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Morphological and functional *in vitro* and *in vivo* characterization of the mouse corpus cavernosum

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1 In normal mice, the distribution of adrenergic, cholinergic, some peptidergic, and neuronal nitric oxide synthase (nNOS)-containing nerves were investigated. Functional *in vitro* correlates were obtained. An *in vivo* model was developed in which erectile haemodynamics in response to drugs or nerve-stimulation were studied.

2 Immunoreactivities for vesicular acetylcholine transporter protein (VAChT), nNOS-, and vasoactive intestinal polypeptide (VIP), co-existed in nerve fibres and terminal varicosities. Immunoreactivities for neuropeptide Y (NPY) and tyrosine hydroxylase (TH) were found in the same nerve structures.

3 Chemical sympathectomy abolished TH- and NPY-IR nerve structures in cavernous smooth muscle bundles. The distribution of calcitonin gene-related peptide (CGRP)-, nNOS-, VAChT- and VIP-IR nerve structures was unchanged.

4 In endothelial cells of the central and helicine arteries, veins and venules, intense immunoreactivity for endothelial NOS (eNOS) was observed. No distinct eNOS-IR cells were found lining the cavernous sinusoids.

5 *In vitro*, nerve-induced relaxations were verified, and endothelial NO/cyclic GMP-mediated relaxant responses were established. VIP and CGRP had small relaxant effects. A functioning adenylate cyclase/cyclic AMP pathway was confirmed.

6 Neuronal excitatory responses were abolished by prazosin, or forskolin. VIP and CGRP counteracted contractions, whereas NPY and scopolamine enhanced excitatory responses.

7 *In vivo*, erectile responses were significantly attenuated by L-NAME (50 mg kg^{-1}) and facilitated by sildenafil (200 μg kg^{-1}).

8 It is concluded that the mouse is a suitable model for studies of erectile mechanisms *in vitro* and *in vivo*.

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Introduction

Penile erection is regulated by coordinated activities in the central and peripheral nervous systems (Andersson & Wagner, 1995). In humans and many animals, the erectile response has been shown to be dependent on nitric oxide (NO), released from nerves, but also from vascular endothelium. In mice, the NO-producing enzyme, NO-synthase (NOS), has been located to the dorsal penile nerve and its branches in the mouse penis (Burnett *et al.*, 1996) as well as in intrinsic nerves of the erectile tissue (Hedlund *et al.*,

2000a). Upon release from nerves, NO activates soluble guanylyl cyclase (sGC) in adjacent smooth muscle cells, resulting in an increased production of cyclic GMP, and cyclic GMP conveys signals by modification of ion channels, phosphodiesterases, and protein kinases (Lincoln & Cornwell, 1993). In mouse corpus cavernosum (CC) smooth muscle, cyclic GMP-dependent protein kinase I (cyclic GKI) has been shown to be of major importance for erectile function (Hedlund *et al.*, 2000a). However, other signalling pathways have also been implicated in smooth muscle relaxant functions in the erectile tissue (Andersson & Wagner, 1995). Immunoreactivity for the vesicular acetylcholine transporter protein (VAChT) has been demonstrated in nerve terminals

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exhibiting close relations to tyrosine hydroxylase (TH)-immunoreactive (-IR) nerve fibres, an arrangement favouring a cholinergic modification of the release of noradrenaline (NA) from adjacent adrenergic nerves. Muscarinic receptor-mediated release of relaxant factors from vascular endothelium has been proposed as a cholinergic proerectile effect (Andersson & Wagner, 1995). Vasoactive intestinal polypeptide (VIP)-IR nerve fibres have been demonstrated in CC tissue, and it is believed that VIP, by receptor-mediated activation of the adenylyl cyclase/cyclic AMP system, relaxes isolated CC tissue, and is able to induce erectile responses *in vivo* (Andersson & Wagner, 1995). NOS-IR nerves have been shown also to contain immunoreactivity for VIP and VAChT, and these nerves have been suggested to represent a distinct population of penile parasympathetic cholinergic nerves (Hedlund *et al.*, 1999; 2000b).

The development of genetically modified animals, for which the mouse is the species of choice, is receiving attention in most fields of research. The use of such mice will most probably be a valuable additional tool for the study of molecular mechanisms involved in erectile function and various forms of dysfunction. NO/cyclic GMP-mediated inhibitory responses have been shown to be absent in CC tissue from cyclic GKI deficient mice, and a preserved cyclic AMP signalling pathway did not compensate for the erectile dysfunction or the reduced fecundity in these animals (Hedlund *et al.*, 2000a). In mice lacking neuronal NOS (nNOS), the NO-producing enzyme, a compensatory increase in the endothelial isoform of NOS was suggested to account for a preserved mating behaviour with erections (Burnett *et al.*, 1996).

Mouse erectile tissue has been used previously for *in vitro* studies of erectile mechanisms (Burnett *et al.*, 1996; Gocmen *et al.*, 1997; 1998). Monitoring of intracavernous pressure in the mouse *in vivo* has been described by Sezen & Burnett (2000). However, to the best of our knowledge, a thorough characterization of the erectile tissue from mice has not been made. The aim of the present study was therefore to describe the innervation patterns of adrenergic, cholinergic, some peptidergic, and NOS-containing nerves, and to present functional correlates in an isolated CC preparation from normal mice. In addition, we present a model in which erectile haemodynamics, in response to drugs or nerve-stimulation, were studied *in vivo* by intracavernous pressure (ICP) measurements.

Methods

Animals

Ninety-five adult male NMRI mice (Möllegård, Denmark) were used. Prior to experiments, the mice were kept and cared for in standard cages under clean conditions in separate quarters in a 12–12 h light–darkness cycle with free access to water and pellets. The experiments performed were approved by the Animal Ethics Committee of Lund University.

