Evidence for increased tissue androgen sensitivity in neurturin knockout mice

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Abstract

Neurturin (NTN) is a member of the glial cell line-derived neurotrophic factor (GDNF) family and signals through GDNF family receptor alpha 2 (GFRα2). We hypothesised that epithelial atrophy reported in the reproductive organs of Ntn (Nrtn)- and Gfra2 (Gfra2)-deficient mice could be due to NTN affecting the hormonal environment. To investigate this, we compared the reproductive organs of Ntn- and Gfra2-deficient male mice in parallel with an analysis of their circulating reproductive hormone levels. There were no significant structural changes within the organs of the knockout mice; however, serum and intratesticular testosterone and serum LH levels were very low. To reconcile these observations, we tested androgen sensitivity by creating a dihydrotestosterone (DHT) clamp (castration plus DHT implant) to create fixed circulating levels of androgens, allowing the evaluation of androgen-sensitive endpoints. At the same serum DHT levels, serum LH levels were lower and prostate and seminal vesicle weights were higher in the NTN knockout (NTNKO) mice than in the wild-type mice, suggesting an increased response to androgens in the accessory glands and hypothalamus and pituitary of the NTNKO mice. Testicular and pituitary responsiveness was unaffected in the NTNKO males, as determined by the response to the human chorionic gonadotrophin or GNRH analogue, leuprolide, respectively. In conclusion, our results suggest that NTN inactivation enhances androgen sensitivity in reproductive and neuroendocrine tissues, revealing a novel mechanism to influence reproductive function and the activity of other androgen-dependent tissues.

Key Words
- glial cell line-derived neurotrophic factor (GDNF) family
- TGFβ family
- neurotrophic
- hypothalamic-pituitary-testicular axis
- urogenital tract

Introduction

Neurotrophic factors control the development of peripheral nerve circuits by promoting the survival of sensory and motor neurons and targeting their axons to form functional connections (Huang & Reichardt 2003, da Silva & Wang 2011). Neurotrophic factors and their receptors continue to be expressed in adults and are important mediators of axon regeneration after injury (Chen et al. 2007, Navarro et al. 2007). They also have important effects on neurophysiological properties. For example, in nociceptive sensory neurons, many neurotrophic factors promote sensitisation, which underlies inflammatory and neuropathic pain states (Pezet & McMahon 2006).

Neurturin (NTN) is a member of the glial cell line-derived neurotrophic factor (GDNF) family of
neurotrophic factors (Buj-Bello et al. 1995, Kotzbauer et al. 1996, Airaksinen & Saarma 2002) that is produced by many peripheral tissues, including the reproductive organs (Golden et al. 1999, Xian et al. 1999, Widenfalk et al. 2000, Meng et al. 2001). The GDNF family of factors mediate their actions through a receptor complex consisting of a ligand, a high-affinity binding component, a GPI-linked GDNF family receptor alpha (GFRα) and a common signalling component, a receptor tyrosine kinase, Ret (Airaksinen & Saarma 2002). From four GFRα subunits, GFRα2 functions as a specific NTN receptor, and mice deficient in Ntn (Ntn(a) or Gfra2 (Gfra2) bear similar phenotypes (Heuckeroth et al. 1999, Rossi et al. 1999, Wanigasekara et al. 2004).

The best-known action of NTN on reproduction is via its trophic effects on pelvic parasympathetic neurons (Wanigasekara et al. 2004, Yan & Keast 2008). These autonomic neurons are essential for initiation of penile erection and stimulation of glandular secretion. For example, penile erection is highly impaired in adult mice deficient in Ntn or its receptor, Gfra2, largely due to many fewer parasympathetic axons making connections with the cavernosal vessels during the early postnatal period (Laurikainen et al. 2000, Nangle & Keast 2006). NTN also has powerful direct trophic effects on adult pelvic parasympathetic neurons (Laurikainen et al. 2000, Wanigasekara & Keast 2005, Bella et al. 2007), so reduced NTN signalling in adulthood would further impair signalling to their target organs.

