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Loss of nitrergic neurotransmission to mouse corpus cavernosum in the absence of neurturin is accompanied by increased response to acetylcholine

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1 The neurotrophic factor, neurturin (NTN), plays an important role in parasympathetic neural development. In the penis, parasympathetic nitrergic/cholinergic nerves mediate the erectile response. However, despite reduced parasympathetic penile innervation in mice lacking the NTN receptor, glial cell line-derived neurotrophic factor family receptor α (GFRα)2, they are capable of erection and reproduction.

2 Our aim was to assess neural regulation of erectile tissues from mice lacking NTN. Responses of cavernosal smooth muscle were studied *in vitro*, monitoring agonist- and nerve-evoked changes in tension.

3 Frequency-dependent nerve-evoked relaxations in the presence of guanethidine were markedly reduced in the mutant mice compared to wild types (19 vs 72% of phenylephrine pre-contraction). Atropine reduced the amplitude in wild-type mice to 61%, but abolished relaxations in knockout mice. In wild-type and knockout animals, nitric oxide synthase inhibition abolished neurogenic relaxations.

4 In NTN knockout animals, EC_{50} values for nitric oxide-dependent relaxations to acetylcholine and muscarine were increased approximately 0.5 log units. In contrast, contractions to electrical stimulation or phenylephrine, and relaxations to bradykinin or the nitric oxide donor, sodium nitroprusside, were unaltered.

5 Immunohistochemistry confirmed that nerves immunoreactive for nitric oxide synthase, vesicular acetylcholine transporter and vasoactive intestinal polypeptide were substantially reduced in cavernosum of NTN knockout mice.

6 Parallel immunohistochemical and pharmacological studies in GFRα2 knockout animals showed the same changes from their wild types as the NTN knockout animals.

7 The data demonstrate that NTN is essential for normal development of penile erection-inducing nerves and that its absence leads to increased responsiveness to muscarinic agonists, possibly as a compensatory mechanism.
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Abbreviations: eNOS, endothelial nitric oxide synthase; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF family receptor α; nNOS, neuronal nitric oxide synthase; NTN, neurturin; RET, rearranged in translation receptor tyrosine kinase; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal polypeptide

Introduction

Discovery of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), GDNF (Lin *et al*., 1993), neurturin (NTN) (Kotzbauer *et al*., 1996) and artemin (Baloh *et al*., 1998), has been essential for understanding peripheral neural development. These growth factors preferentially bind glycosylphosphatidylinositol-anchored GDNF family receptor α (GFRα) components 1, 2 and 3, respectively, in order to activate a common signalling component, the rearranged in translation (RET) receptor tyrosine kinase (Baloh *et al*., 2000; Enomoto, 2005). Generation of mice with targeted gene deletions against the GFLs and their respective GFRα receptors has provided an invaluable experimental tool for understanding the role of GFLs in the development and plasticity of autonomic ganglion neurons. For example, RET (Schuchardt *et al*., 1994), GDNF (Moore *et al*., 1996; Pichel *et al*., 1996; Sanchez *et al*., 1996) and GFRα1 (Cacalano *et al*., 1998; Enomoto *et al*., 1998) knockout mice fail to develop enteric neurons distal to the stomach, and show substantial loss of neurons in some cranial ganglia. These mice die soon after birth owing to renal agenesis and intestinal dysfunction.

In contrast, NTN (Heuckeroth *et al*., 1999) and GFRα2 (Rossi *et al*., 1999) knockout mice have relatively modest phenotypes and reach adulthood; GFRα2 knockouts are, for example, slightly smaller than their wild-type littermates. However, they show notable deficits in some areas of the

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parasympathetic nervous system. For example, neuronal atrophy occurs in the otic, sphenopalatine and submandibular ganglia (Enomoto, 2005). Furthermore, sacral pelvic ganglia are smaller in NTN and GFRα2 knockout animals and the innervation of a number of pelvic organs is deficient (Wanigasekara *et al*., 2004). Notably, in GFRα2 knockout mice, fewer parasympathetic nerve fibres are present in the cavernosal tissue of the penis (Laurikainen *et al*., 2000a). There are also markedly reduced parasympathetic nerves innervating glandular tissues of reproductive organs (e.g. prostate, seminal vesicles, uterine cervix) but, interestingly, the parasympathetic innervation of the smooth muscle of these organs appears normal (Wanigasekara *et al*., 2004).

These deficits could be owing to failure of parasympathetic pelvic ganglion neurons to survive, to project to some pelvic tissues or to maintain their projections. Although these first two possibilities cannot yet be discounted, there is good evidence for NTN playing a maintenance role in adult pelvic parasympathetic neurons. For example, NTN is highly expressed in the reproductive organs of adult mice (Golden *et al*., 1999), and is also expressed by the smooth muscle of penile blood vessels and corpus cavernosum of adult rats (Laurikainen *et al*., 2000a). All penis-projecting pelvic neurons express neuronal nitric oxide synthase (nNOS), and in adult rats most of these neurons also express GFRα2 (Laurikainen *et al*., 2000a). Moreover, nNOS-positive pelvic ganglion somata are smaller in NTN and GFRα2 knockout mice than in age-matched wild-type animals (Wanigasekara *et al*., 2004), consistent with NTN maintaining neuronal structure. A recent study has also shown that NTN stimulates neurite initiation and extension in cultured pelvic nNOS-immunoreactive (IR) neurons from adult rats (Wanigasekara & Keast, 2005).

In this study, we investigated the role of NTN signalling to maintain adequate parasympathetic neurotransmission in the penis. Despite a dramatic reduction in parasympathetic nerves within the cavernosum (Laurikainen *et al*., 2000a), GFRα2 knockout mice are still able to perform erections and reproduce (Rossi *et al*., 1999). This observation leads to the primary aim of our study, which was to determine the neural mechanisms underlying the erectile response in NTN knockout animals, as they too are fertile (Heuckeroth *et al*., 1999). Given the loss of parasympathetic nerves from the cavernosum of GFRα2-deficient animals, it is possible that the smooth muscle in NTN (and GFRα2) knockouts may show altered sensitivity to one or more transmitters. Although this has been commonly observed in many smooth muscles, the plastic properties of cavernosal smooth muscle following denervation have been poorly examined. Therefore, the second aim of our study was to determine if a reduction in parasympathetic innervation of cavernosum in the knockout animals had any effect on responses to transmitters or relevant agonists. Clinically, a functional understanding of erectile physiology in these animals may be relevant for developing therapeutic strategies to restore erectile function following pelvic surgical procedures such as prostatectomy, where, despite nerve-sparing techniques, long-lasting erectile dysfunction remains a common complication (Burnett, 2005).

