Antagonism of proNGF or its receptor p75^{NTR} reverses remodelling and improves bladder function in a mouse model of diabetic voiding dysfunction

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Abstract

Aims/hypothesis Although 80% of diabetic patients will suffer from voiding difficulties and urinary symptoms, defined as diabetic voiding dysfunction (DVD), therapeutic targets and treatment options are limited. We hypothesise that the blockade of the pro-nerve growth factor (NGF)/p75 neurotrophin receptor (p75^{NTR}) axis by an anti-proNGF monoclonal antibody or by a small molecule p75^{NTR} antagonist (THX-B) can restore bladder remodelling (represented by bladder weight) in an animal model of DVD. Secondary outcomes of the study include improvements in bladder compliance, contractility and morphology, as well as in voiding behaviour, proNGF/NGF balance and TNF- α expression.

Methods In a streptozotocin-induced mouse model of diabetes, diabetic mice received either a blocking anti-proNGF monoclonal antibody or a p75^{NTR} antagonist small molecule as weekly systemic injections for 4 weeks. Animals were tested at baseline (at 2 weeks of diabetes induction), and after 2 and 4 weeks of treatment. Outcomes measured were voiding function with voiding spot assays and cystometry. Bladders were assessed by histological, contractility and protein expression assays.

Results Diabetic mice showed features of DVD as early as 2 weeks after diabetes diagnosis (baseline) presented by hypertrophy, reduced contractility and abnormal cystometric parameters. Following treatment initiation, a twofold increase ($p < 0.05$) in untreated diabetic mouse bladder weight and thickness compared with non-diabetic controls was observed, and this change was reversed by p75^{NTR} antagonism (37% reduction in bladder weight compared with untreated diabetic mice [95% CI 14%, 60%]) after 4 weeks of treatment. However, blocking proNGF did not help to reverse bladder hypertrophy. While diabetic mice had significantly worse cystometric parameters and contractile responses than non-diabetic controls, proNGF antagonism normalised bladder compliance (0.007 [Q1–Q3; 0.006–0.009] vs 0.015 [Q1–Q3; 0.014–0.029] ml/cmH₂O in untreated diabetic mice, representing 62% reduction [95% CI 8%, 110%], $p < 0.05$) and contractility to KCl, carbachol and electrical field stimulation ($p < 0.05$ compared with the diabetic group) after 2 weeks of treatment. These effects were not observed after 4 weeks of treatment with proNGF antagonist. p75^{NTR} antagonism did not show important improvements in cystometric parameters after 2 weeks of treatment. Slightly improved bladder compliance (0.01 [Q1–Q3; 0.009–0.012] vs 0.013 [Q1–Q3; 0.011–0.016] ml/cmH₂O for untreated diabetic mice) was seen in the p75^{NTR} antagonist-treated group after 4 weeks of treatment with significantly stabilised contractile responses to KCl, carbachol and electric field stimulation ($p < 0.05$ for each) compared with diabetic mice.

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Research in context

What is already known about this subject?

- The mechanism of diabetic voiding dysfunction is still poorly understood and its management represents an unmet therapeutic goal
- Nerve growth factor and its receptors (TrkA and p75^{NTR}) are expressed in the bladder and are related to different bladder pathologies
- The precursor form of nerve growth factor was found to activate p75^{NTR} to induce inflammatory and degenerative pathways, and its concentration increases in diabetes

What is the key question?

- Does the proNGF/p75^{NTR} axis play a degenerative role in the development of diabetic voiding dysfunction, similar to the recent evidence of its impact on other diabetic complications, such as retinopathy and angiopathy?

What are the new findings?

- Bladders of diabetic mice had an increased proNGF/NGF ratio, proNGF/p75^{NTR} co-expression in the mucosa, and high levels of TNF- α , which accompanied hypertrophy, impaired function and impaired contractile responses
- The pharmacological inhibition of proNGF improved bladder function and contractility after two weeks of treatment, without changing hypertrophy or hyperglycaemia
- p75^{NTR} antagonism normalised bladder wall thickness and contractility with improvement in bladder function. Both treatments reduced significantly the tissue expression of TNF- α .

How might this impact on clinical practice in the foreseeable future?

- The proNGF/p75^{NTR} axis contributes to the development of diabetic voiding dysfunction, and provides a potential therapeutic target

Bladder dysfunction observed in diabetic mice was associated with a significant increase in bladder proNGF/NGF ratio (3.1 [$\pm$ 1.2] vs 0.26 [$\pm$ 0.04] ng/pg in control group, $p < 0.05$ at week 2 of treatment) and TNF- α ($p < 0.05$). The proNGF/NGF ratio was partially reduced (about 60% reduction) with both treatments (1.03 [$\pm$ 0.6] ng/pg for proNGF antibody-treated group and 1.4 [$\pm$ 0.76] ng/pg for p75^{NTR} blocker-treated group after 2 weeks of treatment), concomitant with a significant decrease in the bladder levels of TNF- α ($p < 0.05$), despite persistent hyperglycaemia.

Conclusions/interpretation Our findings indicate that blockade of proNGF and the p75^{NTR} receptor in diabetes can impede the development and progression of DVD. The reported improvements in morphological and functional features in our DVD model validates the proNGF/p75^{NTR} axis as a potential therapeutic target in this pathology.

