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Functional and Molecular Characterization of the Bilateral Pelvic Nerve Crush Injury Rat Model for Neurogenic Detrusor Underactivity.

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ABSTRACT

Objectives: To create a rat model for neurogenic detrusor underactivity (DU) by bilateral pelvic nerve crush injury (BPNI) and to study temporal changes in detrusor contractility and morphology.

Methods: Male Sprague-Dawley rats were subjected to BPNI or sham surgery and evaluated 1, 3 and 9 weeks after surgery. Bladder function was determined *in vivo* by awake cystometry, micturition pattern analysis and 24-hours urine collection. Bladders were harvested for *in vitro* pharmacological investigation by isometric tension recording. Bladders and major pelvic ganglia were investigated by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and histochemistry.

Results: Overflow incontinence was observed 1 week after BPNI. At 3 and 9 weeks after BPNI, rats showed a bladder phenotype characteristic for DU with increased post-void residuals, reduced voiding efficiencies and lower maximum pressures. In isolated bladder strips, contractile responses to potassium chloride (KCl), carbachol and α,β -methylene adenosine 5'-triphosphate (α,β -mATP) were preserved. On the other hand, neural induced contractility was reduced after BPNI, in line with reduced expression of protein gene product 9.5 and choline acetyltransferase in the major pelvic ganglion 1 week after BPNI. Bladder-to-body weight ratio and detrusor thickness increased following BPNI, indicating detrusor hypertrophy to compensate for the reduced neural input.

Conclusions: BPNI induces a rat model for neurogenic DU. In this model, the detrusor maintains its contractility but denervation of the detrusor was observed.

INTRODUCTION

Detrusor underactivity (DU) is defined as a voiding dysfunction that occurs when the bladder cannot contract long enough and/or with sufficient strength to achieve complete bladder emptying within a normal time span.(1)

DU occurs in 9%-48% of men and 12%-45% of women (2) and has multiple etiologies. Pelvic surgery is frequent complication of iatrogenic DU. Eighteen months after radical hysterectomy, 16% of patients present with a 30% increased post-void residual (PVR) and 2% of patients develop an acontractile bladder.(3) Of patients undergoing total mesorectal excision (TME), 26% of patients with preoperative normal bladder function develop a long-term bladder emptying disorders.(4) Despite the increasing interest in the topic of DU, its pathophysiology remains mostly undetermined.(2,5) This leads to a paucity of available treatment options. Currently, urinary catheterization is the standard therapy for DU. Therefore, a recent plea for novel research initiatives has been launched.(5-7) However, only 2 clinical trials investigate experimental therapies for the treatment of DU. (www.clinicaltrials.gov, NCT03175029 and NCT02463448).

Normal bladder function depends on a perfect interplay between sympathetic, parasympathetic and somatic input to the lower urinary tract. During pelvic surgery, autonomic nerves to the bladder can be injured.(2) Even in nerve-sparing surgery, pelvic surgery causes neuropraxia of the autonomic bladder innervation by traction, crushing or coagulation. Targeting the autonomic nerve supply in animals could mimic neural injury commonly observed in pelvic surgery. In rats, the pelvic plexus is organized in a single, bilateral ganglion: the major pelvic ganglion (MPG). The MPG is supplied by the hypogastric and pelvic nerve that contain the sympathetic and parasympathetic input, respectively.(8)

Recently, the International Consultation on Incontinence Research Society emphasized the need for comprehensive animal models for DU.(5) A crush injury of the pelvic nerves could provide a convenient animal model for neuropraxia and as a consequence, pelvic surgery induced neurogenic DU. Few studies investigated the impact of bilateral pelvic nerve injury (BPNI) on bladder function. Complete transection of the pelvic nerves in rats results in overflow incontinence and detrusor acontractility till 4 and 6 weeks after transection. (9-11) Castiglione et al. described overflow incontinence in 73% of female rats 10 days after a BPNI.(12) However, hallmark parameters such as residual volume (RV) and voiding efficiency (VE) were not reported and the natural evolution and long-term results after pelvic nerve crush injury are not studied yet. To date, the pathophysiologic mechanisms of neurogenic DU are still unknown.(2)

In this manuscript, we will characterize a BPNI model in rats as a model for neurogenic DU and study temporal changes in detrusor contractility and morphology.