Morphological studies

Chemical sympathectomy   As previously described for the rat (Hedlund *et al.*, 1999), chemical sympathectomy was

performed in six mice which were given two doses (100 mg.kg^{-1}) of 6-hydroxydopamine (6-OHDA; Sigma Chemical Co, St Louis, MO, U.S.A.).

Tissue handling   For immunocytochemistry (see below), 20 mice were anaesthetized and perfusion-fixed as previously described (Hedlund *et al.*, 1999). The mouse penis (Figure 1) was removed by cutting the crura of the CC at the point of adhesion to the lower pubic bone, and the CC were then dissected free and immersion-fixed, and further prepared for immunohistochemical staining (Hedlund *et al.*, 1999).

Immunocytochemistry   Cryostat sections (8 μm) were further processed and subsequently incubated (+4°C, 24 h) with rabbit antisera against protein gene product 9.5 (PGP), VAChT, nNOS, endothelial NOS (eNOS), neuropeptide Y (NPY), or TH, or sheep antisera to nNOS or NPY, or guinea-pig antisera to VIP- or calcitonin gene-related peptide (CGRP), or goat antiserum to VAChT. The main protocol followed was the the same as described previously (Hedlund *et al.*, 1999).

For the simultaneous demonstration of three antigens, some sections were incubated with rabbit antiserum to VAChT, rinsed, and incubated with sheep antiserum to nNOS, rinsed, and incubated with antisera raised in guinea-pig to VIP. After rinsing, the sections were incubated with FITC-conjugated donkey anti rabbit IgG, rinsed and incubated with TR-conjugated donkey anti sheep IgG, rinsed and incubated with Cy5-conjugated donkey anti guinea-pig IgG.

All antisera (primary and secondary) used are listed in Table 1. Control experiments and the characteristics of the respective primary antibodies have been previously described (Hedlund *et al.*, 1999). As cross reactions to antigens sharing similar amino acid sequences cannot be completely excluded, the structures demonstrated are referred to as CGRP-, nNOS-, NPY-, PGP-, TH-, VAChT-, and VIP-IR (immunoreactive).

The immunoreactive structures were evaluated as described previously (Hedlund *et al.*, 1999). In double and triple immunolabelled sections, when three or more varicosities with intervening fibre segments showed similar patterns when examined in the two fluorophores, the corresponding nerve terminals were regarded as displaying coinciding profiles.
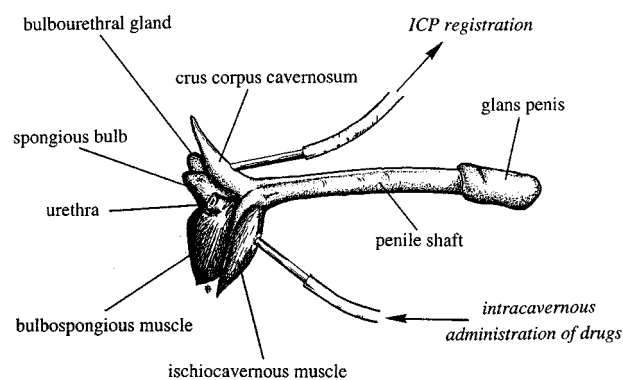

Figure 1 The mouse penis and technical landmarks for the registration of intracavernous pressure *in vivo*.

Table 1 Primary and secondary antibodies used for the immunocytochemical demonstration of different antigens as listed in left column

Antiserum	Host	Dilution	Source
Primary			
CGRP	guinea-pig	1:640	Euro-Diagnostika, Sweden
nNOS	rabbit	1:2400	Euro-Diagnostika, Sweden
nNOS	sheep	1:6000	P. Emson, Cambridge, U.K.
eNOS	rabbit	1:500	Euro-Diagnostika, Sweden
NPY	sheep	1:1800	Chemicon, Sweden
NPY	rabbit	1:640	Euro-Diagnostika, Sweden
PGP	rabbit	1:2000	UltraClone, U.K.
VAChT	goat	1:1600	Chemicon, Sweden
VAChT	rabbit	1:1600	Euro-Diagnostika, Sweden
VIP	guinea-pig	1:1280	Euro-Diagnostika, Sweden
TH	rabbit	1:200	Pel-Freez, U.S.A.
Secondary			
Cy5	donkey anti guinea-pig	1:400	Jackson ImmunoResearch, U.S.A.
FITC	goat anti guinea-pig	1:80	Sigma, U.S.A.
FITC	donkey anti goat	1:80	Jackson ImmunoResearch, U.S.A.
FITC	donkey anti guinea-pig	1:80	Jackson ImmunoResearch, U.S.A.
FITC	donkey anti sheep	1:80	Jackson ImmunoResearch, U.S.A.
FITC	swine anti rabbit	1:80	Dakopatts, Sweden
Texas Red	donkey anti goat	1:125	Jackson ImmunoResearch, U.S.A.
Texas Red	donkey anti guinea-pig	1:125	Jackson ImmunoResearch, U.S.A.
Texas Red	donkey anti rabbit	1:125	Jackson ImmunoResearch, U.S.A.
Texas Red	donkey anti sheep	1:125	Jackson ImmunoResearch, U.S.A.

Confocal microscopy Sections were inspected by confocal microscopy using an MRC-1024 laser scanning confocal equipment (Bio-Rad, Hemel Hempstead, U.K.) attached to an Eclipse E800 upright microscope (Nikon, Tokyo, Japan). Fluorochromes were illuminated with three different wavelengths of light (488 nm, 568 nm and 647 nm) and emitted light was detected in three separate photo multiplier tubes after filtration through a 605 ± 32 nm (red light), a 522 ± 32 nm (green light), and a 680 ± 32 nm (far red light) bandpass filter.