Keywords Bladder · Cystometry · Diabetic complications · Diabetic voiding dysfunction · Nerve growth factor · p75^{NTR} · proNGF · Streptozotocin · Target validation · TNF- α

Abbreviations

Dm	Diabetic mouse/mice (untreated)
DVD	Diabetic voiding dysfunction
EFS	Electric field stimulation
FSG	Fasting serum glucose
mAb	Monoclonal antibody
NGF	Nerve growth factor
p75 ^{NTR}	p75 neurotrophin receptor
proNGF	Precursor of nerve growth factor
TrkA	Tropomyosin kinase A receptor
VSA	Voiding spot assay

Introduction

Diabetic voiding dysfunction (DVD) occurs in almost 80% of diabetic patients [1, 2], resulting in abnormalities of bladder storage and emptying. There is a considerable need to develop therapeutic solutions to control disease progression and its costly and debilitating complications [3]. Several factors contribute to DVD pathogenesis, including hyperglycaemia and polyuria with local oxidative stress and inflammation [4]. These changes lead to significant alteration in innervation and vasculature [5, 6], prompting distorted physiology of

detrusor smooth muscle [3, 7] and urothelium. Resulting urothelial damage compromises its barrier function and cell signalling [8–10], which impacts mucosal communication with the underlying tissues [11]. DVD is widely reported to follow a two-phase progression; early compensated phase with storage symptoms such as urgency and bladder overactivity, followed by a late non-compensated phase with mainly hyporesponsive bladder and voiding symptoms [7, 12].

Recent evidence of the role of nerve growth factor precursor (proNGF) in the pathophysiology of diabetic retinopathy and angiopathy strongly suggests its involvement in DVD. Nerve growth factor (NGF) and its receptors (tropomyosin kinase A receptor [TrkA] and p75 neurotrophin receptor [p75^{NTR}]) are widely expressed in bladder tissues and expression has been correlated to different bladder pathologies including DVD [13]. In diabetes, bladders have an increased expression of *NGF* mRNA early in the disease [14]. However, protein levels of mature NGF are low or normal in later stages, a finding that could be attributed to reduced protease conversion of proNGF to mature NGF [15, 16] due to diabetic oxidative stress conditions [17]. While the mature NGF activates TrkA to mediate trophic signals, proNGF binds exclusively to the p75^{NTR} receptor [18, 19] and causes cell death and abnormal angiogenesis in diabetes, by increasing the expression of TNF- α and RhoA activation [13, 20–23]. In a bladder pathology resulting from spinal cord injury, activation of the proNGF/p75^{NTR} axis was found to promote urothelial cell apoptosis and was detrimental to bladder function [24, 25]. Furthermore, penile injection of anti-proNGF antibody helped to restore erectile dysfunction in a diabetic mouse model [26].

Based on this suggestive evidence, the potential pathological effect of proNGF/p75^{NTR} in bladder dysfunction, particularly in DVD, could identify a novel therapeutic target for a disease of broad impact, such as diabetes. Here, we hypothesise that the blockade of proNGF/p75^{NTR} axis by an anti-proNGF monoclonal antibody (mAb) or by a small molecule p75^{NTR} antagonist (THX-B) [27–29] can restore bladder remodelling (represented by bladder weight) in an animal model of DVD. Secondary outcomes of the study include improvements in bladder compliance, contractility and morphology, as well as in voiding behaviour, proNGF/NGF balance and TNF- α expression.

Methods

Animal housing and treatments All experiments received the ethical approval from the Lady Davis Institute Ethics Board (No. 7859), Montreal, Canada. Housing and handling conformed with the Canadian Council for Animal Care (CCAC). One hundred and forty-four 9-week-old C57BL/6 female mice were kept on a 12 h light/dark cycle with free access to food (standard Purina chow, Teklad Global, WI,

USA) and water. Diabetes was induced by i.p. injection of streptozotocin (60 mg/kg in citrate buffer, 10 mmol/l, pH 4.5 on 5 consecutive days) after 4–5 h fasting. Fasting serum glucose (FSG) was checked after 5–7 days using a glucometer (Contour next EZ, Bayer, ON, Canada) from the tail. FSG above 14 mmol/l was considered diabetic. Mice were divided into four groups without pre-considering their weight changes or glucose levels (for diabetic groups): control (42 mice); untreated diabetic (hereafter referred to as Dm; 42 mice); anti-proNGF mAb-treated diabetic mice (30 mice); and small molecule p75^{NTR} antagonist (THX-B)-treated diabetic mice (30 mice). The anti-proNGF mAb has a molecular mass of 150 kDa and inhibits binding of the ligand to p75^{NTR} but does not inhibit binding of the ligand to other TrkA receptor. This inhibitor antagonises ligand-dependent p75^{NTR} functions and, different from other anti-NGF mAbs, it is free of the off-target side effects resulting from inhibiting TrkA. We have observed that the molecule is detectable in systemic circulation for 2 weeks [27]. The THX-B small molecule ((1,3-diisopropyl-1-[2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-acetyl]-urea); molecular mass 540 Da) is a synthetic chemical with an estimated systemic circulation half-life in vivo of 6–8 h. It inhibits proNGF binding to p75^{NTR} and also inhibits ligand-independent signals by p75^{NTR}. Thus, we consider THX-B to be a pure antagonist. Dose calculations were based on previous studies [27–29].

Without blinding, i.p. injections of the drug molecules or the vehicle (PBS) were given weekly for 4 weeks, starting after 2 weeks of diabetes confirmation. A single starting proNGF mAb i.p. injection of 100 μ g/mouse in 150 μ l PBS was followed by 60 μ g/mouse in 100 μ l PBS for the following three injections. THX-B was injected i.p. at a weekly dose of 50 μ g/mouse in 125 μ l PBS. Experimental time points (to carry out cystometry, contractility, protein extraction and histology) were baseline (2 weeks after diabetes diagnosis included control and Dm groups only), week 2 after treatment (following two injection cycles) and week 4 after treatment (following four injection cycles) (Fig. 1). Body weight was measured weekly and FSG fortnightly for all groups. Six mice from each group were kept for another 2 weeks after the end of the study duration to observe the washout/prolonged effect of the molecules.