MATERIAL AND METHODS

Animals & Ethical Approval

Animal care was conducted in accordance with the Belgian and European laws, guidelines and policies for animal experimentation, housing and care. All experiments were approved by the Animal Ethics Committee of the KU Leuven under license numbers P191/2014 and P083/2016. One hundred and two male Sprague-Dawley rats of 10-12 weeks (Strain RjHan:SD, Janvier, Le Genest-Saint-Isle, France) were housed in standard laboratory conditions with a 12-hour light/dark cycle with access to food pellets and tap water ad libitum. Thirty-six rats were used for in vivo investigations: 16 rats were followed with micturition pattern analyses and 24-hours urine collections. They were subsequently evaluated by cystometry at 9 weeks.

Additionally 20 rats were used for the remaining cystometry-groups, 30 rats were used for isometric tension recordings and 36 rats were used for morphological investigations.

Study Design

Rats were randomly assigned to 5 experimental groups: sham 1w, 9w and BPNI 1w, 3w and 9w according to the type of surgery and time between surgery and evaluation. The primary outcome of this study was voiding efficiency on awake cystometry. Second, consecutive evaluation by non-invasive assessment (spotting test and 24-hours urine collection) was planned to evaluate the temporal aspects of bladder function following sham surgery or BPNI. Third, bladders and MPGs were harvested at the respective time points for *in vitro* functional studies, molecular and histological examination.

Pelvic Nerve Crush Injury Model

Under Isoflurane anesthesia and buprenorphine analgesia, a midline laparotomy was performed and the prostate was bilaterally exposed. The Major Pelvic Ganglion (MPG) and Pelvic Nerve (PN) were identified posterolateral to the prostate. The PN was isolated and crushed 1 cm lateral of the MPG by applying a Dumont #5 forceps for 3x15s (fine Science Tools, Foster City, CA, USA). In sham animals, the PN was exposed but not injured. The abdomen was closed in 2 layers. Bladders were emptied daily until euthanasia by gentle abdominal pressure. No rats died following sham surgery, while 3 rats died in the first week following BPNI and 1 rat was euthanized due to bladder perforation during manual expression.

Awake Cystometry

Cystometry was performed as previously described.(13) Briefly: under 1,5% Isoflurane anesthesia, a midline laparotomy was performed. A purse-string suture was placed in the bladder dome using Prolene 6-0. A small cystostomy was made inside the purse string and a polyethylene catheter (PE50) was inserted in the bladder dome. The abdomen was closed in 2 layers and the catheter was tunneled to the interscapular region. Catheters were checked for complete drainage of the bladder and animals with dysfunctional catheters were excluded for cystometry studies. After a recovery phase of 3 hours in a metabolic cage, bladders were emptied through the catheter before the experiment and were then filled with room temperature saline solution at a constant filling rate of 100 μ L/min. After a 30 min equilibration period, intravesical pressure and urine weight were recorded for 60 min. Subsequently, residual volumes (RV) were measured during 3 consecutive micturition cycles by disconnecting the catheter and allow spontaneous drainage of the intravesical volume on a balance. This technique was validated using high-frequency micro ultrasound (figure S1). Cystometric parameters were determined as defined by Andersson et al.(14) and Abrams et al.(15)

Micturition Pattern Analyses

Rats were individually placed in a 21x15cm cage with grid surface. After a habituation period of 12 hours, urine output was measured during 6 hours on filter paper. Papers were photographed under ultraviolet light, blinded and the number and area of spots were analyzed.

24-hours Urine Collection

Rats were placed in a metabolic cage above a balance with food pellets and tap water ad libitum. After a 24-hour habituation period, urine output (weight per time) was measured during 24 hours. Data was blinded and the number and weight of voiding events were analyzed. Voiding events were classified in <0,1g and >0,1g as all voiding events in non-operated rats were >0,1g.

High-Frequency Micro-Ultrasound

Under Urethane-anesthesia, the lower abdomen was scanned with the Vevo 2100 system (VisualSonics Inc., Toronto, Canada) with the MS 250 probe.

