mu$M resulted in a rightward parallel shift in the concentration response curve to BAY41-2272. In the presence of 500 $\mu$M L-NAME $EC_{50}$ values of BAY41-2272 were 836.7±46.7 nM in the rabbit and 429.1±30.9 nM in human tissue (n=8; Figures 25 and 26, Tables 4 and 5). In both rabbit and human tissues these $EC_{50}$ values were not significantly different from $EC_{50}$ values obtained in the absence of L-NAME ($p>0.05$; Tables 3 and 4).

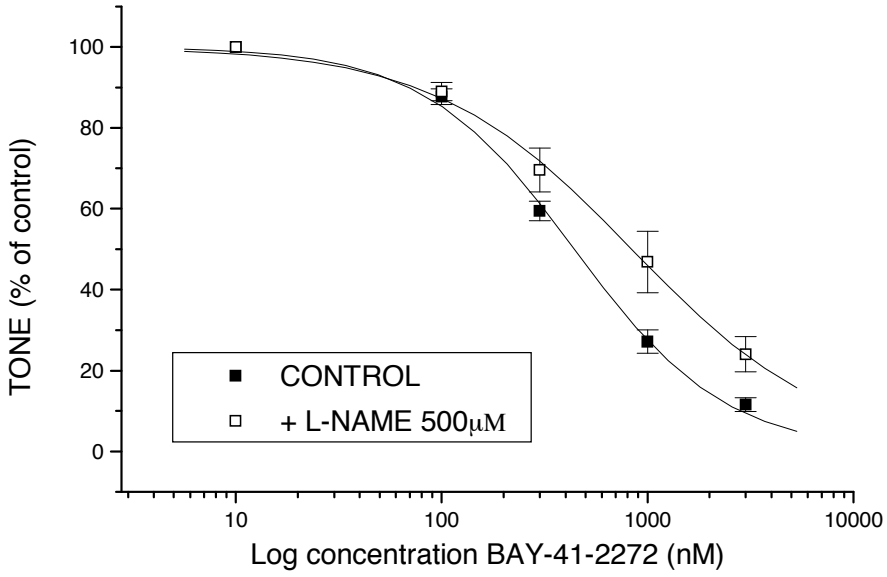


**Figure 25:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

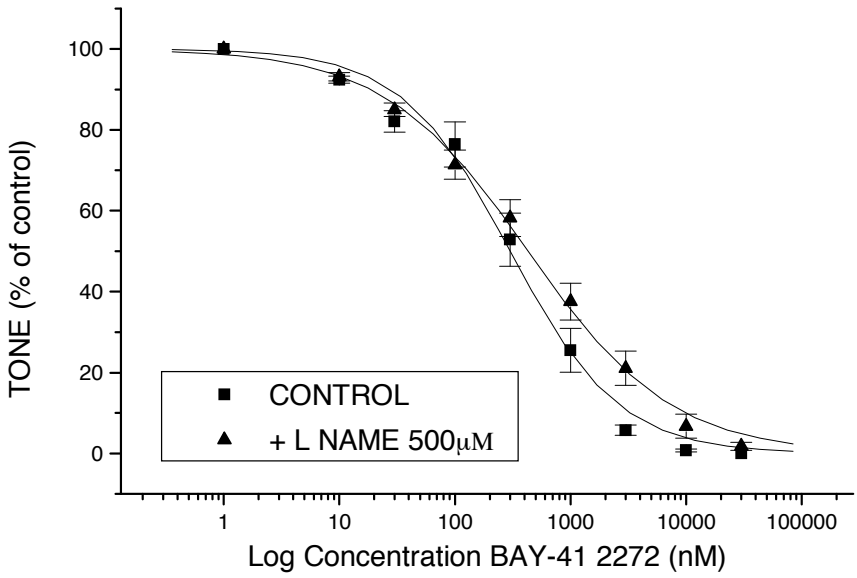


**Figure 26:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.34 Investigation of the effect of YC-1 on phenylephrine-induced tone in rabbit and human corpus cavernosum

The administration of another sGC activator YC-1 resulted in a concentration-dependent reduction of phenylephrine-induced tone in rabbit and human corpus cavernosum with $EC_{50}$ values of 13173.2±2018.4 nM and 25335.9±3332.8 nM respectively (n=8; Figure 27).

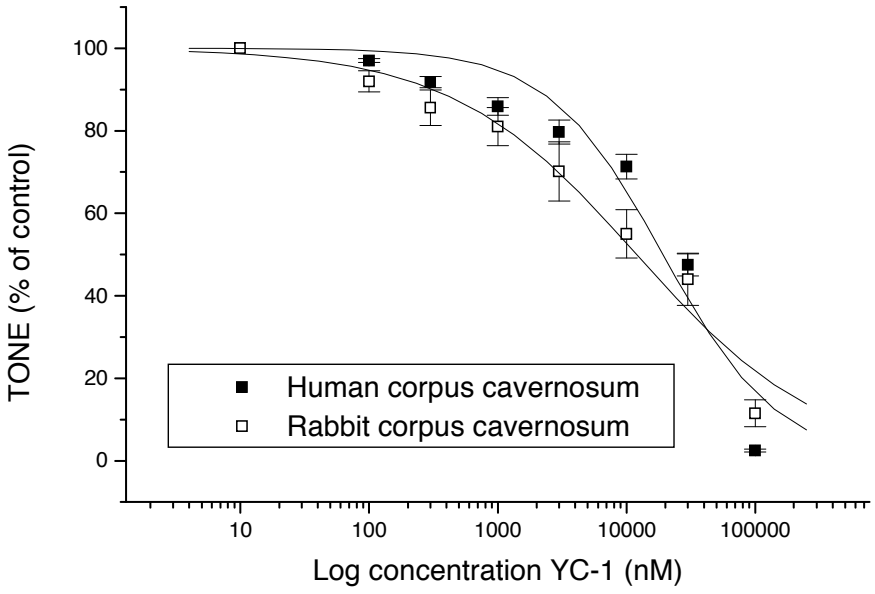


**Figure 27:** The effect of YC-1 in reducing the phenylephrine-induced tone in human (closed squares) and rabbit corpus cavernosum (open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

Significantly higher concentrations of YC-1 were required to induce relaxation of phenylephrine-induced tone compared to BAY41-2272. In comparison to BAY41-2272 there was a 32-120 fold difference in the potency with respect to the $EC_{50}$ ($p< 0.0001$ vs. BAY41-2272) (Figure 28).

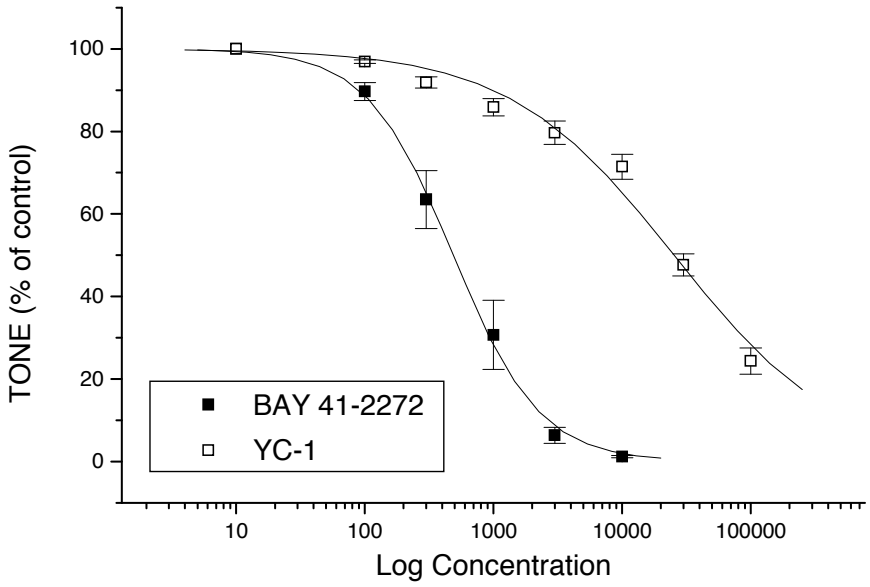


**Figure 28:** The effect of YC-1 in reducing the phenylephrine-induced tone in human corpus cavernosum (open squares) in comparison to BAY41-2272 (closed squares) ($p>0.05$). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.35 Investigation of the effects of spermine-NONOate on phenylephrine-induced tone in rabbit and human corpus cavernosum in the presence and absence of L-NAME

The addition of increasing concentration of the NO donor spermine-NONOate resulted in a concentration dependent relaxation of phenylephrine-induced tone with $EC_{50}$ values of 843.3±54.4 nM in rabbit (Figure 29) and 1220.54±19.3 nM in human corpus cavernosum (Figure 30). The addition of L-NAME at 500$\mu$M did not result in any significant change in the efficacy or potency of spermine-NONOate in rabbit tissues ($EC_{50}$=698.42±61.9 nM P=0.1002) (Figure 29). The experiment with L-NAME was not repeated on human tissues because the results in rabbit tissues were easily reproducible and conclusive and the cost and availability of the NO donor spermine-NONOate was a significant limiting factor. Furthermore, there were no significant differences in the responses to spermine-NONOate between the rabbit and human tissues in the absence of L-NAME.

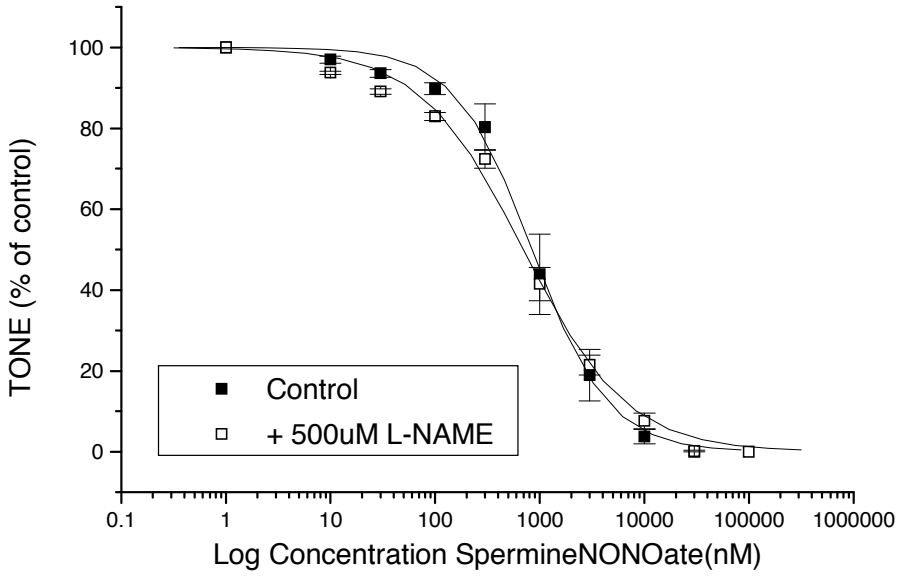


**Figure 29:** The effect of spermine-NONOate in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares) ($p>0.05$). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

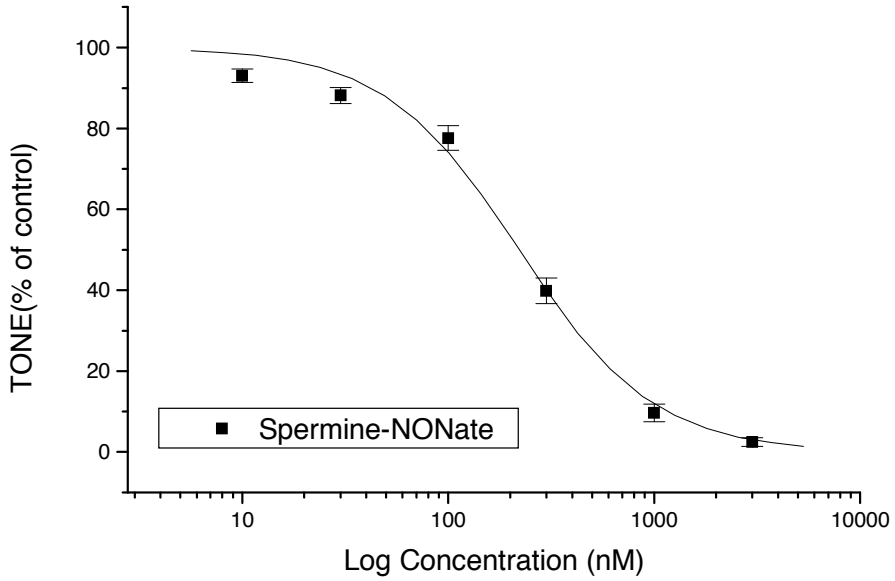


**Figure 30:** The effect of spermine-NONOate in reducing the phenylephrine-induced tone in human corpus cavernosum. Each data point represents mean of ten separate experiments, error bars represent the standard error of the mean.

### 3.36 Investigation of the effects of spermine-NONOate on phenylephrine-induced tone in rabbit corpus cavernosum in the presence and absence of ODQ

In the presence of ODQ at 10 and 30 $\mu$M respectively, there was a concentration-dependent significant right ward shift in the concentration-response curve in rabbit corpus cavernosum (Figure 31). $EC_{50}$ values for spermine-NONOate in the presence of 10 $\mu$M and 30 $\mu$M ODQ were 6091.2±1718.6 nM and 41479.0±17711.2 nM respectively (both $p<0.0001$ vs. control).

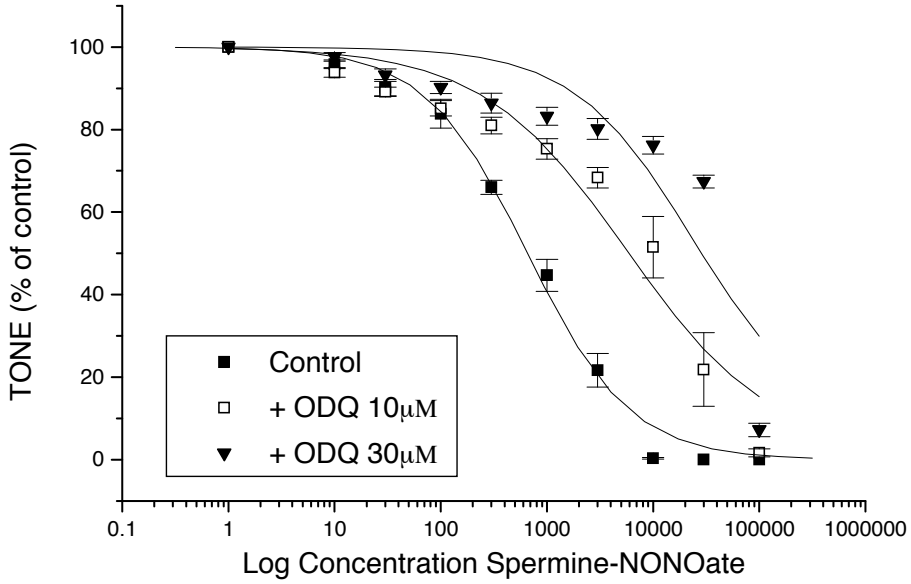


**Figure 31:** The effect of spermine-NONOate in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 10 $\mu$M ODQ (+ ODQ, open squares) ($p<0.001$) and 30 $\mu$M ODQ (+ ODQ, triangle) ($p<0.001$). Each data point represents mean of six separate experiments, error bars represent the standard error of the mean.

In comparison to spermine-NONOate, the potency of BAY41-2272 was at least 2 fold greater in reducing phenylephrine-induced tone in both rabbit and human corpus cavernosum respectively ($p<0.001$; Tables 3 and 4, Figure 32).

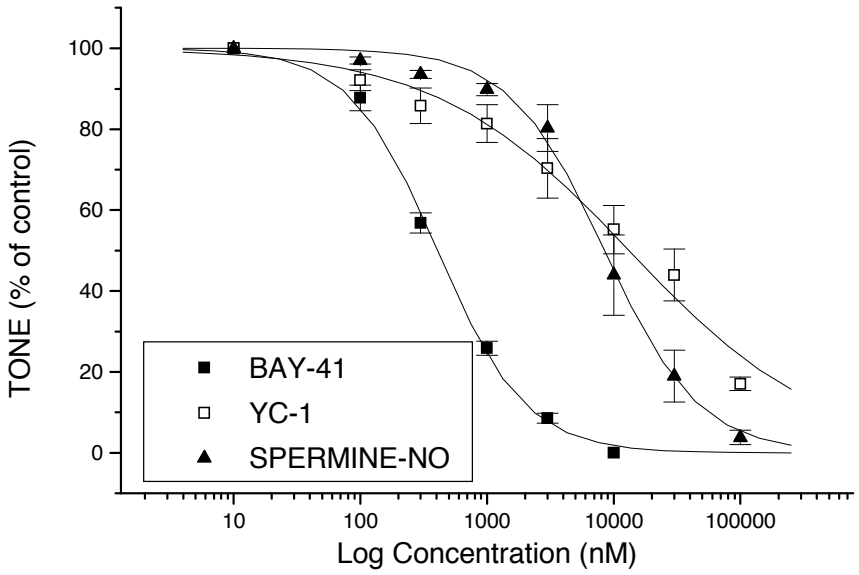


**Figure 32:** The effect of BAY41-2272 (solid squares), YC-1 (open squares) and spermine-NONOate (triangles) in reducing the phenylephrine-induced tone in rabbit corpus cavernosum. Each data point represents mean of six separate experiments, error bars represent the standard error of the mean.

| Rabbit | $EC_{50}$ mean ± s.e.m.(nM) | N |
|---|---|---|
| BAY41-2272 | 407.5±22.7 | 12 |
| + ODQ 30 $\mu$M | 1905.3±14.4* | 8 |
| + L-NAME 500 $\mu$M | 836.7±46.7 | 8 |
| YC-1 | 13173.2±2018.4** | 8 |
| Spermine-NONOate | 843.3±54.4 | 6 |
| +ODQ 30 $\mu$M | 41479±17711.2*** | 6 |
| + L-NAME 500 $\mu$M | 698.4±61.9 | 8 |

**Table 3:** The $EC_{50}$ values of BAY41-2272, YC-1 and spermine-NONOate for lowering phenylephrine induced tone in rabbit corpus cavernosum in the absence or presence of ODQ or L-NAME. n denotes the number of animals used for each set of experiments. *$P$<0.05 vs. BAY 41-2272 in the absence of L-NAME or ODQ, ** $P$<0.05 vs. BAY 41-2272, *** $P$<0.05 vs. spermine-NONOate in the absence of L-NAME or ODQ.

| Human | $EC_{50}$ mean ± s.e.m.(nM) | N |
|---|---|---|
| BAY41-2272 | 300.3±33.8 | 12 |
| +ODQ 30 $\mu$M | 1407.3±158.0* | 8 |
| + L-NAME 500 $\mu$M | 429.1±30.9 | 8 |
| YC-1 | 25335.9±3332.8** | 8 |
| Spermine-NONOate | 1220.5±19.27*** | 6 |

**Table 4:** The $EC_{50}$ values of BAY41-2272, YC-1 and spermine-NONOate for lowering phenylephrine induced tone in human corpus cavernosum in the absence or presence of ODQ or L-NAME. n denotes number of patients used for each set of experiments. *$P$<0.05 vs. BAY 41-2272 in the absence of ODQ, ** $P$<0.05 vs. BAY 41-2272, *** $P$<0.05 vs. BAY 41-2272.

