NEUROTROPHIN THERAPY IMPROVES RECOVERY FROM POSTPARTUM STRESS

URINARY INCONTINENCE FOLLOWING SIMULATED CHILDBIRTH INJURY IN RATS

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Neurotrophin Therapy Improves Recovery from Postpartum Stress Urinary Incontinence Following Simulated Childbirth Injury in Rats

Abstract
Bradley C Gill

Aims: Stress Urinary Incontinence (SUI) affects women both acutely and chronically after vaginal delivery. Current SUI treatments assume the neuromuscular continence mechanism, comprised of the pudendal nerve (PN) and external urethral sphincter (EUS), is either intact or irreparable. This study investigates the ability of neurotrophin therapy to facilitate recovery of the neuromuscular continence mechanism.

Methods: Virgin, Sprague Dawley rats received simulated childbirth injury or sham injury and treatment with continuous infusion of brain derived neurotrophic factor (BDNF) or saline placebo to the site of PN injury. Continence was assessed by leak point pressure (LPP) and EUS electromyography (EMG) 14 and 21 days after injury. Structural recovery was assessed histologically. Molecular assessment of the muscular and neuroregenerative response was determined via measurement of EUS BDNF and PN βII-tubulin expression respectively, 4, 8, and 12 days after injury.

Results: Following injury, LPP was significantly reduced with saline compared to either BDNF treatment or sham injury. Similarly, compared to sham injury, resting EUS EMG
amplitude and firing rate, as well as amplitude during LPP were significantly reduced with saline but not with BDNF treatment. Histology confirmed improved EUS recovery with BDNF treatment. EUS BDNF and PN βII-tubulin expression demonstrated that BDNF improved the neurogenerative response and may facilitate sphincteric recovery.

**Conclusions:** Continuous, targeted, neurotrophin therapy accelerates continence recovery after simulated childbirth injury likely through stimulating neuroregeneration and facilitating EUS recovery and re-innervation. Neurotrophins or other therapies targeting neuromuscular regeneration may be useful for treating SUI related to failure of the neuromuscular continence mechanism.
Introduction

Approximately 25%-46% of women suffer from urinary incontinence, with stress urinary incontinence (SUI) 250% more prevalent amongst women who experienced vaginal childbirth; a process associated with damage to the tissues of the pelvic floor.\textsuperscript{1, 2}

Numerous surgical procedures and symptom management techniques for SUI exist, but none target its underlying neuromuscular pathophysiology.\textsuperscript{3, 4} Current therapies assume the neuromuscular continence mechanism is either intact (i.e. pelvic floor physiotherapy or pharmacotherapy) or irreparable (i.e. urethral slings or bulking injections).

Animal models of simulated vaginal birth demonstrate that skeletal muscle of the external urethral sphincter (EUS) is injured during simulated parturition.\textsuperscript{5} Injury to the pudendal nerve (PN), which innervates the EUS, has also been observed clinically and in animal models.\textsuperscript{6, 7} Thus, childbirth inflicts a double insult to the neuromuscular urinary continence mechanism: damage to the muscles responsible for preventing leakage and interruption of the nerve supply controlling them. PN damage likely contributes appreciably to the pathogenesis of SUI, not only via directly impairing muscular function but also through limiting EUS recovery because of persistent denervation.\textsuperscript{8}

Following peripheral nerve injury, upregulation of brain-derived neurotrophic factor (BDNF) occurs in innervated target organs and axons distal to injury sites.\textsuperscript{9} BDNF is required for neuroregeneration and local administration to nerve injury sites reduces motoneuron death following transection.\textsuperscript{10} In contrast, BDNF is inhibitory to and thus reduced during neuromuscular junction formation and restoration as well as myogenic
The concurrent injury of EUS muscle and the PN during childbirth results in BDNF downregulation to facilitate EUS recovery, likely impairing the PN neuroregenerative response since isolated PN crush without EUS injury requires upregulation of BDNF for neuroregeneration.\textsuperscript{13,14}

EUS electromyography (EMG) and PN electroneurography (ENG), as well as urinary leak point pressure (LPP), show slowed recovery when the EUS and PN are simultaneously injured compared to either injury in isolation.\textsuperscript{13,14} More severe loss of NMJ integrity at the EUS has also been demonstrated in this combined injury model.\textsuperscript{15} Therefore, exogenous BDNF may overcome the muscle injury-induced BDNF downregulation in the EUS and improve both PN recovery and EUS re-innervation, leading to improved continence following childbirth. This study aimed to determine if local continuous administration of BDNF improves the regenerative response of the PN as well as both structural and functional recovery of the EUS following simulated childbirth injury.
Materials and Methods