Isometric Tension Recording

After euthanasia, bladders were harvested, denuded and circular detrusor strips of 4 x 1.5 mm were prepared in ice-cold Krebs solution (in mM: 118.3 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 5.5 glucose, 2.5 CaCl₂). Strips were placed in an organ bath filled with Krebs and attached with a 5/0 Silk ligature to a force transducer (Harvard Apparatus, Holliston, MA, USA) and a fixed hook. The bathing solution was aerated with a mixture of 95% O₂ and 5% CO₂ with a controlled pH of 7.4 and temperature of 35-37°C. Force transducers were calibrated before each experiment. After a 45 mins habituation period, preload of 2 grams was applied every 10 mins for 50 mins. After stabilization, contractile force was recorded to KCl 120 mM, cumulative doses of carbachol (10⁻⁸-10⁻⁴M), non-cumulative doses of α,β -methylene adenosine 5'-triphosphate (α,β -mATP, 10⁻⁷-3x10⁻⁴M) and electrical field stimulation (EFS, square wave pulses of 50V amplitude, 1-32Hz frequency, 0.5ms pulse width, 10s train) in separate detrusor strips. EFS induced contractions were abolished by 1 μ M tetrodotoxin (TTX) (Figure S2).

Tension was digitally recorded using custom-made software and analyzed with OriginPro 8.6 (OriginLab Corp.).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

MPGs were snap-frozen and RNA was extracted using TriPure Reagent (Roche Diagnostics, Mannheim, Germany). RNA quantity was checked with the Thermo Scientific NanoDrop 1000 Spectrophotometer and RNA quality was determined by the Experion RNA Analysis kit (Bio-rad, Belgium). Samples with an RNA quality indicator > 7 were used for cDNA synthesis. qPCR was performed using the StepOnePlus system (Applied biosystems, Foster City, CA, USA). Taqman gene expression assays for protein gene product 9.5 (Rn00568258_m1), choline acetyltransferase (Rn01453446_m1) were used, with glyceraldehyde-3-phosphate dehydrogenase (Rn01775763_g1), beta-actin (Rn00667869_m1) and ribosomal protein S18 (Rn01428913_gH) as endogenous control (Applied biosystems, Foster City, CA, USA). All experiments were used with triplicate technical replicate qPCR reactions per sample.

Histology

Bladders and MPGs were harvested at respective time points, fixed in formalin 4% and embedded in paraffin. Paraffin sections (4µm) were dewaxed in xylene and rehydrated with ethanol. Slides were subsequently stained with haematoxylin and eosin.

Masson Trichome Stain: After deparaffinization and rehydration, bladder slides of 4µm were stained with Weigert's iron haematoxylin for 10 mins. After rinsing, application of Biebrich scarlet-acid fuchsin solution was carried out for 15 mins. Slides were washed, putted in phosphomolybdic-phosphotungstic acid solution for 15 mins and subsequently in aniline blue

solution for 10 mins. Bladders were analyzed by a pathologist and scored for different parameters according to the following score: 0=absent, 1=mild, 2=moderate, 3=severe manifestation.

CHAT stain: MPGs were stained for choline acetyltransferase (CHAT). Endogenous peroxidase was blocked by H₂O₂ 0.5% for 30 mins and subsequently washed with Tris-buffered saline (TBS). Slides were placed in heated Tris/EDTA buffer (pH 9.0) during 60 mins for epitope retrieval, followed by blocking for 60 mins using BSA2%/Tween10%/NFDM1%/NGS4% in TBS. Slides were incubated overnight on 4°C with CHAT antibody 1/2000 (ab178850, Abcam, Cambridge, UK). Next, slides were blocked for 15 mins and subsequently incubated with peroxidase-conjugated 2e antibody (111-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The reaction product was developed by DAB for 10 mins, followed by Mayer haematoxylin counter stain (Sigma-Aldrich, St. Louis, MO, USA).

Statistical Analyses

Statistical analyses were performed with Graphpad Prism 5 (GraphPad Software, San Diego, CA, USA). Analysis of Variance with Bonferroni post-hoc test was used to compare the group means. Non-parametric data was compared with the Kruskal-Wallis test with Bonferroni post-hoc. Experiments with repeated measures design were analyzed by the repeated-measures ANOVA or Friedman test with Bonferroni post-hoc test according to data distribution. Categorical variables were compared by Chi-square test. A p-value of < 0.05 was considered as statistically significant.