Studies of Ntn- or Gfra2-deficient mice have also revealed epithelial atrophy of male accessory sex organs (Wanigasekara et al. 2004), and although it is generally believed that the mechanism may be neurotrophic, the specific mechanism of this defect has not been defined. In these mice, the parasympathetic innervation of glandular tissue fails to develop properly, so it is possible that a loss of normal, regular neuronal activation leads to cellular loss. However, NTN may have a more direct action on these and other non-neuronal tissues. This is supported by the expression of NTN, GFRα2 and Ret in the testis, prostate, pituitary, and hypothalamus (Widenfalk et al. 1997, 2000, Golden et al. 1998, 1999, Xian et al. 1999, Meng et al. 2001). Moreover, there is a transient disruption of spermatogenesis in NTN-overexpressing mice (Meng et al. 2001), NTN stimulates DNA synthesis in spermatogonia (Viglietto et al. 2000) and GFRα2-positive cells are important for the differentiation of secretory pituitary cells including the gonadotrophin-secreting cells (Garcia-Lavandeira et al. 2009).

The initial goal of our study was to determine whether NTN maintains epithelial integrity of male accessory sex organs by regulating their hormonal environment. To investigate this, we compared the reproductive organs of Ntn knockout (NTNKO) and Gfra2 knockout (GFRα2KO) mice, in parallel with an analysis of their circulating sex steroid hormone levels. The results from this study show an unexpected mismatch between androgen-dependent accessory sex organs and androgen environment, prompting us to investigate whether these mice had the ability to respond normally to hormonal stimulation. Our results also show that NTN deprivation leads to a heightened response to androgens. This reveals a novel and powerful way for NTN to influence not only reproductive function but also the activity of other androgen-sensitive tissues. It also provides an additional mechanism by which NTN may influence androgen-sensitive peripheral neurons that regulate the function of male reproductive organs.

Materials and methods

Mouse colonies and sample collection

The generation of NTNKO and GFRα2KO mice has been described elsewhere (Heuckeroth et al. 1999, Rossi et al. 1999). The mice were genotyped as described previously (Rossi et al. 1999, Enomoto et al. 2000). The mice were killed by cardiac exsanguination under anaesthesia (60 mg/kg ketamine and 10 mg/kg xylazine, i.p.), and serum was stored frozen at −20°C. Male reproductive organs analysed included prostate lobes, seminal vesicles (SVs) and testes. Prostate lobes (ventral prostate (VP), dorsolateral prostate (DLP) and anterior prostate (AP)), SVs and testes were dissected free of fat and connective tissue and weighed separately. Non-reproductive organs (kidney, spleen and heart) were also weighed. Prostate tissues were either snap−frozen with liquid nitrogen and stored at −80°C for RNA extraction or fixed in Bouin’s solution for 4 h at room temperature for studying histology. The NTNKO and GFRα2KO males were compared with their respective wild-type (WT) littermates. All procedures were approved by the Animal Care and Ethics Committee of the Royal North Shore Hospital and University of Sydney.

Experimental design

Experiment 1: intact males

Sexually mature (12±4 (mean ± s.d.) weeks of age) homozygous NTNKO and GFRα2KO male mice were compared with their respective WT littermates. Endpoints analysed were serum
testosterone and gonadotrophins as well as weights of male reproductive organs and selected non-reproductive organs (see above).

**Experiment 2: androgen sensitivity**  Androgen sensitivity in the male mice was determined following castration to remove the endogenous source of androgens and treatment with fixed androgen levels produced by dihydrotestosterone (DHT) implants (DHT clamp; Simanainen et al. (2011)). DHT was chosen as a non-aromatisable androgen. The WT and NTNKO male mice were orchidectomised via scrotal sac under ketamine/xylazine anaesthesia (anaesthetic doses as given above) and treated by subdermal implantation of 0.5 cm silastic tubing filled with ~5 mg crystalline DHT (Rossi et al. 1999, Simanainen et al. 2009) for 7 days. Treatment for 7 days was chosen based on previous experience in analysing androgen sensitivity using the same type of DHT implants (Simanainen et al. 2011). Endpoints analysed were serum DHT, 3α-diol and 3β-diol and gonadotrophins as well as male reproductive organ weights (organs listed above).

