

## Original Research

# A comparative study of sildenafil, NCX-911 and BAY41-2272 on the anococcygeus muscle of diabetic rats

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We compared the effects of a nitric oxide (NO)-releasing sildenafil (NCX-911), NO-independent soluble guanylate cyclase activator (BAY41-2272) and sildenafil on the anococcygeus muscle from streptozotocin-induced 16-weeks diabetic rats. NCX-911, BAY41-2272 and sildenafil reduced the phenylephrine-induced tone in the control group ( $EC_{50} = 1088.8 \pm 165.0$ ,  $151.6 \pm 9.3$  and  $827.1 \pm 167.3$  nM, respectively). The potencies of NCX-911 and BAY41-2272 were not altered, but that of sildenafil was significantly reduced in the diabetic group.  $EC_{50}$  values for NCX-911, BAY41-2272 and sildenafil in the diabetic group were  $1765.9 \pm 303.5$ ,  $209.7 \pm 27.3$  and  $2842.2 \pm 640.3$  nM, respectively ( $P < 0.05$  for sildenafil). Nitroergic relaxation responses were significantly decreased in the diabetic group. The remaining nitroergic relaxation responses were potentiated by BAY41-2272 but not by sildenafil or NCX-911. These results confirm that endogenous NO derived from nitroergic nerves is significantly decreased in diabetes, and suggest that NO-releasing PDE5 inhibitors and NO-independent soluble guanylate cyclase activators could be more useful than PDE5 inhibitors in the treatment of ED in long-term diabetes.

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## Introduction

Erectile dysfunction (ED) is a significant male health problem reported to affect the lives of 35–54% of men aged over 40 y.<sup>1</sup> The management of ED was revolutionised with the introduction of the PDE5 inhibitor sildenafil citrate (Viagra<sup>®</sup>).<sup>2</sup> PDE5 inhibitors are highly effective in a wide range of patient subtypes; however, certain groups of patients such as long-term diabetic patients do not gain the same degree of benefit.<sup>3,4</sup> This has been postulated to be due to a lack of endogenous nitric oxide (NO).<sup>5–7</sup>

Therefore, new therapeutic options, which are able to induce relaxation independent of endogenous NO, are being developed. NO-independent soluble guanylate cyclase (sGC) activators (BAY41-

2272) and an NO-releasing derivative of sildenafil (sildenafil nitrate; NCX-911) are two such potential approaches to the medical management of ED.<sup>8,9</sup>

Although the rat is an excellent model for diabetes research, our experience with its penile corpus cavernosum is that it is a difficult tissue to work with (ie spontaneous contractions and the size of the tissue) compared to the anococcygeus muscle. Anococcygeus and retractor penis muscles have been widely accepted as ideal smooth muscle models to study not only nitroergic neurotransmission but also other nonadrenergic noncholinergic neurotransmitters.<sup>10,11</sup> Since their vascular supply is not as rich as the corpus cavernosum, the involvement of eNOS is also limited. We have previously characterised the nitroergic neurotransmission in the anococcygeus muscle<sup>12</sup> and defective nitroergic neurotransmission has been well documented in diabetes models using this tissue.<sup>5,7,13</sup>

The aim of this study was to compare the potencies of sildenafil, NCX-911 and BAY41-2272 in reducing the elevated tone and in potentiating the nitroergic responses in the anococcygeus muscle obtained from nondiabetic and diabetic rats.

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## Materials and methods

### *Induction of diabetes*

Male Wistar rats 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.<sup>5,7</sup> The other group was injected with the vehicle (saline). At 16 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 Mannheim, Germany) and bilateral anococcygeus muscles were isolated for functional studies as described previously.<sup>5,7</sup> All animal studies were conducted according to the rules outlined by the Home Office, Animals (Scientific Procedures) Act 1986 (Project No. 70/5161).