Functional *in vitro* studies

Isometric tension measurements For the functional experiments, 19 mice were sacrificed by carbon monoxide asphyxia followed by exsanguination. The penises were immediately taken out as described above and placed in chilled Krebs solution (for composition see below). As previously described for the rat (Hedlund *et al.*, 1999), the tunica albuginea was carefully opened from its proximal extremity of the CC towards the penile shaft and the erectile tissue within the CC was microsurgically dissected free. One crural strip preparation $(0.3 \times 0.3 \times 3$ mm$)$ was obtained from each CC. All preparations were used immediately after removal.

The procedures for mounting, of the preparations and recording of isometric tension and electrical field stimulation (EFS) were the same as described previously (Hedlund *et al.*, 1999).

Experimental procedure During an equilibration period of 40 min, tension was adjusted until a mean stable tension of 1.37 ± 0.08 mN $(n=34$, $N=19)$ was obtained. In order to verify the contractile ability of the preparations, a K^+ solution (124 mM) was added to the organ baths at the end of the equilibration period. The mean contractile response amounted to 1.23 ± 0.23 mN $(n=34$, $N=19)$. Concentration-response curves for NA were performed in control animals $(n=6$, $N=6)$. The agonist concentration used $(3 \times 10^{-6}$ M$)$ corresponded to the approximate EC_{70} value, and produced stable and reproducible contractions. The effects of NO, carbachol, VIP, NPY, CGRP and forskolin were investigated in NA-contracted preparations.

Frequency-response relationships were investigated at supramaximum voltage in all preparations stimulated electrically, and the effects of prazosin, VIP, NPY, CGRP, and forskolin on electrically-induced excitatory responses were studied. A preparation was regarded as stable when the amplitude of three consecutive electrically-induced contractions did not differ by more than 5%. The investigated drugs were then added cumulatively. The degree of inhibition was expressed as a percentage of the contraction elicited prior to the addition of the lowest concentration of the drugs. Inhibitory responses to transmural stimulation of nerves were investigated in NA-activated preparations. The degree of relaxation was expressed as percentage of the NA-induced contraction. Some preparations were pretreated for 20 min with the NO synthesis inhibitor N^G-nitro-L-arginine (L-NOARG; 10^{-4} M), or the guanylyl cyclase inhibitor, ODQ $(10^{-6}$ M$)$. Effects of EFS were then investigated as described above.

Experimental *in vivo* procedure

Fifty-five mice were anaesthetized with pentobarbital sodium $(50$ mg kg^{-1}, Sigma Chemical Co, St Louis, MO, U.S.A.) and ketamine (Ketalar®, Parke Davis, Barcelona, Spain; 10 mg kg^{-1}) given intraperitoneally (i.p.). During the experiment, the mice breathed spontaneously and were placed on a thermally isolating blanket. Body temperature and circulatory volume were kept optimal by frequent i.p. administration of body-warm saline, which prior to nerve stimulation was removed. Through a lower midline abdominal incision, access

was given to the femoral artery and the pelvic viscera. A stretched heparinized (100 IE ml^{-1}) polyethylene catheter (Clay Adams PE-10, Parsippany, NJ, U.S.A.) was introduced into the femoral artery. With a midline incision in the perineum, the base of the penis, enclosed by the striated bulbospongious and ischiocavernous muscles, was made visible. By blunt dissection, the ischiocavernous muscle covering the CC was divided on one side, and entrance to the underlying tunica albuginea of the crus of the CC was given. A 27 gauge needle attached to a heparinized (100 IE.ml^{-1}) polyethylene catheter, was inserted into the crus of the CC. In some of the mice, another 27 gauge needle was placed into the other crus for administration of drugs (Figure 1). Continuous direct measurements of mean arterial and ICP were performed with pressure transducers (OHME-DA, Model P23 XL-1, Singapore) and registered by a Grass Polygraph 7E (Grass Instrument Co, MA, U.S.A.).

By moving intestine, seminal vesicles and bladder aside, the cavernous nerve, situated on the lower lateral portion of the prostate, was visualized. Electrical stimulation of nerves was performed with a slender bipolar platinum contact electrode. Square wave pulses were delivered with a duration of 1.0 ms by a Grass S48 stimulator (Grass Instrument Co, MA, U.S.A.). Each stimulation had a duration of 60 s. Prior to the next stimulation, a resting interval of 15 min was allowed. Frequency-response relationships were determined at different voltage amplitudes. In some animals, sildenafil, L-nitro-arginine-methyl-ester (L-NAME), or forskolin were administered systemically or into the CC. Submaximal voltage, producing approximately 70% of maximal ICP, were used when investigating the effects of drugs on nerve-induced erectile responses. Before stimulation, basal ICP (BICP) was noted. During tumescence, the peak ICP (PICP) and time (T80) for the ICP to reach 80% of maximal increase (PICP-BICP) were recorded. At this point, the increase in ICP per second (ΔT80) was evaluated. After stimulation, during detumescence, the time (D20) and the rate (ΔD20) for ICP to decrease to 20% of maximal increase were determined (Figure 2). Circumcision was performed in some animals in order to evaluate penile lengthening and cuffing of the glans penis during erectile responses.
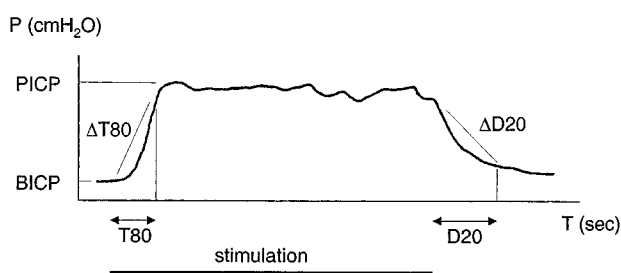

Figure 2 Typical curve showing the increase in intracavernous pressure (ICP) induced by nerve stimulation *in vivo*. During stimulation, the time for ICP to reach 80% of maximal increase (peak ICP−basal ICP) was recorded (T80). At this point, the increase per second (ΔT80) was evaluated. After stimulation, the time for and the rate by which a decrease to 20% of maximal pressure occurred (D20 and ΔD20) were determined.