**Experiment 3: testicular and pituitary responsiveness**  The WT and NTNKO male mice were administered an i.p. injection of 10 IU human chorionic gonadotrophin (hCG; Pregnyl, Organon Pty Ltd, Lane Cove, NSW, Australia) in saline to analyse the testicular responsiveness to LH/hCG (Ingman & Robertson 2007) or with 1 μg of the GnRH analogue, leuprolide (Lucrin, Abbott), to analyse the pituitary and testicular responsiveness to GnRH stimulation (Handelsman et al. 1985a, Bergh & Damber 1988). Serum and tissues were collected 1 h after injection. Endpoints analysed were serum LH (leuprolide) and testosterone (hCG and leuprolide).

**Histology and immunohistochemistry**

Testes were embedded in resin, and representative sections were analysed after 0.5% toluidine blue staining of 5 μm sections. Prostates were embedded in paraffin, cut into 5 μm sections and stained with haematoxylin and eosin (H&E).

Immunohistochemistry for AR was performed on 5 μm dewaxed paraffin sections. Rabbit anti-AR (N-20; 1:100 dilution; Santa Cruz Biotechnology, Inc.) antibody was used to detect AR (Mulholland et al. 2011). Microwave-induced (high-power) antigen retrieval was performed with 0.01 M citrate buffer, pH 6, for 12 min. Signal was visualised using a Vectastain Elite anti-rabbit ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. Colour development was done using the 3,3′-diaminobenzidine tetrahydrochloride chromogenic substrate (Dako Australia Pty Ltd, Campbellfield, VIC, Australia). Negative control sections were incubated with non-immune rabbit IgG (Santa Cruz Biotechnology). The sections were counterstained with Harris haematoxylin.

Cell proliferation was determined using a proliferating cell nuclear antigen (PCNA) kit (Zymed, San Francisco, CA, USA). This kit provides ready-to-use biotinylated anti-PCNA primary antibodies, which were used according to the manufacturer’s instructions. Microwave antigen retrieval was done using 10 mM citric acid buffer (pH 6) for 10 min at high power. Negative control sections were incubated with non-immune mouse IgG (Santa Cruz Biotechnology). Mouse small intestine sections were used as positive controls. Prostate epithelial proliferating cell index and prostate epithelial height were quantified using the CASTGRID V1.10 (Olympus Corp., Albertslund, Denmark) software as described previously (Simanainen et al. 2007).

Mean tubular diameter per testis was determined from fixed testes embedded in methacrylate resin, sectioned and stained with 0.5% toluidine blue as described previously. Mean tubular diameter per testis was calculated using the Cast software (Olympus) to trace the perimeter of 80–100 tubular cross sections, where each tubular diameter was derived from the formula \( P = \pi d \).

**Hormone assays**

**Serum**  Mouse serum LH levels were measured by an immunofluorometric assay as described previously (Jimenez et al. 2005), using specific antibodies for mLH as validated previously (Simanainen et al. 2011). Mouse serum FSH levels were determined using a specific immunofluorometric assay as described previously (Jimenez et al. 2005). All assays were performed in a single batch.

Serum levels of testosterone, DHT, 3α-diol and 3β-diol were measured in extracts of 100 μl of mouse serum by liquid chromatography–tandem mass spectrometry (LC–MS/MS; Harwood & Handelsman 2009) as adapted for mouse serum (McNamara et al. 2010). The quantitation limits for testosterone, DHT, 3α-diol and 3β-diol were 20, 100, 400 and 400 pg/ml respectively. The diols were measured separately, but presented as a sum of 3α-diol and 3β-diol.

The androgen sensitivity index (ASI) was calculated as a product of serum LH and testosterone (Hiort et al. 2000).
with the increased androgen sensitivity demonstrated by a lower ASI value.

**Testis** Intratesticular testosterone was analysed by stable isotope dilution LC–MS/MS (Harwood & Handelsman 2009) as adapted for mouse serum and reproductive tissue (McNamara et al. 2010). Briefly, whole frozen testis was homogenised in 500 μl PBS buffer (containing 0.5% BSA (w/v) and 5 mM EDTA, pH 7.4) and centrifuged (2000 g 10 min, 4 °C) to separate insoluble debris, and then 200 μl of supernatant were extracted with 1 ml hexane:ethyl acetate (3:2 ratio) fortified with testosterone-1,2,3-d₃ (d₃-T) as the internal standard. The organic layer, separated by freezing the aqueous layer, was dried and reconstituted in 1.2 ml of 20% methanol in PBS prior to injection onto the C8 column for analysis (1 ml). In a separate experiment using radiolabelled steroid as a tracer, no detectable steroid losses or metabolism was detected when the testis homogenate was processed for analysis (data not shown), eliminating the need for a correction factor so that data are expressed as ng/testis.