### *Functional studies*

Each anococcygeus muscle was cleaned of adherent tissues and mounted horizontally between two ring electrodes (5 mm diameter) in superfusion chambers (37°C) as described previously.<sup>5,7,9</sup> The chambers were perfused with modified Krebs' solution at a constant flow of 1.0 ml/min by means of peristaltic pumps (Miniplus 2, Gilson). One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Linearcororder WR 3101 (Graphtec, Tokyo, Japan) for the registration of isometric changes in tension. The preparations were stretched until they reached approximately the *in situ* length with a tension of 0.5 g and were allowed to equilibrate for 90 min. The preparations were stimulated electrically (electrical field stimulation; EFS) for 5 s with trains of rectangular pulses of 50 V, 0.3 ms pulse duration and at a frequency range of 0.5–25 Hz, delivered by Grass S88 stimulators. The mechanical responses were also recorded on a computer by a specialised data acquisition system (Axon Instruments, USA).

### *Chemicals and solutions*

The composition of the modified Krebs' solution was (mM): NaCl 136.9, KCl 2.7, CaCl<sub>2</sub> 1.8, MgSO<sub>4</sub> 0.6, NaHCO<sub>3</sub> 11.9, KH<sub>2</sub>PO<sub>4</sub> 0.5, glucose 11.5, indomethacin 0.01, dexamethasone 0.01 and gassed with 5% CO<sub>2</sub> in O<sub>2</sub> (pH 7.0–7.2). Dexamethasone, guanethidine monosulphate, indomethacin, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) and scopolamine hydrochloride were obtained from Sigma, UK.

Tetrodotoxin was obtained from Calbiochem, UK. BAY41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]-pyrimidin-4ylamine) was a gift from Bayer AG, Germany. NCX-911 was kindly provided by Nicox S.A. (Sophia Antipolis, France). Both compounds were dissolved in DMSO and were applied directly to the medium reservoir. DMSO on its own has been shown to have no effect on either tone or nitrenergic responses at the concentrations used in this study (not shown). The sildenafil was purified from Viagra<sup>®</sup> tablets by Medicinal Chemistry Department, Wolfson Institute for Biomedical Research. Viagra<sup>®</sup> tablets were crushed and the coating material was removed. The remaining powder was suspended in water and carefully neutralised. The drug substance was extracted into ethylacetate and the solution was dried and evaporated to provide a white powder. This material had <sup>1</sup>H-NMR, mass spectroscopy, melting point and microanalysis characteristics in agreement with the structure of sildenafil. It was then resuspended in water and one equivalent of citric acid was added to provide a solution of the drug as its citrate salt, which was then isolated by freeze drying. The resulting white powder was analysed by microanalysis to confirm the presence of one equivalent of citrate.

### *Statistical analysis and presentation of the results*

The effect of drugs on phenylephrine-induced tone was expressed as the percentage of the height of contraction of the same tissue under control conditions (prior to addition of the drugs). The effect of drugs on EFS-induced relaxation responses was calculated as percentage of area over the relaxation curve of the same tissue under control conditions. Results are expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using Student's unpaired two-tailed *t*-test. A probability value (*P*) less than 0.05 was considered as statistically significant; *n* denotes the number of animals.

## Results

### *Body weight and serum glucose concentrations*

The average body weight and serum glucose concentrations were similar between the two groups before the STZ injection. At 16 weeks after the STZ injection in comparison to the control group, it was found that the diabetic group failed to gain weight and their serum glucose concentrations were significantly higher (Table 1).

**Table 1** Body weight (upper value; g) and serum glucose concentrations (lower value; mM) of control (saline-injected) and diabetic (streptozotocin-injected) rats at 0 (before the injection) and at 16 weeks

	Control group, n = 16	Diabetic group, n = 16
<i>0 week</i>		
Weight (g)	227.8 ± 5.9	228.9 ± 8.7
Serum glucose (mM)	5.2 ± 0.5	4.9 ± 0.5
<i>16 weeks</i>		
Weight (g)	552.4 ± 20.6	264.3 ± 17.8*
Serum glucose (mM)	5.1 ± 0.8	50.7 ± 2.7*