Drugs and solutions

A saline solution, containing 154 mM NaCl, and a Krebs solution of the following composition were used (mM): NaCl 119, KCl 4.6, CaCl$_2$ 1.5, MgCl$_2$ 1.2, NaHCO$_3$ 15, NaH$_2$PO$_4$ 1.2, glucose 5.5. A high K$^+$ solution (124 mM) was used, in which the NaCl in the normal Krebs solution was replaced by equimolar amounts of KCl was used. The following drugs were used: noradrenaline (NA; Aldrich-Chemie GmbH & Co, Germany), carbachol (carbamylcholine chloride), VIP, NPY, CGRP, forskolin, L-NOARG, L-NAME (Sigma Chemical Co, St Louis, MO, U.S.A.), ODQ (1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; Tocris Cookson, UK), sildenafil (Pfizer, Sandwich, U.K.). Stock solutions were prepared and then stored at −70°C and subsequent dilutions of the drugs were made with 0.9% NaCl (NA; with ascorbic acid added as an antioxidant). NO was freshly prepared at each experiment. An air-tight glass-beaker, containing 20 ml of distilled water, was deoxygenated for 1 h with helium gas. The beakers were then bubbled with medical NO gas (purity >99.5%) for 15 min until saturated solutions were obtained (3×10^{-3} M).

Calculations

Student's paired or unpaired two-tailed *t*-tests were used for statistical comparison of two means. A probability of $P < 0.05$ was accepted as significant. ANOVA with Bonferroni correction was used for the comparison of multiple means. When appropriate, results are given as mean values $\pm$ standard error of the mean (s.e.m.). Small *n* denotes the number of strip preparations, and capital *N* denotes the number of individuals. All statistical calculations were based on *N*.

Results

Morphological findings

Neuronal NOS-, and TH-IR coarse nerve trunks were uniformly distributed in the erectile tissue. No trunks expressing CGRP-, NPY-, VAChT- and VIP-immunoreactivity could be detected.

The smooth muscle septa, surrounding the cavernous spaces, were supplied with varicose terminals, which ran along the bundles of smooth muscle cells. TH-IR terminals were most numerous (large number), whereas nNOS-, NPY-, VAChT- and VIP-IR terminals were slightly less abundant (moderate number). In contrast, only few CGRP-IR terminals were found.

Arteries in the corpus cavernosum were surrounded by nNOS-, NPY-, TH-, VAChT- and VIP-IR varicose terminals, which formed dense plexuses in the adventitia of the vessels. CGRP-IR varicose terminals also formed similar plexuses, which were less dense than those formed by the other populations of nerve terminals. Generally, CGRP-IR nerve fibres exhibited different profiles than nerve fibres of the other investigated transmitters.

In double and triple stained sections, VAChT-, nNOS-, and VIP-IR nerve fibres and terminal varicosities showed identical coinciding profiles (Figure 3). With confocal laser scanning sectioning, co-existence of immunoreactivities for VAChT, nNOS, and VIP was observed in nerve fibres and
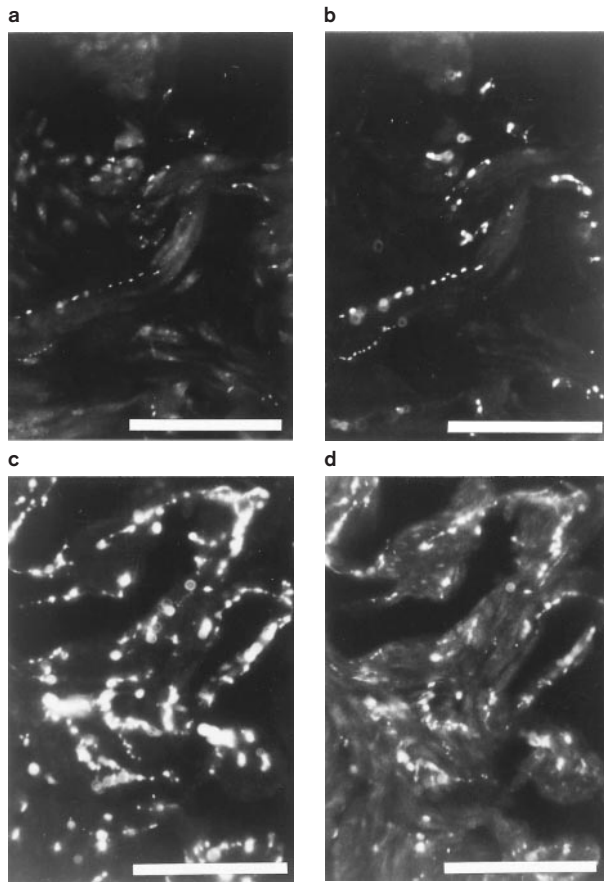
Figure 3 Mouse corpus cavernosum. Double immunohistochemistry. (a) Vesicular acetylcholine transporter (VAChT)-immunoreactive (IR) nerve terminals along smooth muscle bundles. FITC immunofluorescence. (b) Same section as in (a). NO synthase-IR nerve terminals displaying coinciding profiles with VAChT-IR varicosities. (c) Tyrosine hydroxylase (TH)-IR nerve terminals. FITC immunofluorescence. (d) Neuropeptide Y-IR nerve terminals exhibiting coinciding profiles with TH-IR nerve terminals. Same section as in (d). Texas red immunofluorescence. Bars = 100 μm

varicosities (Figure 4), interspersed in trabecular smooth muscles of the erectile tissue and in the adventitial layers of vessels. TH- and NPY-IR nerve fibres also had coinciding profiles, and terminal varicosities were observed to contain immunoreactivities for TH and NPY (Figure 3). VAChT- and TH-, VAChT- and NPY-, VAChT- and CGRP-, and TH- and VIP- IR terminals sometimes displayed intimate and parallel profiles, although never coinciding.