**RNA extraction and real-time RT-PCR**

For RT-PCR and real-time RT-PCR, total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and cDNA was synthesised with Omniscript reverse transcriptase (Qiagen) from 250 ng of total RNA using oligo dT primers, and cDNA was then dilated 1:5 for storage at −80 °C. The primer sequences, product size and annealing temperature were as described previously (Lin et al. 2002, 2007). Histone-H3, β-actin and NTN KO and GFR a2KO males were slightly (~10%) but significantly (P=0.041) smaller than their respective WT littermates (WT vs KO (mean ± S.D.): NTN, 27.1 ± 2.7 vs 25.6 ± 2.6 g; GFR a2, 30.3 ± 2.4 vs 27.1 ± 2.5 g), so organ weights are reported relative to body weights. The relative testis weight was slightly increased (P=0.044) in both the NTNKO and GFR a2KO males compared with the respective WT controls (Fig. 1A), while the weights of epididymis and SVs were not statistically different between the genotypes (Fig. 1B and C). The weights of the separated prostate lobes were not significantly different in the NTNKO and GFR a2KO males when compared with their respective WT littermates (Fig. 1D, E and F). The weights of the non-reproductive organs (kidney, spleen and heart) in the NTNKO or GFR a2KO males did not differ from that of the non-reproductive organs in their respective WT littermates (Fig. 1G, H and I). The results for absolute organ weights (data not shown) were directionally similar to the body weight relative organ weights.

**Statistical analysis**

Statistical analysis was performed using two-way ANOVA with treatment groups and genotype as the fixed main effects. In case of non-homogenous variances (according to Levene’s test, P<0.01), the non-parametric Kruskal–Wallis ANOVA was used, followed by the Mann–Whitney U test. The statistical analysis was performed using SPSS, Inc. and NCSS (Kaysville, UT, USA) software. Data are expressed as median and 95% CIs for serum hormone levels due to high variability of serum hormones in adult male mice and as mean and S.E.M. for others unless otherwise specified. P values <0.05 were considered statistically significant.

**Results**

**Experiment 1: intact males**

**Body weights and organ weights** Adult NTNKO and GFR a2KO males were slightly (~10%) but significantly (P=0.041) smaller than their respective WT littermates (WT vs KO (mean ± S.D.): NTN, 27.1 ± 2.7 vs 25.6 ± 2.6 g; GFR a2, 30.3 ± 2.4 vs 27.1 ± 2.5 g), so organ weights are reported relative to body weights. The relative testis weight was slightly increased (P=0.044) in both the NTNKO and GFR a2KO males compared with the respective WT controls (Fig. 1A), while the weights of epididymis and SVs were not statistically different between the genotypes (Fig. 1B and C). The weights of the separated prostate lobes were not significantly different in the NTNKO and GFR a2KO males when compared with their respective WT littermates (Fig. 1D, E and F). The weights of the non-reproductive organs (kidney, spleen and heart) in the NTNKO or GFR a2KO males did not differ from that of the non-reproductive organs in their respective WT littermates (Fig. 1G, H and I). The results for absolute organ weights (data not shown) were directionally similar to the body weight relative organ weights.

**Histology** Testis morphology was not affected by NTN or GFR a2 inactivation and was similar among the NTNKO, GFR a2KO and WT males, with each testis demonstrating complete spermatogenesis and tubules containing elongated spermatids (Fig. 2A) and qualitatively normal Leydig cells (Supplementary Figure 1A and B, see section on supplementary data given at the end of this article). Tubular diameter per testis was further analysed in the WT and NTNKO males and was found to be similar between the genotypes (Supplementary Figure 1C). Prostate morphology under light microscopy with H&E staining appeared to be normal in the NTNKO and GFR a2KO males when compared with the respective WT males (Fig. 2B, shown for ventral lobe only), and the epithelial height was similar between the genotypes (Supplementary Figure 1D). Prostate epithelial cell proliferation as analysed by PCNA-positive epithelial cells (as percentage of epithelial cells counted) was similar in the NTNKO or GFR a2KO males when compared with the WT males (Fig. 3A).
Molecular analysis  
AR protein expression in prostate epithelia appeared to be similar in the KO and WT males (Supplementary Figure 1E), and the Ar mRNA levels in the prostate (VP) were not significantly modified by GFRα2 or NTN inactivation (Fig. 3B). Similarly, the mRNA levels of androgen-dependent MP25 were not significantly modified by GFRα2 or NTN inactivation (Fig. 3C). The expression of Ar and MP25 was quantified relative to that of cyclophilin and β-actin as housekeeping genes. The results were not affected by the housekeeping genes, and the data are shown relative to cyclophilin.