RESULTS

BPNI establishes a rat model for neurogenic DU

Sham rats exhibited regular micturition cycles at 1 and 9 weeks after surgery with VE of 100% and 96%, respectively. One week after BPNI, a pattern of continuous dribbling without micturition cycles with VE of 0.01% was seen, which indicates overflow incontinence. At 3 and 9 weeks after BPNI, partial functional recovery was observed with VE of 22% and 29%, respectively (figure 1). Oscillations of baseline intravesical pressure, which were never present following sham surgery, emerged 1 week after BPNI. Recovery to stable baseline pressure was observed at 9 weeks following injury (figure 1A). Frequency analysis by Fourier transformation revealed a peak frequency of $0.0668 \pm 0.0013\text{Hz}$ 1 week after BPNI, which corresponds to 4 autonomic contractions/min. Cystometric parameters are summarized in table 1.

Following BPNI, the bladder initially decompensates, with partial functional recovery at intermediate and long-term follow up

Urine spotting analysis revealed an increased number ($p=0.0008$) of significantly smaller spots ($\sim 80\%$, $p<0.0001$) dispersed around the cage one week after BPNI compared to pre-surgery. We observed partial functional recovery at 3 and 9 weeks (figure 2A and 2C) with a reduced number of spots, whereas the number and mean area of spots were not significantly different after sham surgery (figure 2B and 2D).

Twenty-four hours continuous urine output recording revealed a shift in voiding events after BPNI. Whereas voiding events of $<0.1\text{g}$ were not present before BPNI, 84% of voiding events were $<0.1\text{g}$ 1 week following BPNI. Partial functional recovery was seen with 18% of voiding events $<0.1\text{g}$ at 9 weeks after BPNI. ($p<0.0001$, figure 2E-F). In contrast to the shift observed following BPNI, voiding events were $>0.1\text{g}$ before and after sham surgery. (figure 2G-H). Water intake was not different between time points in both study groups (figure S3).

Detrusor contractility was preserved after BPNI

Contractile force in response to depolarization of the detrusor smooth muscle by KCl 120mM was not reduced after BPNI compared to sham ($p=0.47$, figure 3A). The bladder-to-body weight ratio was more than doubled following BPNI compared to sham ($p<0.0001$, figure 3B), in line with increased detrusor thickness observed 3 and 9 weeks after BPNI ($p=0.0003$, figure 3C). Vacuolization of the detrusor and subserosal fibrosis were observed following BPNI, but was not present after sham surgery (figure 3D).

Detrusor contractility to cholinergic and purinergic stimulation was preserved following BPNI

Although the maximum contractile forces in response to carbachol were unaltered following BPNI (figure 4A), the sensitivity to carbachol was significantly increased at one week and normalized towards 9 weeks after BPNI ($p<0.0001$, figure 4A inset).

The normalized contractile forces in response to α,β -mATP were increased (from 10 μ M) 1 week after BPNI compared to sham with recovery at 3 and 9 weeks ($p=0.0015$, figure 4B).

Nerve-mediated detrusor contractility was reduced after BPNI

Contractile responses to EFS were decreased by 40% at 1 week after BPNI compared to sham surgery. Partial recovery was observed at 3 and 9 weeks at 32Hz with a persistent 18% reduction 9 weeks following BPNI compared to sham surgery ($p=0.0051$, figure 4C).

Denervation of the pelvic plexus was observed 1 week following BPNI

To investigate molecular neural alterations, we investigated the cell bodies of the post-ganglionic neurons to the bladder at the MPG. One week after BPNI, relative expression of the neural marker protein gene product 9.5 (PGP9.5, $p=0.0012$) and the cholinergic marker choline acetyltransferase (CHAT, $p=0.0004$) were significantly reduced compared to sham. Normalization was observed at 9 weeks (figure 5A-B). No clear morphological changes were observed between the different study groups with comparable total number of neurons between study groups. No differences were observed in the proportion of CHAT expression neurons (figure 5C).