Voiding spot assay For the voiding spot assay (VSA), mice were placed individually with access to food without water for 2 h. The cage was fitted with a chromatography paper, grade 3 mm CHR (Whatman, GE Healthcare, UK) covered by a fine metallic mesh (2–3 mm pore size). The urine spot pattern was assessed by imaging the chromatography paper with ultraviolet light using SynGene Bioimaging system (USA) connected to GeneSnap software (Version 7.02, USA) and analysed by ImageJ software (Version: 2.0.0-rc-66/1.52n, NIH, USA) using a calibration curve and Void Whizzard free software

Fig. 1 Study timeline: the study started with 144 mice; diabetes was induced by streptozotocin (STZ) injection in 102 mice at the beginning of the study. Diabetes diagnosis (DM Dx) confirmation was carried out after 5–7 days. Study time points were baseline (2 weeks after diabetes

diagnosis) to confirm the development of DVD before initiating treatment; 2 weeks (W2) after treatment (Rx) and 4 weeks (W4) after treatment. Treatment included a single weekly injection. Drug washout was studied 3 weeks after the last injection

plugin for FIJI (distribution of ImageJ, Version: 2.0.0-rc-66/1.52n, NIH, USA) [30]. Mice were acquainted to the procedure once to get accustomed, then the experiment was done once to obtain the voiding data.

Bladder catheter implantation and conscious cystometry At each time point, six mice from each group were anaesthetised under isoflurane (2.5%) and injected with subcutaneous slow release buprenorphine (1 mg/kg). A lower abdominal midline incision was made to expose the bladder and the dome was punctured to insert a polyethylene PE10, 0.28/0.61 mm catheter (Stoelting, Wood Dale, IL, USA). The catheter was secured with a suture and tunnelled subcutaneously, leaving the exposed end through an incision in the back of the neck then sealed thermally.

Conscious cystometry was performed in metabolic cages 48 h after catheter implantation as previously described [31]. The exposed end of the catheter was unsealed and connected to a pressure transducer (Grass Technologies, USA) and an infusion pump instilling saline (154 mmol/l NaCl) to the bladder at 1.5 ml/h (for 1 h). Voided urine and saline were collected in a container connected to a force displacement transducer. The recorded parameters were maximal pressure (highest pressure during micturition), threshold pressure (pressure immediately before micturition), basal pressure (lowest pressure between voids), intermicturition pressure (mean calculated pressure between voids), micturition volume (volume per micturition), and inter-contraction interval (time between two voids). These parameters were used to compute: spontaneous activity (intermicturition pressure minus basal pressure), bladder capacity (instillation rate $\times$ inter-contraction interval), and bladder compliance (bladder capacity/[threshold pressure – basal pressure]) [32]. The data were analysed using the LabScribe2 Data Recording and Analysis Software (iWorx, Dover, NH, USA). Animals were then euthanised by exsanguination under 3% isoflurane.

Contractility studies (organ bath) At each time point, three mice from each group were selected randomly (without pre-considering their weight changes or glycaemic levels), their bladders were removed under anaesthesia, and then they were euthanised by exsanguination. Bladders were emptied and weighed, and placed in ice-cold Krebs–Ringer solution. After removing the bladder dome and base, longitudinal strips 2×5 mm with intact urothelium were pinned in wells of a Tissue Bath System – 720MO (DMT-USA, Ann Arbor, MI, USA) containing 6 ml of Krebs–Ringer solution pH 7.4 at 37°C, under 95% O₂/5% CO₂ bubbling. Strips were kept at a tension of 0.5 g for 1 h, changing Krebs every 15 min. After stabilisation, the strips were stimulated with 60 mmol/l KCl twice (60 mmol/l of KCl was obtained by adding 120 μ l of 3 mol/l KCl solution to 6 ml of Krebs), carbachol (3 nmol/l to 100 mmol/l) and electrical field stimulation (EFS; 1, 2, 4, 8, 16 and 32 Hz) using a Grass Technologies S88 Stimulator (West Warwick, RI, USA) with washing cycles between stimulations. All values were normalised to respective strip weight (to reflect the collective effect of bladder wall increased thickness and weight on its contractility) and analysed using LabChart 7 (ADInstruments, CO, USA).

Tissue and protein extraction At each time point, three animals from each group that did not undergo surgical manipulation were euthanised and the collected bladders were cut into equal halves; one half was snap frozen in dry ice then transferred to -80°C until protein extraction, and the second was immersed in 4% formaldehyde in PBS, 10%, 20% and 30% sucrose in PBS for 24 h each. Fixed tissues were then processed for cryosectioning at 10 μ m thickness. Frozen bladder samples were homogenised in RIPA buffer with antiprotease cocktail (Complete Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN, USA).

Histology: Masson trichrome stain and immunofluorescence Tissue sections were stained using Masson's trichrome according to the standard protocol [33]. Bladder wall

thickness measurements (detrusor vs mucosa) in the different groups were not performed, so as to avoid the bias of area selection and variations created by tissue fixation and bladder condition at the time of collection. For immunofluorescence, tissue sections were blocked (0.6% BSA in PBS, 0.3% Triton, 10% goat serum) for 30 min, then placed with the primary antibody p75^{NTR} ECD (1:500, MC192, Osenses, CA, USA), and proNGF (1:500, OriGene Tech. Rockville, MD Lot: ANT005AN0802) overnight at 4°C. Then, sections were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) 1X, and incubated with secondary antibodies (anti-rabbit conjugated to Dylight 488 and anti-mouse Rhodamine conjugated, ThermoFisher Scientific, IL, USA) for 30 min. After this, slides were washed and mounted in DAPI. Staining was examined using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Negative controls omitting the primary antibodies showed non-specific binding to the blood vessels (see electronic supplementary material [ESM] Fig. 1).