Steroid hormone and gonadotrophin levels
Serum testosterone was detectable in all the males, but its levels were significantly reduced in both the NTNKO (P=0.013) and GFRα2KO (P=0.029) males compared with their respective WT littermates (Fig. 4A). As has been observed for serum testosterone levels, intratesticular testosterone levels were significantly reduced in both the NTNKO (P=0.012) and GFRα2KO (P=0.05) males compared with their respective WT littermates (Fig. 4B). Similarly, intratesticular levels of DHT and DHT metabolites 3α-diol and 3β-diol were significantly lower in the NTNKO and GFRα2KO males when compared with their respective WT littermates (Supplementary Figure 2, see section on supplementary data given at the end of this article). Serum DHT levels were low but detectable in all the males and not significantly affected by the genotype (Fig. 4C). Serum and intratesticular E2 was undetectable in all the males.

Serum LH levels were significantly lower than the respective WT levels in both the GFRα2KO (P=0.012) and NTNKO (P=0.001) males (Fig. 4D), while serum FSH levels were not significantly affected by either NTN or GFRα2
inactivation (Fig. 4E). The ASI was markedly reduced in both the GFRα2KO \( (P=0.029) \) and NTNKO \( (P=0.024) \) males when compared with their respective WT littermates (Fig. 4F), indicating increased androgen sensitivity.

**Experiment 2: DHT clamp (androgen sensitivity)**

**Serum steroid hormone and gonadotrophin levels** Androgen sensitivity was further examined in the NTNKO males using the DHT clamp, where the mice were castrated to remove endogenous androgens and then provided with DHT implants to deliver comparable non-aromatisable androgen levels. DHT treatment following castration resulted in similar circulating DHT levels as well as levels of two main DHT metabolites, 3α-diol and 3β-diol (combined as diols), in the NTNKO and WT males (Fig. 5A and B), demonstrating the validity of the DHT clamp. Following castration with or without DHT implant, serum testosterone was non-detectable (Fig. 5A and B).

Serum LH levels were significantly influenced by the treatments \( (P<0.001) \). Castration increased serum LH levels almost twofold from intact WT levels in the WT and NTNKO males (Fig. 6A). DHT treatment following castration suppressed the elevated post-castration LH levels to levels comparable to those of the intact males in the WT mice, whereas the NTNKO males demonstrated much greater serum LH suppression, to less than half that of the intact WT males (Fig. 6A). The LH response to castration with or without DHT treatment was significantly \( (P=0.043) \) dependent on the genotype (WT vs NTNKO), with greater suppression being observed in the NTNKO males (significant interaction between genotype and treatment; \( P=0.005 \)).

**Organ weights** Prostate and SV weights were significantly influenced by the treatments \( (P<0.001) \) and genotype \( (P=0.03) \), with a significant interaction being observed between genotype and treatment \( (P<0.01) \). Castration reduced prostate and SV weights compared with those of the intact mice, whereas DHT treatment following castration increased the weights in both the WT and NTNKO males (Fig. 6B and C). In both the castrated and DHT-treated castrated males, the prostate and SVs were significantly heavier in the NTNKO males than in the WT males (Fig. 6B and C). All prostate lobes responded similarly to castration or to castration and DHT treatment (data not shown).