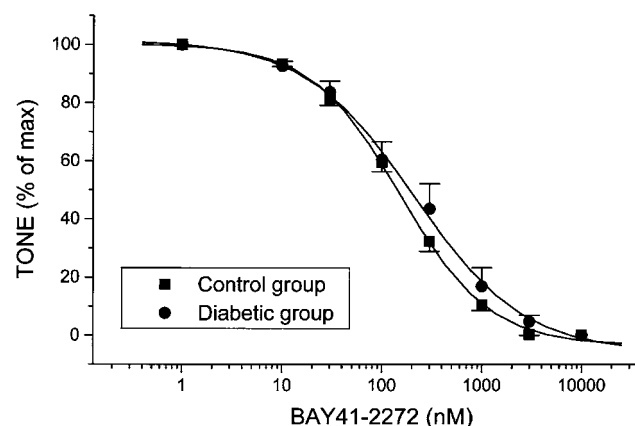
\**P* < 0.05 significantly different from the control group at the 16th week.

### Phenylephrine-induced tone

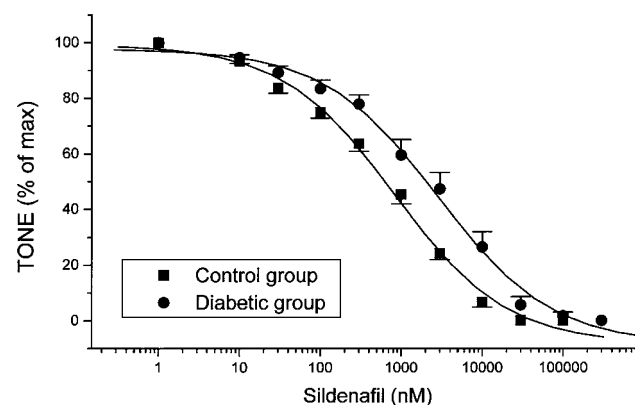
Exogenous application of phenylephrine (0.01–30 μM) produced concentration-dependent elevation of the tone of the tissue. The response to phenylephrine was similar in both groups. EC<sub>80</sub> for phenylephrine was 1 μM in both the control and diabetic groups.

### Effect of sildenafil, NCX-911 and BAY41-2272 on phenylephrine-induced tone

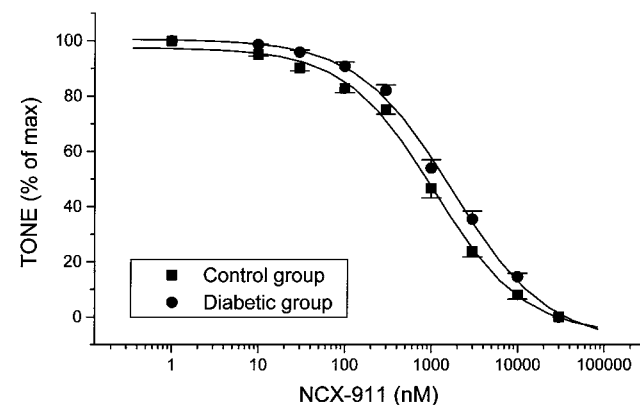
NCX-911 (0.001–30 μM), BAY41-2272 (0.001–10 μM) and sildenafil (0.001–300 μM) elicited concentration-dependent reductions of the phenylephrine-induced tone (elevated with 1 μM) in tissues obtained from control animals (Figures 1–3). In diabetic animals, the potency of sildenafil to reduce the phenylephrine (1 μM)-induced tone was significantly reduced whereas the potencies of NCX-911



**Figure 2** Effect of BAY41-2272 (1 nM–10 μM) on phenylephrine-induced tone in anococcygeus muscles from control (nondiabetic; squares) and 16-week diabetic (circles) rats.



**Figure 3** Effect of sildenafil (1 nM–300 μM) on phenylephrine-induced tone in anococcygeus muscles from control (nondiabetic; squares) and 16-week diabetic (circles) rats.



**Figure 1** Effect of NCX-911 (1 nM–30 μM) on phenylephrine-induced tone in anococcygeus muscles from control (nondiabetic; squares) and 16-week diabetic (circles) rats.