Western blotting Protein concentration in bladder lysate was measured by Micro BCA assay kit (Boster Biological Technology, CA, USA). Equal amounts of protein (20 µg) were loaded on 12% polyacrylamide gel and electro-transferred to PVDF membranes. After blocking in 5% skimmed (non-fat) milk in TBST for 1 h, overnight incubation with TNF-α (1:2000, EMD Millipore, CA, Lot: 3143184) (4°C) was followed by 1 h incubation with anti-rabbit antibody (Millipore, CA, 1:5000 at room temperature). Bands were revealed with Luminata Crescendo horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA) and quantified by ImageJ (Version: 2.0.0-rc-66/1.52n, NIH, USA). β-actin was used as control of protein loading.

ELISA measurement for proNGF and NGF Bladder extracts were used to quantify the proNGF and NGF levels using rapid ELISA kits (proNGF RapidTM ELISA kit, Biosensis, catalogue number: BEK-2236-1P, SA, Australia) and NGF RapidTM ELISA kit, Biosensis, Catalogue number: BEK-2213-1P, SA, Australia) as per the manufacturer's protocol.

Statistical analyses Data are presented as mean (± SD) if parametric; or mean (Q1–Q3) when non-parametric (decided upon Levene's test of normality and histogram of distribution curve). Comparisons between groups at each time point were carried out by independent sample *t* test (for baseline data; control vs Dm) and one-way ANOVA with post hoc Tukey test (for other time points) or Kruskal–Wallis when non-parametric. Reported *p* values of bladder weight changes are for the purpose of hypothesis testing, while the reported *p* values for the other secondary outcomes are descriptive; *p* values always reflect two-tailed tests. Sample size calculation was based on α = 0.05, power 80%, SD of bladder size change in

streptozotocin-induced DVD models was 48% (0.48) and the effect size of the bladder weight change is 80% (0.8) (half reduction in 164% increase in bladder weight [hypertrophy] reported in streptozotocin-induced DVD models) [34, 35]. The sample size estimated was 5.6 (rounded to six mice per group at each time point). Statistics were performed in IBM SPSS Statistics ver.23.0 (IBM, Armonk, NY, USA).

Results

p75^{NTR} antagonism normalised the bladder weight and reversed bladder hypertrophy An increased bladder weight in diabetic rodent models reflects bladder hypertrophy. We hypothesised that proNGF/p75^{NTR} antagonism would normalise this finding. To test this, we injected weekly either anti-proNGF mAb or a small molecule p75^{NTR} blocker (THX-B) [27–29] in mice with streptozotocin-induced diabetes, for 4 weeks. At the end of the study, the bladder weight of Dm (33.5 [±2.11] mg) almost doubled compared with control mice (18.2 [±1.47] mg, *p* < 0.05) (Fig. 2a, b). Diabetic mice treated with proNGF mAb showed a 16% reduction (95% CI 1%, 32%) in bladder weight after 2 weeks of treatment (26 [±0.96] mg, *p* < 0.05 compared with the Dm group, one-way ANOVA between diabetic groups only), but this effect disappeared after 4 weeks (31.93 [±2.42] mg bladder weight, *p* > 0.05 compared with Dm). However, p75^{NTR} antagonism with THX-B prevented bladder weight increase, which was 18% (95% CI 3%, 32%) and 37% (95% CI 14%, 60%) lower after 2 and 4 weeks of treatment, respectively, compared with Dm (25.6 [±1.63] mg and 21.1 [±1.6] mg bladder weight for week 2 and 4, respectively, *p* < 0.05 compared with Dm) (Fig. 2a, b; ESM Table 1).

The bladder weight differences in the diabetic treated groups were observed despite persistent hyperglycaemia. The FSG of diabetic treated and untreated groups remained above 15 mmol/l throughout the study duration (Fig. 2c). All diabetic mice had lower body weight compared with control animals at all time points after diabetes diagnosis (Fig. 2d; ESM Table 2).

Morphological bladder differences of the THX-B treated mice correspond to their lower bladder weight In addition, to demonstrate the increased collagen content in diabetic bladders, Masson trichrome stained photomicrographs (Fig. 2e–n) allowed us to observe increased bladder wall thickness in Dm and proNGF mAb-treated mice with prominent mucosal folding and detrusor thickening. However, THX-B-treated mice did not show histological changes of hypertrophy after 2 and 4 weeks of treatment, a finding that is consistent with the observed bladder weight changes (Fig. 2e–n).

Fig. 2 Bladder weight and morphology with concomitant FSG and body weight changes. **(a, b)** Bladder weight changes **(a)** and bladder/body weight ratio **(b)**; Dm bladders were significantly heavier than control throughout the study. THX-B-treated mice had significantly lighter bladders than Dm and proNGF mAb-treated mice at 4 weeks. $n = 5-6$ at each time point; **(c)** FSG: THX-B-treated mice showed steady glucose level throughout the study duration, which was significantly different from Dm and proNGF mAb-treated mice after 4 weeks of treatment. $n \approx 49, 49, 36$ and 24 at Dx, baseline, week 2 and week 4, respectively. **(d)** Body weight follow-up: diabetic mice (Dm, mAb and THX-B) underwent loss of weight and slower rate of weight gain. In **(c, d)**: ‘at DM Dx’ indicates the start of group allocation after diabetes diagnosis, ‘Start’ indicates week 0 of the study. * $p < 0.05$ vs control, ** $p < 0.01$ vs control,

† $p < 0.05$ vs THX-B; in **(c)** † indicates that CTR, Dm and mAb were all different from THX-B at week 4; all comparisons were done at a particular time point; for baseline **(a, b)** only independent t test; for all other time points one-way ANOVA with post hoc Tukey test. Data represent mean (SD). **(e–n)** Histology of bladders of the four groups (columns) at different time points (rows): the mucosa (yellow arrows) of the Dm group is thicker with more collagen deposition and prominent folding compared with control and diabetic treated groups. The detrusor layer (black arrows) of the THX-B-treated groups looks comparable in thickness to the control. Red (detrusor), pink folded (epithelium), blue (collagen); images are representative for three animals per group at each time point, scale bars, 200 μm . CTR, control