Animal Model: All experiments were pre-approved by the local Institutional Animal Care and Use Committee. Female, virgin, Sprague-Dawley rats (200-225g) were separated into 3 groups. The first (N=32) received vaginal distention (VD) and PN crush (PNC) followed immediately by 4-21 days of BDNF treatment. The second (N=31) received identical injuries but was treated with saline placebo. The third (N=30) underwent sham PNC and received no treatment. VD and PNC were done as previously described, using intraperitoneal ketamine (100mg/kg) and xylene (10mg/kg) or inhaled isofluroane anesthesia for functional and molecular analyses, respectively. Briefly, VD was created using a de-tipped Foley catheter filled with 3ml water and secured in the vagina with a 3-0 vicryl suture through the labia majora for 4 hours. Bilateral PNC was performed subsequently by dorsally isolating the PN in the ischiorectal fossa and crushing it with a Castro-Viejo needle twice, consecutively for 30 seconds each. Isolation alone was performed for sham injury.

Intervention: Treatment was provided using bilateral, dorsally placed, subcutaneous miniature-osmotic pumps with vinyl catheters (V/3A, Durect) to the nerve injury site and secured to nearby paraspinal musculature with 5-0 prolene suture. Following an overnight incubation in 37°C physiologic saline, pumps provided a constant 0.5μl/hr efflux for 1 (Alzet Mini-Osmotic Pump, Model 1007D, Durect) or 2 (Model 2002) weeks. The albumin solution consisted of 0.25g albumin (Albumin from Rat Serum, A6414, Sigma-Aldrich) dissolved in 100ml sterile saline. This both acted as placebo treatment
and served to stabilize the BDNF solution, which was created by adding 1mg BDNF (Recombinant Human BDNF, CYT-207, ProSpec) to 6ml of solution. Overall, this provided the injury site a local, targeted BDNF dose of 2μg/day.

**Functional Assessment:** Continuous EUS EMG was recorded during LPP 2 and 3 weeks after injury and treatment initiation, as described previously. Specifically, via pubic symphysectomy, the EUS was revealed and caudal extension of the incision exposed the bladder. A plane was then developed laterally to expose the right PN in the ischiorectal fossa and catheter placement and patency were confirmed. Next, polyethylene tubing was placed transurethrally into the bladder and platinum bipolar parallel rod electrodes rested ventrally on the EUS for EMG recording. Direct bladder compression was performed using a cotton-tipped applicator that was rapidly removed when leakage at the urethral meatus occurred. One second segments of electrophysiological and bladder pressure recordings from baseline and at LPP were analyzed using previously described techniques. Mean EUS EMG amplitude and firing rate, as well as mean bladder pressures were calculated for each animal from a minimum of 3 separate LPP maneuvers.

**Tissue Procurement:** Spinal cord and urethra were procured from 5 animals in each of the 3 injury and treatment groups 4, 8, and 12 days after injury and treatment initiation. Following ketamine-xylazine anesthesia, sternotomy and intracardiac perfusion of heprinated, phosphate-buffered saline was performed. After an L2-S3 laminectomy, the
spinal cord was frozen in-situ with liquid nitrogen and sharply transected at L3 and S2. The isolated segment was placed into a pre-cooled cryotube and stored in liquid nitrogen until embedded (Tissue-Tek OCT Compound, 4583OCT, Sakura Finetek) for cryostat sectioning. The anterior vaginal wall, urethra, and bladder neck were then removed en bloc after symphysectomy, embedded, flash-frozen, and stored at -80°C. Cryostat sectioning of the spinal cord (12µm) at L6 containing Onuf’s Nucleus (ON) and urogenital tissues at the EUS was performed. Sections were collected on alternating standard glass slides and laser microdissection (LMD) slides (Frame Slides PET-Membrane, 11505151, Leica Microsystems).

Relative Gene Expression Measurement: Thionin staining and ethanol-xylene fixation identified PN cell bodies that were collected via isolating the ON region using LMD. Striated EUS muscle was collected similarly. Collected cells were lysed, ribonucleic acid (RNA) isolated (RNAqueous-Micro, Applied Biosystems) for reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) into complimentary deoxyribonucleic acid (cDNA) using pre-packaged kits. Polymerase chain reaction (PCR) pre-amplification (TaqMan PreAmp Master Mix, Applied Biosystems) specific for βII-tubulin (TaqMan Gene Expression Assay, Rn01435557_g1, Applied Biosystems) in PN samples or BDNF (TaqMan Gene Expression Assay, Rn02531967_s1, Applied Biosystems) in EUS samples was performed. Using standard reagents (TaqMan Gene Expression Master Mix, Applied Biosystems) quantitative-PCR (Q-PCR) normalized to 18S ribosomal RNA (TaqMan Gene Expression Assay, Rn03928990_g1, Applied Biosystems) was
performed to assess β_{II}-tubulin expression in ON and BDNF in the EUS relative to that in the sham-injured group at 12 day after injury.