**Table 2** EC<sub>50</sub> values (nM) of NCX-911, BAY41-2272 and sildenafil to reduce the phenylephrine-induced tone in the anococcygeus muscle from control and diabetic rats

		EC <sub>50</sub> (nM)
NCX-911	Control group	1088.8 ± 165.0
	Diabetic group	1765.9 ± 303.5
BAY41-2272	Control group	151.6 ± 9.3
	Diabetic group	209.7 ± 27.3
Sildenafil	Control group	827.1 ± 167.3
	Diabetic group	2842.2 ± 640.3*

\**P* < 0.05 significantly different from the control group for the same compound.

and BAY41-2272 were not altered (Figures 1–3). EC<sub>50</sub> values for sildenafil, NCX-911 and BAY41-2272 to reduce the phenylephrine-induced tone in control and diabetic animals are shown in Table 2.

### Eliciting nitrenergic relaxations

EFS (50 V, 0.3 ms pulse duration, 0.5–25 Hz, for 5 s, every 120 s) elicited reproducible contractions of the tissues in both control and diabetic groups. After treatment of the tissues with scopolamine (10 μM) and guanethidine (10 μM) and elevation of tone with phenylephrine (1 μM, EC<sub>80</sub>), EFS elicited reproducible frequency-dependent relaxation responses with an optimum frequency of 5 Hz in both experimental groups (not shown). These relaxations were completely inhibited with an inhibitor of sGC (ODQ; 10 μM), an inhibitor of NO synthase (L-NAME; 500 μM) or tetrodotoxin (1 μM) in both groups, indicating that the relaxations were nitrenergic in nature and neurogenic in origin (not shown).

### The effect of diabetes on nitrenergic relaxations

Compared to controls after 16 weeks of diabetes, there was a significant reduction in the magnitude of nitrenergic relaxations at all frequencies (Figures 4 and 5). At the highest frequency (25 Hz), almost 80% of the relaxation response was lost in the diabetic group (Figures 4 and 5).

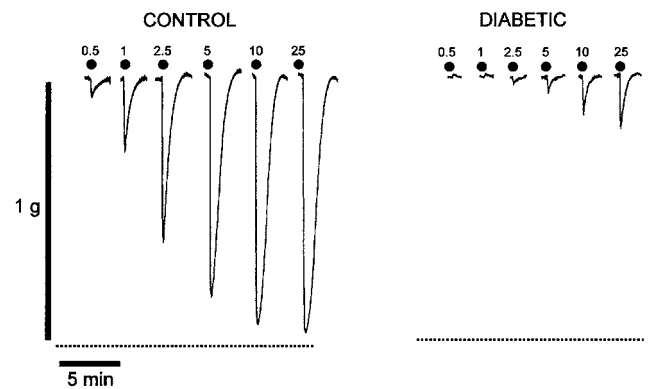
### Effect of sildenafil, NCX-911 and BAY41-2272 on nitrenergic relaxations

Since above a certain concentration all three compounds were able to reduce the phenylephrine-induced tone and it is not possible to elucidate the nitrenergic relaxation responses at reduced tone, two subthreshold concentrations (10 and 30 nM) of each compound were chosen to study the effect of the compound on nitrenergic responses at optimum frequency (5 Hz). At these subthreshold concentrations, the compounds did not reduce the tone, but potentiated both the duration and magnitude of the nitrenergic relaxation responses in the control group (not shown).

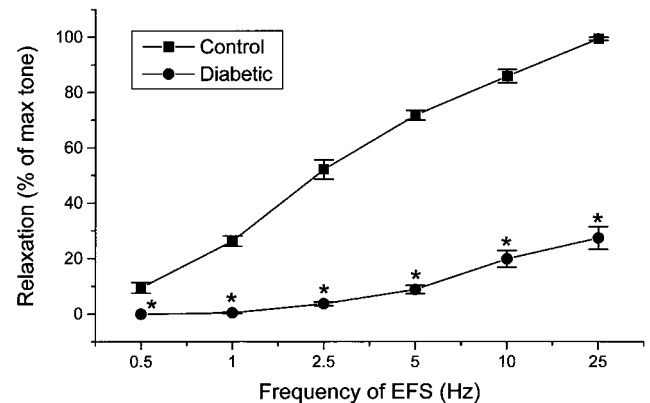
In the diabetic group, the magnitude and duration of the remaining nitrenergic response was potentiated by BAY41-2272 but not by sildenafil or NCX-911 at both 10 and 30 nM (Figure 6; 10 nM not shown). The average potentiation of the nitrenergic response at 5 Hz by 30 nM BAY41-2272 was 183.5 ± 22.1% when the area under the curve of relaxation was considered.