ProNGF or p75^{NTR} antagonism did not modify voiding behaviour Having shown that bladder hypertrophy was reversed by p75^{NTR} antagonist but not with proNGF mAb, we evaluated changes in bladder function using VSA (Fig. 3) and conscious

cystometry (Fig. 4; ESM Table 3). The micturition behaviour of Dm assessed by the VSA showed a statistically significant increase in voiding frequency, total voided volume and mean volume per micturition compared with control mice at all time

Fig. 3 VSA over a 2 h period. (a) Number of spots, reflecting voiding frequency; (b) Total voided volume after 2 h; (c) Mean volume per spot; $n = 4–8$ mice in each group at each time point; * $p < 0.05$ vs control, † $p < 0.05$ vs THX-B; all comparisons were done at a particular time point, for baseline independent t test, for all other time points one-way ANOVA with post hoc Tukey test. Data represent mean (SD). (d–k) Representative images of the VSA paper for the four groups after 2 weeks of treatment (d–g) and after 4 weeks of treatment (h–k); scale bars, 5 cm. CTR, control

points (Fig. 3a–c). Both treatments (proNGF mAb and THX-B) had minimal effects on these parameters, except for a 50% lower total voided volume in the THX-B-treated mice after 2 weeks of treatment ($372 \pm 73 \mu\text{l}$ vs $802.5 \pm 123 \mu\text{l}$, $p < 0.05$) compared with Dm (Fig. 3a–c). Given that this experiment was done without access to water and these mice maintained FSG levels above the kidney glucose reabsorption threshold, we cannot infer whether this was due to decreased water intake or due to a direct effect on urine production. Abnormal voiding patterns were not observed in any group, and mice from the four groups showed a normal peripheral pattern of voiding (Fig. 3e–k).

ProNGF mAb treatment improved conscious cystometry parameters Conscious cystometry parameters for Dm confirmed the development of DVD at all time points as they showed higher inter-contraction intervals, micturition volume, bladder capacity and bladder compliance, compared with control mice (Fig. 4; ESM Table 3). Despite the persistent presence of bladder hypertrophy in proNGF mAb-treated mice, conscious cystometry in this group showed improved bladder capacity and compliance (0.007 [Q1–Q3; $0.006–0.009$] vs 0.015 [Q1–Q3; $0.014–0.029$] ml/cmH₂O in Dm, representing 62% reduction compared with Dm [95% CI

8% , 110% , $p < 0.05$) after 2 weeks of treatment. These effects were not observed after 4 weeks of treatment with proNGF antagonist. Consistent with the absence of bladder hypertrophy after 4 weeks of treatment, p75^{NTR} antagonism did not show important improvements in cystometric parameters after 2 weeks of treatment. Slightly improved bladder compliance (0.01 [Q1–Q3; $0.009–0.012$] vs 0.013 [Q1–Q3; $0.011–0.016$] ml/cmH₂O for Dm; $p > 0.05$) was seen in the THX-B group after 4 weeks of treatment (Fig. 4; ESM Table 3).

ProNGF or p75^{NTR} antagonism improved the in vitro contractility of bladder strips of treated diabetic mice To determine whether the improved functionality of the treated diabetic bladders could be reproduced in vitro, contractile responses to receptor-specific (carbachol) and non-receptor-specific stimulations (KCl and EFS) were evaluated (Fig. 5). Bladder strips from Dm gave significantly weaker responses to KCl (60 mmol/l), increasing intensities of electrical field (from 1 to 32 Hz) and carbachol (3 nmol/l to 100 mmol/l) at all time points compared with control mice ($30–50\%$ decrease in contractile intensities for different stimulations). THX-B treatment protected responses that were diminished in Dm, such that they were not different from control mice after 2 and 4 weeks of treatment. The protected contractile responses were also observed in the proNGF mAb-treated mice at

2 weeks; however this did not persist after 4 weeks (Fig. 5a–g).

Prevention of bladder proNGF/p75^{NTR} mucosal co-expression and TNF- α accumulation by proNGF and p75^{NTR} antagonism

To correlate molecular changes with functional observations, we studied proNGF expression and its receptor p75^{NTR} in the

bladder, by immunofluorescence labelling (Fig. 6). In control bladders, p75^{NTR} expression was mainly confined to the lamina propria and the exterior of detrusor muscle bundles, while proNGF was localised to the urothelial layer and to a lesser extent in the detrusor layer. Dm bladders showed overexpression of p75^{NTR} in all layers. p75^{NTR} was co-expressed with proNGF in the urothelium of Dm bladders at all time points as

Fig. 4 Conscious cystometry. (a–j) cystometry tracings for intravesical pressure (IVP) and voided volume (VV) for the four groups at different time points; a remarkable gradual increase in the inter-contraction interval (ICI), distance between contraction peaks, in the Dm group (d–f) with time, with corresponding increase in VV. proNGF mAb-treated mice (g, h) showed reduced ICI and VV at 2 weeks of treatment and this effect disappeared after 4 weeks of treatment; however, an opposite pattern was seen with THX-B-treated mice (i, j), which showed improved parameters mainly after 4 weeks of treatment. (k–n) Cystometry parameters: (k) Spontaneous activity (SA) showed increase in Dm and proNGF mAb groups after 2 weeks of treatment; (l) ICI was significantly longer in Dm mice at baseline and week 2, the mAb group had shorter ICI compared with Dm. The ICI in week 4 was apparently shorter in control, mAb and THX-B than in Dm mice, although not reaching significance ($p = 0.058$); (m) Bladder capacity (Bcap) significantly increased in Dm throughout the study time points. proNGF mAb showed lower Bcap compared with Dm at week 2, an effect that disappeared at week 4. THX-B showed lower Bcap compared with Dm only at week 4, although it was not statistically different from control or Dm. (n) Bladder compliance (BC) changes at weeks 2 were similar to those of Bcap while at weeks 4 all diabetic groups were significantly different from control group. * $p < 0.05$ vs control, † $p < 0.05$ vs mAb. Data represent mean (SD); all comparisons were done at a particular time point; baseline independent t test, for all other time points non-parametric Kruskal–Wallis test with post hoc t test; $n = 5–6$ each group each time point. CTR, control