The mechanisms underlying penile erection have been well described previously, and the innervation of cavernosum is readily studied *in vitro* (Andersson & Wagner, 1995; Andersson, 2001). Penile erection involves nerve-driven relaxation of the cavernosum vasculature and trabecular smooth muscle concurrent with morphologic restriction of venous outflow. During this process, pelvic parasympathetic neurons release the potent vasodilator, nitric oxide, and corelease of acetylcholine stimulates further nitric oxide production from the endothelium (Andersson & Wagner, 1995; Andersson, 2001). Nitric oxide released from nerves or the endothelium mediates the production of cGMP *via* activation of gaunylyl cyclase in adjacent smooth muscle cells, reducing intracellular Ca^{2+} and causing vasodilation (Ghalayini, 2004). In the current study, isolated strips of cavernosal tissue were electrically stimulated to drive nerve-activated responses, and standard pharmacological approaches to relevant agonists were used to dissect cholinergic and nitrergic components of these responses. For brevity, we have referred to cholinergic/nitrergic penile nerves as 'parasympathetic' throughout, even though a small proportion of the pelvic ganglion neurons supplying these nerves will receive lumbar spinal inputs (Dail *et al*., 1985).

Methods

Animals and anaesthesia

All procedures were approved by the Animal Care and Ethics Committee of the University of Sydney, which complied with guidelines of the National Health and Medical Research Council of Australia. Every effort was made to avoid animal suffering and to minimise the numbers of animals. Male NTN and GFRα2 gene-deficient mice (NTN−/− and GFRα2−/−) were generated as described previously (Heuckeroth *et al*., 1999; Rossi *et al*., 1999; Wanigasekara *et al*., 2004). Tissues were studied from wild-type (NTN, $n=14$; GFRα2, $n=10$) and knockout animals (NTN, $n=13$; GFRα2, $n=10$) aged between 3 and 6 months. All mice had access to water and standard laboratory chow *ad libitum*. Mice were anaesthetised ($20\,mg\,kg^{-1}$ xylazine, $80\,mg\,kg^{-1}$ ketamine i.p.) before intracardiac perfusion with fixative for immunohistochemistry or removal of penile tissue for *in vitro* pharmacological studies. For the latter group, animals were then killed by exsanguination.

In vitro *pharmacology*

The penis was excised at its base and the glans penis and connective tissues surrounding the shaft were removed. The corpora cavernosum were then separated using a scalpel and each strip was mounted in a 15 ml organ bath containing modified Krebs–Ringer solution (144 NaCl, 5 KCl, 1.1 $MgSO_4$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 1.25 CaCl and 5.5 glucose; in mM), maintained at 37°C (pH 7.35) and gassed with a 95% O_2 : 5% CO_2 mixture. Resting tension was set at 0.5 g and monitored by isometric force transducers (AD Instruments, Castle Hill, NSW, Australia). Tissues were allowed to equilibrate for 45–60 min with frequent changing of the bathing media. Cumulative concentration–response curves to the α_1-adrenoceptor agonist, phenylephrine, and nitric oxide donor, sodium nitroprusside, were established against a 10 μM phenylephrine pre-contraction. Non-cumulative response curves for endothelium-dependent relaxation to acetylcholine, muscarine and bradykinin were established against individual 10 μM phenylephrine pre-contractions. Transmural electrical field stimulation (30–90 s duration;

2–30 Hz; 2 ms pulses; 10 V) was delivered using platinum wire ring electrodes *via* an isolated stimulator (Digitimer, Welwyn, Herts, U.K.) connected to a PowerLab system (AD Instruments); data were recorded using Chart 5.0 software. In the presence (15 min preincubation) of the blocker of noradrenergic transmission, guanethidine (3 μM), and/or muscarinic cholinoceptor antagonist, atropine (1 μM), nerve-evoked relaxations were examined in response to electrical stimulation against phenylephrine pre-contraction. In addition, some experiments were repeated in the presence of the nonspecific nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NNA; 10 μM), or the acetylcholinesterase inhibitor, neostigmine (10 μM). Tissues were weighed at the end of experiments.

Fixation and immunohistochemistry

Mice were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) following a 0.1 ml 5000 IU ml^{-1} heparin injection. The penile shaft was dissected and refrigerated overnight in the same fixative, washed in phosphate-buffered saline (PBS) and cryoprotected in 30% sucrose–PBS before sectioning. Cryosections (14 μm) were collected on 1% gelatinised slides, and following a 1–2 h blocking incubation with 10% non-immune horse serum in 0.1% Triton X–PBS, sections were incubated overnight with a selection of primary antibodies raised against the endothelial (eNOS) or neuronal (nNOS) nitric oxide synthase; the pan-neuronal marker, protein gene product 9.5 (PGP 9.5); the catecholamine synthesis marker, tyrosine hydroxylase (TH); the cholinergic nerve marker, vesicular acetylcholine transporter (VAChT); and vasoactive intestinal polypeptide (VIP). Following a PBS wash, sections were further incubated for 2–3 h with appropriate indocarbocyanine (Cy3)- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and coverslipped using 0.5 M bicarbonate-buffered glycerol media (pH 8.6). Immunoreactivity for each of the markers was abolished in the absence of primary or secondary antibody. Endothelial NOS immunoreactivity was abolished in a dose-dependent manner (2–200 μg ml^{-1}) by the antibody manufacturer's blocking peptide. Images were captured by an RT Spot camera (Diagnostic Instruments, Sterling Heights, MI, U.S.A.) mounted on an Olympus BX51 fluorescence microscope and using Image Pro Plus 5.0 software (Media Cybernetics, Carlsbad, CA, U.S.A.). Figures were created using GraphicConverter 4.5 and Adobe Illustrator 11.0 software; brightness and contrast of micrographs were optimised to best represent immunostaining as seen under the microscope.

Materials

Acetylcholine chloride, atropine sulphate, guanethidine monosulphate, muscarine chloride, neostigmine bromide, L-NNA, paraformaldehyde and phenylephrine hydrochloride were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Other chemicals used were bradykinin (Auspep, Parkville, Vic., Australia), heparin sodium (Mayne Pharma, Melbourne, Vic., Australia), ketamine hydrochloride (Parnell Laboratories, Alexandria, NSW, Australia), sodium nitroprusside (Fluka Chemika, Steinham, Germany) and xylazine hydrochloride (Troy Laboratories, Smithfield, NSW, Australia).