### 3.4 Investigation of the effects of BAY41-2272 on nitrergic relaxations

My preliminary experiments have demonstrated that BAY41-2272 was able to reduce the phenylephrine-induced tone at concentrations greater than 50 nM. At concentrations greater than 50 nM it was therefore not possible to evaluate the EFS-induced relaxation responses since reduction in the tone would alter the magnitude of the neurogenic relaxation responses. Therefore, two concentrations (30 and 50 nM) were chosen to study the effect of BAY41-2272 on nitrergic relaxation responses. At these sub-threshold concentrations BAY41-2272 potentiated both the duration and magnitude of the relaxation responses especially at low frequencies without significantly altering the tone (n=4) (Figure 33).

I also investigated whether BAY41-2272 would restore nitrergic relaxation responses after they were inhibited with an inhibitor NO synthase. In order to achieve this, firstly I blocked the nitrergic relaxations using L-NAME (500 $\mu$M). Administration of BAY41-2272 after complete inhibition of nitrergic responses caused a reduction in the phenylephrine-induced tone. Concentrations above 0.3 $\mu$M of BAY41-2272 caused partial restoration of the nitrergic responses even at reduced tone such that 12.3±3.6% of nitrergic responses were recovered after addition of 3 $\mu$M BAY41-2272 (n=4) (Figure 34).

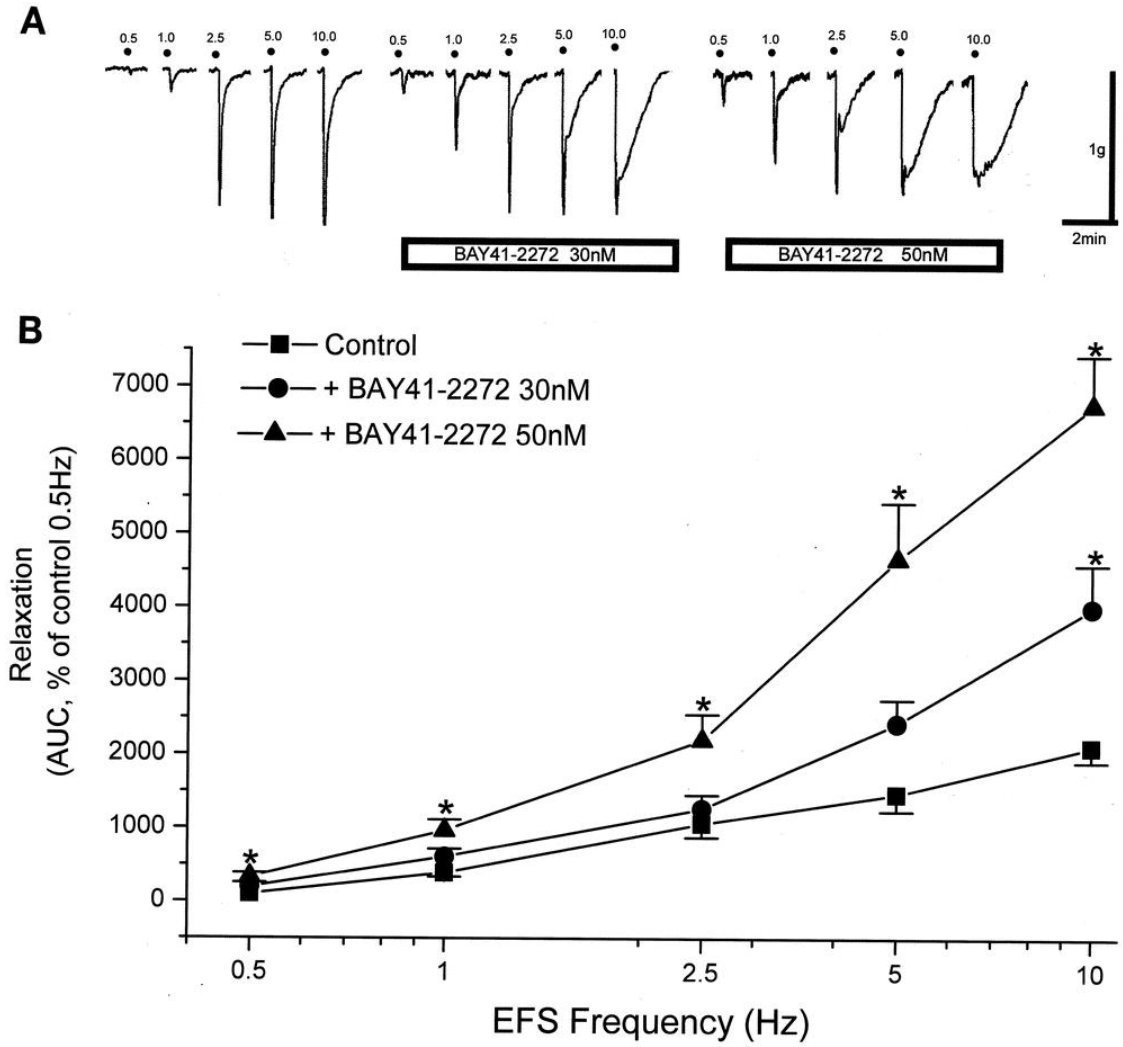


**Figure 33:** Electrical field stimulation (EFS; 50 V, 0.3 ms pulse duration for 5 seconds at 0.5 to 10 Hz) elicited nitrergic relaxations in rabbit corpus cavernosal strips (n=4). *A*, relaxations were enhanced in magnitude and duration by 30 and 50 nM BAY41-2272. Values above circles indicate stimulation frequencies. *B*, area under curve (*AUC*) of each EFS-induced relaxation was measured and expressed as percent of relaxation induced at 0.5 Hz under control conditions in same tissues.

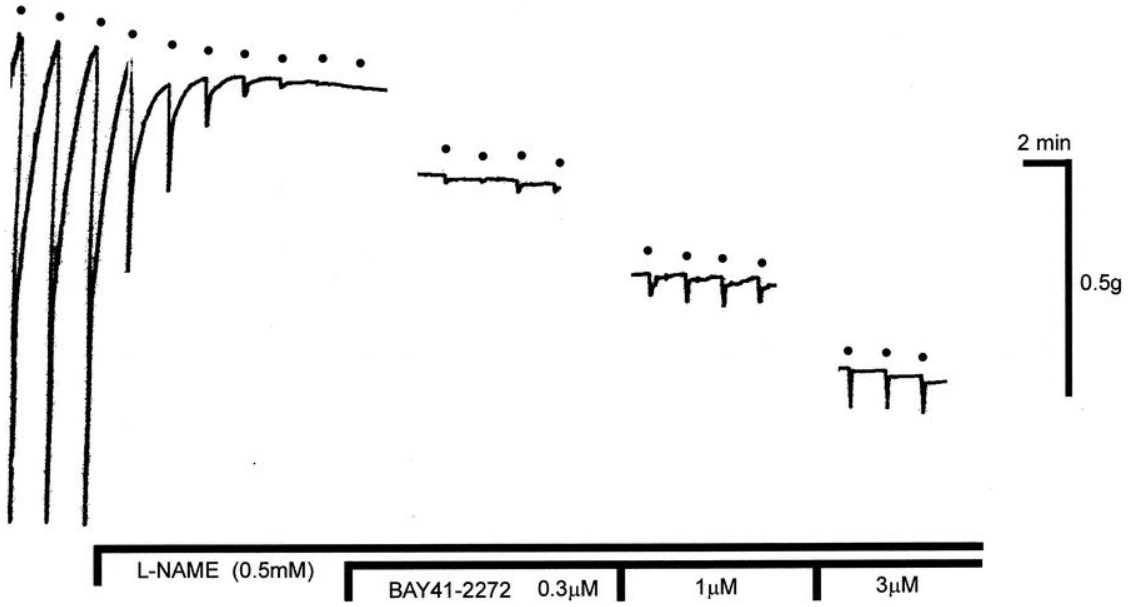


**Figure 34:** Electrical field stimulation (circles) at 50 V with 0.3 ms pulse duration for 5 seconds at 5 Hz elicited nitrergic relaxations in rabbit corpus cavernosal strips (n=4). 500 $\mu$M L-NAME abolished these relaxations. Under these conditions treatment with (0.3 to 3 $\mu$M BAY41-2272) reduced the tone and partially restored inhibited relaxations.

### 3.5 Investigation of the effect of sildenafil on rabbit and human corpus cavernosum

Increasing cumulative concentrations of sildenafil (1 nM – 300 $\mu$M) were added to construct the concentration-response curves. Sildenafil caused reduction of phenylephrine-induced tone in both rabbit and human cavernosal strips in a concentration-dependent manner with a similar potency. $EC_{50}$ values for rabbit and human corpus cavernosal tissues were 1000.5±140.8 nM and 800.7±155.8 nM (n=10 for both) respectively (Figure 35 and Tables 5 and 6).

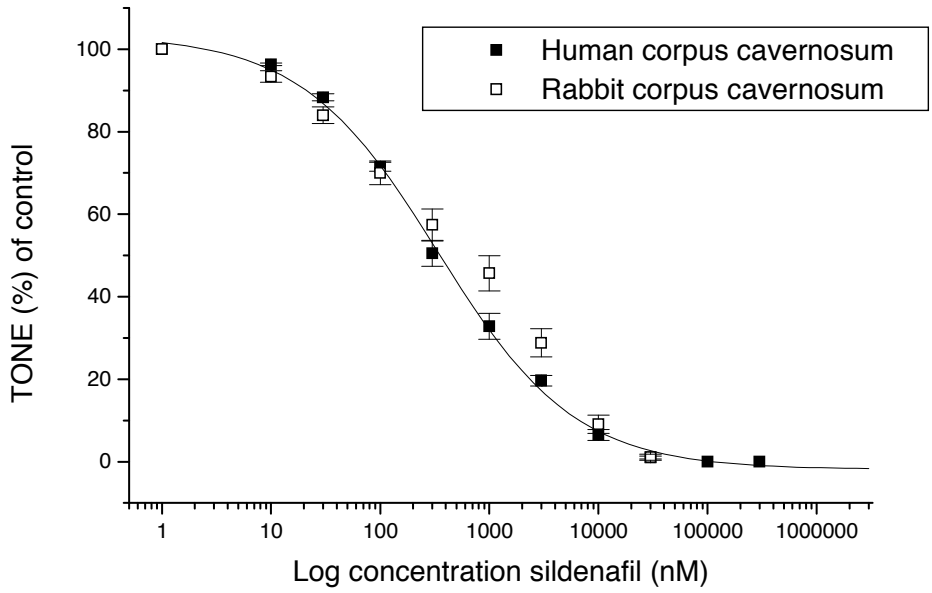


**Figure 35:** The effect of sildenafil in reducing the phenylephrine-induced tone in human (solid squares) ($EC_{50}$=800.7±155.8 nM) and rabbit corpus cavernosum (open squares) ($EC_{50}$=1000.5±140.8 nM). Each data point represents mean of ten separate experiments, error bars represent the standard error of the mean. Please note that the two curves were identically overlaid on each other.

### 3.51 Investigation of the effect of sildenafil on phenylephrine-induced tone in the presence of L-NAME

The addition of L-NAME (500$\mu$M) resulted in a significant rightward parallel shift in the concentration response curve to sildenafil. The $EC_{50}$ of sildenafil was 4959.1±882.1 nM in rabbit (n=8, P<0.05 vs. control) and 2446.7±256.8 nM in human tissues (n=6, P<0.05 vs. control) in the presence of 500 $\mu$M L-NAME (Figures 36 and 37, Tables 5 and 6).

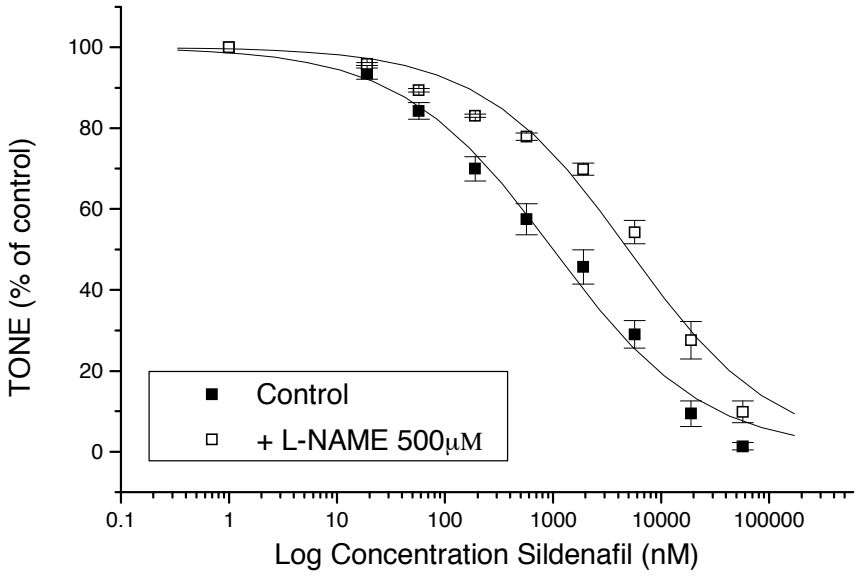


**Figure 36:** The effect of sildenafil in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

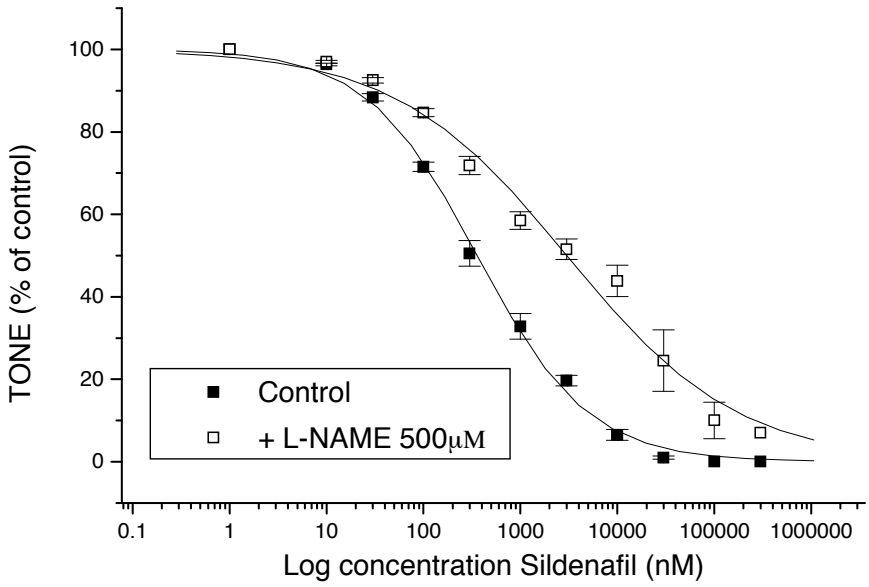


**Figure 37:** The effect of sildenafil in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of six separate experiments, error bars represent the standard error of the mean.

### 3.52 The effect of sildenafil on phenylephrine-induced tone in human corpus cavernosum in the presence and absence of ODQ

Once the tone was established, increasing concentrations of sildenafil (1 nM–300 $\mu$M) were added to construct the concentration–response curves in the absence or presence of an inhibitor of soluble guanylate cyclase, ODQ (10 $\mu$M) (Figure 38).

In the presence of 10 $\mu$M ODQ, sildenafil failed to induce a significant relaxation below 1 $\mu$M. Even above 1 $\mu$M, sildenafil could only reach ~50% of the tone resulting in a significant rightward and upward shift in the concentration–response curve. The $EC_{50}$ value for sildenafil in the presence of ODQ was 6488.0±938.0 nM (n=6) (Figure 38 and Table 6).

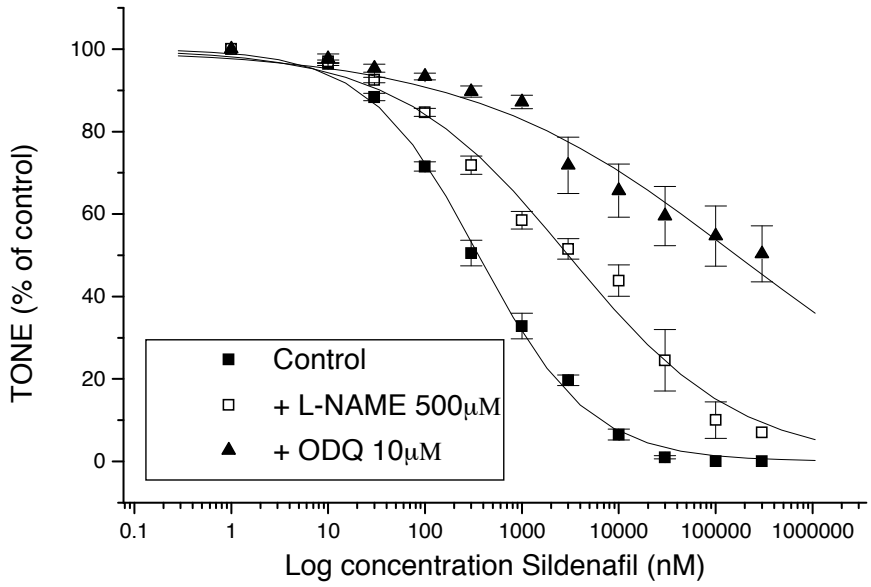


**Figure 38:** The effect of sildenafil in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (control, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares) or 10 $\mu$M ODQ (+ODQ, triangles).

### 3.53 Investigation of the effect of NCX-911 on phenylephrine-induced tone in rabbit and human corpus cavernosum

Administration of increasing concentrations of NCX-911 resulted in relaxation of rabbit and human cavernosal strips in a concentration-dependent manner (n=8 for both, $EC_{50}$=997.8±195.7 nM and 733.1±94.4 nM in rabbit and human respectively) (Figure 39 and Tables 5 and 6).