early as the baseline, compared with the absence of co-expression in the urothelium in the bladders of control mice. The p75^{NTR} expression pattern seen in the Dm bladders was not clearly different from the proNGF mAb-treated ones. On the other hand, there was reduced p75^{NTR} expression in all bladder layers in the THX-B-treated mice (Fig. 6). This was expected due to the positive feed-forward loop of p75^{NTR} activity, while reduced p75^{NTR} activity leads to reduced p75^{NTR} mRNA levels [28].

On the basis of the evidence of proNGF accumulation in diabetes [17, 28], we hypothesised that proNGF levels relative to mature NGF in the bladder would be higher in Dm (Fig. 7a–c). We demonstrated that the bladder proNGF/NGF ratio of Dm was significantly higher at all time points compared with control mice (more than tenfold, $3.1 [\pm 1.2]$ vs $0.26 [\pm 0.04]$ ng/pg in control group, $p < 0.05$ at week 2 of treatment). Treatment with proNGF or p75^{NTR} antagonism caused lower proNGF/NGF ratio than that of the Dm (to less than half of the untreated Dm mice), yet without statistical significance ($1.03 [\pm 0.6]$ ng/pg for proNGF mAb-treated group and $1.4 [\pm 0.76]$ ng/pg for THX-B-treated group after 2 weeks of treatment) (Fig. 7a–c).

As proNGF/p75^{NTR} axis activation in diabetes causes degenerative changes by increasing TNF- α expression [21, 22, 28], similarly we identified a significant increase in transmembrane TNF- α in Dm bladders at 2 and 4 weeks, compared with controls. Interestingly, this increase was prevented by both treatments ($p < 0.05$) (Fig. 7d, e). Taken together, the increase in proNGF levels in the diabetic bladder was accompanied by overexpression of transmembrane

TNF- α , a finding prevented by blocking proNGF/p75^{NTR} activation.

Prolonged treatment decreases bladder hypertrophy, and the effect is sustained after drug washout The FSG in both treatment groups was significantly lower than those in the Dm after a 3 week washout period ($p < 0.05$). Bladder weight in the THX-B group maintained a significant difference when compared with Dm ($p < 0.05$), while mean bladder weight of proNGF mAb-treated mice was not statistically different from Dm despite maintaining similar pre-drug washout bladder weight. (Fig. 8b) (ESM Table 1) In addition, photomicrographs of the bladders showed that hypertrophy was absent in both treatment groups, suggesting a prolonged effect on blood glucose and bladder morphology (Fig. 8). In both the proNGF mAb and the THX-B treatments, the prolonged anatomical physiological effects on the bladder are likely to be due to a prolonged therapeutic effect rather than to the drugs remaining in circulation at therapeutic levels after a 3 week period, given that we have observed that anti-proNGF mAb is detectable in systemic circulation only for 2 weeks in previous studies [27].

Discussion

The pathophysiology of DVD is complex, where several tissue disturbances orchestrate the deterioration of bladder function. In this study, we establish the involvement of proNGF and its receptor p75^{NTR} in the structural and functional changes of DVD. We have shown that proNGF and p75^{NTR} expression increased in diabetic bladders and that blocking p75^{NTR} receptor reversed bladder hypertrophy, an effect that persists after drug washout. Furthermore, interfering with the proNGF/p75^{NTR} axis by proNGF mAb or p75^{NTR} antagonist improved bladder contractility in vitro and reduced voiding frequency and bladder capacity, with a significant reduction in bladder TNF- α expression (see Table 1 for results summary).

We anticipated differences in the therapeutic effect of blocking the ligand (proNGF mAb) or the receptor (THX-B) owing to the specificity of proNGF binding exclusively to p75^{NTR}, while p75^{NTR} itself can be activated by diverse ligands, including neurotrophins and other pro-neurotrophins [36, 37], as well as ligand-independent actions. In addition, interpretation of the effect size in animal models is subjective and setting such endpoints can be difficult to achieve in the absence of a standard treatment considered a positive control.

Bladder wall hypertrophy and increased bladder capacity and compliance seen here are commonly seen in studies on streptozotocin-induced diabetic and type 2 diabetic rodent models that attain high blood glucose levels [34, 35, 38–40]. However, in this study, the in vitro bladder contractility

Fig. 5 In vitro contractility responses to different stimuli; KCl response (a), carbachol (b–d) and EFS (e–g): bladder strips from the Dm gave weaker response to KCl (60 mmol/l), carbachol and EFS throughout the study duration. The THX-B-treated group had comparable contractions to control at week 2 and week 4 of treatment for all stimuli. All the responses of proNGF mAb-treated mice were comparable to control only after 2 weeks of treatment, subsequently responses were comparable to Dm group. * $p < 0.05$ vs control; † $p < 0.05$ vs Dm; ‡ $p < 0.05$ vs proNGF mAb. (d, g) brackets on the right indicate that both control and THX-B are different from Dm and mAb, as indicated. All comparisons were done at a particular time point; for baseline, independent t test; for all other time points, comparisons were done with one-way ANOVA with post hoc Tukey test. Data represent mean (SD) of the tension presented as Newton (N) per gram of bladder strip weight. $n = 6$ –9 strips from three different mice for each group at each time point. CTR, control

showed early weak responses. This is inconsistent with the two-phase theory, which proposes that the uncompensated reduced contractility starts usually after 9 weeks of diabetes [12]. Weaker responses were reported mainly at late stages of diabetes, beyond 9–12 weeks [35, 38, 40]. These differences may be due to different animal species, experimental settings (conscious vs unconscious cystometry and the influence of

anaesthetic agents on urethral tone in the later approach), glycaemic levels or duration of diabetes. Another factor is the sex (female in our study, compared with the majority of published studies using male mice). Nonetheless, the reduced responsiveness of bladder strips in our study is compatible with the observed cystometry pattern of increased bladder compliance, reflecting reduced bladder responsiveness.