The following primary antibodies were used, with host species and working dilution indicated: eNOS (rabbit, 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), nNOS (sheep, 1 : 5000; gift from Dr Piers Emson, Cambridge, U.K.), PGP9.5 (rabbit, 1 : 800; Ultraclone, Isle of Wight, U.K.), TH (rabbit, 1 : 500; Chemicon, Temecula, CA, U.S.A.), VAChT (goat, 1 : 3000; Chemicon), VIP (rabbit, 1 : 2000; DiaSorin, Stillwater, MN, U.S.A.). Cy3-conjugated goat (1 : 1000) and rabbit (1 : 1500) and FITC-conjugated sheep (1 : 200) and rabbit (1 : 100) secondary antibodies were all raised in donkey and were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Statistical analysis

Data are presented as means ± standard error (s.e.m.). Data were analysed using paired or unpaired two-tailed Student's *t*-tests, as appropriate; significance was accepted at $P<0.05$. Concentration–response curves were fitted by sigmoid curves using the least squares method to calculate EC_{50}. Frequency–response curves were compared by paired *t*-tests (within group) or two-way ANOVA with Bonferroni post-test (between group; Prism 4.0 software, Graphpad, San Diego, CA, U.S.A.).

Results

Although our primary focus was to investigate neural regulation and anatomy of corpus cavernosum from NTN−/− mice, we also performed some studies in heterozygous NTN littermates and GFRα2−/− mice and their respective wild types.

Animal and penile tissue weights

As previously reported (Rossi *et al*., 1999; Wanigasekara *et al*., 2004), adult GFRα2−/− mice had reduced body weight (g) compared to their respective wild-type controls (26.2 ± 0.7, $n=8$, vs 31.4 ± 0.4, $n=6$, $P<0.001$). However, cavernosum tissue weights (mg) did not significantly differ (17.4 ± 0.6 vs 19.4 ± 1.5). For NTN−/− mice, body and cavernosum weights did not significantly differ compared to their respective wild-type controls (25.9 ± 0.7 g and 17.7 ± 0.5 mg, $n=11$, vs 26.9 ± 0.8 g and 19.1 ± 0.6 mg, $n=13$). Despite similar housing and husbandry, wild-type mice from the GFRα2 colony were significantly heavier than wild-type mice from the NTN colony ($P<0.001$). The reason for this discrepancy did not reflect variations in age (111.8 ± 11.8 for GFRα2 and 96.7 ± 3.1 days for NTN wild types, respectively).

In vitro *pharmacology*

Electrical stimulation of corpus cavernosum elicited frequency-dependent contractions (Figure 1a) that were abolished by guanethidine (3 μM) or tetrodotoxin (1 μM), indicating that they were owing to activation of noradrenergic nerves. Expressed relative to tissue weight, responses of cavernosum from NTN knockout animals tended to be slightly greater than those of wild types; however, this did not achieve statistical significance (maximum tensions at 30 Hz: 0.034 ± 0.004 mN mg^{-1}, $n=4$, vs 0.025 ± 0.004 mN mg^{-1}, $n=5$). Similarly, responses from GFRα2−/− mice did not significantly differ from their wild-type littermates (0.027 ± 0.005, $n=4$, vs

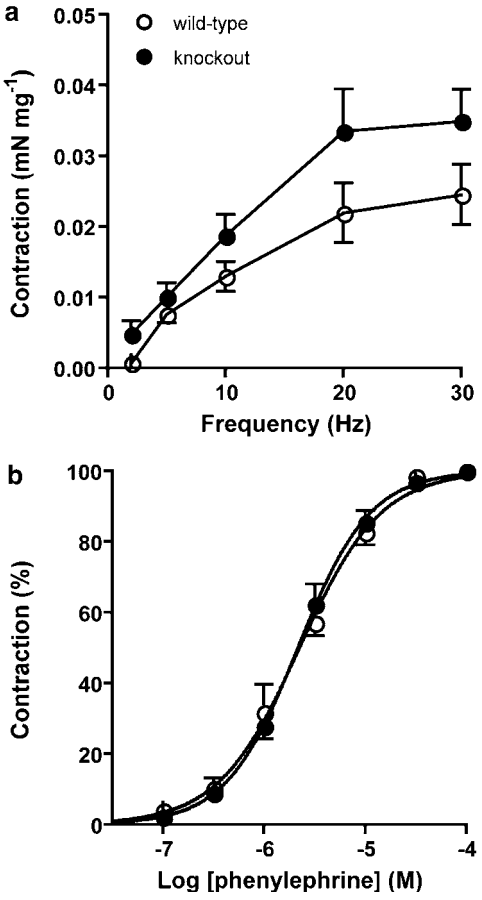

Figure 1 Contractile responses of cavernosal tissues. (a) Frequency–response curves for contraction to electrical stimulation (10 V, 2–30 Hz). (b) Cumulative concentration–response curves for contraction to phenylephrine. Wild type (a, $n=4$; b, $n=6$); NTN knockout (a, $n=5$; b, $n=7$). There were no significant differences between tissues from wild-type and NTN knockout mice ($P>0.05$).

0.025 ± 0.006, $n=7$). In addition, contractile responses to phenylephrine (Figure 1b) were not altered in NTN knockout animals; maximum tension (mN) and sensitivity ($-\log EC_{50}$) were 0.66 ± 0.06 and 5.66 ± 0.07 M, $n=7$, for knockout mice, and 0.57 ± 0.09 and 5.66 ± 0.09 M, $n=6$, for wild types, respectively. Similar results were noted for GFRα2 knockouts and their wild types (0.51 ± 0.04 and 5.59 ± 0.07 M, $n=6$, vs 0.64 ± 0.14 and 5.75 ± 0.05 M, $n=4$).