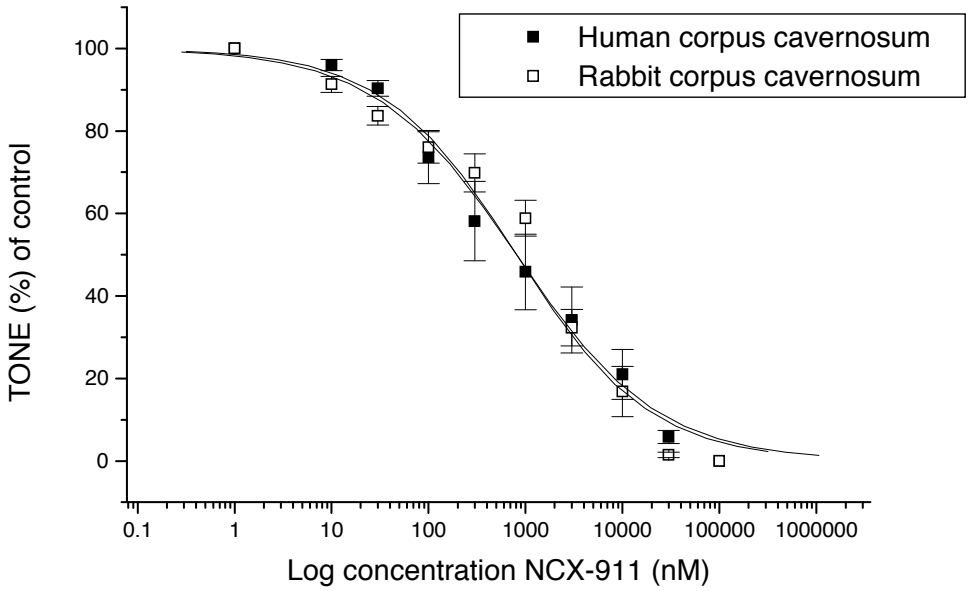


**Figure 39:** The effect of NCX-911 in reducing the phenylephrine-induced tone in human (solid squares) and rabbit corpus cavernosum (open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.54 The effect of NCX-911 on phenylephrine-induced tone in rabbit and human corpus cavernosum in the presence and absence of L-NAME

The NO synthase inhibitor L-NAME resulted in a slight rightward parallel shift in the concentration response curve to NCX-11. In the presence of 500 $\mu$M L-NAME, $EC_{50}$ values of NCX-911 were 1281.2±268.3 nM (n=8, p=0.41 vs. control) and 980.4±106.7 nM (n=8, p=0.1132 vs. control) in rabbit and human tissue respectively (Figures 40 and 41, Tables 5 and 6). Even when the concentration of L-NAME was increased to 1 mM there was no significant effect in rabbit cavernosal tissues (p=0.72; not shown).

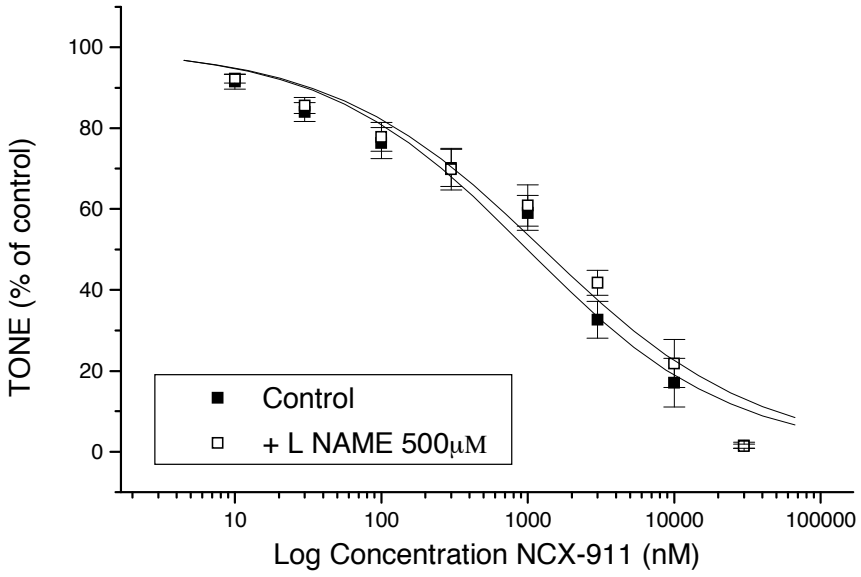


**Figure 40:** The effect of NCX-911 in reducing the phenylephrine-induced tone in rabbit corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

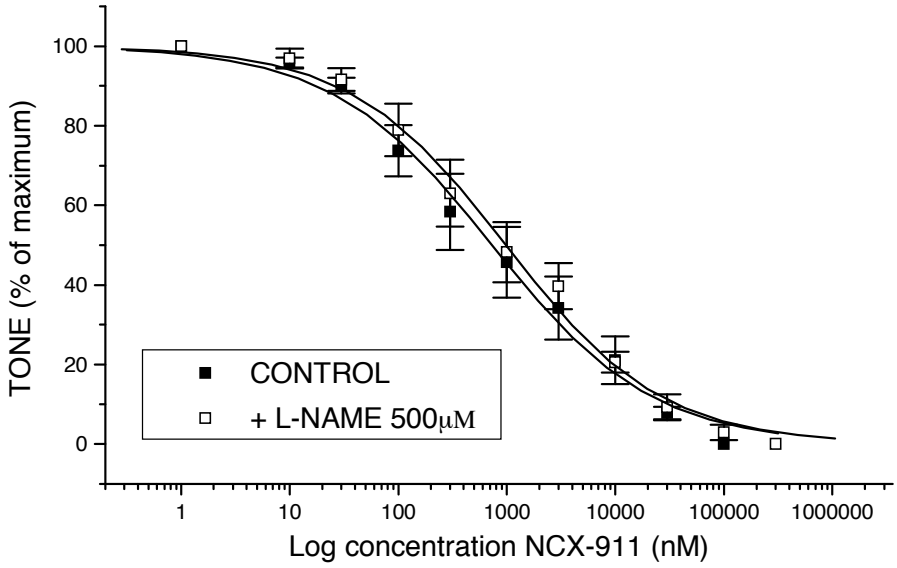


**Figure 41:** The effect of NCX-911 in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (CONTROL, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares). Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.55 The effect of NCX-911 on phenylephrine-induced tone in human corpus cavernosum in the presence and absence of ODQ

Increasing concentrations of NCX-911 (1 nM - 300 $\mu$M) were added to construct the concentration–response curves in the absence or presence of an inhibitor of an inhibitor of soluble guanylate cyclase, ODQ (10 $\mu$M) (Figure 42 and Table 4). In the presence of 10 $\mu$M ODQ, NCX-911 failed to induce a significant relaxation below 1 $\mu$M. Above 1 $\mu$M, NCX-911 could only reach ~50% of the tone resulting in a significant rightward and upward shift in the concentration–response curve. The $EC_{50}$ value for NCX-911 in the presence of ODQ was 6578.0±1150.0 nM (n=6, Figure 42 and Table 6).

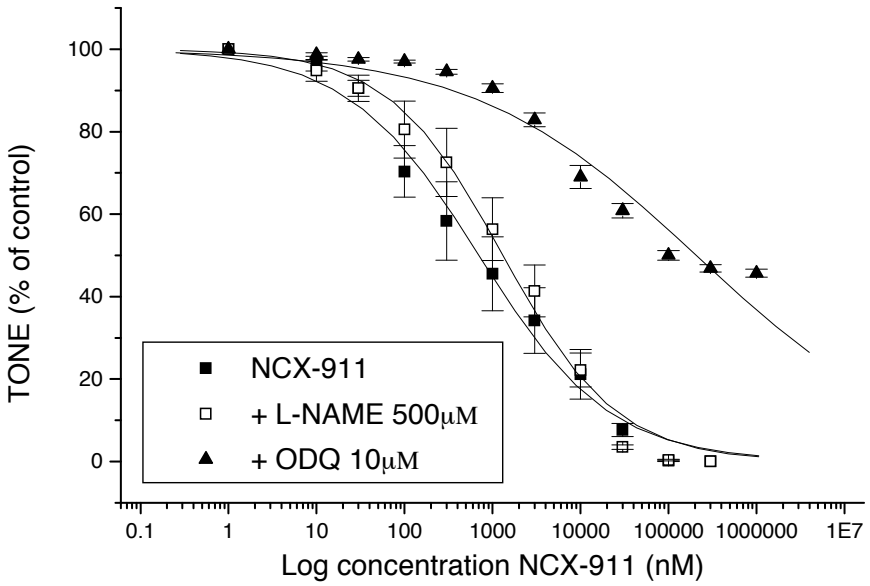


**Figure 42:** The effect of NCX-911 in reducing the phenylephrine-induced tone in human corpus cavernosum in the absence (control, solid squares) or presence of 500 $\mu$M L-NAME (+L-NAME, open squares) or 10 $\mu$M ODQ (+ODQ, triangles). Each data point represents mean of at least six separate experiments, error bars represent the standard error of the mean.

| Rabbit | $EC_{50}$ mean ± s.e.m. (nM) | n |
|---|---|---|
| NCX-911 | 997.8 ± 195.7 | 8 |
| + L-NAME 500 μM | 1281.2 ± 268.3 | 8 |
| Sildenafil | 1000.5 ± 140.8 | 10 |
| + L-NAME 500 μM | 4959.1 ± 882.1* | 8 |
| Spermine-NONOate | 843.3 ± 54.4 | 8 |
| + L-NAME 500 μM | 698.4 ± 61.8 | 8 |

**Table 5:** $EC_{50}$ values of NCX-911, sildenafil and spermine-NONOate for reducing phenylephrine-induced tone in the rabbit corpus cavernosum. n denotes the number of animals used for each set of experiments. *$P$<0.05 vs. sildenafil in the absence of L-NAME.

| Human | $EC_{50}$ mean ± s.e.m. (nM) | n |
|---|---|---|
| NCX-911 | 733.09 ± 94.4 | 8 |
| + L-NAME 500μM | 980.4 ± 106.7 | 8 |
| + ODQ 10$\mu$M | 6578 ± 1150** | 6 |
| Sildenafil | 800.7 ± 155.8 | 10 |
| + L-NAME 500μM | 2446.7 ± 256.8* | 6 |
| + ODQ 10$\mu$M | 6488 ± 938** | 6 |
| Spermine-NONOate | 1220.54 ± 19.27 | 6 |

**Table 6:** $EC_{50}$ values of NCX-911, sildenafil and spermine-NONOate for reducing phenylephrine-induced tone in human corpus cavernosum. n denotes the number of patients used for each set of experiments. *$P$<0.05 vs. sildenafil in the absence of L-NAME. ** $P$<0.05 vs. sildenafil or NCX-911 in the absence ODQ (10 $\mu$M)

### 3.6 Investigation of the effect of NCX-911 on nitrergic relaxations

Since above 100 nM NCX-911 was able to reduce the phenylephrine-induced tone and it is not possible to elucidate the nitrergic relaxation responses at reduced tone, two concentrations (10 and 30 nM) were chosen to study the effect of the compound on nitrergic responses. At those sub-threshold concentrations NCX-911 was found to potentiate both the duration and magnitude of the relaxation responses (n=6) (Figure 43A and 44; 30 nM shown).

### 3.61 Investigation of the effects of sildenafil on nitrergic relaxations

At sub-threshold concentrations of sildenafil (10 and 30 nM) the duration and magnitude of the nitrergic relaxation responses were potentiated (n=6) (Figure 43B and 44; 30 nM shown).

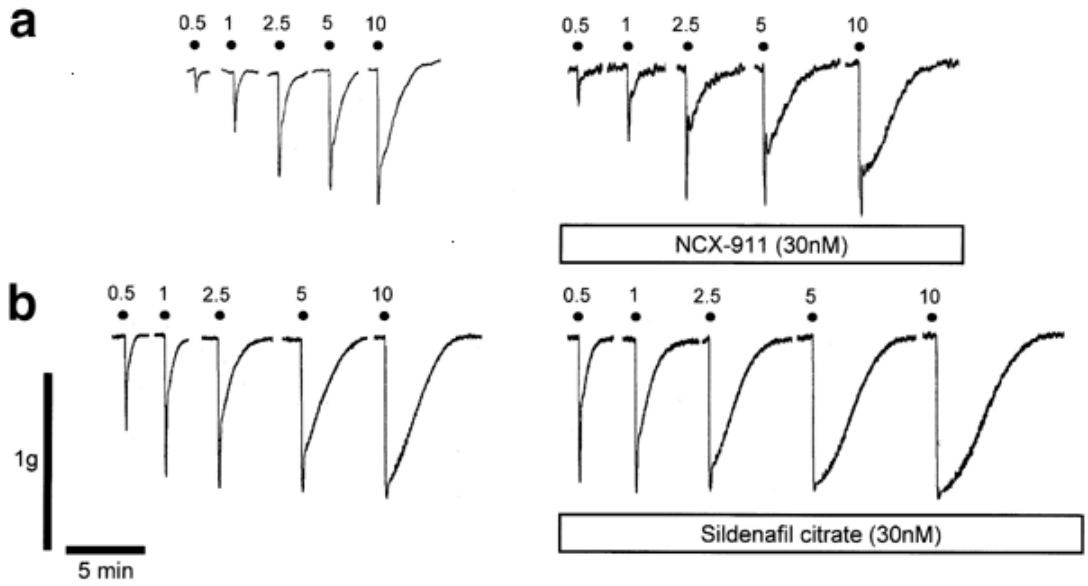


**Figure 43:** The effect of NCX-911 (a) or sildenafil (b), both at 30 nM, on the nitrergic responses of the rabbit corpus cavernosum. Each dot represents EFS (50 V, 0.3 ms pulse duration, for 5 s) at indicated frequencies.

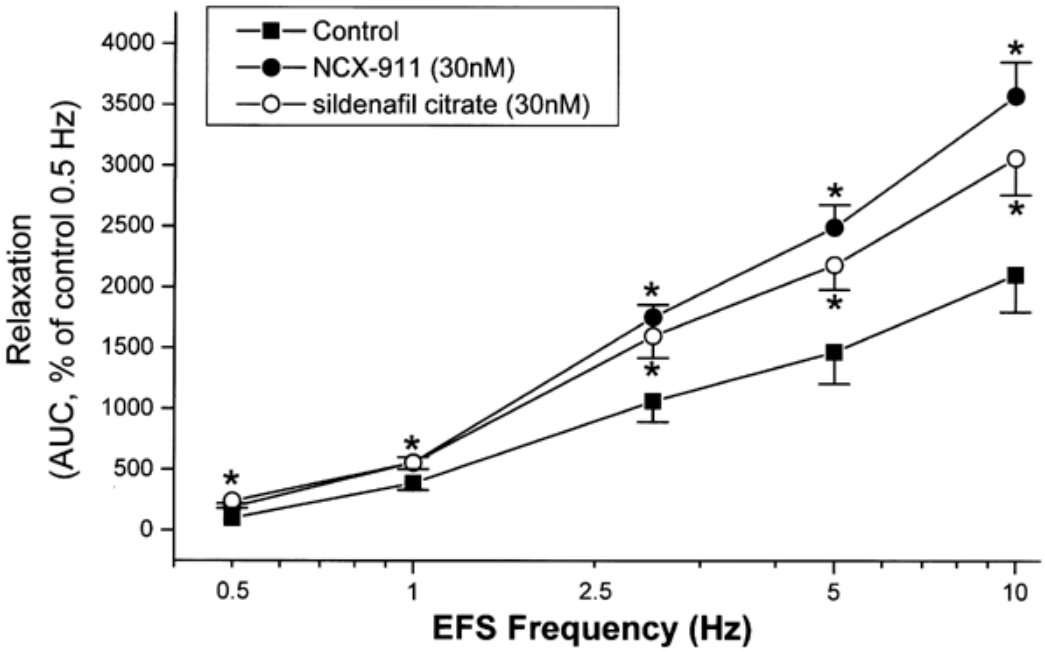


**Figure 44:** The effect of NCX-911 (solid circles), sildenafil (open circles) or control (solid squares), all at 30 nM, on the nitrergic relaxation responses of the rabbit corpus cavernosum. *P<0.05, significantly different from control. The difference between NCX-911- and sildenafil-induced potentiation was not significant.

### 3.62 Investigation of the effects of sildenafil and NCX-911 on blocked nitrergic responses

After the nitrergic relaxations were blocked by L-NAME (500 $\mu$M), the administration of increasing concentrations of both sildenafil and NCX-911 were found to result in a decrease in the phenylephrine-induced tone as mentioned above, however nitrergic responses were not observed to appear again (n=6) (Figure 45).

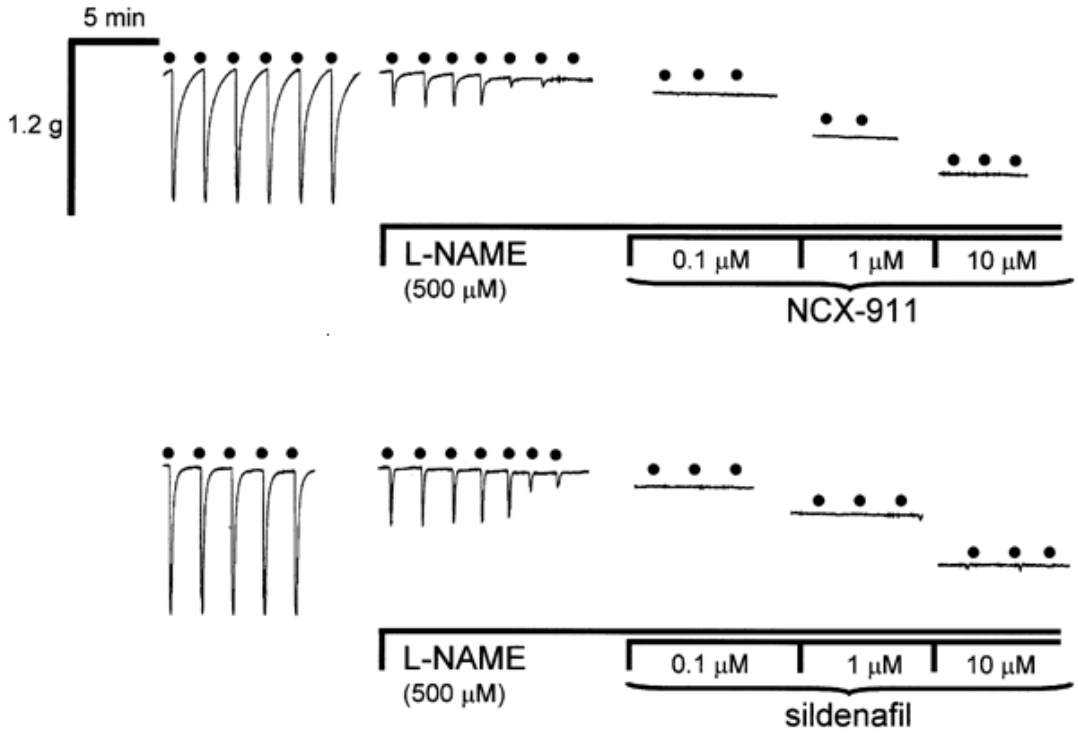


**Figure 45:** Two representative tracings showing the effect of NCX-911 (upper panel) or sildenafil (lower panel) on the tone and blocked nitrergic responses of the rabbit corpus cavernosum. EFS (indicated by dots; 50V, 0.3ms pulse duration, 5 Hz, for 5 s, every 120 s) elicited nitrergic responses that were completely inhibited by L-NAME (500 $\mu$M). NCX-911 or sildenafil lowered the tone; however they failed to reverse the inhibition of the nitrergic responses.