Fig. 6 Immunofluorescence study of p75^{NTR} and proNGF in the bladder mucosa and detrusor at baseline (a–d), 2 weeks of treatment (e–l) and 4 weeks of treatment (m–t). proNGF (green fluorescence) is mainly localised to the urothelial layer (UT) with low to absent signal in the lamina propria (LP) in the control mice, while in Dm and THX-B-treated mice the signal can also be detected in the LP. The proNGF signal in the detrusor muscle fibres (white arrow) does not show a specific change across groups and different time points. The p75^{NTR} signal (red fluorescence) in the control mice is expressed in the LP and around the muscle bundles (in detrusor). In Dm, p75^{NTR} shows expression mainly in the UT layer at baseline. This pattern changes with time to show more expression in the UT and LP, which is co-expressed with proNGF. In proNGF mAb-treated mice the p75^{NTR} expression pattern is not different from Dm, while the expression in the THX-B-treated mice looks similar to the control group. In the detrusor, the p75^{NTR} signal becomes stronger around and within muscle bundles in Dm, a finding that is attenuated in the diabetic treated groups. Blue colour (DAPI) indicates nuclei; images are representative of sections from three different animals; scale bars 50 μ m. CTR, control

Our results reveal that bladders from a mouse model of diabetes express higher levels of proNGF at the expense of mature NGF. The imbalance between proNGF and NGF has been proven to play a detrimental role in other diabetic conditions such as retinopathy, through the activation of p75^{NTR} [41]. No previous studies have made observations on the proNGF/NGF imbalance in DVD [13]. In this study, the increase in proNGF/NGF ratio in Dm was associated with structural and functional changes of DVD, which were controlled variably by proNGF or p75^{NTR} antagonism.

Diabetic mice treated with proNGF mAb showed restoration in bladder contractility and cystometry after 2 weeks of treatment with no remarkable change in blood glucose levels and bladder hypertrophy. This suggests that bladder hypertrophy is not the main contributor in DVD. A similar effect was observed by Jiang et al, who found that decreasing the

oxidative stress by α -lipoic acid restored bladder function in diabetic rats without affecting bladder hypertrophy or glucose level [42]. Findings from our study and Jiang et al's indicate that diabetic bladder function improvements can be achieved despite the presence of hypertrophy [35].

Previous studies aimed to restore mature NGF levels to rescue bladder function, based on the observation of lower NGF levels in streptozotocin-induced DVD models. [43–45] The deterioration of functional and contractile parameters in the proNGF mAb-treated mice after 4 weeks of treatment can be attributed to the potential detrimental effect of chronic administration of proNGF mAb on NGF levels required to restore bladder function, or to the possibility that p75^{NTR} was activated by other ligands accumulating in the bladder wall, such as the precursor to brain derived neurotrophic factor (proBDNF).

Fig. 7 ELISA and western blot analysis of proNGF (**a**), NGF (**b**), proNGF/NGF ratio (**c**) and TNF-α (**d**, **e**), in bladder tissue extracts of the four groups at different time points. The proNGF/NGF ratio (**c**) was higher in the Dm group at all time points compared with control; it appeared lower in the treatment groups, but the values were not significantly different from either control or Dm. Membrane bound TNF-α (**d**, **e**) was significantly lowered in both treated diabetic mice at 2 and 4 weeks (significantly lowered in proNGF mAb group at week 2 and in the THX-

B group at week 4). β-actin is shown to reflect amount of protein loading (normalisation was carried out to total protein content as well). * $p < 0.05$ vs control; † $p < 0.05$ mAb; ‡ $p < 0.05$ vs THX-B; All comparisons were done at a particular time point; for baseline independent t test; for all other time points, comparisons were done with one-way ANOVA (non-parametric Kruskal–Wallis) with post hoc Tukey test. Data represent mean (SD); $n = 3$ for each group at each time point. BL, baseline; CTR, control

p75^{NTR} antagonist-treated mice showed reversal in bladder hypertrophy and improved in vitro contractility after 2 and 4 weeks of treatment. Similar to the NGF trend in late DVD, p75^{NTR} was expressed at lower levels in streptozotocin-induced diabetic bladders and fructose-fed obese rodents, with no data available on its expression at early stages [46, 47]. Therefore, blocking p75^{NTR} was not a therapeutic option in the advanced DVD models (beyond 9 weeks of diabetes). P75^{NTR} blockage was found to increase bladder activity and improve urothelial and neuronal integrity in cystitis and neurogenic bladder models, respectively [24, 48]. Observed bladder improvements in THX-B-treated mice can be attributed to the restoration of NGF levels at 4 weeks of treatment and/or bladder structural uniformity.

It is worth mentioning here that Dm showed higher transmembrane TNF-α levels and both treatments caused significant inhibition at 2 and 4 weeks. It is known that p75^{NTR} receptor exerts its toxic effect by causing increased production of TNF-α [21, 22, 49],

and blocking the proNGF/p75^{NTR} axis helped reduce the degenerative consequences of diabetic retinopathy and microangiopathy [28] through reduced inflammation [27].