DISCUSSION

In this study, we characterized the BPNI rat model as a model for pelvic surgery induced neurogenic DU. Additionally, our study provides insights into the pathophysiologic mechanisms underlying this disease.

Detrusor underactivity is a voiding dysfunction characterized by a reduced VE, decreased detrusor contraction and/or decreased or absent flow on urodynamics.(1,16) As such, one of our primary outcomes in this animal model was VE. Voiding efficiency was defined as $(\text{voided volume (VV)} / (\text{VV} + \text{RV})) \times 100$.(15) Therefore, precise measurement of RV is essential. Residual volume was measured by disconnecting the intravesical catheter and allowing spontaneous drainage of the bladder. We validated this technique in sham and BPNI animals (figure S1). Cystometry was performed under conscious conditions as anesthesia, such as urethane, increases RV and decreases VE.(17)

Voiding efficiency was markedly reduced at all timepoints after BPNI, with urinary retention at one week and partial recovery thereafter. Rats emptied 29% of the bladder 9 weeks after BPNI compared to 96% following sham surgery. Maximum pressures were 70% reduced 9 weeks after BPNI. Interestingly, this BPNI animal model mimics the clinical evolution of bladder dysfunction following pelvic surgery. The percentage of patients reporting difficulty to empty their bladder increases initially after TME, but gradually decreases thereafter and stabilizes 18 months postoperatively.(4) Our rats suffered from overflow incontinence 1 week after BPNI, with subsequent partial recovery at later time points. These results are comparable to a previously published study demonstrating overflow incontinence in 73% of rats 10 days postoperative.(12) To our knowledge, this study is the first to report RV, VE or long-term follow-up cystometric results after PN crush injury. In contrast to previous studies(12,18), we used male rats to eliminate the influence of hormonal fluctuations on the micturition pattern.(19) Given the markedly reduced VE and the similarities in the evolution of bladder function between the BPNI model and the clinical state after pelvic surgery, the BPNI model seems a suitable model for neurogenic DU.

Following peripheral neural injury, Schwann cells and inflammatory cells phagocytize the degenerated nerve and promote neural regeneration within the first days after injury. (20,21) In our study, voiding function did not improve between week 3 and 9 after BPNI, suggesting this might be a suitable follow up period for future experiments studying DU. Improvement of bladder function after 9 weeks is not expected, since axons regenerate at 1 mm/day(20) and they should regenerate over 10-20mm in the BPNI model. Similarly to our findings, Payne et al. recently investigated the regeneration of pelvic motor and sensory autonomic axons and found no additional recovery between 4 and 12 weeks post-injury.(22)

The existence of autonomous bladder micromotility has been described in many species and its relation to functional bladder disorders, including DU, has recently been hypothesized.(23) We observed an increase in the autonomic bladder activity on cystometry 1 week after BPNI with a frequency of approximately 4 contractions/min. Recently, Persyn et al. found a similar frequency of non-voiding activity (NVA) in anesthetized rats.(24) Interestingly, the amplitude of NVA increased immediately after removal of the MPG but not after removal of the hypogastric or pelvic nerve.(24) Similar conclusions were made by a study of Sugaya et al., where ganglion blockage increased the amplitude of spontaneous bladder contractions in contrast to transection of the dorsal and ventral roots.(25) These studies imply an inhibitory influence of the MPG on the bladder autonomic activity. Following BPNI, dysfunction of the MPG caused by a reduced input of the pelvic nerve could therefore cause a reduced inhibition on bladder autonomic activity.

Contractile responses to direct depolarization of the SMC by KCl were unaltered after BPNI, which is also observed 4 weeks after pelvic nerve cut injury.(11) A degenerative pattern of the detrusor is described in ultrastructural studies in aging related hypocontractility and DU.(26) An important degenerative sign was vacuolization of the sarcoplasm, which was markedly more frequent in BPNI bladders. However, we describe an increased detrusor thickness and increased bladder to body weight after injury, suggesting detrusor hypertrophy, similar to previous studies.(12,18) Therefore, we hypothesize that detrusor hypertrophy compensates for the degenerative changes of the muscle we observed following BPNI.