### 3.7 Investigation of the effects of sildenafil, NCX-911 and BAY 41-2272 on the rat anococcygeus muscle

#### 3.71 Body weight and serum glucose concentrations

The average body weight and serum glucose concentrations were measured at the beginning and at the end of 16 weeks. Both the weight and glucose concentrations were found to be similar between the two groups before the STZ injection.

Sixteen weeks after the STZ injection, in comparison to the control group, the STZ injected group gained less weight and had higher serum glucose concentrations (Table 7).

| Time | Control Group n=16 | Diabetic Group n=16 |
|---|---|---|
| **0 week** | 227.8±5.9 g<br>5.2±0.5 mM | 228.9±8.7 g<br>4.9±0.5 mM |
| **16$^{th}$ week** | 552.4±20.6 g<br>5.1±0.8 mM | 264.3±17.8 g *<br>50.7±2.7 mM * |

**Table 7:** The body weight (upper value) and serum glucose concentrations (lower value) of control (saline-injected) and diabetic (STZ-injected) rats at 0 (before the injection) and at 16$^{th}$ week. *P<0.05 significantly different from control group at 16$^{th}$ week.

### 3.8 Investigation of phenylephrine-induced tone

The application of phenylephrine (0.01-30 $\mu$M) on rat anococcygeal strips produced concentration-dependent elevation of the tone of the tissue. The $EC_{80}$ for phenylephrine was 1 $\mu$M in both the control and diabetic groups (n=8 for both) (Figure 46). This concentration of phenylephrine was used in the following experiments to elevate the tone.

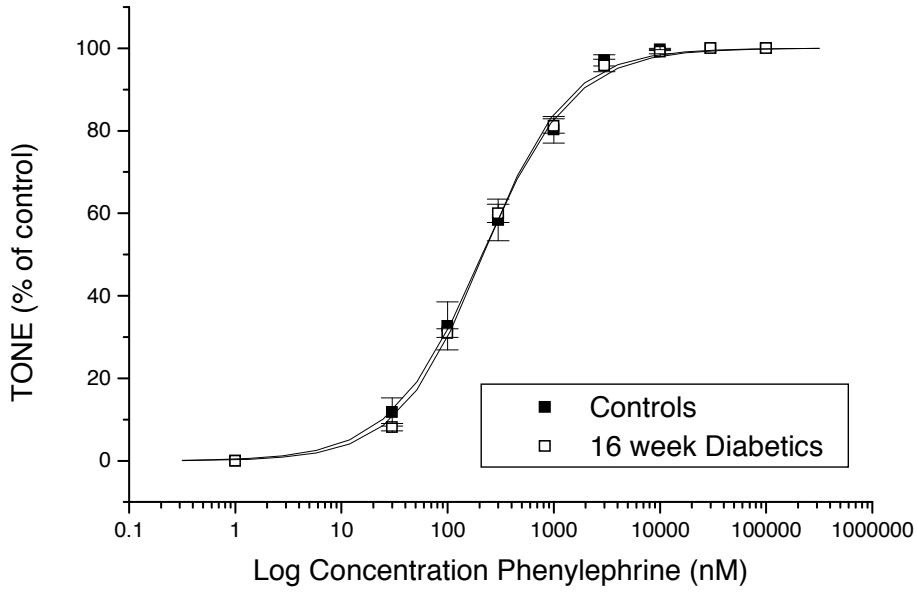


**Figure 46:** Concentration-response curve to phenylephrine in strips of anococcygeus muscles from control (CONTROL, solid squares) and STZ-injected (16 WEEK DIABETICS, open squares) rats. $EC_{80}$ values for phenylephrine (1 $\mu$M for both groups) were calculated using this curve. Each data point represents mean of eight separate experiments, error bars represent the standard error of the mean.

### 3.81 Investigation of the effect of sildenafil on phenylephrine-induced tone

The addition of increasing concentrations of sildenafil (0.001-300 $\mu$M) elicited concentration-dependent reductions of the phenylephrine-induced tone in tissues obtained from control animals with an $EC_{50}$ of 827.1±167.3 nM (n=6) (Figure 47 and Table 8).

In the diabetic animals the potency of sildenafil to reduce the phenylephrine-induced tone was significantly lower than the control animals (n=6) (Figure 47). The $EC_{50}$ values for sildenafil to reduce the phenylephrine-induced tone in diabetic animals was 2842.2±640.3 nM ($p<0.05$ vs. control) (Figure 47 and Table 8).

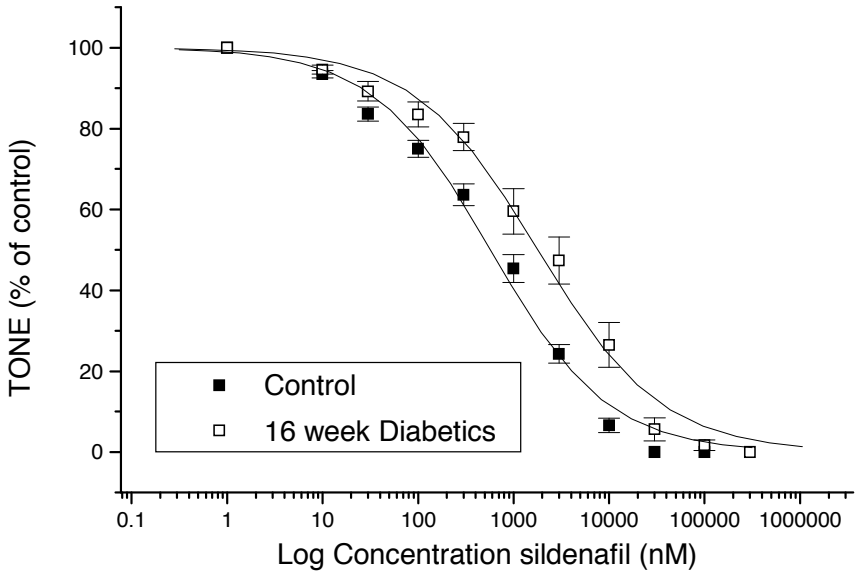


**Figure 47:** The effect of sildenafil in reducing the phenylephrine-induced tone in anococcygeus muscles from control (non-diabetic; solid squares) and 16 weeks diabetic (open squares) rats. Each data point represents mean of at least six separate experiments, error bars represent the standard error of the mean.

### 3.82 Investigation of the effect of NCX-911 on phenylephrine-induced tone

The addition of increasing concentrations of NCX-911 (0.001-30 $\mu$M) reduced the phenylephrine-induced tone in a concentration-dependent manner in tissues obtained from control animals with an $EC_{50}$ of 1088.8±165 nM (n=6) (Figure 48). In the diabetic animals the potency of NCX-911 was not significantly different from control animals ($EC_{50}$ = 1765.9±303.5 nM, n=6, Figure 48, Table 8).

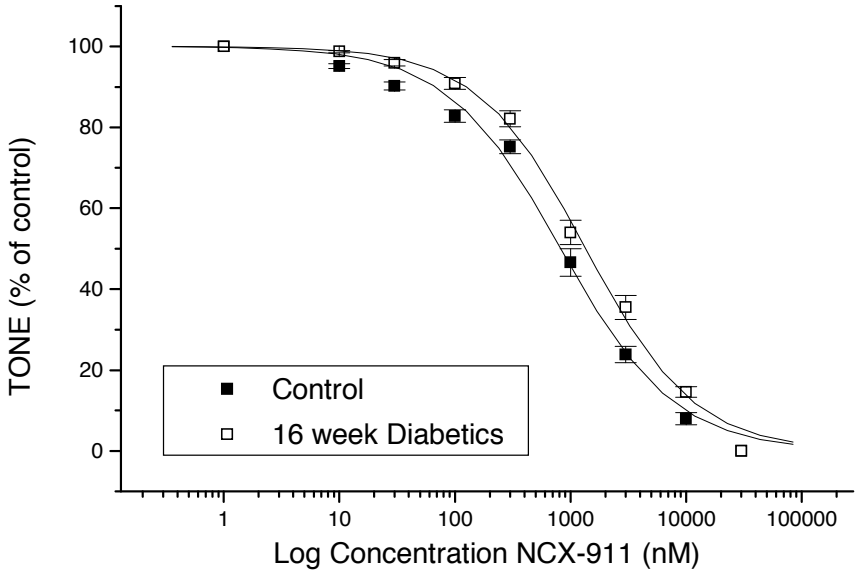


**Figure 48:** The effect of NCX-911 in reducing the phenylephrine-induced tone in anococcygeus muscle from control (non-diabetic; solid squares) and 16 weeks diabetic (open squares) rats. Each data point represents mean of at least six separate experiments, error bars represent the standard error of the mean.

### 3.83 Investigation of the effect of BAY41-2272 on phenylephrine-induced tone

The addition of increasing concentrations of BAY41-2272 (1 nM-10 $\mu$M) produced concentration-dependent reductions of the phenylephrine-induced tone in tissues obtained from control animals with an $EC_{50}$ of 151.6±9.3 nM (n=6) (Figure 49).

In the diabetic animals the potency of BAY41-2272 was not significantly different from the control animals. The $EC_{50}$ value for BAY41-2272 to reduce the phenylephrine-induced tone in diabetic animals was 209.7±27.3 nM (n=6, $p>0.05$ vs. control; Figure 49, Table 8).

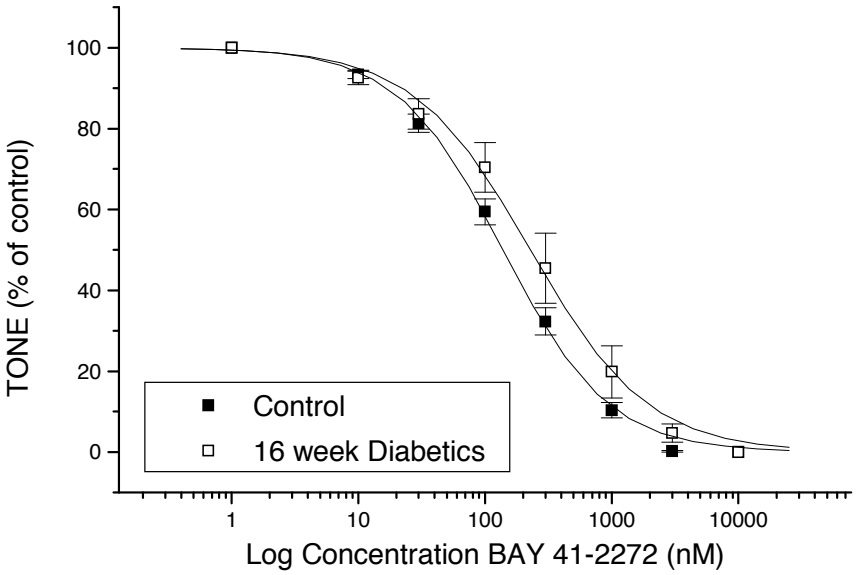


**Figure 49:** The effect of BAY41-2272 in reducing the phenylephrine-induced tone in anococcygeus muscles from control (non-diabetic; solid squares) and 16 weeks diabetic (open squares) rats. Each data point represents mean of at least six separate experiments, error bars represent the standard error of the mean.

| Compound | Group | $EC_{50}$ ± s.e.m. (nM) | n |
|---|---|---|---|
| NCX-911 | Control | 1088.8±165.0 | 6 |
| | Diabetic | 1765.9±303.5 | 6 |
| BAY41-2272 | Control | 151.6±9.3 | 6 |
| | Diabetic | 209.7±27.3 | 6 |
| Sildenafil | Control | 827.1±167.3 | 6 |
| | Diabetic | 2842.2±640.3* | 6 |

**Table 8:** $EC_{50}$ values (nM) of NCX-911, BAY41-2272 and sildenafil to reduce phenylephrine-induced tone in the anococcygeus muscle from control and diabetic rats. n denotes the number of animals used for each set of experiments. *P<0.05 significantly different from control group for the same compound.

### 3.9 Nitrergic relaxation responses in the rat anococcygeus muscle

After treatment of the tissues with scopolamine (10$\mu$M) and guanethidine (10$\mu$M) and elevation of tone with phenylephrine (1$\mu$M, $EC_{80}$), electrical field stimulation (EFS) (50 V, 0.3 ms pulse duration, 0.5-25 Hz, for 5 s, every 120 s) elicited reproducible contractions of the tissues (Figure 50). The optimum EFS-elicited frequency-dependent relaxation responses were seen at a frequency of 5 Hz (Figure 51). These relaxations were completely inhibited with an inhibitor of sGC (ODQ; 10 $\mu$M), an inhibitor of NO synthase (L-NAME; 500$\mu$M) (Figure 52) or tetrodotoxin (1 $\mu$M) indicating that they were nitrergic in nature and neurogenic in origin.

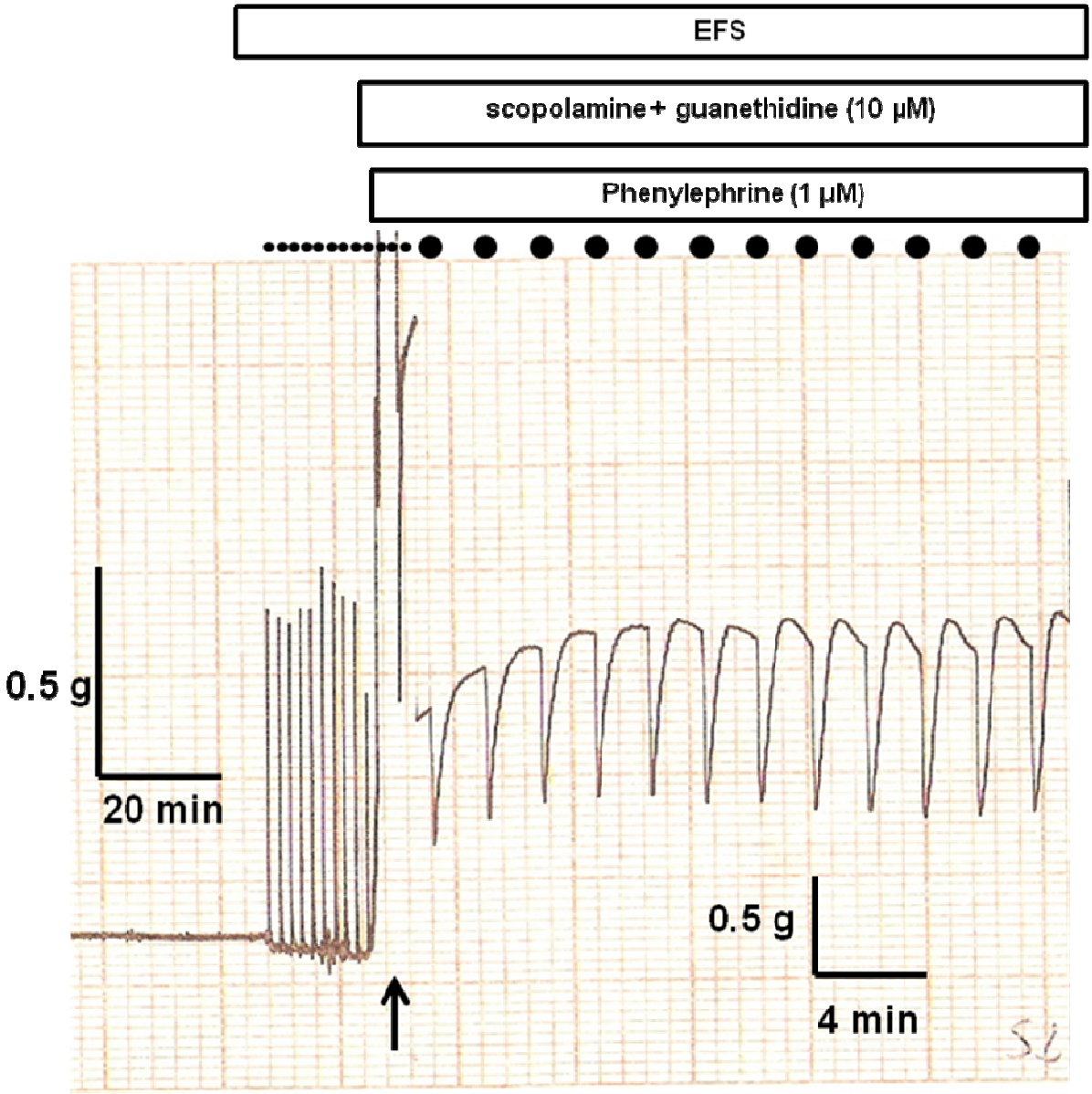


**Figure 50:** EFS (50 V, 0.3 ms pulse duration, 0.5-25 Hz, for 5 s, every 120 s, denoted with solid dots) of rat anococcygeus muscle elicited contractions. The addition of scopolamine and guanethidine at 10 $\mu$M abolished these contractions. The further addition of phenylephrine at 1 $\mu$M ($EC_{80}$) elevated the tone and allowed the demonstration of NANC relaxation responses. In this trace the baseline, sensitivity and the speed of the recorder were adjusted (as denoted by arrow) to allow the full responses to be demonstrated on the trace.