It is well established that the pathogenesis of DVD is attributed to inflammatory changes [4, 40, 50]. Increased expression of inflammatory markers such as TNF-α was found to correlate with different models of DVD in compensated [40] and uncompensated phases [50]. Wang et al reported that inhibiting TNF-α had reduced the overactive DVD at early stages in a type 2 diabetic model in a Rho kinase and myosin light chain phosphorylation related pathways [40]. Furthermore, annihilating the inflammatory response by TLR-4 knockout controlled bladder hypertrophy and restored bladder contractility in a DVD mouse model [51]. Collectively, it is obvious that TNF-α is an important mediator in the diabetes-associated inflammation in the bladder. We show that the proNGF/p75^{NTR} axis is upstream of TNF-α in DVD, and is therefore an attractive pharmacological target.

Fig. 8 FSG and bladder weight and morphology after the drug washout period (3 weeks after the last injection, 8 weeks of diabetes). **(a)** FSG in the diabetic treated mice was significantly lower than in the Dm group despite being significantly higher than control. **(b)** Bladder weight shows persistent absence of bladder hypertrophy in the THX-B group compared with Dm and mAb after the washout period. **(c)** Bladder/body weight ratio showed similar pattern to the bladder weight changes. * $p < 0.05$, ** $p < 0.01$ vs control; † $p < 0.05$ vs mAb; ‡ $p < 0.05$ vs THX-B; one-way

ANOVA with post hoc Tukey test. Data represent mean (SD). **(d–g)** Masson trichrome staining of the bladder after the washout period showing absence of bladder hypertrophy in the THX-B group after the washout period with reduced thickness in the mAb compared with the Dm group, especially in the mucosa (yellow arrow) and detrusor (black arrow); images are representative for three animals per group, scale bars, 200 μ m. CTR, control

Table 1 Summary of DVD changes in our animal model and the observed effects of proNGF/p75^{NTR} axis blockade, compared with Dm

Outcome	Dm	proNGF mAb	THX-B
Bladder weight	↑	↓ (week 2) ⇌ (week 4)	↓ (week 2, 4)
Morphology			
Overall thickness	↑	⇌	↓
Collagen content	↑	↓	↓
Mucosa	↑	↓	↓
Detrusor	↑	↑	↓
VSA			
Number of spots	↑	⇌	↓ (week 2)
Total volume	↑	⇌	↓ (week 2)
Volume per spot	↑	⇌	⇌
Cystometry			
Spontaneous activity	↑	⇌	⇌
ICI	↑	⇌	↓ (week 4)
Bladder capacity	↑	↓ (week 2)	↓ (week 4)
Bladder compliance	↑	↓ (week 2)	⇌
Contractility			
KCl	↓	↑ (week 2)	↑ (week 2, 4)
Carbachol	↓	↑ (week 2)	↑ (week 2, 4)
EFS	↓	↑ (week 2)	↑ (week 2, 4)
Molecular changes			
proNGF/NGF	↑↑	↓	↓
TNF- α	↑↑ (week 2, 4)	↓↓	↓↓

ICI, inter-contraction interval

Streptozotocin-induced diabetic mice are widely used as a DVD model, with bladder changes representing mainly type 1 diabetes [34, 35, 39]. While we could have administered a low insulin dose to avoid very high glucose levels, we instead adopted low dose streptozotocin-induced diabetes, which is known to avoid complete ablation of pancreatic β -islets. In addition, insulin administration showed a direct effect improving NGF levels and bladder function in DVD models [46]. Second, studies carried out outside the urology field have shown that the streptozotocin model has limited translational value to studying type 1 diabetes due to its cytotoxicity [52]. Third, streptozotocin can induce alterations independent of beta cell destruction, such as the activation of TRAP1 ion channels in organs other than the bladder [53]. Despite the questionable applicability of this model to human DVD, studies on this model have extensively provided information to understand DVD pathogenesis. Our Dm manifested several features of DVD, including bladder hypertrophy and polyuria, with increased voiding frequency, spontaneous activity and bladder compliance with weak contractility.

Studying DVD in type 1 diabetic mice with systemic treatments that target a widely expressed neurotrophin and its receptor poses several limitations. In addition, the multisystem involvement of a condition like diabetes makes it difficult to isolate local from systemic effects of our proposed therapeutic intervention. For instance, an effect on the kidney or the blood glucose level cannot be excluded. However, a systematic review showed that bladder hypertrophy was not correlated to glucose levels in a model of type 1 diabetes [34]. Type 2 diabetes models did not show a remarkable increase in bladder

weight, which was particularly attributed to the relatively lower glycaemic levels in these models [35]. The FSG levels in treated and untreated diabetic mice in our study stayed above the renal glucose threshold that would cause polyuria (above 10 mmol/l) [35]. This would exclude a direct effect of the treatment on urine production, suggesting a local effect on bladder.

In summary, our study demonstrated the involvement of the proNGF/p75^{NTR} axis in the development of DVD as early as 4 and 6 weeks of streptozotocin-induced diabetes in mice. Interruption of this axis reversed bladder structural and functional changes, such as bladder hypertrophy and reduced responsiveness, with an associated significant reduction in TNF- α . Further studies to understand the underlying pathophysiological mechanisms and the balance between proNGF and NGF in DVD will have direct clinical implications in validating this axis as a potential therapeutic target in this pathology.

Data availability Data are available on request from the corresponding author.

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Contribution statement All authors have participated in the conception of the design, acquisition of data, analysis interpretation of data, drafting and revising of the manuscript and the final approval of the text. Specific contribution is highlighted for each author as follows. AHM: designing research studies, conducting experiments, acquiring and analysing data and writing the manuscript; AG: production, characterisation and quality control of reagents, optimisation of immunohistochemical techniques, editing the manuscript; PGC: conducting experiments and writing; MVF: conducting experiments; SS: acquiring and analysing data; PB: production, characterisation and quality control of reagents; HUS: designing research studies, data analyses, providing reagents and writing the manuscript; LC: designing research studies, acquiring and analysing data and writing the manuscript and is responsible for the integrity of the work.

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