**Experiment 3: pituitary and testicular responsiveness**

**Serum steroid hormone and gonadotrophin levels** To determine the responsiveness of the pituitary
and testis to GNRH analogue stimulation as well as the testicular steroidogenic response to LH, the WT and NTNKO males were administered the GNRH analogue, leuprolide, or hCG respectively. Serum testosterone levels were significantly influenced by the treatments ($P < 0.001$) and genotype ($P < 0.01$), while serum LH levels were significantly influenced by the treatments only ($P < 0.001$). The administration of hCG reduced (31 and 11% compared with intact WT levels in the WT and NTNKO males respectively) serum LH levels and increased (790 and 1125% compared with intact WT levels in the WT and NTNKO males respectively) serum testosterone levels in both the NTNKO and WT males, while that of leuprolide caused a marked increase in serum LH and testosterone levels (Fig. 7).

**Discussion**

The GDNF family of neurotrophic factors has well-known roles in the differentiation and maintenance of the nervous system (Airaksinen & Saarma 2002). On the other hand, while GDNF signalling is important for kidney development and spermatogenesis (Sariola & Saarma 2003), the role of other GDNF families of neurotrophic factors outside the nervous system is not well understood. In humans and rodents, the prostate gland has a rich sympathetic and parasympathetic innervation (Vaalasti & Hervonen 1979, 1980), and previous rodent studies of autonomic denervation have suggested significant (Martinez-Pineiro et al 1993, Lujan et al 1998, Diaz et al 2010) but complex and ill-defined roles of autonomic nerves in the regulation of glandular structure and function. Therefore, the original aim of this study was to utilise transgenic mouse models lacking either a
ligand (NTNKO) or a receptor (GFRζ2KO) for the NTN signalling pathway to characterise the role and signalling mechanisms of parasympathetic nerves in prostate development.

While only a minor role for NTN signalling was revealed in prostate development, we found a novel role for NTN via GFRζ2 in the regulation of tissue androgen sensitivity. The inactivation of NTN signalling had only a modest effect on prostate growth with statistically normal prostate weights, although the VP and DLP weights were consistently lower than those of the respective WT mice in both the NTNKO and GFRζ2KO mice. A previous study using these mice has suggested atrophy of the prostate epithelial layer. However, following a more detailed non-biased stereological analysis of the epithelial height, we did not find major differences in the histological appearance or height of prostate epithelium between the WT and KO prostates. The minor reduction observed in the present study supports a relatively minor role of parasympathetic activity in prostate growth, suggested previously by a small decrease in prostate weight observed in pre-pubertal rats following preganglionic parasympathectomy, but a greater reduction following preganglionic sympatheticectomy (McVary et al. 1994). These differential effects may also be related to the targeting of each group of axons, with parasympathetic nerves mainly innervating the epithelium and sympathetic axons innervating the smooth muscle (Bruschini et al. 1978, Wang et al. 1991).

NTN and GFRζ inactivation has previously been confirmed to significantly modify the parasympathetic innervations of the prostate with the NTNKO and GFRζ2KO male mice having an almost complete loss of VIP terminals innervating the epithelium of the reproductive organs (Wanigasekara et al. 2004).

In contrast to the grossly normal development of androgen-dependent accessory sex organs, the circulating and intratesticular testosterone and DHT levels were markedly reduced (by 83–97%) in both the NTNKO and GFRζ2KO mice. The significantly reduced circulating androgen levels together with reduced circulating LH levels and grossly normal weights of androgen-dependent accessory sex organs suggested that the inactivation of NTN signalling led to increased responsiveness to androgens. This was subsequently confirmed directly using the DHT clamp.