## Discussion

NO released from sinusoidal endothelium and postganglionic cholinergic (nitrenergic) nerve fibres



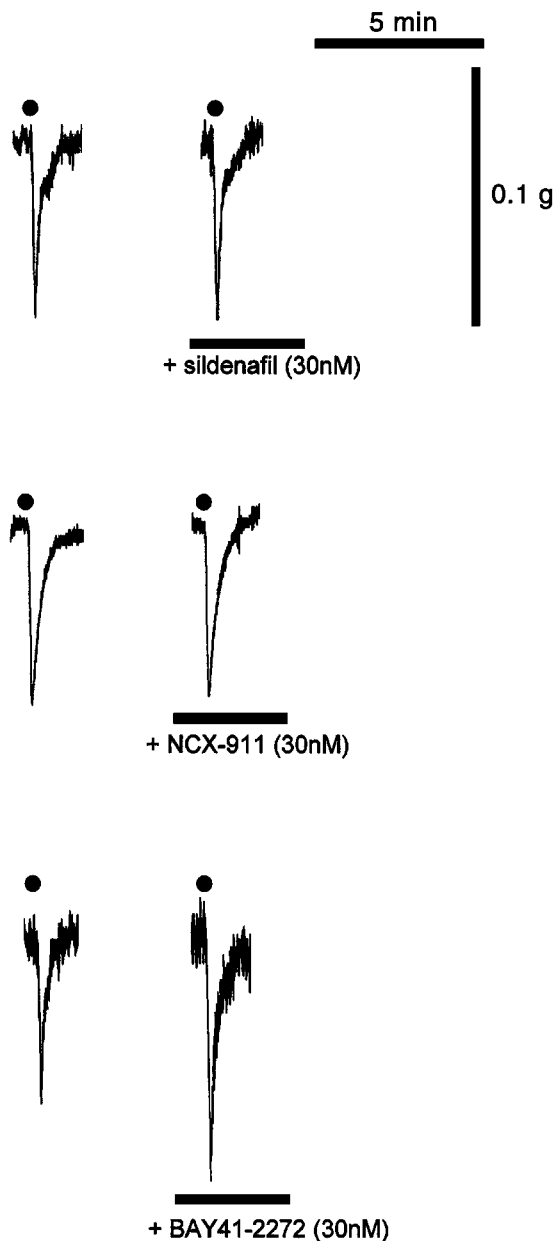
**Figure 4** Electrical field stimulation (50 V, 0.3 ms pulse duration, for 5 s, at frequencies between 0.5 and 25 Hz indicated by solid circles) produced nitrenergic relaxation responses in the anococcygeus muscle from control (left) and 16-week diabetics (right) rats. Dotted lines represent the baseline tone of the tissues.



**Figure 5** Electrical field stimulation (50 V, 0.3 ms pulse duration, for 5 s, 0.5–25 Hz)-induced nitrenergic relaxation responses were significantly attenuated in the diabetic group (circles) compared to the control group (squares). \**P* < 0.05 significantly different from the control group at the same frequency.

is the major mediator of vascular and cavernosal smooth muscle relaxation in the penis.<sup>14</sup> In diabetes, the NO-mediated responses have been shown to be reduced in both human and animal studies.<sup>15–19</sup> This has been attributed either to decreased availability of NO<sup>20</sup> or to decreased NO production secondary to downregulation of eNOS/nNOS expression<sup>21–23</sup> or degeneration of nitrenergic nerves.<sup>5,7,24</sup> The decreased availability and/or production of NO is now widely accepted to be the primary cause for the significant loss of erectile function in diabetic men.