Electrical stimulation of penile erectile tissue, following phenylephrine contraction in the presence of guanethidine (3 μM), produced frequency-dependent relaxations (Figures 2 and 3) that were abolished by tetrodotoxin (1 μM). Maximum relaxation (% reduction of phenylephrine pre-contraction produced at 20 Hz stimulation) was markedly attenuated in tissues from NTN$-/-$ mice (Figures 2b, c and 3a) compared to wild-type controls (Figures 2a and 3a; 19.4 ± 2.8, $n=8$, vs $71.7\pm6.6\%$, $n=6$, $P<0.001$). Similarly, responses from GFRα2$-/-$ mice were markedly reduced compared to their wild-type littermates (10.5 ± 2.1, $n=2$, vs $83.3\pm10.9\%$, $n=3$). Typically, neurogenic relaxations from wild-type tissues reached maximal amplitude within 10–30 s of stimulation. In contrast, maximal relaxations from NTN$-/-$ and GFRα2$-/-$ cavernosum had a slower onset, requiring between 60 and 90 s of stimulation. In many cases, the modest size of the knockout responses was difficult to distinguish from background tissue activity (see Figure 2b). Whereas atropine (1 μM) caused a modest reduction in the maximum relaxation achieved by tissues from wild-type mice (NTN to $61.1\pm6.5\%$, $P<0.01$; GFRα2 to $61.7\pm9.8\%$, $P<0.05$), it abolished relaxations in tissues from NTN and GFRα2 knockout mice (Figures 2b, c and 3a; only NTN is shown). In cavernosum from wild-type mice, the nitric oxide synthase inhibitor, L-NNA (10 μM), abolished the remaining neurogenic component in five out of seven tissues (NTN, three of four; GFRα2, two of three), with modest responses detected in the remaining two strips ($\sim$5–15% at 10–20 Hz). In tissues from NTN knockout mice, neostigmine (10 μM) augmented the maximum nerve-evoked relaxation more than three-fold compared to wild types (to $62.7\pm6.1\%$, $P<0.001$; Figures 2c and 3b). In cavernosum from heterozygous ($+/-$) NTN mice, maximal neurogenic relaxations (20 Hz) did not significantly differ from wild-type controls ($66.7\pm8.2\%$, $n=6$) and similarly, atropine led to a modest reduction in maximal relaxation ($50.3\pm9.0\%$, $P<0.01$). Therefore, deletion of both copies of the NTN gene causes complete loss of nitrergic and partial loss of cholinergic transmission to the cavernosum, but deletion of one copy of the NTN gene appears to have no significant effect on either component of neurotransmission.

Because nitrergic neurotransmission was absent in the cavernosal tissue from NTN knockout animals, we investigated whether the muscle was capable of responding to nitric oxide. Endothelium-independent relaxation to the nitric oxide donor, sodium nitroprusside (Figure 4a), following phenylephrine pre-contraction, did not significantly differ between groups, indicating that the cavernosum from NTN knockout animals was able to respond normally to nitric oxide. Maximum relaxations and sensitivity ($-\log EC_{50}$) were $65.8\pm6.2\%$ and 6.07 ± 0.10 M, $n=6$, for NTN knockout mice, and $65.7\pm7.1\%$ and 6.05 ± 0.13 M, $n=6$, for wild types, respectively. Similarly, responses to nitroprusside between GFRα2$-/-$ mice and their wild types did not significantly differ ($64.3\pm3.7\%$ and 6.19 ± 0.09 M, $n=6$, vs $64.5\pm8.3\%$ and 6.28 ± 0.15 M, $n=4$). Furthermore, responses to bradykinin, which causes endothelium-dependent nitric oxide-mediated relaxation of erectile tissue (Lopes-Martins *et al.*, 1994; Teixeira *et al.*, 1998), did not significantly differ between NTN$-/-$ mice and their corresponding wild types (Figure 4b). Maximum relaxations following bradykinin (300 μM) were $48.6\pm7.4\%$, $n=4$ (NTN$-/-$) and $55.1\pm15.8\%$, $n=4$ (wild type). These responses were abolished by L-NNA. Therefore, the capacity of the endothelium to generate nitric oxide does not appear to be altered in NTN knockout mice.

We then examined the response to cholinoceptor agonists, which cause relaxation by activation of eNOS and the release of nitric oxide from the endothelium (Andersson & Wagner, 1995). As with bradykinin, acetylcholine and muscarine produced relaxations of wild-type cavernosum, following phenylephrine pre-contraction (Figure 5a and c). These relaxations were abolished by incubation with L-NNA (10 μM). In cavernosum from NTN$-/-$ mice (Figure 5b and c), there was a significant increase of greater than 0.5 log units in sensitivity to acetylcholine, compared to wild-type controls ($(-\log- EC_{50})$: 7.40 ± 0.06, $n=5$, vs 6.86 ± 0.17 M, $n=7$, $P<0.05$). Responses to muscarine also showed a significant increase in sensitivity of 0.4 log units in tissues from NTN knockout animals compared to wild types ($(-\log EC_{50})$:

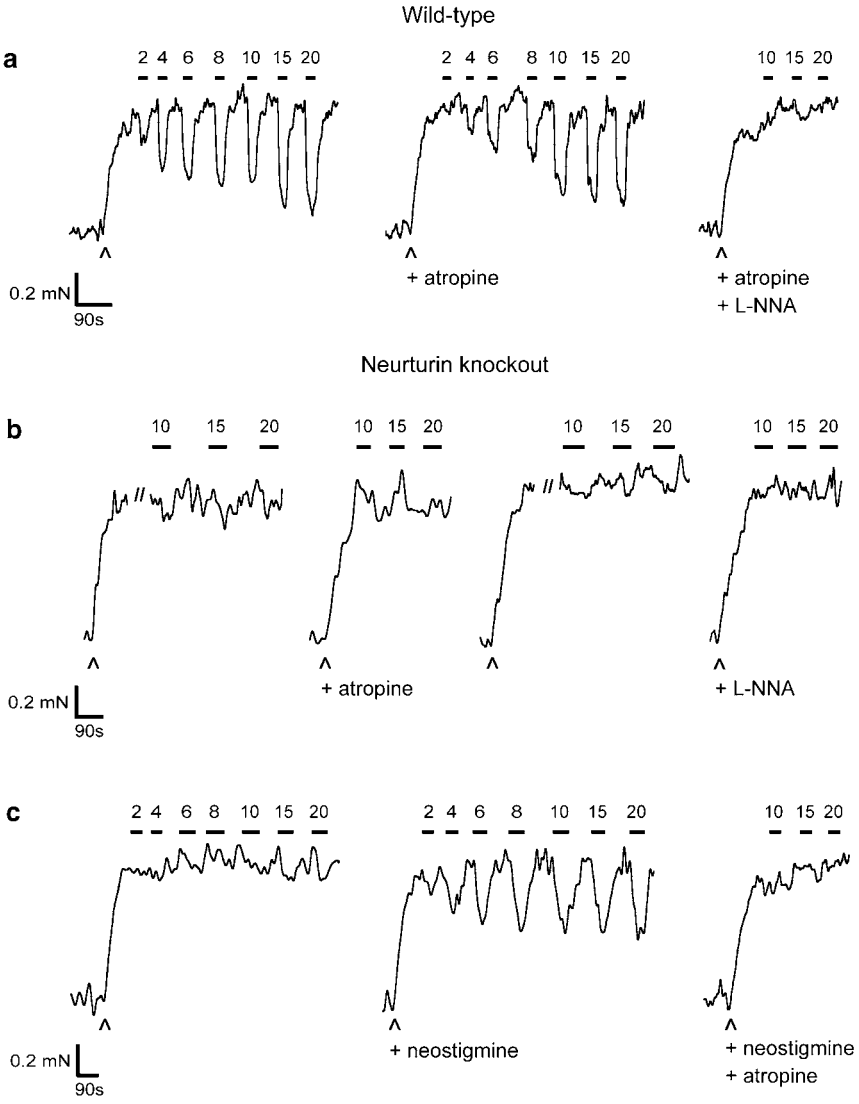

Figure 2 Nerve-evoked relaxation responses of cavernosal tissues. (a–c) Relaxations to nerve stimulation (2–20 Hz, duration indicated by bar length), following phenylephrine (10 μM) pre-contraction in the presence of guanethidine (3 μM), of corpus cavernosum from wild-type (a) and NTN knockout mice (b and c). Atropine (1 μM) caused a reduction (a) or abolition (b) of the nerve-evoked response, which is demonstrated here in the presence of neostigmine (10 μM, c) in tissues from NTN knockout mice. In tissues from wild-type and NTN knockout mice, the response was inhibited by L-NNA (10 μM) with or without atropine coincubation. Between each trace, tissues were washed and drugs applied for 15 min before phenylephrine pre-contraction ($\wedge$ indicates application, // indicates excision of responses to <10 Hz from original traces).

7.87 ± 0.09, $n=4$, vs 7.47 ± 0.08 M, $n=4$; $P<0.01$; Figure 5d). Similar shifts in EC_{50} were noted for GFRα2$-/-$ cavernosum against their wild-type controls (acetylcholine: 7.36 ± 0.23, $n=3$, vs 6.85 ± 0.23, $n=4$, but $P>0.05$; muscarine: 7.71 ± 0.14, $n=3$, vs 7.33 ± 0.01, $n=3$, $P<0.05$). In contrast, maximum responses to cholinoceptor agonists did not significantly differ between NTN or GFRα2 knockouts and their respective wild types (acetylcholine: 79.4 ± 3.3 and 81.1 ± 1.8 vs 66.8 ± 5.9 and 82.8 ± 12.2%, respectively; muscarine: 93.4 ± 6.5 and 97.1 ± 10.1 vs 85.1 ± 10.1 and 97.5 ± 6.7%, respectively). The maximal relaxation and sensitivity of cavernosal tissues to acetylcholine did not significantly differ between NTN wild-type and heterozygous animals (72.9 ± 7.4% and 6.99 ± 0.10 M, $n=5$). In summary, this group of experiments showed that under conditions where nitrergic neurotransmission is absent and cholinergic transmission is compromised, there appears to be a compensatory increase in tissue sensitivity to exogenous muscarinic ligands.

Immunohistochemistry

Arterioles of the corpus cavernosum were surrounded by TH-IR varicosities, which formed dense perivascular plexuses (Figure 6). As previously reported (Mizusawa *et al*., 2001), VAChT, nNOS and VIP immunoreactivities were coexpressed, whereas TH-IR fibres expressed none of these other substances but were frequently located in close proximity to these fibres (Figure 6). Although both types of nerve fibres were also interspersed in the trabecular smooth muscle of the erectile tissue, TH-IR fibres were more prevalent. In penis sections from NTN$-/-$ and GFRα2$-/-$ mice, no apparent changes in TH-IR were observed (Figure 6; NTN$-/-$ only); however

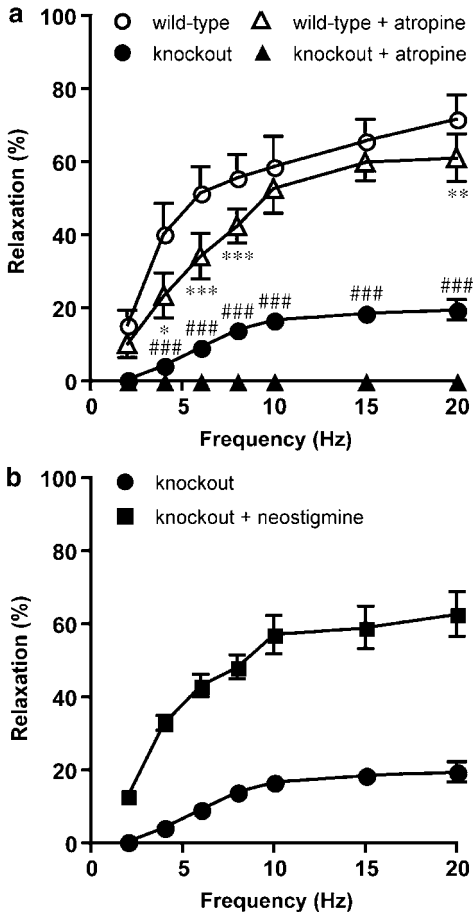

Figure 3 Frequency–response curves for nerve-stimulated tissues from wild-type ($n=6$) and NTN knockout mice ($n=8$). (a) Responses between 4 and 20 Hz were significantly reduced in NTN knockout animals ($^{###}P<0.001$). Atropine significantly attenuated wild-type responses at 4, 6, 8 and 20 Hz ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) and abolished NTN knockout responses. (b) Frequency–response curves for nerve-stimulated tissues from NTN knockout mice before and after neostigmine incubation ($P<0.001$ for all frequencies).

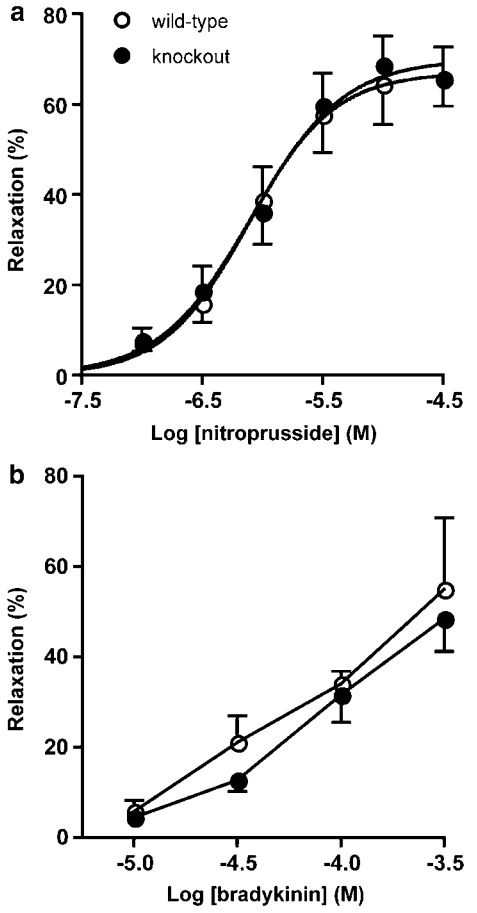

Figure 4 Relaxation responses of cavernosal tissue to sodium nitroprusside and bradykinin. (a) Cumulative concentration–response curves to sodium nitroprusside. (b) Non-cumulative concentration responses to bradykinin. Both agonists were tested following phenylephrine (10 μM) pre-contractions. Wild-type mice (a, $n=6$; b, $n=4$); NTN knockout mice (a, $n=6$; b, $n=4$). There were no significant differences between responses of wild-type and knockout mice ($P>0.05$).

there was a profound loss of VAChT, nNOS and VIP-IR, which in most sections were rare or absent (Figures 6 and 7; NTN−/− only).