Chemical sympathectomy abolished TH- and NPY-IR nerve structures in smooth muscle bundles of the trabecular erectile tissue. Single TH- and NPY-IR varicose terminals remained in the perivascular plexuses, and in nerve trunks, single TH-IR nerve fibres remained. In contrast, the number and distribution patterns of CGRP-, nNOS-, NPY-, VAChT- and VIP-IR nerve structures were unchanged compared to non-sympathectomized animals.

In endothelial cells, covering the lumen of the central and helicine arteries and veins and venules, intense immunoreactivity for eNOS was observed. Endothelial NOS-IR cells exhibited a non-stained central nucleus with surrounding immunoreactive cytoplasm, which extended sideways into thin cytoplasmic processes. No distinct eNOS-IR cells with

central non-stained nuclei and cytoplasmic processes were found lining the cavernous sinusoids.

Functional in vitro *studies*

Relaxation of noradrenaline-contracted preparations Spontaneous contractile activity was not observed in any of the mouse isolated CC preparations ($n = 34$, $N = 19$). Reproducible, contractile responses to NA, amounting to 0.52 ± 0.04 mN ($n = 19$, $N = 12$) were obtained.

In NA-contracted preparations, VIP ($n = 6$, $N = 6$) and CGRP ($n = 5$, $N = 5$) had concentration-dependent small relaxant actions, and at 10^{-6} M, the highest peptide concentration used, mean relaxations of $22 \pm 3\%$ and $32 \pm 2\%$ was registered.

NO ($10^{-6} - 10^{-4}$ M, $n = 7$, $N = 7$), produced concentration-dependent, rapid and almost complete ($97 \pm 2\%$, pIC_{50} 5.8 ± 0.1) relaxations which were significantly counteracted by ODQ (10^{-6} M). Carbachol-induced (10^{-6} M) relaxations ($54 \pm 4\%$, $n = 12$, $N = 12$), were significantly attenuated by L-NOARG (10^{-4} M) or ODQ (10^{-6} M).

Forskolin, added cumulatively ($10^{-9} - 10^{-6}$ M), produced concentration-dependent relaxations (pIC_{50} 7.1 ± 0.1), which amounted to $98 \pm 3\%$ at the highest concentration used ($n = 7$, $N = 7$).

In NA-contracted preparations, EFS generated frequency-dependent and TTX-sensitive ($n = 9$, $N = 9$) relaxant responses. At any investigated frequency, pretreatment with 10^{-4} M L-NOARG ($n = 6$, $N = 6$), or 10^{-6} M ODQ ($n = 5$, $N = 5$) abolished the electrically-induced inhibitory activity. Inhibitory responses were unaffected by scopolamine.

Electrically-induced responses Stimulation of preparations at baseline level produced frequency-dependent contractile responses ($n = 6$, $N = 6$), which were abolished by TTX 10^{-6} M. Prazosin ($n = 6$, $N = 6$) effectively counteracted the contractions produced by EFS, and at 10^{-7} M, the electrically-induced responses were abolished. The pIC_{50} value for prazosin amounted to 9.3 ± 0.1. Forskolin also concentration-dependently inhibited contractile responses induced electrically (Figure 5), and at 10^{-6} M, contractions were not obtained. A pIC_{50} value of 7.5 ± 0.1 was calculated for forskolin on EFS-induced contractions.

Contractions induced by electrical stimulation were to a limited degree counteracted by VIP ($n = 6$, $N = 6$), and at 10^{-6} M a mean inhibitory effect of $36 \pm 6\%$ was registered. CGRP ($n = 4$, $N = 4$) effectively counteracted EFS-induced contractions with a maximum inhibitory effect of $70 \pm 8\%$ at 10^{-5} M, and with a pIC_{50} value of 7.7 ± 0.8. NPY enhanced contractions produced by EFS; the effect amounted to $29 \pm 7\%$ at 10^{-6} M ($n = 4$, $N = 4$). Scopolamine ($10^{-11} - 10^{-6}$ M) also enhanced electrically-induced contractions, and at 10^{-6} M this effect amounted to $68 \pm 20\%$ ($n = 4$, $N = 4$). Addition of prazosin, VIP, NPY, CGRP, forskolin or scopolamine did not affect baseline tension in the preparations.