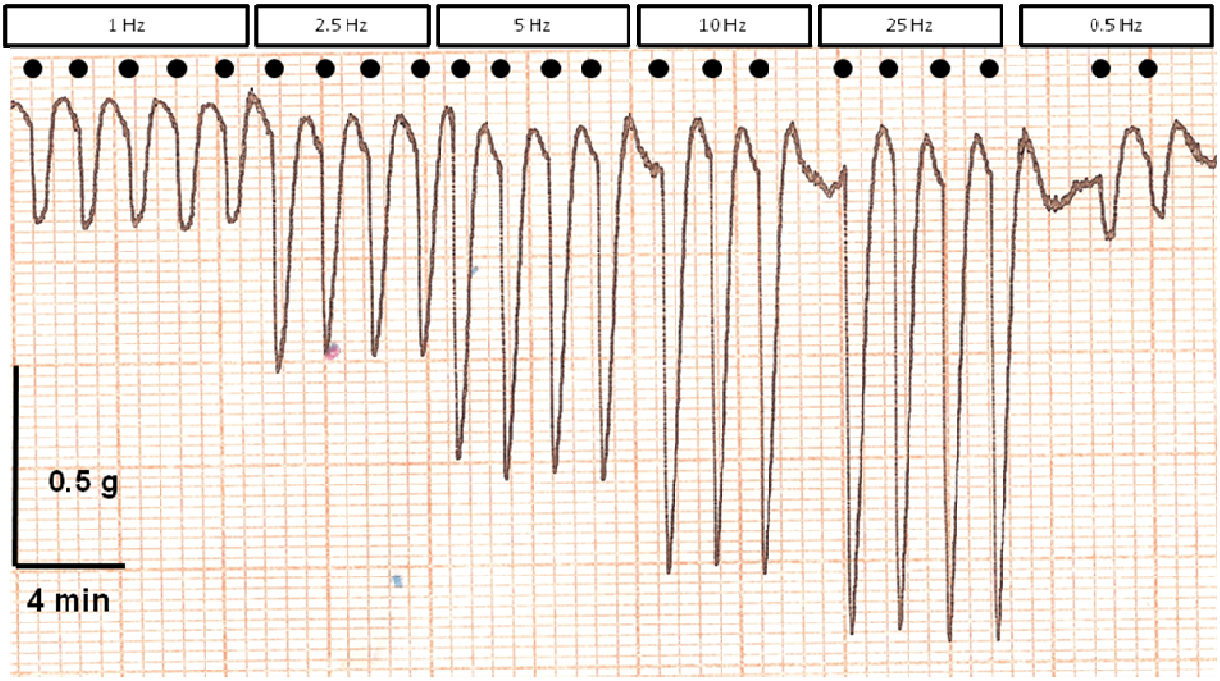


**Figure 51:** After the addition of scopolamine and guanethidine at 10 $\mu$M and phenylephrine at 1 $\mu$M, EFS elicited NANC relaxation responses. Different frequencies of EFS (0.5-25 Hz) were then applied to assess the optimum frequency for EFS-induced relaxation responses. The black dot indicates when the EFS was applied.

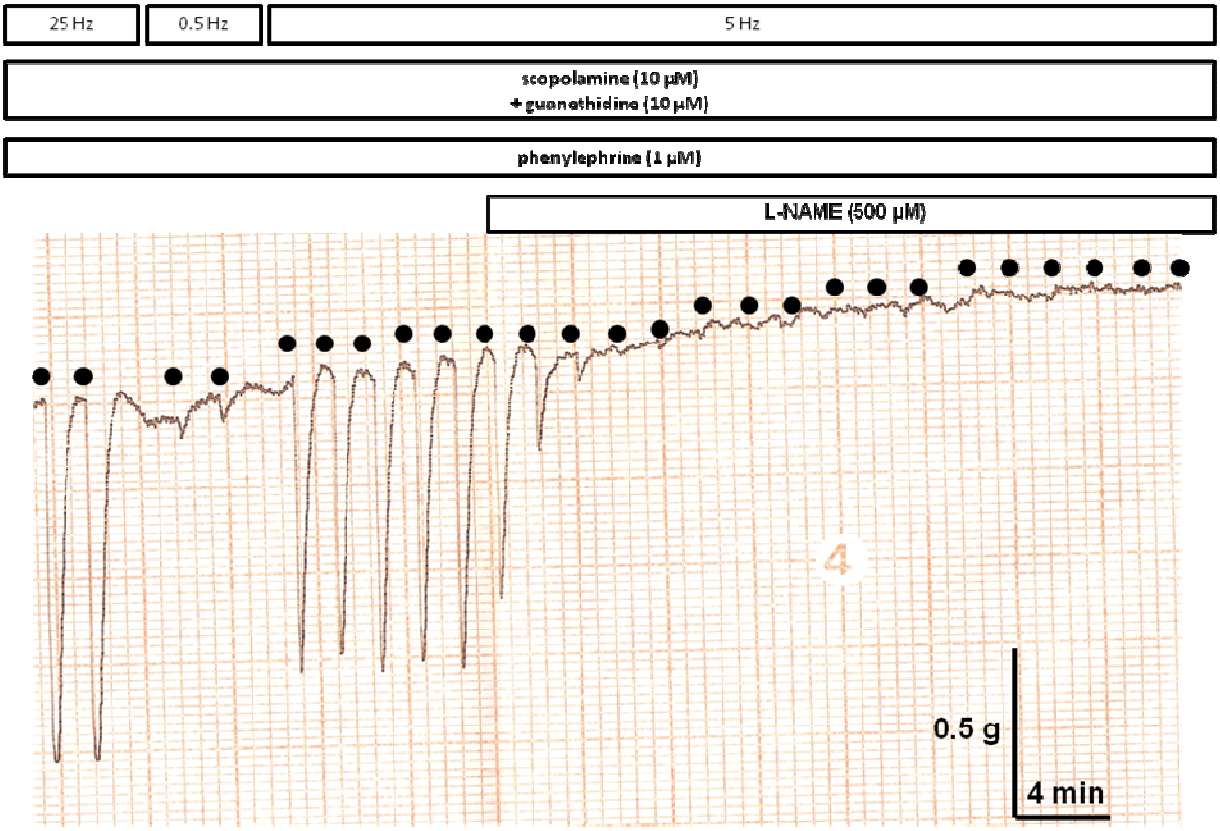


**Figure 52:** The addition of scopolamine and guanethidine at 10 $\mu$M and phenylephrine at 1 $\mu$M ($EC_{80}$) allowed EFS (50 V, 0.3 ms pulse duration, 0.5-25 Hz, for 5 s, every 120 s) to produce reproducible frequency-dependent relaxations. In the trace EFS at 25, 0.5 and 5 Hz frequencies is shown. The black dot indicates when the EFS was given. The addition of L-NAME at 500 $\mu$M resulted in complete inhibition of nitrergic relaxations and elevation of the tone.

In the anococcygeus muscle from diabetic animals, there was a significant reduction in the magnitude of nitrergic relaxations at all frequencies in comparison to control animals (Figures 53 and 54). At the highest frequency (25 Hz) almost 80% of the relaxation response was lost in the diabetic group (Figures 53 and 54).

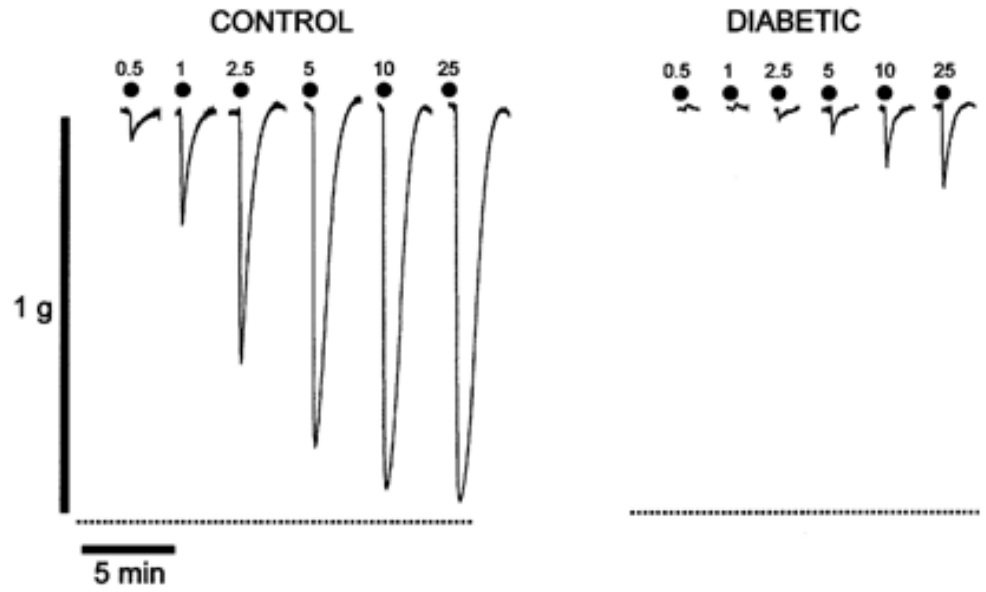


**Figure 53:** Electrical field stimulation (50 V, 0.3 ms pulse duration, for 5 s, indicated by solid circles) at variable frequencies (0.5-25 Hz; as indicated) elicited nitrergic relaxation responses in the anococcygeus muscle from control (left) and 16 weeks diabetics (right) rats. The dotted line represents the basal tone.

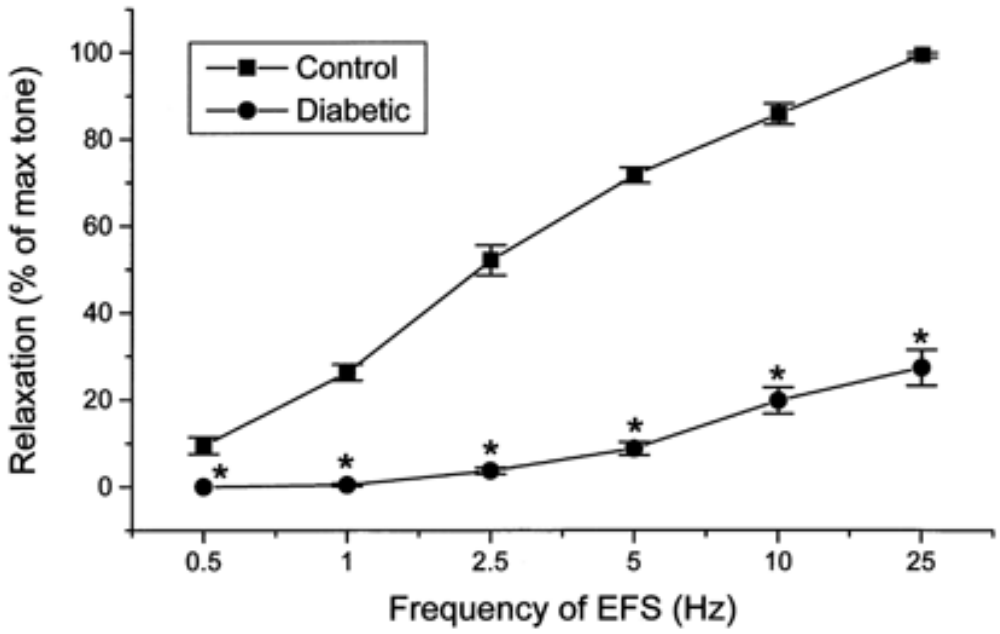


**Figure 54:** Electrical field stimulation (50 V, 0.3 ms pulse duration, for 5 s)-induced nitrergic relaxation responses were significantly attenuated in the diabetic group (circles) compared to control group (squares). *P<0.05 significantly different from control group at the same frequency.

### 3.91 Investigation of the effect of sildenafil, NCX-911 and BAY41-2272 on nitrergic relaxations in the rat anococcygeus muscle

Since above certain concentration all three compounds were able to reduce the phenylephrine-induced tone and it is not possible to elucidate the nitrergic relaxation responses at reduced tone, two sub-threshold concentrations (10 and 30 nM) of each compound were chosen to study the effect of the compound on nitrergic responses. At these sub-threshold concentrations the compounds were found not to reduce the tone but were found to potentiate both the duration and magnitude of the nitrergic relaxation responses in the control group (not shown).

In the diabetic group the magnitude and duration of the remaining nitrergic response was potentiated by BAY41-2272 (30 nM) but not by sildenafil (30 nM) or NCX-911 (30 nM) (Figure 55). The average potentiation of the nitrergic response at 5 Hz by 30 nM BAY41-2272 was found to be 183.5±22.1% when the area under the curve of relaxation was evaluated (n=6).

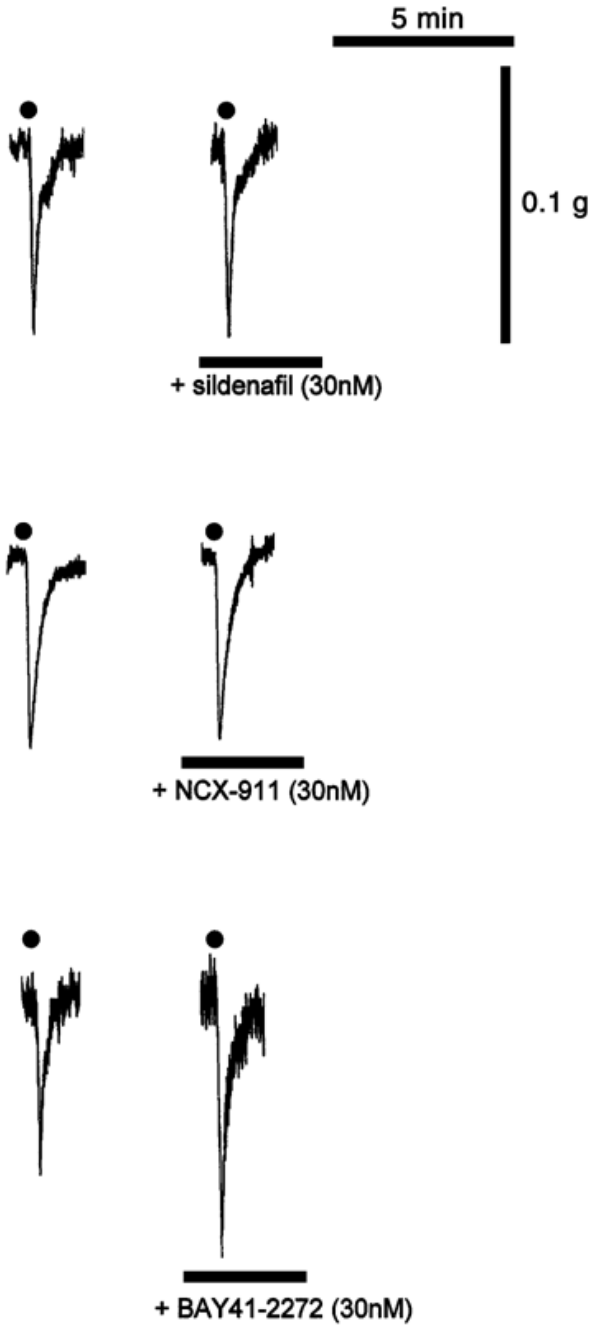


**Figure 55:** The effect of sildenafil, NCX-911 and BAY41-2272 (all at 30 nM) on the nitrergic responses in the anococcygeus muscle from diabetic rats. Electrical field stimulation (50 V, 0.3 ms pulse duration, 5 s, 5 Hz) is denoted by solid circles. Note the potentiating effect of BAY41-2272 on both magnitude and duration of the response (n=6).

# CHAPTER 4.

# DISCUSSION

## 4.0 Discussion

Despite major advances in the understanding of the physiology of penile erection and the pathophysiology of ED, together with an increase in the armamentarium of medications available, ED remains a significant global male health problem. This condition greatly affects patient quality of life and self-esteem. Moreover there is also evidence that ED negatively impacts on the ability to maintain intimate relationships.

New oral medications especially the phosphodiesterase type 5 (PDE5) inhibitors have revolutionalised the treatment of ED by decreasing reliance on more invasive options. Three potent selective PDE5 inhibitors, sildenafil (Viagra; Pfizer), tadalafil (Cialis; Lilly), and vardenafil (Levitra; Bayer) are currently available in the UK. Although large multi-centre clinical trials have shown the efficacy and tolerability of these drugs in ED with various aetiologies and a broad range of severity, 30-35% of patients fail to respond (Moore et al., 2005). Studies which have assessed the long term uptake of PDE5 inhibitors report a renewal rate of 62% for prescriptions at three to four months of follow-up. However this level fell back to around 30% by 6-12 months (McMahon et al., 2006).

The possible reasons for acute or delayed failure include severe ED at presentation, worsening of endothelial dysfunction, ED after radical prostatectomy or diabetes, unrecognised or untreated hypogonadism, inadequate patient education or incorrect drug usage and the development of drug tolerance.

**ED and diabetes**

There is now ample evidence to suggest that ED is common in men with diabetes. It has been reported to affect up to 32% with type 1 and 46% of type 2 diabetic men (Vickers and Wright, 2004). Analysis of the results from the Massachusetts Male Ageing Study (MMAS) demonstrated that diabetics are three times as likely to have ED than non-diabetic men (Feldman et al., 1994). In the study, the prevalence of ED varied from 33 to 75% depending on age, the glycaemic control and the presence of additional risk factors (e.g. smoking, hypertension, hyperlipidaemia) (Feldman et al., 2000). Importantly, it is now recognised that ED can be the first manifestation of previously undiagnosed diabetes (Sun et al., 2006). This of course further supports the importance of risk factor screening on first presentation.

As previously discussed, the pathophysiology of ED in diabetes is multifactorial (See 1.3.5). The proposed mechanisms in diabetic patients includes elevated advanced glycation end-products (AGEs) (Cartledge et al., 2001a) and increased levels of oxygen free radicals (Cartledge et al., 2001a), impaired NO synthesis (Cartledge et al., 2001b), increased endothelin B receptor binding sites and ultra structural changes (Sullivan et al., 1997), an up regulated RhoA/Rho-kinase pathway (Bivalacqua et al., 2004), NO-dependent selective nitrergic nerve degeneration (Cellek et al., 1999) and impaired cyclic guanosine monophosphate (cGMP)-dependent kinase-1 (PKG-1) (Chang et al., 2004).

It is now well established that nitrergic function is impaired in diabetic patients with ED (Saenz, I et al., 1989), in the corpus cavernosum of alloxan-induced diabetic rabbits (Azadzoi and Saenz, I, 1992) and in the corpus cavernosum and anococcygeal smooth muscle of streptozotocin-induced diabetic rats (Rehman et al., 1997).

Furthermore, it has been demonstrated that the activity and amount of nNOS protein is reduced in functional and structural studies involving the corpus cavernosum of spontaneously diabetic BB/WOR$_{dp}$ rats (Vernet et al., 1995). Strong subsequent evidence also demonstrates that the reduction in nNOS activity and protein is most likely to be secondary to selective nitrergic degeneration as this may be prevented by administration of an NOS inhibitor (Cellek et al., 1999). Further investigations have clarified that the defective nitrergic relaxation responses were not due to changes in the responsiveness of the smooth muscle to NO. The addition of an NO donor resulted in responses which were very similar between the control and diabetic tissues (Cellek et al., 1999).

It is however likely that the processes of selective nitrergic degeneration and oxidative stress work closely together to produce the pathophysiological changes which result in diabetes-induced ED. Indeed some authors have suggested that the generation of the powerful pro-oxidants such as peroxynitrite in diabetes may directly result in ED by the process of selective nitrergic nerve loss (Lyall et al., 1998). Evidence from studies assessing the timing of insulin replacement in diabetes, shows that the loss of nNOS content is potentially reversible with early insulin replacement. This finding provides support to the theory that early on in the disease process there are non-structural reasons for nNOS loss such as a reduction in necessary growth factors or dysfunction axonal transport of nNOS protein from the neuronal cell body (Cellek et al., 2003). From the same study, when the diabetes is allowed to progress with time, underlying structural damage to nitrergic nerves occurs. Unfortunately, these changes are not reversible with insulin treatment. Moreover, as these results suggest a degenerative process in the nerve ganglia, this further supports a role for oxidative stress. The accumulation of factors that

cannot be reversed by insulin treatment and may ultimately result in irreversible nerve damage include the aforementioned advanced glycation end-products (AGEs). As described previously (See 1.3.5b), AGEs have been shown to accumulate during the course of diabetes and may therefore act together with endogenous NO to produce neuronal apoptosis and ultimately nitrergic degeneration (Cellek et al., 2003).