This dramatic loss of nitrergic/cholinergic nerves in knockout mice was not readily detectable when the total nerve population was immunostained using PGP 9.5-IR (a pan-neuronal marker). This is likely to be because the majority of nerves are TH-IR and are unaffected by either gene deletion. Numerous PGP 9.5-IR nerves were observed in the major dorsal nerve bundles, but the vast majority of these structures did not express TH- or VAChT/nNOS/VIP-IR and are presumably sensory afferents of the pudendal nerve. There was no obvious effect of gene deletion on the dorsal nerve immunostaining or general structure of tissue sections.

Endothelial NOS-IR was detected in many cell types within the penis. Endothelial NOS-IR was located in the endothelium and smooth muscle of small arterioles. A modest level of eNOS expression was also observed within the dorsal nerve bundles, possibly reflecting their vascular supply and/or glial expression. Relatively rich immunostaining was observed within the urethral lining of the corpus spongiosum. No differences in eNOS-IR distribution or intensity were observed between NTN or GFRα2 knockout mice and their respective wild types.

Discussion

This study has elucidated the functional implications of impaired penile innervation due to loss of NTN signalling. The profound anatomical effects of this loss, namely reduced innervation by nitrergic/cholinergic nerves, was confirmed in both NTN and GFRα2 knockout mice. However, our results show that this deficit appears to be partly compensated for by an increased sensitivity to muscarinic receptor agonists. Although this compensation is relatively modest in the context of its impact on neurotransmission (owing to the small number of nerves present in the cavernosal tissues from the knockout animals), it leads to impressive relaxations of cavernosal tissues in response to exogenous agonists. This is likely to reflect an increased responsiveness at the level of the endothelium, as nitric oxide-mediated endothelium-dependent bradykinin-induced vasodilatation and smooth muscle respon-

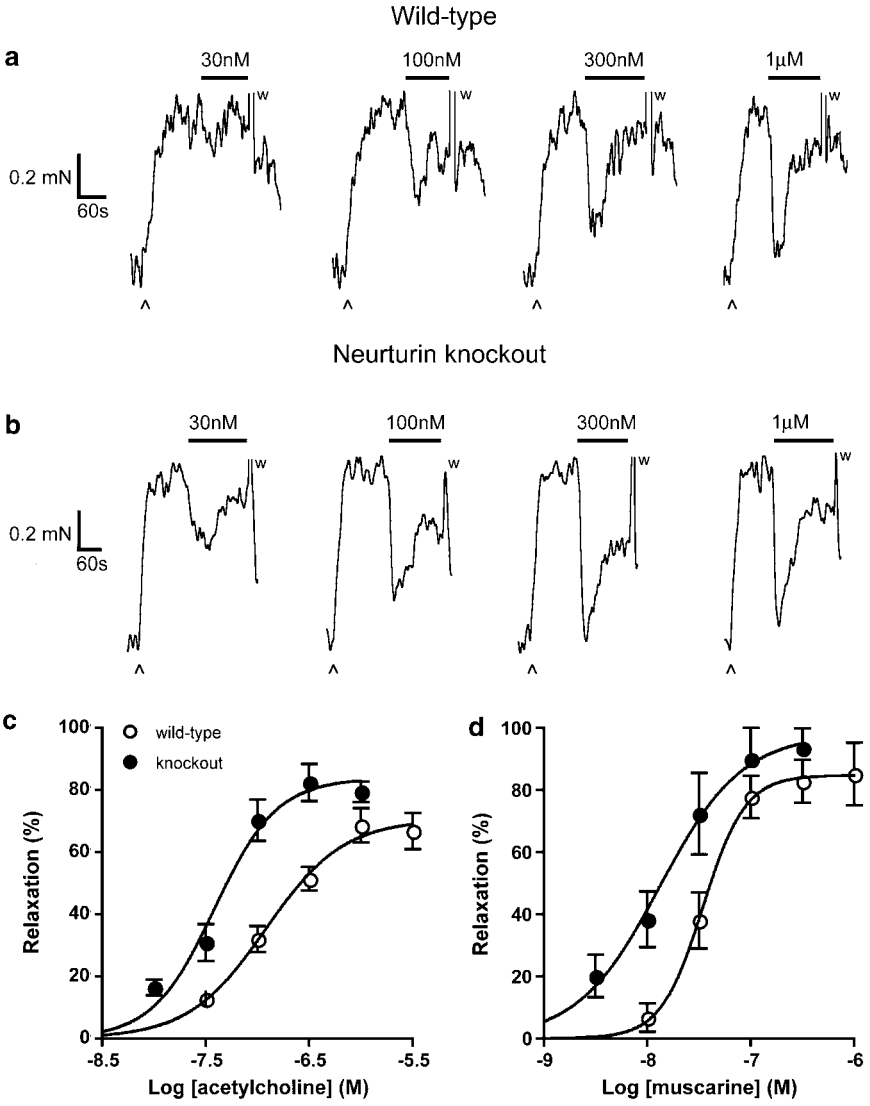

Figure 5 Relaxation responses of cavernosal tissue to acetylcholine and muscarine. (a and b) Concentration-dependent relaxations to acetylcholine, following phenylephrine (10 μM) pre-contraction, of corpus cavernosum from wild-type (a) and NTN knockout (b) mice. Between each trace, tissues were washed until resting tension was restored and were equilibrated for a further 10 min (w indicates commencement of wash period). (c and d) Non-cumulative concentration–response curves to acetylcholine (c) and muscarine (d). Wild-type mice (c, $n=7$; d, $n=4$); NTN knockout mice (c, $n=5$; d, $n=4$). For both (c) and (d), EC_{50} is significantly different between wild types and NTN knockouts (c, $P<0.05$; d, $P<0.01$).

siveness to the nitric oxide donor, sodium nitroprusside, were unaltered in NTN or GFRα2 knockout mice.