In vivo *studies*

No spontaneous increases in ICP were registered. BICP was 13 ± 1 cm H_2O and mean arterial blood pressure 131 ± 3 cm H_2O ($N = 51$). Stimulation of the cavernous nerve produced
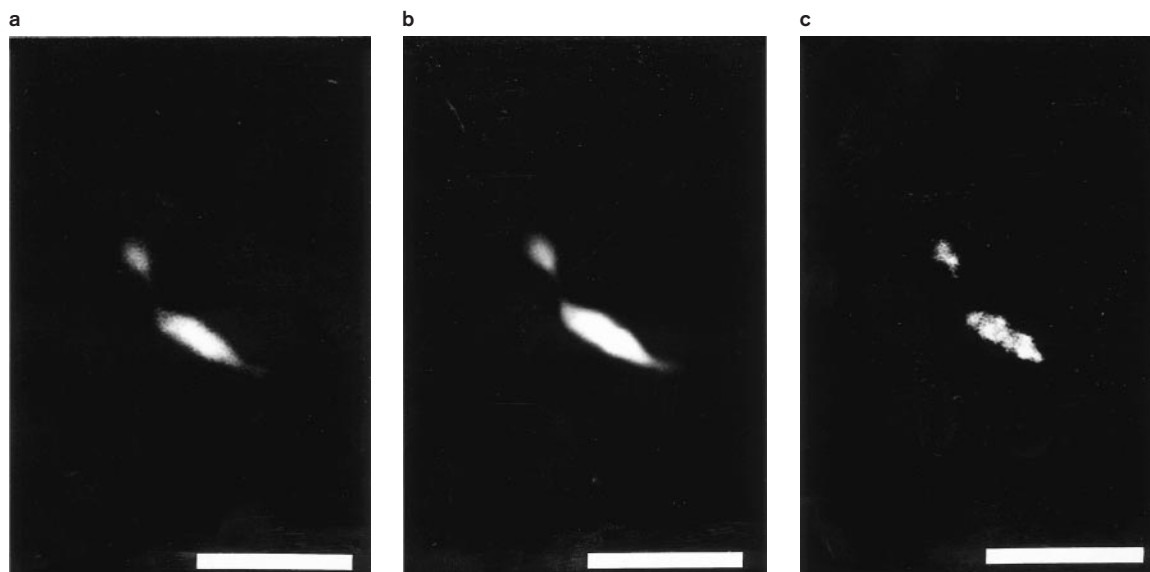
Figure 4 Mouse corpus cavernosum. Triple immunocytochemistry. Confocal microscopy. (a) Vesicular acetylcholine transporter – immunoreactive (IR) nerve terminals. FITC- immunofluorescence. (b) Same section as in (a). NO synthase-IR nerve varicosities. Texas red immunofluorescence. (c) Same section as in (a) and (b). Vasoactive intestinal polypeptide-containing varicosities. Cy-5 immunofluorescence. Each image consists of 19 consecutive sections with 0.30 μm between adjacent sections. Bars = 10 μm.

voltage- and frequency-dependent erectile responses, observed as penile tumescence, increases in shaft length of the penis, and engorgement of the glans penis, and simultaneously, reproducible increases in ICP were registered (Table 2). The highest values for PICP were recorded at 6 V and 20 Hz, and amounted to a mean value of 116 ± 5 cm H_2O ($N=6$). At these stimulation parameters, the fastest and most effective increases in ICP were also registered, and as defined by T80 and ΔT80, mean values of 11 ± 1 s and 8 ± 1 cm H_2O s^{-1} were calculated. Corresponding values at e.g. 1.5 V and 5 Hz were 28 ± 4 cm H_2O, 38 ± 5 s and 0.5 ± 0.1 cm H_2O s^{-1} ($N=6$; for PICP $P<0.0001$, for T80 $P<0.001$, and for ΔT80 $P<0.0001$, respectively). During detumescence, after assessment of cavernous nerve stimulation, small values for ΔD20 were observed at the lowest stimulation parameters (Table 2). No statistical differences were found in the values for D20 at any investigated frequency, and mean values were kept between 11 and 14 s for D20.

At submaximal stimulation (3 V, 5–10 Hz), intraperitoneally administered L-NAME ($N=5$, 50 mg kg^{-1}) effectively counteracted the nerve-induced increase in ICP (Figure 6), and PICP decreased from 74 ± 11 cm H_2O to 32 ± 8 cm H_2O ($N=5$; $P<0.05$). Upon stimulation, T80 and ΔT80 were not significantly altered by L-NAME, and during detumescence no differences in the D20 and ΔD20 values were registered. After intraperitoneal administration of 200 μg kg^{-1} of sildenafil ($N=9$, Figure 7), submaximal stimulation increased PICP from 68 ± 6 cm H_2O to 90 ± 9 cm H_2O ($P<0.05$). T80 and ΔT80 were not significantly affected by the drug, whereas D20 was significantly ($P<0.05$) prolonged from 11 ± 1 s to 16 ± 1 s. The value for ΔD20 was not significantly reduced by the drug. Sildenafil, at the investigated concentration, did not affect systemic blood pressure.

Intracavernous administration of forskolin (0.2, 2, 20 μg; $N=5$–6 at each concentration) produced concentration-dependent increases in ICP (Figure 5), with the highest PICP

of 127 ± 9 cm H_2O at 20 μg. The duration of the ICP increase was 4 ± 1, 6 ± 1, and 21 ± 6 min for 0.2, 2, and 20 μg forskolin, respectively. Forskolin did not affect systemic blood pressure. The response to forskolin (5 μg) was not changed after blockade of NO synthesis by L-NAME (50 mg kg^{-1}, $N=5$; Figure 6).