**Treatment of diabetic ED**

To achieve optimum results in this difficult to treat patient group, diabetic ED treatment should ideally involve a multimodal approach. Patients need to be carefully counselled with respect to treatment of the underlying hyperglycaemia and co-morbidities. Studies have reported an improvement in erectile function after glycaemic control is improved and other co-morbidities treated (Awad et al., 2009;El-Sakka et al., 2009).

According to the Second Princeton Consensus Conference (Jackson et al., 2006) it was recommended that the cardiovascular system should be assessed in all patients with ED prior to the initiation of any treatment. If this advice is followed, each patient may be assigned to one of three risk levels based on their cardiovascular risk.

As previously mentioned (See 1.4.1), the PDE5 inhibitors are now the mainstay of oral medical treatment of ED in diabetic patients. These agents successfully inhibit PDE5, the primary phosphodiesterase enzyme in the corpus cavernosum tissue responsible for the degradation of cGMP (Beavo, 1995;Wallis et al., 1999). During sexual stimulation and arousal, the released NO activates soluble guanylate cyclase, which in turn catalyzes guanosine triphosphate to cGMP. Cyclic GMP causes by way of many processes a

decrease in cytosolic calcium concentration and the relaxation of smooth muscle (Kalsi et al., 2002). Thus, by inhibiting PDE5, there is a prolonged elevated concentration of intracellular cGMP and enhanced smooth muscle relaxation.

In a study comparing sildenafil versus placebo in type 1 diabetics, there was reported to be significant improvements from baseline using several end points. When the ability to achieve erections (IIEF Q3) was assessed 35.7% were successful with sildenafil versus placebo (19.9%) whereas with the ability to maintain erections (IIEF Q4) 68.4% of patient had a positive outcome with sildenafil versus placebo (26.5%). Moreover, when a more crude measure of success was used such as the Global Assessment Question (GAQ) the results were 66.6% *vs*. 28.6% for sildenafil and placebo respectively (Stuckey et al., 2003).

In a more recent but similar study with vardenafil, the baseline erectile function (EF) domain of IIEF score was 11.2, and it increased to 19.0 (69.6%), 17.1 (52.7%) and 12.6 (12.5%) for vardenafil 20 mg, 10mg and placebo, respectively. In this study the results with the GAQ was 72% for vardenafil 20 mg, 57% for vardenafil 10 mg, and 13% for placebo. A more detailed evaluation of patients based on severity of baseline ED demonstrated that patients with severe ED (EF score < 11) had a 40% rate of successful intercourse with vardenafil 20 mg versus 11% for placebo. This compared with a higher rate of 75% success with vardenafil at 20 mg versus 47% for placebo success in patients with mild ED (EF score 22-25) had a (Goldstein et al., 2003).

In a retrospective analysis from 12 placebo-controlled trials evaluating tadalafil, it was reported that patients with diabetes had a baseline of 12.6 for the EF domain of IIEF. Treatment with tadalafil at 10 mg and 20 mg was found to result in an increase in the

IIEF EF domain score of 6.2 and 7.4, respectively, versus 0.9 for placebo. The corresponding scores for the GAQ was 74.5% for tadalafil 20 mg 60.6% for 10 mg, and 29.7% for placebo, respectively (Fonseca et al., 2004).

In summary, all three PDE5 inhibitors have been evaluated in diabetic patients with similar levels of efficacy averaging 60-70%. However, there are at present no head-to-head comparative trials and hence, efficacy comparisons cannot be performed due to different populations studied. In my clinical experience, diabetic men require the top dose of each agent, sildenafil 100 mg, vardenafil 20 mg and tadalafil 20 mg. Despite this a significant number of patients have poor results or fail therapy. Therefore, new therapeutic targets which may potentially relax cavernosal smooth muscle without the need of endogenous NO are needed. Therefore, sGC activators such as BAY41-2272 and NO releasing PDE5 inhibitors such as NCX-911 were investigated in this thesis in order to assess whether they could relax cavernosal or anococcygeal smooth muscle in NO deficiency.

**The rabbit animal model**

In 1990, it was first reported that the relaxation of isolated rabbit corpus cavernosum smooth muscle by electrical stimulation was accompanied by the production of NO and cyclic GMP. This relaxation was prevented by treatment of tissues with NO synthase inhibitors, haemoglobin and methylene blue. However, treatment with indomethacin was not found to inhibit relaxation responses (Ignarro et al., 1990). These results were the first to suggest that relaxation of corpus cavernosum was mediated by non-adrenergic non-cholinergic (NANC) neurotransmitters and attributed to the generation and release of NO as the primary neurotransmitter.

Since that time, the rabbit corpus cavernosum has been confirmed to be an excellent model for the investigation of erectile smooth muscle physiology (Cellek and Moncada, 1997b). The results obtained are reproducible and the tissue is easy to handle and mount. However, despite this reports suggest that rabbit corpus cavernosum shows a different phosphodiesterase profile to human cavernosal tissue (Qiu et al., 2000). Because of these differences, the experiments were repeated in human corpus cavernosal tissues for comparison and validation.

**Human corpus cavernosum**

Following on from the work on rabbit corpus cavernosum, which demonstrated that penile erection is mediated by neuronal NO, additional studies revealed the same physiological mechanism for penile erection exist in human (Rajfer et al., 1992) and canine (Trigo-Rocha et al., 1993) corpus cavernosum. The main problems of using human corpus cavernosum tissue to perform *in-vitro* studies are associated with its limited availability further complicated by the heterogeneous source of the tissues used (Peyronie's disease, ED, penile cancer).

In this study I used human corpus cavernosum from patients undergoing gender re-assignment surgery. The men had been maintained on long-term oestrogen therapy before surgery to aid the development of secondary feminine characteristics. However oestrogen treatments were stopped 6 weeks before surgery in all patients. It has previously been demonstrated that gender reassignment is a reliable source of human tissue (Goepel et al., 1999). As the entire corpus cavernosum is obtained at the time of surgery, this allows a standardized procedure to prepare human corpus cavernosum strips. Furthermore, studies have also shown that the human tissue from gender re-assignment patients, if kept refrigerated does not lose its ability to contract to phenylephrine, angiotensin II and high potassium for many days (Mirone et al., 2000). Moreover, the human corpus cavernosum has been demonstrated to relax to endothelium-dependent and independent mechanisms as well as direct K(ATP) channel openers (cromakalim) after pre-contraction with phenylephrine (Mirone et al., 2000). The results obtained were very similar to those from human cavernosal tissue from non-gender change patients (Mirone et al., 2000).

Previous studies which have evaluated the relationship between the degree of corporeal smooth muscle contraction and the magnitude of the observed relaxation response (nitroglycerine, nitroprusside, and prostaglandin E1) suggest that the relationship between contraction and relaxation is linear with an inverse relationship. However, statistical analysis indicated that the slope of the regression line was significantly greater than unity in corporeal tissues obtained from patients with organic ED versus controls (Taub et al., 1993). These results provided evidence implicating heightened adrenergic tone as a significant aetiological factor in ED. To make sure that there was no significant difference in adrenergic tone between the human and rabbit corporeal tissues, concentration response curves in response to phenylephrine were constructed. The results showed that both tissues had very similar responses with an $EC_{80}$ to phenylephrine of 3μM.

**Diabetic animal model**

Both the rabbit and rat are good and reliable models for diabetes research. The cost and husbandry is significantly greater with rabbit when directly compared with rats. However, the rat penile corpus cavernosum is a difficult tissue to work with due to spontaneous contractions compared to the rat anococcygeus muscle. In contrast, the anococcygeus muscle (Cheah et al., 2002;Rand and Li, 1992;Rand and Li, 1993) has been widely accepted as ideal smooth muscle models to study not only nitrergic neurotransmission but also other NANC neurotransmitters (Kasakov et al., 1994;Rand and Li, 1992;Rand and Li, 1993). In the rat, the corpus cavernosum and the anococcygeus muscles receive motor sympathetic innervations via the lumbar sympathetic nerves and parasympathetic innervations via the sacral parasympathetic nerves. They therefore share a common origin for nitrergic and sympathetic pathways. Furthermore, the anococcygeus muscle, unlike the corpus cavernosum, is a purer smooth muscle preparation containing very few blood vessels making pharmacological experiments easier to interpret. Moreover, the anococcygeus was the first tissue in which a neurotransmitter role for NO was firmly established (Gillespie et al., 1989).

By developing a rat anococcygeal model of diabetes, the potencies of the PDE5 inhibitor (sildenafil), the NO-releasing sildenafil (NCX-911) and the sGC activator (BAY41-2272) could be assessed and compared in non-diabetic and diabetic rats.

**Soluble guanylate cyclase activators**

Our knowledge of the function of soluble guanylate cyclase (sGC) has increased significantly during the past decade. The best studied class of sGC activators are organic nitrates which mimic the action of endogenous NO by bioconversion to NO or NO-related compounds. Organic nitrates are widely used in the treatment of angina pectoris. However, the use of such compounds is limited by a potential lack of response due to insufficient biometabolism (Li et al., 2006), development of tolerance following prolonged administration (Munzel et al., 2005) and non-specific interactions of NO with other biological molecules, including peroxynitrite-mediated tyrosine nitration (Dikalov et al., 1998). These reactions are difficult to control owing to the spontaneous release of NO from nitro-vasodilators and its free diffusion in biological systems. Furthermore, although there is symptomatic improvement in patients with cardiovascular disease treated with organic nitrates, there is no evidence that such treatment reduces mortality (Csont and Ferdinandy, 2005). Therefore compounds that activate sGC in an NO-independent manner might offer considerable advantages over current therapies.

The recent discoveries of compounds that stimulate sGC independently of NO allow this pharmacological target to be investigated more thoroughly. NO-independent but haem-dependent stimulators of sGC, as well as NO- and haem-independent sGC activators, are emerging as valuable tools that could help to elucidate the physiology and pathophysiology of the NO–sGC–cGMP pathway in many conditions including ED.

In 1994, Ko and co-workers described an indazole derivative YC-1 (Ko et al., 1994), which was subsequently characterized as an NO-independent, haem-dependent stimulator of highly purified sGC (Mulsch et al., 1997). It was found to stimulate sGC

directly *via* a distinct mechanism and sensitize the enzyme towards its native activator NO (Friebe and Koesling, 1998). YC-1 has also been shown also to act as a non-specific phosphodiesterase inhibitor (Friebe et al., 1998) and to stimulate the synthesis and release of NO (Wohlfart et al., 1999).

YC-1 has been shown to relax rabbit cavernosal strips ($EC_{50}$ = 8.5 μM) via increasing cGMP levels (Nakane et al., 2002). Systemic injection of YC-1 has been shown to enhance penile erection in the rat and to induce erection when injected intracavernously (Mizusawa et al., 2002). *In vitro* YC-1 enhances EFS-induced relaxations in the rat corpus cavernosum (Nakane et al., 2002) and enhances relaxations induced by an NO donor in rabbit corpus cavernosum (Mizusawa et al., 2002).

Using YC-1 as a lead compound, BAYER AG embarked upon a chemical optimization programme of 2,000 newly synthesized compounds in search for more potent NO-independent sGC activators. This resulted initially in the discovery of a pyrazolopyridine derivative, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-Hpyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine (BAY41-2272) which was identified using a photo affinity label on the purified enzyme (Stasch et al., 2001). The cysteine 238 and cysteine 243 spanning regions in the $\alpha_1$-subnunit of sGC were subsequently identified as part of the target site. Because of the structural similarity between BAY41-2272 and YC-1, it was initially thought that both compounds may act by the same mechanism. However, in contrast to YC-1, BAY41-2272, up to 10$\mu$M, is devoid of any PDE5 inhibitory activity (Stasch et al., 2001). More recently a further pyrazolopyridine derivative BAY41-8543 has also been identified (Stasch et al., 2002a).

**Effect of BAY41-2272 on human and rabbit corpus cavernosum**

In this study I have shown that BAY41-2272 lowers the phenylephrine-induced tone in the human and rabbit corpus cavernosum with an $EC_{50}$ of approximately 400-500 nM. This is in agreement with a previous study where $EC_{50}$ of the compound to relax rabbit aortic rings was 304±63 nM (Stasch et al., 2001). The potency of BAY41-2272 on lowering phenylephrine-induced tone was twice more than an NO donor (spermine-NONOate) and 32 times more than YC-1. These results confirm that BAY41-2272 has a similar potency to NO and is significantly more potent than YC-1 as shown previously (Stasch et al., 2001) . It has been suggested that metabolites of BAY 41-2272 may retain the ability to activate sGC, and may therefore contribute to the greater *in vivo* activity (Straub et al., 2002).

Other groups have reported results with BAY41-2272 in rabbit and human cavernosal tissues with significantly different potencies to my observations (Baracat et al., 2003). They ascribe this to the use of cavernosal tissue from healthy multiple organ donors and suggest that my results are because of the use of oestrogenised tissues. However, their results are significantly different to mine in both human and rabbit tissues so the differences are most likely to be methodological.

My results have shown that the relaxant effect of BAY41-2272 is significantly inhibited by a sGC inhibitor (ODQ) suggesting that the effect of BAY41-2272 is sGC-dependent. However, in both rabbit and human corpus cavernosum, the relaxations elicited by BAY41-2272 were only partially affected by ODQ at concentrations which can block the nitrergic relaxations (30 $\mu$M). Interestingly, even in the purified enzyme,

ODQ has previously been shown to fail to abolish the BAY41-2272-induced sGC activation (Stasch et al., 2001). My results are also in accordance with previous studies where the activation of sGC by YC-1 (Martin et al., 2001) and BAY41-2272 (Becker et al., 2001b) was not completely blocked by ODQ. This may be attributed to the possibility that the binding of ODQ and sGC activators to the sGC are not mutually exclusive processes and that their binding sites do not overlap. Moreover, non-NO-based sGC activators have haem-dependent and -independent activities (Becker et al., 2001b;Martin et al., 2001). Moreover, ODQ has been described to oxidize the haem moiety of sGC. It may also have an allosteric mechanism by interfering with the binding of BAY 41-2272, through changes in the secondary structure of the recombinant enzyme when ODQ is added to the NO-stimulated sGC (Kosarikov et al., 2001).

In contrast, in my study the relaxation response to the NO donor, spermine-NONOate was found to be completely blocked by ODQ. This is in accordance with previous studies which have demonstrated that relaxation of the corpus cavernosum by exogenous or endogenous NO was abolished by ODQ (Nakane et al., 2002). In these studies, responses elicited by an NO donor, glyceryl trinitrate and by acetylcholine, which releases NO from the sinusoidal endothelium, were fully prevented by ODQ (Baracat et al., 2003). It has been suggested that the inhibitory effect of ODQ on NO-stimulated sGC is as a result of changes in the oxidation state of the haem moiety, without significant effects in the catalytic activity of the enzyme (Zhao et al., 2000).

My results demonstrated that the concentration-relaxation response curve to BAY41-2272 was shifted to the right with the addition of an inhibitor of NO synthase suggesting that the endogenous basal NO levels could be potentiating the effect of the

sGC activator. Indeed stimulation of purified sGC by BAY41-2272 has previously been shown to be enhanced by an NO donor (Stasch et al., 2001). Interestingly, my results indicate that BAY41-2272 enhances nitrergic relaxations at concentrations known to have no PDE-inhibitory activity. These findings further support the concept that BAY41-2272 synergizes with endogenous NO. When I completely blocked nitrergic relaxations with the NO synthase inhibitor, only high concentrations of BAY41-2272 could reverse the inhibition confirming the PDE-inhibitory action of the compound at high concentrations. This in accordance with a recent *in vitro* study where concentrations of BAY41-2272 several orders of magnitude above those needed for sGC stimulation resulted in some degree of PDE5 inhibition ($IC_{50}$ = 3 μM) (Mullershausen et al., 2004). However, this remains many orders of magnitude higher than that required to cause relaxation in a number of isolated tissues including aorta (Priviero et al., 2005), vaginal wall (Cellek, 2003), and corpus cavernosum (Kalsi et al., 2003) ($EC_{50}$ = 0.06–0.49 μM). Furthermore, studies using recombinant sGC revealed that concentrations of BAY41-2272 as low as 0.01–0.1 μM stimulate sGC to a level that would usually be expected to cause biologically and clinically important increases in cGMP (Stasch et al., 2001). Moreover, in vivo studies provide further evidence against a role for PDE-5 inhibition in the action of BAY 41-2272 (Evgenov et al., 2004).

In conclusion, my results clearly demonstrate that the sGC activator BAY41-2272 can relax human and rabbit corpus cavernosum. The relaxant effects of BAY 41-2272 synergize with endogenous NO. In the total absence of endogenous NO the compound is still able to relax the smooth muscle and to reverse the inhibition of nitrergic relaxations.

Therefore, sGC activators may be a novel way of treating male ED particularly in patients where endogenous NO production is dysfunctional.

**Nitric oxide and PDE5 inhibitors**

It is now generally accepted that the most critical step in penile erection is relaxation of penile cavernosal and vascular smooth muscle (Andersson and Wagner, 1995). Moreover, the role of NO is of fundamental importance in this process. NO has been shown to induce an increased production of the second messenger cGMP via stimulation of the enzyme sGC in the penile smooth muscle. In turn cGMP has been shown to activate a number of downstream pathways, ultimately resulting in relaxation of the cavernosal smooth muscle (Andersson, 2001a).

Several synthetic NO donors have been tested in clinical trials or on experimental animal studies, on the premise that the release of exogenous NO would allow corporal smooth muscle relaxation. Although sodium nitroprusside (SNP) substantially relaxes pre-contracted preparations *in vitro*, contrasting results have been obtained *in vivo* by directly injecting NO donors into the corpora cavernosum in erectile dysfunction (Truss et al., 1994;Wang et al., 1994;Wegner and Knispel, 1993).

A new class of NO donors has recently been synthesized, which consists of nitro-derivates of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin (NO-NSAIDs) (Fiorucci et al., 2004). A common chemical feature of these compounds is a link of an effective moiety with nitrate through a spacer. It has been reported that these agents are chemically stable, but are able to subsequently release NO enzymatically after exposure to biological tissues (Keeble & Moore, 2002). One such example is NCX-4050, which has been shown to allow the release of low concentrations of NO in a controlled manner (Filippi et al., 2003a).