While the striking reduction in circulating and intratesticular testosterone levels is suggested to be due to reduced Leydig cell synthesis and secretion of testosterone, it may be attributable to either intrinsic defects in Leydig cell steroidogenesis or reduction in Leydig cell stimulation drive by circulating LH. An intact hypothalamic–pituitary–testicular axis (HPT axis) would normally respond to low circulating testosterone levels with an increase in pituitary gonadotrophin secretion due to a lack of androgenic negative feedback. Yet, in the NTNKO and GFRζ2KO males, both of which lacked NTN signalling, the very low circulating androgen levels were significantly reduced compared to intact WT males. By two-way ANOVA, serum LH levels as well as prostate and SV weights were significantly influenced by the treatments (P < 0.001) and genotype (P ≤ 0.043). Data are shown as median ± 95% CI for LH levels and as mean ± s.e.m. for organ weights, n ≥ 5.
accompanied by reduced serum LH levels but unchanged serum FSH levels. This implies impaired hypothalamic–pituitary LH secretion and/or an enhanced negative testosterone feedback set point (Aiman et al. 1979, Handelsman et al. 1985a, Hiort et al. 2000, Simanainen et al. 2011). The preserved HPT responses to stimulation with hCG (pituitary responsiveness) and the GNRH analogue leuprolide (pituitary and testicular responsiveness) exclude any significant functional defect in the testis or pituitary. These findings together with largely unchanged prostate and SV weights and AR expression in the NTNKO and GFRzKO males (Widenfalk et al. 2000). The preservation of spermatogenesis despite markedly reduced circulating and intratesticular androgen levels is consistent with the fact that intratesticular testosterone levels required for induction (Singh et al. 1995) or maintenance (Handelsman et al. 1999) of murine spermatogenesis are much lower than ambient levels prevailing in the testis or bloodstream with analogous findings reported in rats despite a significantly reduced number of seminiferous tubules and interstitial fluid testosterone levels (Zirkin et al. 1989). Hence, increased androgen sensitivity in the testis of the NTNKO mice could support the maintenance of normal spermatogenesis despite markedly lowered circulating and intratesticular levels. Although E₂ can induce murine spermatogenesis (Ebling et al. 2000) in gonadotrophin-deficient hpg mice by a mechanism requiring a functional AR (Lim et al. 2008) and stimulation of pituitary FSH secretion (Allan et al. 2010), serum E₂ was not detectable in either the WT or KO mice by LC–MS/MS consistent with our previous findings (McNamara et al. 2010). Hence, the remarkable maintenance of spermatogenesis in this study is unlikely to involve E₂ due to the absence of sufficiently increased circulating FSH and E₂ levels involved in E₂ induction of spermatogenesis. Similarly, it is unlikely that another unknown testicular androgen could explain the presence of normal spermatogenesis despite serum and testicular testosterone and DHT concentrations that, together with reduced serum LH levels, indicate inactive Leydig cell steroidogenesis, the only plausible source of such a hypothetical unknown androgen. Analogous findings of histologically normal testis in Tgfβ1⁻/⁻ KO (Tgfβ1⁻/⁻/KO) mice despite 95% reduction in intratesticular testosterone levels compared with the WT mice (Ingman & Robertson 2007) have been reported.

To directly test androgen responsiveness, we used an open-loop DHT clamp, whereby in castrated mice, steady-state DHT delivery stabilises circulating androgen levels, allowing for the direct examination of the suppression of pituitary gonadotrophin secretion and androgen-dependent organ weights. While castration reduced the circulating androgen (testosterone and DHT) levels to non-detectable levels (compared with detectable androgen levels in intact males), DHT treatment produced similar circulating DHT levels in both the WT and NTNKO males, thus validating the DHT clamp. DHT-induced suppression of serum LH levels was significantly greater in the NTNKO males when compared with the WT males. Similarly, the response of the prostate and SVs to DHT was significantly greater in the NTNKO males, despite them having similar prostate
Ar expression. While AR expression appeared to be normal in target tissues such as the prostate, we did not look at the AR co-factors that could be modified by NTN. Interestingly, the differences between the WT and NTNKO males appeared to remain even in the androgen-deprived environment of castrated males. Comparable observations of changes in androgen responsiveness have been reported previously in uraemic rats (Handelsman et al. 1985b) as well as in humanised mice with variable-length CAG repeats in the AR (Simanainen et al. 2011). It is speculated that the latter might be an adaptive phenotype termed ontogenic regression (Handelsman & Dong 1992, Handelsman 2004), for example, by epigenetic modifications, with the promoter activity of androgen-sensitive genes being enhanced to maintain the same activity as ARs operating at higher sensitivity (Zhang & Ho 2011).