Sympathetic noradrenergic nerves provide tonic contraction of corpus cavernosum smooth muscle, maintaining penile flaccidity (Andersson & Wagner, 1995). Immunoreactivity for TH revealed an apparently normal development of sympathetic nerves in the cavernosum of mice lacking NTN, as has been demonstrated for GFRα2 knockouts (Laurikainen *et al.*, 2000a). In addition, noradrenergic nerve-mediated contractions of corpus cavernosum in response to electrical stimulation, and smooth muscle responsiveness to phenylephrine, did not significantly differ between NTN- and GFRα2-deficient mice and their wild-type controls. This suggests that there is no significant compensatory effect of either gene deletion on sympathetic nerves, despite the profound effect on parasympathetic nerves.

Neurogenic relaxations in the presence of guanethidine were markedly attenuated in cavernosum from NTN and GFRα2 gene-deficient mice, compared to wild-type controls. This concurs with the greatly reduced density of cholinergic/nitrergic nerve fibres, as demonstrated by immunostaining for nNOS, VAChT or VIP. As these relaxations were abolished by coincubation with atropine, this suggested that at least functionally, neuronal-derived nitric oxide was absent and that the residual component was mediated by acetylcholine released from parasympathetic nerves. In the endothelium of cavernous tissue, the M_3 cholinergic receptor subtype predominates (Traish *et al.*, 1995); its activation evokes calcium influx, which, in turn, activates eNOS and causes dilatation of the adjacent cavernosal smooth muscle *via* nitric oxide production (Andersson & Wagner, 1995; Andersson, 2001). Indeed, acetylcholine-mediated relaxations of cavernous

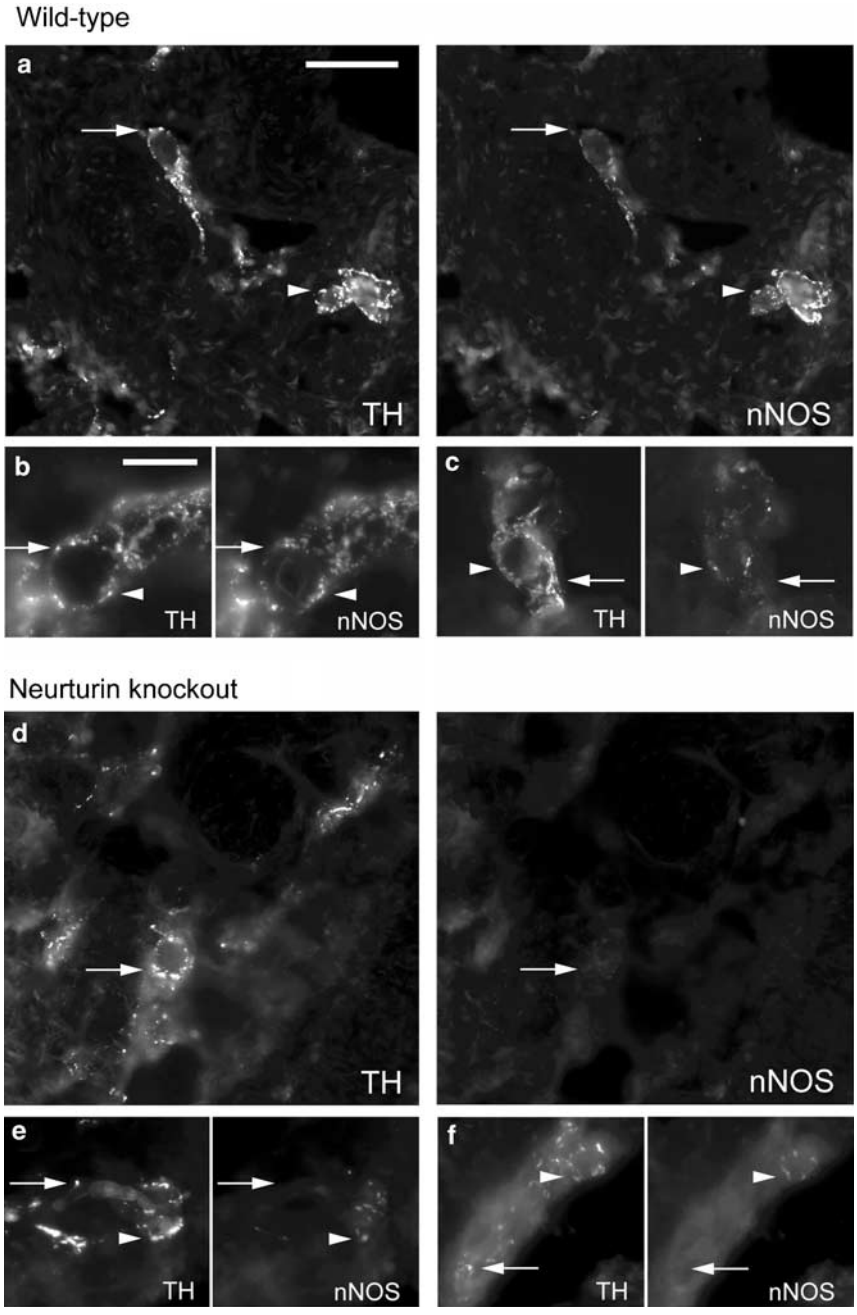

Figure 6 TH and nNOS immunoreactivity in corpus cavernosum from wild-type and NTN knockout mice. Matching arrows indicate examples of TH-positive, but nNOS-negative, immunostaining (surrounding blood vessels in a, b, d and e). Matching arrowheads indicate examples of adjacent TH- and nNOS-positive nerve fibres, but no coexpression of the two markers (confirmed by focusing under a high-power objective). Scale bars = 50 μm in (a) and (d), 20 μm in (b), (c), (e) and (f).

tissue are abolished by chemical disruption of the endothelium (Okamura *et al*., 1999). In support of this mechanism, the residual neurogenic component in knockouts was also abolished by the NOS inhibitor, L-NNA, and was augmented more than three-fold by the cholinesterase inhibitor, neostigmine, in NTN−/− tissues. Furthermore, whereas neurogenic relaxations in wild types approached maximal amplitude well within a 30 s stimulus period, the maximal response in NTN- and GFRα2-deficient cavernosum required between 60 and 90 s stimulation, indicating possible involvement of receptor-dependent mechanisms. Whereas neuronal nitric oxide is released and stimulates guanylyl cyclase independent of membrane-bound receptors (Ghalayini, 2004), cholinergic neurotransmission requires vesicular release and endothelial cholinoceptor activation; these processes are likely to slightly delay the onset of smooth muscle relaxation relative to wild types.