It has therefore been suggested that the administration of an NO donor with sildenafil may compensate for reduced "NO drive" in vasculopathic states (Jeremy et al., 1997;Kalsi et al., 2004;Kalsi et al., 2005;Seidler et al., 2002).

Using the same basic principles as above, novel agents which are combinations of a PDE5 inhibitor and NO-releasing compounds have been produced. NCX-911 (sildenafil nitrate) is one such agent. It has been shown to have a similar potency to sildenafil and release NO spontaneously (Riffaud JP, 2001). It was my aim to investigate the effect of NCX-911 on the tone and nitrergic responses of rabbit and human corpus cavernosum and compare the effects with that of sildenafil and an NO donor (spermine-NONOate).

**The effect of NCX-911 and sildenafil on human and rabbit corpus cavernosum**

The results of this study have revealed that NCX-911 lowers the phenylephrine-induced tone in rabbit and human corpus cavernosum with an $EC_{50}$ of approximately 733-997 nM. This is in agreement with a previous study where the $EC_{50}$ of the NCX-911 to relax human corpus cavernosum was in the range of 1-10 μM (Seidler et al., 2002).

My results also demonstrate that the potency of NCX-911 on lowering phenylephrine-induced tone was similar to both sildenafil and the NO donor in control tissues. These results are in disagreement with a previous study where NCX-911 was reported to be more potent than sildenafil at relaxing human cavernosal strips from gender reassignment surgery pre-contracted with noradrenaline (Seidler et al., 2002). However, since the potencies of NCX-911 and sildenafil were not detailed with exact $EC_{50}$ values in that study, the degree of separation between the two compounds is difficult to evaluate. The disparity between the results of that study and mine could be as a result of different techniques used. Seidler et al (Seidler et al., 2002) used organ baths whereas I used superfusion chambers. It is possible that superfusion of corpus cavernosum might cause a higher shear stress compared to the organ bath and therefore cause a greater release of NO from the endothelium.

In this study, I established that the treatment of human and rabbit cavernosal strips with an inhibitor of NO synthase (L-NAME), at a concentration known to block endogenous NO production (Cellek and Moncada, 1997b;Kalsi et al., 2003), resulted in a significant rightward shift of the concentration-response curve to sildenafil (a 5-fold reduction in potency), but not that of NCX-911 or the NO donor. These results suggest that sildenafil requires endogenous NO to relax cavernosal smooth muscle. This is in

accordance with a previous study where sildenafil was shown to be unable to potentiate the reduced erectile responses in mice lacking the neuronal NO synthase enzyme (Cashen et al., 2002). However, they are in disagreement with another study which demonstrates that sildenafil citrate may have a direct relaxant effect on cavernosal tone at concentrations of 100 nM or greater (McAuley et al., 2001). These authors proposed this effect was independent of the NO–cGMP pathway, as the effect was not able to be inhibited with L-NAME and only partially prevented by oxadiazolo quinoxaline (ODQ), a guanylate cyclase inhibitor (McAuley et al., 2001). Other studies suggest that the mechanism of sildenafil-induced relaxation may entail, in part, the suppression of superoxide formation (Shukla et al., 2005). However, the possible direct relaxation of cavernosal tissue by sildenafil may be actually be a result of basal production of cGMP which may accumulate to exert a significant modulating effect on cavernosal smooth muscle tone in the presence of PDE5 inhibition. This basal cGMP production may be produced by the basal release of NO. Unfortunately the group investigating this phenomenon could not answer whether the basal cGMP production is due to stimulation by NO as they did not treat cavernosal tissue strips with both L-NAME and ODQ (McAuley et al., 2001).

As there was no change in the potency of NCX-911 in either rabbit or human cavernosal tissues with an inhibitor of NO synthase (L-NAME), the results of this study clearly demonstrate that NCX-911 does not require endogenous NO to cause relaxation of human and rabbit corpus cavernosum. My results further confirm the difference between the two compounds NCX-911 and sildenafil.

The results seen with NCX-911 and sildenafil in my study are in accordance with a previous study where endogenous NO production was reduced in a hypercholesterolemia rabbit model and NCX-911 was reportedly 5-10 times more potent than sildenafil citrate in reducing phenylephrine-induced tension (Riffaud JP, 2001). Furthermore, in anaesthetized rats, cavernous nerve-stimulation resulted in increases in intracavernous pressure. This pressure rise was shown to be enhanced by intravenous administration of both sildenafil and NCX-911, however at sub-maximal stimulation NCX-911 was found to be more potent than sildenafil (Riffaud JP, 2001).

In the presence of an inhibitor of sGC (ODQ), my results demonstrated that the concentration-response curves to both NCX-911 and sildenafil were both shifted to the right and upward. These results suggest that both compounds require an active sGC enzyme to exert their effects. However, the inhibitory effect of ODQ was only partial; at concentrations above 1 μM both NCX-911 and sildenafil induced relaxation responses reached up to 50% of the maximum. This is in accordance with other previous studies where ODQ partially inhibited sildenafil-induced relaxation responses in human corpus cavernosum (Baracat et al., 2003) and failed to completely inhibit intracavernous sildenafil-induced erection in rabbits (McAuley et al., 2001).

The NO donor NCX4050 belongs to a novel class of drugs called NO-NSAIDs characterized by the ability to release low concentrations of NO, in a controlled manner. This is in contrast to NCX-911 where NO is released spontaneously. A common chemical feature of this novel drug class is a link of an effective moiety with nitrate through a spacer. As previously discussed, it is reported that they are chemically stable agents but

are able to release NO after enzymatic change following exposure to biological tissues (Keeble and Moore, 2002).

In rabbit and human corpus cavernosum, NCX-4050 has previously been shown to be able to relax cavernosal tissues with an efficacy similar to sodium nitroprusside (SNP) ($IC_{50}$ = 21 ± 1.7 μM, $IC_{50}$ = 19 ± 2.3 μM, in rabbit and human respectively) (Filippi et al., 2003a). In contrast to SNP the relaxant responses to NCX-4050 were reported to develop more slowly. In accordance to the results seen with NCX-911 in my study, it was also observed that ODQ antagonized the relaxant response induced by NCX-4050. However, even at high concentrations of ODQ, NCX-4050 was still able to produce some relaxation responses ($IC_{50}$ = 2587 ± 312 μM) (Filippi et al., 2003a). Again, these observations suggest that there may an alternative direct relaxing effect of these compounds which is not related to amplification of the NO-cGMP system.

I found that both NCX-911 and sildenafil increased the magnitude and the duration of nitrergic relaxations with similar potencies in the rabbit corpus cavernosum. These results suggest that both compounds have similar mechanisms of action and properties under control conditions despite the differences in chemical structure. In my study, the release of NO from the NCX-911 compound did not seem to further potentiate the nitrergic relaxations in rabbit corpus cavernosum.

In my study, in the presence of an inhibitor of NO synthase (L-NAME), the nitrergic relaxations were completely inhibited in the rabbit corpus cavernosum. Increasing concentrations of NCX-911 or sildenafil were not able to reverse the loss of nitrergic relaxations. This is in contrast to the effect of a NO-independent sGC activator, BAY41-2272 (Kalsi et al., 2003). This compound is able to reverse the loss of nitrergic

relaxation at concentrations greater than 300 nM. BAY41-2272 has been shown to bind to an allosteric site on the sGC enzyme different to the binding site for NO (Stasch et al., 2001). It has been suggested that this binding at the allosteric site may lead to a conformational change such that there is an increased affinity for NO. The conformational change may be responsible for the reversal of inhibition of nitrergic relaxations. In contrast, there are no reported conformational changes associated with the addition of either a PDE5 inhibitor or an NO donor.

In conclusion, my results suggest that NCX-911 is a potent relaxant with a similar potency to NO. It is equally efficacious with respect to relaxation of rabbit and human corpus cavernosum independent of NO availability. Like PDE5 inhibitors, NCX-911 potentiates both the duration and magnitude of the nitrergic relaxation. Therefore, NCX-911 may be a novel way of treating male ED particularly in patients where endogenous NO production is dysfunctional.

**NO and diabetes mellitus**

Nitric oxide released from sinusoidal endothelium and postganglionic cholinergic (nitrergic) nerve fibres is the major mediator of vascular and cavernosal smooth muscle relaxation in the penis (Andersson, 2001b). In diabetes the NO-mediated responses have been shown to be reduced in both human and animal studies (Azadzoi and Saenz, I, 1992;Pickard et al., 1994;Pickard et al., 1995;Rehman et al., 1997;Saenz, I et al., 1989).

As previously described (See 1.3.5b), this has been attributed to elevated advanced glycation end-products (AGEs) and increased levels of oxygen free radicals (Cartledge et al., 2001a) which may lead to decreased bioavailability of NO (Jones et al., 2002), impaired NO synthesis due to down-regulation of eNOS/nNOS expression (Akingba and Burnett, 2001;Podlasek et al., 2001;Vernet et al., 1995), decreased and impaired cyclic guanosine monophosphate (cGMP)-dependent kinase-1 (PKG-1) (Chang et al., 2004), increased endothelin B (ETB) receptor binding sites and ultrastructural changes (Sullivan et al., 1997), up-regulated RhoA/Rho-kinase pathway (Bivalacqua et al., 2004), and NO-dependent selective nitrergic nerve degeneration (Cellek et al., 2003;Cellek et al., 1999;El-Sakka et al., 1999).

The decreased production and/or availability of NO are widely accepted to be the primary cause for the significant loss of erectile function in diabetic men. Although the pathophysiology of diabetic induced ED is multifactorial, I believe that the most important factors are related to a reduction in NO production from dysfunctional nitrergic nerves and reduced NO bioavailability through the generation of AGEs and oxygen free radicals.

PDE5 inhibitors require endogenous NO in order to produce relaxation of smooth muscle in the penis (Cashen et al., 2002). It has therefore been postulated that the reduced rate of success of PDE5 inhibitors in diabetic men (Rendell et al., 1999;Stuckey et al., 2003) may be due to lower NO production from the endothelium and nerves of these men.

To test this hypothesis, new therapeutic targets which are potentially able to induce relaxation independent of endogenous NO (BAY 41-2272 and NCX-911) were compared in control and diabetic tissues versus the PDE5 inhibitor sildenafil.

**The effect of sildenafil, NCX-911 and BAY 41-2272 on the anococcygeus muscle**

It has previously been suggested that compounds which do not require endogenous NO would be more successful than PDE5 inhibitors to relax penile smooth muscle in diabetes (Cellek et al., 2002). The two compounds I used (NCX-911 and BAY41-2272) fit this description. NCX-911 has been previously shown to release NO spontaneously and increase cGMP concentrations by activating sGC and inhibiting PDE5 (Seidler et al., 2002). Furthermore, NCX-911 has been shown to increase cGMP in a concentration-dependent manner in the absence of endogenous NO whereas sildenafil has little effect on cGMP concentrations under the same conditions (Seidler et al., 2002). As previously discussed, in a hypercholesterolemia rabbit model, NCX-911 was shown to be 5-10 times more potent than sildenafil at reducing the phenylephrine-induced vascular tone (Riffaud JP, 2001). BAY41-2272 is a non-NO-based compound (does not release NO) which activates sGC (Stasch et al., 2001). This activation does not require endogenous NO however the potency of the compound is elevated if there is endogenous NO in the environment. I previously showed that BAY41-2272 potentiates the nitrergic responses in the rabbit corpus cavernosum.

In my study, in control (non-diabetic) tissues BAY41-2272 ($EC_{50}$ = 151.6±9.2 nM) was significantly more potent at relaxing anococcygeal tissues pre-contracted with phenylephrine compared to both NCX-911 and sildenafil. However, there was no significant difference between the NCX-911 and sildenafil ($EC_{50}$ = 1088.8±165 nM and 827.1±167.3 nM respectively). The $EC_{50}$ for sildenafil in this study however was significantly different from that of a previous study in the anococcygeus of mice ($EC_{50}$ =

30 nM) (Frith and Gibson, 2000). This may be accounted for by differences in methodology i.e. use of carbachol and species differences i.e. mouse versus rat.

I compared BAY41-2272 and NCX-911 to sildenafil with respect to their potency to relax the anococcygeus muscle of diabetic rats. I found that the potency of BAY 41-2272 and NCX-911 was not altered by diabetes whereas sildenafil was significantly less potent in the diabetic group. This suggests that compounds which do not require endogenous NO to relax the penile smooth muscle could be more efficacious than PDE5 inhibitors in the treatment of ED in diabetic men.

In contrast, a previous study reported that relaxation of the corpus cavernosum to exogenous NO donors and endogenous nitrergic nerve stimulation was enhanced by sildenafil with same potency in the control and the diabetic group in rabbit corpus cavernosum (Thompson et al., 2001). Although the authors did not study the effect of sildenafil on phenylephrine-induced tone in that study, they noted that their model represented only the early phases of autonomic neuropathy and suggested that the potency of sildenafil might be decreasing in the later phases. Furthermore, it has been recently shown that severe nitrergic degeneration starts after 12 weeks of diabetes in the rat model (Cellek et al., 2003) in contrast to earlier studies which showed that there was no loss of NOS content up to 8 weeks (Way et al., 1999). The decrease in the potency of sildenafil seen in this study may therefore be dependent on the severity of nitrergic nerve loss, which is time-dependent in diabetic animal models.

In this study, although the $EC_{50}$ value for sildenafil was higher in the diabetic group than in the control group, $E_{max}$ values were similar. Hence the shift in the concentration–response curve was parallel, suggesting that the target (PDE5) is still

active but its sensitivity was altered during diabetes. This may be explained by either the enzyme was desensitised or the substrate of the enzyme (cGMP) was less available. Since there was no shift in the curves to NCX-911 and BAY41-2272, the desensitisation possibility is doubtful and it is more likely that the available NO-cGMP input is reduced in diabetes. Although there was no significant difference between the potencies of NCX-911 and sildenafil in control tissues, the difference became significant in diabetic animals, suggesting that NO supplied by NCX-911 accounted for the preserved relaxant effect in diabetic tissue. In the control tissues, NO released from NCX-911 did not cause any additional relaxant effect probably because endogenous NO was higher than the NO released by NCX-911. A comparative measurement of NO released from NCX-911 and nitrergic nerves locally would be required to support this theory.

Interestingly I found that NCX-911 and sildenafil failed to potentiate the remaining nitrergic relaxation responses in the diabetic group. This suggests that a threshold concentration of endogenous NO might be required to stimulate the cGMP pathway. When the endogenous NO levels are below this threshold, PDE5 inhibitors are potentially without effect. In the long-term diabetes model I used, NO production is reported to be so low (Cellek et al., 2003) that even in the presence of an NO-releasing compound (e.g. NCX-911), the threshold may not be reached. Therefore, the compounds fail to potentiate the remaining nitrergic response. However, further studies measuring cGMP concentrations are required to confirm this.

In the case of sGC activator BAY41-2272 however, the compound was able to potentiate the remaining nitrergic responses in the diabetic group. This could be due to the compound's ability to synergise with the remaining very low levels of endogenous

NO. A similar phenomenon in the rabbit corpus cavernosum, where the inhibition of nitrergic responses with an inhibitor of NO synthase was reversed by BAY41-2272 was previously demonstrated (Kalsi et al., 2003).

My results further confirm the different pharmacological profiles of these compounds. The systemic administration of NCX-911 and BAY41-2272 has been shown to be without major side effects (Bischoff et al., 2003;Riffaud JP, 2001), although at high doses both compounds can affect the systemic blood pressure (Riffaud JP, 2001;Stasch et al., 2001). Nevertheless, I believe that further research in higher animals with different durations of diabetes is required to compare the effects of similar compounds in the corpus cavernosum to further validate these results.

In conclusion, in long-term diabetes with severe endogenous NO deficiency the NO-releasing sildenafil (NCX-911) and the sGC activator (BAY41-2272) have higher potency than sildenafil to relax the anococcygeus muscle. The reduction in nitrergic responses is reversed by BAY41-2272 but not by sildenafil and NCX-911. Further research is required to characterise the pharmacological profiles of these compounds in human tissue.

**Update of sGC activators**

Following on from my results using the sGC activators YC-1 and BAY41-2272, the knowledge of the function of sGC has continued to increase as has the number of putative new agents. The available agents can now be divided into those which are NO-independent but haem-dependent stimulators and those which are NO- and haem-independent.

**Haem-dependent sGC stimulators**

In 1994, scientists at Bayer HealthCare AG started a search for substances that could induce an increase in NO dependent pathways by stimulating sGC. This led to the discovery of 5-substituted-2-furaldehydehydrazone derivatives as direct NO-independent sGC stimulators. Unfortunately, the potency of these agents increased when exposed to light. However, another group working in parallel, in 1994 described a structurally related compound, the indazole derivative YC-1 (Ko et al., 1994), which was subsequently characterized as an NO-independent, haem-dependent stimulator of highly purified sGC (Mulsch et al., 1997). Furthermore, its potency was unaffected by the prevailing light conditions (Friebe et al., 1996).

More recently, various compounds that activate sGC in an NO-independent fashion have been identified (Lee et al., 2001;Selwood et al., 2001;Straub et al., 2001). These include BAY41–2272, BAY41–8543, CMF-1571 and A-350619 and now BAY 63-2521. They are all haem-dependent sGC-stimulators as they share a crucial dependency on the presence of the reduced prosthetic haem moiety and strong synergistic enzyme activation when combined with NO.

Abbott laboratories developed the novel sGC activator A-350619. Activation of sGC by A-350619 was demonstrated to be partially inhibited by ODQ whereas pre-treatment with N-omega-nitro-L-arginine, (an NO-synthase inhibitor) resulted in a dose-dependent right ward shift in the dose-response curve in corporeal smooth muscle. Moreover, in their study the addition of sodium nitroprusside (SNP) was found to potentiate the relaxation effect of A-350619. Further work in a conscious rat model, also showed that A-350619 (1 μmol/kg) could induce penile erection (Miller et al., 2003).

In subsequent studies the same group of investigators, examined the effects of a derivative of acrylamide (A-778935) ((+/-)-cis-3-[2-(2,2-dimethyl-propylsulfanyl)-pyridin-3-yl]-N-(3-hydroxy-c yclohexyl)-acrylamide) on corpus cavernosal smooth muscle. A-778935 was found to activate sGC synergistically with SNP over a wide range of concentrations, inducing up to 420-fold activation. Once again ODQ was found to competitively inhibit the activation by A-778935. There was no reported activation of haem-deficient sGC, indicating that the activation of sGC by A-778935 is fully haem-dependent. A-778935 was also found to increase intracellular cGMP levels concentration-dependently in smooth muscle. However, in the presence of 1 μM SNP, a lower concentration of A-778935 was required to increase cGMP versus A-778935 alone. A-778935 was reported to relax cavernosal tissue strips in a concentration-dependent manner. The addition of 1 μM SNP resulted in shifting the concentration-response curve to the left and increased potency (Nakane et al., 2006).