Parasympathetic nerve terminals in the bladder release neurotransmitters like acetylcholine and ATP that cause the detrusor to contract. We did not observe reduced contractile responses to carbachol following BPNI. In contrast, BPNI bladders showed increased sensitivity to carbachol. In literature, increased cholinergic contractility 1 week after injury to the pelvic

plexus with subsequent recovery is reported after BPNI.(12,18) Following a cut injury of the pelvic nerve, a similar increased sensitivity to carbachol was observed. However, the maximum contractile responses to carbachol were reduced after a cut injury which is in line with the higher severity of the lesion.(11) Conserved responses to carbachol are also described in the lumbar canal stenosis model for DU.(27). We also investigated the responsiveness of the bladder to purinergic agonists, which has not been reported in literature. At one week, contractile responses to α,β -mATP are significantly increased, but this hypersensitivity normalizes at 3 and 9 weeks. Hence, it seems that the detrusor SMC temporarily tries to compensate for the reduced excitatory signals but loses this ability again once activation partially restores.

Although detrusor contractility to direct depolarization and parasympathetic stimulation was preserved, nerve-induced contractility was reduced during overflow incontinence with only partial recovery in the DU-phase. This is in line with previous reports of preganglionic neural crush injury where partial functional recovery was observed but morphologic recovery could not be objectified.(12,18) Of interest, Payne et al. observed recovery of sensory neurons, but not motor neurons following pelvic neural injury.(22) Taken together, our results suggest that the bladder SMC try to increase its ability to react to the reduced neural input during overflow incontinence. However, the SMC lose this ability once partial neural recovery has occurred in the DU-phase.

Besides preganglionic parasympathetic fibers, the pelvic nerve also contains an important part of the sensory fibers innervating the pelvic organs.(8) In this study, we did not evaluate sensory dysfunction after BPNI. However, a recent study of our group investigated sensory dysfunction 4 week after BPNI. Intravesical activation of TRPV4 improved bladder dysfunction by increasing afferent signaling.(28) BPNI bladders were characterized by acute urinary

retention and overflow incontinence the first week after surgery. Beside neural injury, bladder distention itself could therefore have contributed to the observed phenotype. Increased post-void residuals and urinary retention are on the other hand common features of DU(1,2) and are therefore part of the clinical phenotype. Our results are limited to the preclinical setting in rodents. Translational research is needed to evaluate how the findings in this rat model could thrive the quest for novel treatment options for neurogenic detrusor underactivity.

CONCLUSIONS

Bilateral pelvic nerve crush injury in rats represents an interesting model for neurogenic DU. Bladder decompensation with overflow incontinence is observed 1 week after injury with partial functional recovery at 3 and 9 weeks, in which the animals display a phenotype of DU. In this animal model, the detrusor remains contractile to direct and parasympathetic stimulation. However denervation of the detrusor and temporary denervation of the pelvic plexus are important players in the pathophysiology of DU. Hence, optimizing neuroprotection and neural regeneration to improve voiding represents an exciting avenue for further research in DU.

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CONFLICTS OF INTEREST

None declared.

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FIGURES

Figure 1: Conscious Cystometry. **A.** Representative traces of awake cystometry 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. Arrows indicate micturitions. **B-D.** Residual volume, voiding efficiency and maximum pressure at 1, 3 and 9 weeks post-surgery. Values are represented as box plots, whiskers report min-max. (n=6-7 per group) Kruskal-Wallis test with Bonferroni post-hoc test. *p<0.05, **p<0.05.

Table 1: Analyses of Conscious Cystometry. Values are represented as median (IQR). (n=6-7 per group) Kruskal-Wallis test with Bonferroni post-hoc test. *p<0.05 and **p<0.01 compared to

sham 1w; $\$p<0.05$ and $\$\$p<0.01$ compared to sham 9w; $\#p<0.05$ and $\#\#p<0.01$ compared to BPNI 1w.

Figure 2: Consecutive non-invasive *in vivo* assessment of bladder function. **A-B.** Representative photographs of micturition pattern analysis following BPNI (A) and sham surgery (B). **C-D.** Analysis of micturition pattern analyses following BPNI (C) and sham surgery (D). **E, G.** Combined scatter dot plots of the 24-hours urine collections in BPNI-rats (E) and sham rats (G). **F, H.** Bar graph of 24-hours urine collections in BPNI-rats (F) and sham rats (H). (C-D) Repeated measures one-way ANOVA or Friedman test with Bonferroni post-hoc test. (n=7-8 per group) (F, H): Chi-square test with Bonferroni post-hoc test. (n=6-7 per group) $*p<0.05$; $**p<0.01$.