NTN, GDNF, artemin and persephin comprise a group of structurally related neurotrophic factors belonging to the transforming growth factor-β (TGFβ) superfamily (Airaksinen & Saarma 2002). Therefore, it is interesting to consider our results in relation to a recent study characterising Tgfβ1−/− mice (Ingman & Robertson 2007). Comparable to the NTNKO and GFRα2KO males in the present study, these mice had significantly reduced serum LH and testosterone levels but normal weight and morphology of androgen-dependent accessory sex organs. This suggests that the tissue response to androgens may also be increased in Tgfβ1−/− males. The similar phenotypes of the NTNKO and Tgfβ1−/− males suggest either disruption of a common signalling pathway or possible cooperation between NTN and TGFβ signal transduction. The latter is supported by the finding that TGFβ and NTN cooperate in various steps of GDNF signalling, including GFRα2 membrane localisation (Peterziel et al. 2002). To our knowledge, this potential interaction of TGFβ and NTN signalling has not been studied. In addition, while GDNF regulates spermatogenesis as demonstrated by both GDNF-KO and -overexpressing mice (Meng et al. 2000), biologically significant crosstalk between NTN and GDNF is unlikely to be relevant due to significantly different testicular phenotypes between the GDNF- and NRTN-overexpressing mice as well as between the KO mice (Meng et al. 2001).

NTN signalling is mediated by the binding of NTN to GFRα2 and their subsequent interaction with Ret, the extracellular domain. One or all members of NTN signalling are expressed in the HPT axis, allowing direct biological effects. NTN and Ret can be detected in adult pituitary gland and in the large secretory neurons of supraoptic and paraventricular nuclei of the hypothalamus (Widenfalk et al. 1997, Golden et al. 1998, Japon et al. 2002), while the expression of GFRα2 can be observed in the developing pituitary gland and in the hypothalamus (Widenfalk et al. 1997, Golden et al. 1998, Xian et al. 1999). In the hypothalamus, it is suggested that NTN in the supraoptic and paraventricular nuclei may be a source of NTN for a number of neuronal populations that express NTN receptors and have efferent projections to these hypothalamic nuclei, including neurons in the medial preoptic nucleus (Golden et al. 1998). NTN, GFRα2 and Ret are present in the adult murine testis (Golden et al. 1999, Viglietto et al. 2000, Wong et al. 2002), where they are expressed in the testicular Sertoli cells as well as in germ cells at spermatogenic stages IX–XII and I–II of the cycle (Golden et al. 1999, Widenfalk et al. 2000, Meng et al. 2001). GFRα2 and Ret are also expressed in the testis (Cao et al. 1996). The presence of NTN signalling components throughout the HPT axis, together with our data demonstrating the effects of NTN inactivation in intact males (testicular responsiveness) and following DHT clamp (pituitary and reproductive organ responsiveness), suggests that the effect of NTN may be related to AR signalling rather than being tissue specific. This could be at the level of gene transcription or signal transduction.

The loss of circulating androgens with an increase in tissue androgen sensitivity in Ntn-deficient animals also has important implications for the peripheral nervous system. Many of the sensory and autonomic neurons innervating the reproductive organs express ARs, which mediate diverse effects on the growth and physiological properties of these neurons in developing, postnatal and mature animals (Melvin & Hamill 1987, 1989, Keast 2006, Brock et al. 2007, Purves-Tyson et al. 2007). Previous studies in NTN and GFRα2 gene KO mice have revealed a deficit in the innervation of reproductive organs by cholinergic nitricergic neurons, closely matching the expression patterns of GFRα2 within the pelvic ganglia (Laurikainen et al. 2000, Waniagasekara et al. 2004). While it is reasonable to ascribe at least some of this deficit to the absence of NTN signalling, it is also possible that some aspects of the phenotype are obscured by concomitant actions on androgen signalling in these neurons. To our knowledge, lumbosacral sensory neurons and pelvic autonomic neurons have not been investigated as a site of signalling crosstalk between androgens and the GDNF family of neurotrophic factors. The temporal patterns of secretion of these factors by different tissues and organs within the urogenital tract have not been determined, but they could exert a significant influence on androgen actions in this system.
Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0056.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References
Hurtott, O Holterhus PM, Horter T, Schulze W, Kremke B, Bals-Pratsch M, Sinnecker GH & Kruse K 2002 Significance of mutations in the...


Nangle MR & Keast JR 2006 Loss of nitricergic neurotransmission to mouse corpus cavemosum in the absence of neurturin is accompanied by increased response to acetylcholine. British Journal of Pharmacology 148 423–433. (doi:10.1038/journal.2007.06.005)


Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B & Olson L 1997 Neurturin and glial cell line-derived neurotrophic factor receptor-β (GDNFR-β), novel proteins related to GDNF and GDNFR-α with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *Journal of Neuroscience* **17** 8506–8519.


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