CFM-1571 was developed using YC-1 as a lead structure (Selwood et al., 2001). CFM-1571 has been shown to be a weak but specific activator of sGC ($EC_{50}$ =5.5 μM)

that inhibits platelet aggregation ($EC_{50}$ = 2.8 μM). Similar to YC-1, it reportedly synergizes with NO however unlike YC-1 it does not show PDE inhibitory activity and demonstrates minimal inhibition of NOS (Selwood et al., 2001).

More recently, Bayer has developed a further pyrazolopyridine derivative, BAY 41-8543 (Stasch et al., 2002a;Straub et al., 2002). It has been reported to act in a similar mode of action to that of YC-1, but demonstrates an improved potency and specificity for sGC (Stasch et al., 2002a;Straub et al., 2002). It has been proposed that in the absence of NO, BAY41-2272 stimulates sGC activity approximately 20-fold from baseline (Stasch et al., 2001) whereas BAY41-8543 exhibits even greater potency stimulating sGC activity up to 92-fold (Stasch et al., 2002a). Furthermore, BAY41-8543 has also been shown to have about a 3-fold greater potency than BAY41-2272 in various in vitro and in vivo assays (Stasch et al., 2002a;Straub et al., 2002). Like BAY41-2272, it is now known that it synergizes with NO. By stabilizing the nitrosyl-haem complex it is able to stimulate sGC activity up to 200-fold (Schmidt et al., 2003). Furthermore, it also does not cause inhibition of PDE5 (Schermuly et al., 2008;Stasch et al., 2002a). Moreover, BAY41-8543 has no appreciable inhibitory effect on other cGMP specific PDEs, such as PDE1, 2 and 9 (Stasch et al., 2002a) (Bischoff and Stasch, 2004).

*In-vitro* studies also confirm that BAY41-8543 like BAY41-2272, exerts potent vasodilatory, anti-platelet, and anti-proliferative activity (Stasch et al., 2002a). The vasodilatory effects of BAY41-2272 and BAY41-8543 have been observed in isolated systemic, aortic, coronary, and pulmonary arteries and veins, and the compounds potently reduce coronary perfusion pressure in the rat heart Langendorff preparation without any effect on left ventricular pressure and heart rate (Hobbs, 2002;Stasch et al., 2001;Stasch

et al., 2002a). Interestingly, BAY41-8543 has also been reported to stimulate equivalent degrees of vasodilatation in normal and nitrate-tolerant systems (Stasch et al., 2002a), which suggests that it may be devoid of the problem of tachyphylaxis.

BAY63-2521 is the most recent of the optimised compounds following extensive pharmacological and pharmacokinetic profiling of additional compounds in pre-clinical tests (Evgenov et al., 2006;Stasch et al., 2002a). BAY63-2521 has been shown to stimulate sGC activity up to 73-fold in vitro in a concentration-dependent manner and synergize with the NO donors to increase sGC activity up to 112-fold (Schermuly et al., 2008). BAY63-2521 has been reported to possess vasodilatory properties similar to BAY41-8543 *in vitro* and *in vivo*. It has been demonstrated to inhibit contraction of rabbit aortic rings, rabbit saphenous artery rings, porcine coronary artery rings, canine femoral vein rings, and rabbit corpus cavernosum. Furthermore, BAY63-2521 promotes smooth muscle relaxation in arteries isolated from nitrate-tolerant rabbits, and decreases acute pulmonary vasoconstriction in isolated mouse lungs at a concentration of 0.01 μM (Schermuly et al., 2008). Moreover, similar to other novel sGC activators, at concentrations up to 10 μM, BAY63-2521 does not inhibit PDEs 1, 2, 5 or 9 (Schermuly et al., 2008). This compound has now undergone Phase I trials (Frey et al., 2008;Grimminger et al., 2009), and Phase II trials are currently in progress.

**Haem-independent sGC activators**

Using a cell-based assay for cGMP as a screening tool, more than 900,000 compounds were screened. This lead to the identification of the primary hit BAY W 1449, an amino dicarboxylic acid (Stasch et al., 2002a;Wunder et al., 2005). Further modifications resulted in BAY58–2667 to be selected in 2002 as the first NO-independent activator of sGC. This compound showed a completely different range of characteristics to any of the known haem-dependent sGC stimulators. The activation of sGC by this compound was even stronger after oxidation or removal of the prosthetic haem group, indicating a previously unknown mechanism of enzyme activation (Stasch et al., 2002b).

Sanofi-Aventis have also developed compounds with comparable characteristics. They are anthranilic acid derivatives 5-chloro-2-(5-chloro-thiophene-2-sulfonylamino-N-(4-(morpholine-4-sulfonyl)-phenyl)-benzamide sodium salt (HMR1766) and 2-(4-chloro-phenylsulfonylamino)-4,5-dimethoxy-N-(4-(thiomorpholine-4-sulfonyl)-phenyl)-benzamide (S3448). Both compounds have been shown to activate sGC in a concentration-dependent manner ($EC_{50}$ = 0.5–10 $\mu$M). Once again the activation of sGC by these compounds was synergistic with NO donors. However, instead of being inhibited, they were found to be potentiated by the haem-iron oxidant 1H-[1,2,4]-oxdiazolo[3,4-a]quinoxalin-1-one (ODQ). This suggests that these compounds target the ferric- haem sGC complex. Furthermore, it was reported that protoporphyrin IX was found to act as a competitive activator, and zinc-protoporphyrin IX as an inhibitor of activation of haem-oxidized sGC by HMR1766 and S3448. Haem depletion of sGC by

Tween 20 treatment was demonstrated to reduce activation. Furthermore both compounds increased cGMP levels in cultured rat aortic smooth muscle cells; induced relaxation of isolated endothelium-denuded rat aorta, porcine coronary arteries, and human corpus cavernosum ($EC_{50}$ 1 to 10 $\mu$M) and elicited phosphorylation of the cGMP kinase substrate vasodilator-stimulated phosphoprotein at Ser239. An intravenous HMR1766 injection resulted in a decrease in arterial blood pressure in anesthetized pigs. All of these pharmacological responses to the new compounds were enhanced by sGC inhibitors (ODQ and NS2028). These findings suggest that HMR1766 and S3448 preferentially activate the NO-insensitive haem-oxidized form of sGC. This form of sGC may therefore be used as a pharmacological target for newer types of vasodilator drugs (Schindler et al., 2006).

**Further strategies for the treatment of ED**

ED is usually the result of a number of processes acting either in parallel or together to result in a significant reduction in bioavailability of the chief mediator of erection, NO. I believe that future strategies for the treatment of ED should be aimed at correcting or treating the underlying mechanisms involved in the pathogenesis ED as well as finding more specific and effective sGC activators and NO-releasing compounds.

The various targets of investigation include gene therapy with neurotrophic factors, eNOS, nNOS and superoxide dismutase. Through the use of an appropriate vector, diabetic animals have already been successfully transfected with these agents. It has been reported that direct injections into the cavernous sheath of diabetic rats with neurotropohin-3 (NT3) using the herpes simplex virus as the vector have been performed. Subsequent immuno-reactive stains have demonstrated a significant increase in nNOS neurons in the major pelvic ganglia. Moreover, this was associated with significant increases in the cavernous nerve induced rises in the intracavernous pressure (Bennett et al., 2005).

Moreover, diabetic rats which were injected with adenoviruses containing eNOS into their corpus cavernosum, have seen subsequent significant rises in intracavernous pressures secondary to cavernous nerve stimulation. This was further associated with a rise in eNOS (measured by western blot analysis) and an increase in NOS biosynthesis (measured by an increase in cavernous nitrate and nitrite formation) (Bivalacqua et al., 2003).

More recently, the intracavernous injection of adenoviruses containing superoxide dismutase into diabetic rats has been preformed. The results indicate a decrease in

superoxide anion levels, an increase in NO bioavailability and an increase in cGMP levels with ultimately an increase in intracavernous pressure demonstrated (Bivalacqua et al., 2005).

In the field of ion channels, other work was performed using intracavernous injection of *hSlo* (calcium-sensitive potassium channel) in diabetic rats. This resulted in increases in intracavernous pressures after cavernosal nerve stimulation compared with controls. These results were the basis for a potential alteration in these potassium channels secondary to diabetes (Christ et al., 1998).

More recently, the effects of gene transfer on erectile function and sexual behaviour have evaluated in male cynomolgus monkeys with ED (Christ et al., 2008) and an ageing rat model (Melman et al., 2008). The animals were injected intracavernously with a smooth-muscle-specific gene transfer vector (pSMAA-hSlo) encoding the pore-forming subunit of the human large-conductance, calcium-sensitive potassium channels (Maxi-K). The results report that were significant improvements in erectile function and sexual behaviour (Christ et al., 2008) or increased intracavernous pressure (ICP) responses to cavernous nerve stimulation (Melman et al., 2008) after the intracorporeal gene transfer. These results support the concept that intracorporeal Maxi-K-channel gene transfer may be a novel way of improving erectile function.

## Conclusions

1. The sGC activators BAY41-2272 and YC-1 cause potent relaxation of human and rabbit corpus cavernosum. Their effect synergizes with endogenous NO, however the potency of BAY 41-2272 is far superior to YC-1.

2. In the total absence of endogenous NO, BAY41-2272 still potently relaxes the rabbit corpus cavernosum smooth muscle and reverses the inhibition of nitrergic relaxations in corpus cavernosum.

3. The NO releasing PDE5 inhibitor (NCX-911) is a potent relaxant with a similar potency to NO and sildenafil. However, in contrast to sildenafil, it is equally effective with respect to relaxation of rabbit and human corpus cavernosum independent of NO availability suggesting release of NO as well as PDE5 inhibition.

4. NCX-911 like sildenafil potentiates both the duration and magnitude of the nitrergic relaxation, indicating a similar mechanism of action with respect to PDE5 inhibition.

5. In the long-term diabetes model I used, there is a significant reduction in nitrergic function compared to controls.

6. In this long-term diabetes animal model with severe endogenous NO deficiency, the NO-releasing sildenafil (NCX-911) and the sGC activator (BAY41-2272) had a significantly greater potency compared to sildenafil to relax the anococcygeus muscle.

7. In this model, the reduction in nitrergic responses was reversed by BAY41-2272 but not by sildenafil or NCX-911. This suggests that a threshold concentration of

endogenous NO is required to stimulate the cGMP pathway. When the endogenous NO levels are below this threshold, NCX-911 and sildenafil are without effect and fail to potentiate the remaining nitrergic response. However, further studies measuring cGMP concentrations are required to confirm this.

8. PDE5 inhibitors such as sildenafil have reduced efficacy in diabetes. This is likely to be secondary to reduced endogenous NO production. I believe that for a PDE5 inhibitor to be successful a certain degree of residual NO needs to be present.

9. My results support the potential role of sGC activators and NO-releasing PDE5 inhibitors as novel ways of treating male ED particularly where endogenous NO production is dysfunctional such as in severe diabetes. However, further research is required to characterise the pharmacological profiles of these compounds in higher animals and in humans.

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# APPENDICES

## 1. Published Papers

(1) Kalsi JS, Ralph DJ, Thomas P, Bellringer J, Kell PD, Cellek S. A nitric oxide-releasing PDE5 inhibitor relaxes human corpus cavernosum in the absence of endogenous nitric oxide. J Sex Med 2005 Jan;2(1):53-7.

(2) Kalsi JS, Ralph DJ, Madge DJ, Kell PD, Cellek S. A comparative study of sildenafil, NCX-911 and BAY41-2272 on the anococcygeus muscle of diabetic rats. Int J Impot Res 2004 Dec;16(6):479-85.

(3) Kalsi JS, Kell PD, Cellek S, Ralph DJ. NCX-911, a novel nitric oxide-releasing PDE5 inhibitor relaxes rabbit corpus cavernosum in the absence of endogenous nitric oxide. Int J Impot Res 2004 Apr;16(2):195-200.

(4) Kalsi JS, Rees RW, Hobbs AJ, Royle M, Kell PD, Ralph DJ, Moncada S, Ceek S. BAY41-2272, a novel nitric oxide independent soluble guanylate cyclase activator, relaxes human and rabbit corpus cavernosum in vitro. J Urol 2003 Feb;169(2):761-6.

(5) Cellek S, Rees RW, Kalsi J. A Rho-kinase inhibitor, soluble guanylate cyclase activator and nitric oxide-releasing PDE5 inhibitor: novel approaches to erectile dysfunction. Expert Opin Investig Drugs 2002 Nov;11(11):1563-73.

## 2. International Index of Erectile Function Questionnaire (IIEF)

These questions ask about the effects **erection** problems on the patient's sex life, over the past 4 weeks. In answering these questions, the following definitions apply:

***Definitions:***

*Sexual activity includes intercourse, caressing, foreplay and masturbation*

*Sexual intercourse is defined as vaginal penetration of the partner (you entered the partner)*

*Sexual stimulation includes situations like foreplay with a partner, looking at erotic pictures, etc.*

*Ejaculate is defined as the ejection of semen from the penis (or the feeling of this)*

**Mark ONLY one circle per question:**

1. Over the past 4 weeks, how often were you able to get an **erection** during sexual activity?

0 No sexual activity
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

2. Over the past 4 weeks, when you had **erections** with sexual stimulation, how often were your **erections** hard enough for penetration?

0 No sexual stimulation
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

Questions 3, 4 and 5 will ask about **erections** you may have had during sexual intercourse.

3. Over the past 4 weeks, when you attempted sexual intercourse, how often were you able to penetrate (enter) your partner?

0 Did not attempt intercourse
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

4. Over the past 4 weeks, during sexual intercourse, how often were you able to maintain your **erection** after you had penetrated (entered) your partner?

0 Did not attempt intercourse
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

5. Over the past 4 weeks, during sexual intercourse, how difficult was it to maintain your **erection** to completion of intercourse?

1 Did not attempt intercourse
2 Almost always or always
3 Most times (much more than half the time)
4 Sometimes (about half the time) 0 A few times (much less than half the time)
5 Almost never or never

6. Over the past 4 weeks, how many times have you attempted sexual intercourse?

0 No attempts
1 1-2 attempts
2 3-4 attempts
3 5-6 attempts
4 7-10 attempts
5 11 or more attempts

7. Over the past 4 weeks, when you attempted sexual intercourse how often was it satisfactory for you?

0 Did not attempt intercourse
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

8. Over the past 4 weeks, how much have you enjoyed sexual intercourse?

0 No intercourse
1 Very highly enjoyable
2 Highly enjoyable
3 Fairly enjoyable
4 Not very enjoyable
5 Not enjoyable

9. Over the past 4 weeks, when you had sexual stimulation or intercourse how often did you ejaculate?

0 Did not attempt intercourse
1 Almost always or always
2 Most times (more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

10. Over the past 4 weeks, when you had sexual stimulation or intercourse how often did you have the feeling of orgasm or climax (with or without ejaculation)?

0 No sexual stimulation or intercourse
1 Almost always or always
2 Most times (much more than half the time)
3 Sometimes (about half the time)
4 A few times (much less than half the time)
5 Almost never or never

Questions 11 and 12 ask about sexual desire. Let's define sexual desire as a feeling that may include wanting to have a sexual experience (for example, masturbation or intercourse), thinking about having sex or feeling frustrated due to a lack of sex.

11. Over the past 4 weeks, how often have you felt sexual desire?

5 Almost always or always
4 Most times (much more than half the time)
3 Sometimes (about half the time)
2 A few times (much less than half the time)
1 Almost never or never

12. Over the past 4 weeks, how would you rate your level of sexual desire?

5 Very high
4 High
3 Moderate
2 Low
1 Very low or none at all

13. Over the past 4 weeks, how satisfied have you been with you overall sex life?

5 Very satisfied
4 Moderately satisfied
3 About equally satisfied and dissatisfied
2 Moderately dissatisfied
1 Very dissatisfied

14. Over the past 4 weeks, how satisfied have you been with your sexual relationship with your partner?

5 Very satisfied
4 Moderately satisfied
3 About equally satisfied and dissatisfied
2 Moderately dissatisfied
1 Very dissatisfied

15. Over the past 4 weeks, how do you rate your confidence that you can get and keep your **erection**?

5 Very high
4 High
3 Moderate
2 Low
1 Very low

**Scoring Algorithm for IIEF**

All items are scored in 5 domains as follows:

| Domain | Items | Range | Score Max Score |
|---|---|---|---|
| Erectile Function | 1, 2, 3, 4, 5, 15 | 0-5 | 30 |
| Orgasmic Function | 9, 10 | 0-5 | 10 |
| Sexual Desire | 11, 12 | 0-5 | 10 |
| Intercourse Satisfaction | 6, 7, 8 | 0-5 | 15 |
| Overall Satisfaction | 13, 14 | 0-5 | 10 |

**Clinical Interpretation**

I. Erectile function total scores can be interpreted as follows:

| Score | Interpretation |
|---|---|
| 0-6 | Severe dysfunction |
| 7-12 | Moderate dysfunction |
| 13-18 | Mild to moderate dysfunction |
| 19-24 | Mild dysfunction |
| 25-30 | No dysfunction |

II. Orgasmic function total scores can be interpreted as follows:

| **Score** | **Interpretation** |
|---|---|
| 0-2 | Severe dysfunction |
| 3-4 | Moderate dysfunction |
| 5-6 | Mild to moderate dysfunction |
| 7-8 | Mild dysfunction |
| 9-10 | No dysfunction |

III. Sexual desire total scores can be interpreted as follows:

| **Score** | **Interpretation** |
|---|---|
| 0-2 | Severe dysfunction |
| 3-4 | Moderate dysfunction |
| 5-6 | Mild to moderate dysfunction |
| 7-8 | Mild dysfunction |
| 9-10 | No dysfunction |

IV. Intercourse satisfaction total scores can be interpreted as follows:

| **Score** | **Interpretation** |
|---|---|
| 0-3 | Severe dysfunction |
| 4-6 | Moderate dysfunction |
| 7-9 | Mild to moderate dysfunction |
| 10-12 | Mild dysfunction |
| 13-15 | No dysfunction |

V. Overall satisfaction total scores can be interpreted as follows:

| **Score** | **Interpretation** |
|---|---|
| 0-2 | Severe dysfunction |
| 3-4 | Moderate dysfunction |
| 5-6 | Mild to moderate dysfunction |
| 7-8 | Mild dysfunction |
| 9-10 | No dysfunction |

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The international index of erectile function (IIEF) a multidimensional scale for assessment of erectile dysfunction. Urology. 1997 Jun; 49(6):822-30.