PDE5 inhibitors require endogenous NO in order to produce relaxation of smooth muscle in the penis.<sup>25</sup> It has therefore been postulated that the reduced rate of success of PDE5 inhibitors in diabetic men<sup>3,4</sup> may be due to lower NO production in the endothelium and nerves of these men.<sup>6</sup> Further support for this is provided by the demon-



**Figure 6** Effect of sildenafil, NCX-911 and BAY41-2272 (all at 30 nM) on the nitrenergic responses in the anococcygeus muscle from diabetic rats. Electrical field stimulation (50 V, 0.3 ms pulse duration, for 5 s, 5 Hz) is denoted by solid circles. Note the potentiating effect of BAY41-2272 on both magnitude and duration of the response. Also note the force scale in comparison to Figure 4.

stration that nitrenergic relaxation responses are significantly diminished and that the potency of sildenafil is decreased in diabetic animals in this study.

In contrast, a previous study reported that relaxation of the corpus cavernosum to exogenous NO donors and endogenous nitrenergic nerve stimulation was enhanced by sildenafil with the same potency in the control and the diabetic group.<sup>26</sup> 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.<sup>26</sup> Furthermore, we have recently shown that severe nitrenergic degeneration starts after 12 weeks of diabetes in our rat model.<sup>7</sup> Therefore, we conclude that the decrease in the potency of sildenafil is dependent on the severity of nitrenergic nerve loss, which is time-dependent in diabetic animal models.

We have previously suggested that compounds that do not require endogenous NO would be more successful than PDE5 inhibitors to relax penile smooth muscle in diabetes.<sup>8</sup> The two compounds that we have used in the current study—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.<sup>27</sup> 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.<sup>27</sup> In a hypercholesterolaemic rabbit model, NCX-911 was shown to be 5–10 times more potent than sildenafil at reducing the phenylephrine-induced vascular tone.<sup>28</sup> BAY41-2272 is a non-NO-based compound (does not release NO) that activates sGC.<sup>29</sup> This activation does not require endogenous NO; however, the potency of the compound is elevated if there is endogenous NO in the environment.<sup>29</sup> BAY41-2272 potentiated the nitrenergic responses in the rabbit corpus cavernosum<sup>30</sup> and induced penile erection in conscious rabbit model.<sup>31</sup>

In the current study, we have compared the two compounds to sildenafil in their potency to relax the anococcygeus muscle of diabetic rats. Their potency was not altered by diabetes while the sildenafil was less potent in the diabetic group, suggesting that the compounds that 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.

Although the EC<sub>50</sub> value for sildenafil was higher in the diabetic group than in the control group, E<sub>max</sub> values were similar. This implies that the shift in the concentration–response curve was parallel, suggesting that the target (PDE5) is still active but its sensitivity was altered during diabetes. Two possible scenarios can be put forward to explain this. 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, we can rule out the desensitisation possibility; therefore, 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 nitrenergic nerves locally would be required to support this statement.

Interestingly, NCX-911 and sildenafil failed to potentiate the remaining nitrenergic 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 our long-term diabetes model, NO production is so low<sup>7</sup> that even in the presence of an NO-releasing compound (eg NCX-911), the threshold is probably not reached; therefore, the compounds fail to potentiate the remaining nitrenergic response. 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 nitrenergic 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. We have recently observed a similar phenomenon in the rabbit corpus cavernosum, where the inhibition of nitrenergic responses with an inhibitor of NO synthase was reversed by BAY41-2272.<sup>30</sup> Therefore, these results further confirm the different pharmacological profiles of these compounds and warrant further research with these compounds in humans.

Although the information obtained from the anococcygeus muscle is not as directly applicable to erectile physiology as that from corpus cavernosum, the anococcygeus muscle is an ideal smooth muscle preparation to study the effect of diabetes on nitrenergic neurotransmission.<sup>7,12,13</sup> Moreover, in our hands, rat corpus cavernosum is less sensitive to EFS and has more spontaneous contractions than the rat anococcygeus muscle. That is why we chose anococcygeus muscle for this study. Nevertheless, further research in higher animals with different durations of diabetes is required to compare the effects of similar compounds in the corpus cavernosum.

Systemic administration of NCX-911 and BAY41-2272 has been shown to be without major side effects,<sup>28,32</sup> although at high doses both compounds can affect the systemic blood pressure.<sup>28,29</sup> Further studies are required to investigate the systemic effects of these compounds in order to assess their potential as a safe treatment for ED.

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 nitrenergic 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 humans.

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