Discussion

Most neurons originating from the pelvic plexus and supplying the rat penile erectile tissue, have been shown to contain immunoreactivities for NOS and VIP, and are presumed to be cholinergic due to positive acetylcholinesterase staining. VAChT is found in nerves containing acetylcholine, and is considered a special marker for nerves with cholinergic characteristics. In the mouse CC, VAChT-, VIP-, and nNOS-IR nerves were found in moderate numbers, and by double immunolabelling, VAChT- and nNOS-IR, VAChT- and VIP-IR, and nNOS- and VIP-IR nerve fibres showed coinciding profiles. In addition, varicosities with immunoreactivities for VAChT, nNOS, and VIP exhibited identical and overlapping patterns when examined in double filter settings, and co-existence was verified by 3D reconstruction after microscopical confocal laser scanning sectioning. These findings confirm observations made in the CC from rat and cyclic GKI-deficient mice (Hedlund *et al*., 1999; 2000a), and support that NOS-containing nerves are cholinergic. Immunoreactivity for TH was not found in VAChT-, nNOS-, or VIP-IR nerves, but was observed in close relation to the latter structures. Chemical sympathectomy virtually abolished all TH- and NPY-IR nerve structures. NPY was reported to be present in subpopulations of sympathetic adrenergic nerves, and upon release from these nerves, to produce receptor-mediated vasoconstriction (Lundberg, 1996). Thus, as described for the rat CC, an arrangement of separate

sympathetic adrenergic and parasympathetic cholinergic nerves seems likely for the innervation of the mouse CC. In both species, the observed close relation, with similar distribution patterns, between VAChT/NOS/VIP-IR and TH-IR nerves favours a suggested muscarinic modulation of the release of NA from adrenergic nerves (Hedlund *et al.*, 1984). In further support of this mechanism in the mouse CC, scopolamine, an inhibitor of muscarinic receptor-mediated effects, significantly enhanced EFS-induced contractions, whereas the drug had no effect on base-line tension, NA-induced contractions, or inhibitory neurotransmission in isolated preparations.

Penile erection is dependent on cavernous smooth muscle relaxation, and it has been shown that NO, released from nerves, is the major relaxant agent in several mammals (Andersson & Wagner, 1995). This has also been proposed for mouse erectile tissues (Burnett *et al.*, 1996; Gocmen *et al.*, 1997; 1998; Hedlund *et al*, 2000a). With the presently developed isolated precontracted CC preparation, nerve-mediated, frequency-dependent, L-NOARG- and ODQ-sensitive relaxations were confirmed, and this inhibitory neurotransmission was found to be similar to responses described for other species, as well as humans (Holmquist *et al.*, 1992; Hedlund *et al.*, 1995; 1999).

During continuous monitoring of systemic blood pressure, subsystolic erectile responses *in vivo* were recorded as reproducible voltage- and frequency-dependent increases in ICP during stimulation of the cavernous nerve. These responses were also observed as penile engorgement and swelling of the glans penis. The presently observed values for BICP, filling and emptying rates, and the PICP, qualitatively correspond well with findings in rat, rabbit, dog and primates (Lue *et al.*, 1983; Junemann *et al.*, 1989; Holmquist *et al.*, 1991; Martinez-Pineiro *et al.*, 1994; Calabro *et al.*, 1996). In mice, one study has demonstrated measurements of ICP before and during nerve-induced erectile responses (Sezen & Burnett, 2000). In this previous study, the mean BICP corresponded with the findings in our investigation, but the maximal PICP values, obtained in our experimental set-up, without activation of somatic nerve structures, were roughly 100% higher. This difference is noteworthy. Since the same anaesthetic agent was used during similar experimetal
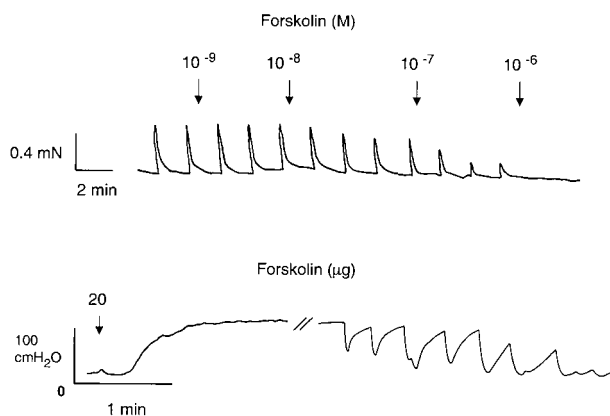

Figure 5 Tracing showing the effects of forskolin on electrical field stimulation-induced contractions in an isolated preparation of mouse corpus cavernosum (top) and on intracavernous pressure in an anaesthetized mouse after intracavernous administration (bottom).
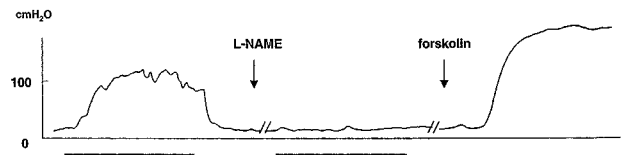

Figure 6 Intracavernous pressure (ICP) changes in the mouse corpus cavernosum in response to cavernous nerve stimulation *in vivo*. After pretreatment with intraperitoneally administered L-NAME (50 mg kg^{-1}), the erectile response was abolished. The increase in ICP induced by forskolin (5 μg kg^{-1}) was unaffected by L-NAME.
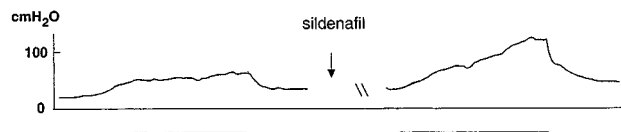

Figure 7 Intracavernous pressure (ICP) changes in the mouse corpus cavernosum in response to cavernous nerve stimulation (3 V, 10 Hz) *in vivo*. Facilitatory effect of sildenafil (200 μg kg^{-1}).