Figure 3: Functional and morphological investigations of the detrusor following BPNI and sham surgery. **A.** Isometric tension recording to KCl 120mM in whole-thickness bladder strips 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. Results are normalized on strip weight. **B.** Bladder-to-body weight ratio 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. **C.** Detrusor thickness 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. **D.** Representative photomicrographs of Masson's Trichome stain at 5x, 40x and 20x magnification. Lines indicate detrusor thickness. Vacuolae were marked by arrowheads. Arrows indicate subserosal fibrosis, while asterisks (*) mark subserosal edema. Kruskal-Wallis test with Bonferroni post-hoc test. (n=6-8 per group) $**p<0.01$.

Figure 4: Functional assessment of cholinergic, purinergic and electrical field mediated contractility 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. **A.** Dose-response curves of carbachol in denuded bladder strips and their respective half maximal effective concentration (EC₅₀, inset). **B.** Dose-response curves of α,β -mATP in denuded bladder

strips. **C.** Dose-response curve of electrical field stimulation (EFS) in whole-thickness bladder strips. (A) One-way ANOVA with Bonferroni post-hoc test. (n=6 per group) **p<0.01. (B-C) Two-way ANOVA with Bonferroni post-hoc test. (n=6 per group) *p<0.05; **p<0.01.

Figure 5: Gene expression and morphological assessment of the major pelvic ganglion (MPG) 1 and 9 weeks after sham surgery and 1, 3 and 9 weeks after BPNI. **A.** RT-qPCR of protein gene product 9.5 (PGP 9.5). **B.** RT-qPCR of Choline Acetyltransferase (CHAT). **C.** Pooled analysis of CHAT-positive and negative neurons in the MPG on immunohistochemistry. (A-B) Kruskal-Wallis test with Bonferroni post-hoc test. (n=6-7 per group) *p<0.05, **p<0.05 (C) Chi-square test. (n=6-8 per group)

	Sham 1w	Sham 9w	PNI 1w	PNI 3w	PNI 9w	p-value
Maximum Pressure (cm H ₂ O)	61.0 (53.4–71.7)	77.2 (74.2–82.6)	n/a	26.2 (23.0–26.9)* ^{\$}	22.5 (20.7–25.7)** ^{,\$\$}	0.0002
Basal Pressure (cm H ₂ O)	9.5 (7.4–12.0)	9.4 (8.1–9.9)	n/a	10.2 (9.3–11.3)	8.2 (7.3–9.4)	0.299
Intercontractile Interval (s)	215 (147–250)	479 (330–726)	n/a	787 (619–852)*	688 (591–923)**	0.0017
Micturition Volume (g)	0.33 (0.23–0.42)	0.83 (0.52–1.26)	n/a	0.76 (0.62–1.21)*	0.96 (0.54–1.11)*	0.0117
Residual Volume (g)	0.00 (0.00–0.10)	0.03 (0.00–0.15)	7.82 (7.12–9.15)* ^{\$}	5.24 (2.72–6.62)*	4.51 (2.54–5.77)* ^{,\$,#}	< 0.0001
Voiding Efficiency (%)	100 (83.97–100)	96.0 (92.1–100)	0.01 (0.00–0.01)* ^{\$}	22.1 (13.3–28.7)* ^{,\$}	28.5 (20.8–41.1)* ^{,\$,##}	< 0.0001
Mean Pressure (cm H ₂ O)	11.7 (10.9–13.3)	13.1 (12.0–14.2)	22.9 (22.3–26.6)* ^{\$}	15.1 (13.6–16.8) [#]	11.3 (10.8–12.0) ^{##}	0.0005
Intermicturition Pressure (cm H ₂ O)	10.2 (9.1–11.9)	12.3 (10.6–12.7)	n/a	15.1 (13.6–17.9)	11.3 (10.4–11.9)	0.0565