In wild-type cavernosum, atropine caused only modest reductions in neurogenic relaxation, indicating that under normal physiological conditions direct neural release of nitric oxide, rather than an indirect activation of the cavernous endothelium, is the key mechanism by which penile erection is initiated. In support of this, intracavernous pressure readings evoked by penile nerve stimulation in anaesthetised dogs are

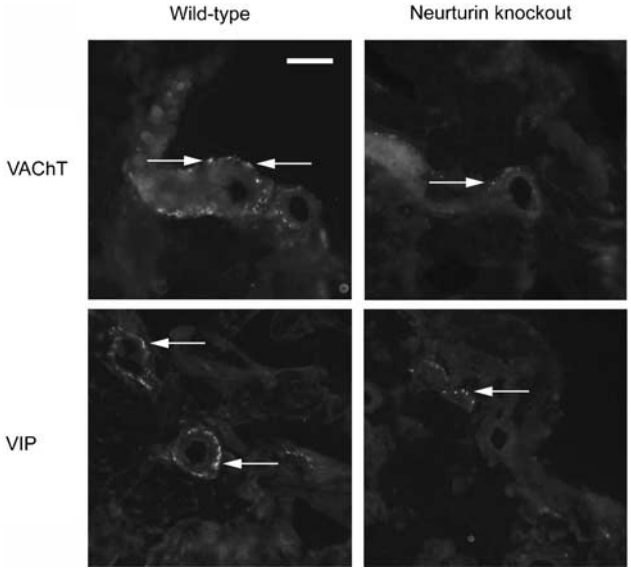

Figure 7 VAChT- and VIP-immunoreactivity in corpus cavernosum from wild-type and NTN knockout mice. IR axons are indicated by arrows and shown surrounding blood vessels in a–c. Each panel shows a single-stained, separate field. Scale bars = 20 μm for all micrographs.

only partially reduced (Trigo-Rocha *et al*., 1993), or unaltered (Ayajiki *et al*., 1997), by atropine. Furthermore, no changes in neurogenic relaxations of isolated monkey erectile tissue occur following muscarinic blockade (Toda *et al*., 2005), and in rats, intracavernous injection of acetylcholine does not produce full penile erection (Suh *et al*., 1995).

Our data strongly suggest that muscarinic cholinoceptor signalling is enhanced in cavernosum of NTN and GFRα2 mice, possibly as a compensatory mechanism in response to loss of neuronal nitrergic signalling. This could be owing to increased expression of muscarinic receptors or increased efficacy of muscarinic signalling pathways in these tissues. As bradykinin-induced relaxations did not differ significantly between NTN knockouts and wild-type controls, there is no evidence that the increased response to acetylcholine or muscarine is owing to increased eNOS expression or activity. This is further supported by our immunohistochemical data showing no obvious differences in eNOS expression between NTN or GFRα2 knockouts and wild types. Responsiveness to nitroprusside was also unaffected by gene ablations, indicating that the ability of the smooth muscle to respond to nitric oxide was not altered.

Physiological compensation mechanisms can account for retained penile erection in mice with gene mutations against recognised erectile promoters. For example, in nNOS gene-deficient mice, upregulation of eNOS has been proposed to retain nitric oxide-dependent functions (Burnett *et al*., 1996; Nangle *et al*., 2004), although cavernous pressure in response to penile nerve stimulation is reduced (Cashen *et al*., 2002). Furthermore, cavernosum isolated from nNOS- and eNOS-deficient mice develop increased sensitivity to nitroprusside, and an L-NNA-resistant neurogenic relaxation is revealed in the nNOS knockouts, whereas L-NNA-sensitive relaxation in eNOS knockouts is increased (Nangle *et al*., 2004). Of particular interest, nNOS/eNOS double-deficient mice display a priapic (prolonged erection) phenotype that has been linked to changes in cGMP-specific phosphodiesterase activity (Champion *et al*., 2005). It is therefore possible that in addition to upregulation of muscarinic mechanisms, the NTN and GFRα2 knockout mice also exhibit compensatory increases in other 'pro-erectile' signalling pathways, such as VIP. Of interest, neurogenic and acetylcholine-mediated responses were comparable in penile tissues from heterozygous NTN mice and their wild types. This demonstrates that the presence of just one allele is sufficient for preservation of parasympathetic nerve function and suggests that in these mice there is no drive to upregulate the muscarinic pathway.

Supersensitivity to muscarinic receptor stimulation has been observed previously in a number of denervated smooth muscle preparations (Chiu-Wei *et al*., 1984; Hasegawa *et al*., 1987; Braverman *et al*., 1998). For example, this occurred in bladder detrusor and urethral smooth muscle of rats following bilateral pelvic ganglionectomy (Ekstrom & Malmberg, 1984a, b); in this case, sensitivity to nitric oxide is unaffected (Persson *et al*., 1998). In the present study, the increased sensitivity to cholinoceptor agonists was not induced by surgical denervation but correlated with a developmental defect in parasympathetic penile innervation. The mechanism underlying this defect is not known, but could be owing to either an early innervation of the penis that is not maintained (due to absence of the appropriate neurotrophic factor) or a failure in the initiation of axon growth towards the penile tissue. The first case could be viewed similarly to denervation supersensitivity, but the second would resemble more closely the 'pre-innervation supersensitivity' observed in the sympathetic innervation of the rodent vas deferens (MacDonald & McGrath, 1984). Further studies on the developmental mechanisms underlying penile innervation and plasticity will be very valuable.

In addition to NTN, the development and maintenance of parasympathetic penile innervation is likely to involve a number of other neurotrophic factors. Although the vast majority of penis-projecting neurons originating from the major pelvic ganglia express GFRα2, 1 and 3 are also expressed, but to a lesser extent (Laurikainen *et al*., 2000a, b). This raises the possibility that GDNF and artemin, the preferred ligands for these receptors, also play a role. There is already good evidence that penile neurons can retrogradely transport GDNF, but so far artemin has not been explored further. Recently, neurotrophin-3 has also been postulated as a target-derived neurotrophic factor for penile parasympathetic neurons (Hiltunen *et al*., 2005), and distinguishing the mechanisms of action and interaction of these various growth factors on penile nerve development, maintenance and responses to injury will be challenging but informative.

In conclusion, mice lacking the neurotrophic factor, NTN, or its preferred receptor, GFRα2, have marked loss of their penile nitrergic/cholinergic nerves, which in turn alters nitric oxide-dependent erectile functions. Nitrergic neuronal responses are severely attenuated, but endothelial sensitivity to muscarinic agonists is increased, partially compensating for a lack of functionally detectable nerve-released nitric oxide. This compensation is likely to be a major contributor to the retained fertility of these mice. The role of NTN in maintaining the optimum structure and function of parasympathetic penile innervation is likely to provide critical clues into understanding possible regenerative mechanisms in this part of the nervous system. Future studies of NTN-deficient animals may also reveal further changes in compensatory mechanisms

within pelvic organs, suggesting potential therapeutic targets for restoring reflex function after injury or disease.

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