Table 2 Intracavernous pressure (ICP) during cavernous nerve stimulation *in vivo*

Volt	Hertz	PICP (cm H$_2$O)	T80 (s)	D20 (s)	ΔT80 (cm H$_2$O s^{-1})	ΔD20 (cm H$_2$O s^{-1})
1.5	5	28±4	38±5	13±3	0.5±0.1	2.0±0.5
	10	65±8	31±4	14±2	1.5±0.3	3.6±0.6
	15	90±14	32±4	14±2	2.1±0.3	6.1±1.5
	20	95±9	26±2	13±2	2.7±0.3	7.7±1.8
3.0	5	78±3	29±5	12±3	2.4±0.5	7.8±2.3
	10	101±9	27±6	12±4	3.4±0.7	6.4±0.5
	15	110±8	22±3	11±2	3.9±0.6	9.4±1.5
	20	111±8	22±2	13±2	3.8±0.5	8.4±1.8
6.0	5	85±9	28±4	13±3	2.4±0.5	8.4±2.9
	10	108±10	15±2	11±2	5.4±0.9	9.2±1.5
	15	111±4	12±1	12±1	6.8±0.5	8.1±0.9
	20	116±5	11±1	14±3	7.7±0.8	7.3±1.3

PICP=peak ICP, T80=time to reach 80% of PICP, D20-time for ICP to decrease to 20% of PICP, ΔT80=increase in ICP per second at T80, ΔD20=decrease in ICP per second at D20

procedures, other methodological differences may exist between the studies. In the present study, erections were attenuated, and registered ICP changes were significantly counteracted by intraperitoneal administration of L-NAME. Systemically given sildenafil facilitated tumescence and prolonged detumescence. This was an expected effect of the drug, and is in accordance with findings *in vivo* by other investigations (Boolell *et al*., 1996; Carter *et al*., 1998; Andersson *et al*., 1999). Taken together, the present study verify that in the mouse, the NO/cyclic GMP pathway is of main importance for penile erectile responses *in vitro* and *in vivo*.

In functional experiments, VIP affected NA- or EFS-induced contractile activities to a minor extent, did not influence the base-line tension in isolated preparations. A direct smooth muscle regulatory function in the mouse penis for the peptide therefore seems unlikely. Still, a functioning adenylyl cyclase (AC)/cyclic AMP pathway *in vitro* has been suggested for mice (Hedlund *et al*., 2000a). This was confirmed in the present study, and was seen as pronounced relaxant and inhibitory effects of forskolin. When administered intracavernously, forskolin caused concentration-dependent and reproducible increases in the ICP *in vivo*, even in the presence of NO synthase inhibition. In mice with null mutations for the gene encoding for cyclic GKI, a preserved AC/cyclic AMP system did not compensate for the erectile dysfunction observed in these animals (Hedlund *et al*., 2000a), and at present, it is unclear if an endogenous transmitter, that may act with cyclic AMP as second messenger, can initiate penile erection.

In the flaccid state, it is generally accepted that CC smooth muscle is kept contracted mainly by NA acting on postjunctional α-adrenoceptors (Andersson & Wagner, 1995). This also seems to be the case in the mouse. Confirming findings in cyclic GKI-deficient mice (Hedlund *et al*., 2000a), large amounts of TH-IR nerve structures were observed in the erectile tissue from normal mice. In non-activated isolated mouse CC preparations, frequency-dependent and α-adrenoceptor-mediated contractions were produced in response to transmural stimulation of nerves. The presence of moderate numbers of NPY-IR nerve fibres, and a 30% potentiation of the EFS-induced contractile activity, suggests a possible function for the peptide in the CC, preferably during detumescence, as previously proposed (Kirkeby *et al*., 1991, Giuliano *et al*., 1993).

Cholinergic agents are shown to release NO from vascular endothelium (Furchgott & Zawadzki, 1980), a finding also described for erectile tissue (Ignarro *et al*., 1987; Palmer *et al*., 1987). It is, however, disputed to what extent the endothelium contributes with NO-mediated effects during erection, and differences between species appears to be present. In the rat, often used for the study of penile erection *in vivo*, NADPH diaphorase staining has been observed in parts of the sinusoidal endothelium (Keast, 1992). Using immunohistochemistry, later investigators found eNOS-IR endothelial cells to be confined to the penile arteries (Schirar *et al*., 1994; Dail *et al*., 1995; Hedlund *et al*., 1999), and functional *in vitro* correlates suggested that endothelial NO was of minor importance for erectile responses in the rat (Hedlund *et al*., 1999). In the presently isolated mouse CC, carbachol produced a 50% reduction in the NA-induced contraction, and this relaxation was almost abolished by L-NOARG or ODQ. These findings correspond well with functional results obtained in cavernous tissue from humans and other mammals (Andersson & Wagner, 1995), and suggest that the endothelium in the mouse CC may provide NO during penile erection. However, it cannot be excluded that an additional release of NO may occur, due to carbachol acting on nicotinic receptors on cholinergic ganglion cells in the preparation.

Endothelial NOS has previously been located to the mouse erectile tissue, and an up-regulation of eNOS has been suggested to compensate for loss of the neuronal isoform in nNOS transgenic mice with normal erectile responses (Burnett *et al*., 1996). In the present study, immunoreactivity for eNOS was found in endothelial cells lining arteries and veins of the erectile tissue, but no distinct eNOS-IR cells were observed in sinusoidal endothelium.

Binding sites and immunoreactivity for CGRP have been described in CC tissue, and when given intracavernously, the peptide can induce erectile responses. Functional effects *in vitro* are previously reported to be small (Andersson & Wagner, 1995). Similar to effects seen in the rat (Hedlund *et al*., 1999). CGRP attenuated α-adrenoceptor mediated contractions in the mouse isolated CC. Based on the present functional results, although only few CGRP-IR nerve fibres were observed, a prejunctional action of CGRP cannot be excluded.

Conclusion

In comparison with results from other mammals, including humans, the mouse corpus cavernosum exhibit similar functions of nerves and endothelium, as well as similar intrinsic systems, i.e the sGC/cyclic GMP and AC/cyclic AMP pathways, for activation of smooth muscle functions. In addition, reproducible pharmaco- and nerve-induced erectile responses were obtained *in vivo*. The mouse is a suitable model for studies of penile erection.

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