**Histology and Immunofluorescence:** Glass slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes and then washed 3 times with PBS. Tissues were blocked using standard universal blocking buffer for 30 minutes and then exposed to primary antibodies at 4°C overnight, targeting BDNF (1:50 Rabbit Polyclonal BDNF, SC546, Santa Cruz Biotechnology) in the EUS and β_{II}-tubulin (1:50 Mouse Monoclonal β_{II}-tubulin, SC47751, Santa Cruz Biotechnology) along with neurofilament (1:50 Rabbit Polyclonal Neurofilament, N4142, Sigma-Aldrich) in PN sections. Following 3 PBS washes, slides were exposed to secondary antibodies (1:400 AlexaFluro 488 Donkey anti-Mouse, A21202, or 1:400 AlexaFluro 488 Donkey anti-Rabbit, A21206, Invitrogen), with an additional antibody (1:1,200 DyLight 594 Goat Anti-Rabbit, 111-515-144, Thermo Scientific) used for PN sections. After 3 more PBS washes slides underwent anti-fade (ProLong Gold, P36930, Invitrogen) treatment. Additional glass slides were fixed in Bouin’s solution and stained with Masson’s trichrome. Images were acquired with standard ultraviolet immunofluorescence and light microscopy.

**Statistical Methods:** Statistical evaluations (SigmaStat, Version 10.0, Systat Software) of normally distributed functional outcomes were performed using a standard One-Way ANOVA followed by Student-Newman-Keuls pairwise comparisons. A Kruskal-Wallis One-Way ANOVA on Ranks followed by Dunn’s pairwise comparisons were used for
non-normally distributed data. Molecular expression data were analyzed using a Two-Way ANOVA followed by Holm-Sidak multiple pairwise comparisons. For all analyses, $p<0.05$ indicated a statistically significant difference between groups. Data is presented as mean ± standard error of the mean. Images were assessed qualitatively by a blinded observer.
Results

**Urinary Leak Point Pressure:** Animals treated with saline demonstrated significantly decreased mean LPP 2 weeks after injury compared to both untreated sham-injured (p=0.032) and BDNF-treated (p=0.033) animals (Fig 1). No significant differences in mean LPP were noted 3 weeks after injury. Baseline bladder pressures did not significantly differ at any time.

**Urethral Sphincter Electromyography:** Saline treatment resulted in significantly lower median resting EUS EMG amplitude 2 weeks after injury compared to sham injury (p<0.05) while BDNF treatment did not (Fig 2). Similarly, 2 weeks after injury median EUS EMG amplitude at LPP was significantly reduced with saline treatment compared to sham injury (p<0.05) but not with BDNF treatment. Three weeks after injury, no significant differences existed in mean EUS EMG amplitude at rest or during LPP. Saline treatment resulted in significantly reduced mean resting EUS EMG firing rate 2 weeks after injury compared to sham injury (p=0.006) while BDNF treatment did not (Fig 2). No significant differences in mean EUS EMG firing rate at LPP existed 2 weeks after injury. Three weeks after injury, no significant differences existed in mean EUS EMG firing rate at rest or during LPP.

**Urinary Sphincter Neurotrophins:** No significant differences in EUS BDNF expression were noted between groups at 4 or 8 days after injury (Fig 3). Significantly higher EUS BDNF expression occurred 12 days after injury and saline treatment compared to BDNF
treatment ($p=0.007$) or sham injury ($p = 0.001$); however, the latter was the basis of relative PCR comparisons and may be artifactual. Significantly less BDNF expression occurred with saline treatment 4 ($p=0.005$) and 8 ($p=0.011$) days after injury compared to 12 days after injury. Levels of EUS BDNF expression with PN BDNF treatment appeared relatively stable while in sham-injured animals, a transient increase in BDNF occurred, although these observations were not statistically significant.

**Pudendal Nerve Neuroregenerative Response:** No significant differences in PN $\beta_{\text{II}}$-tubulin expression were detected (Fig 3). However, saline treatment appeared to reduce $\beta_{\text{II}}$-tubulin expression 4 and 8 days after injury compared to BDNF treatment or sham injury, although non-significantly. A transient increase in PN $\beta_{\text{II}}$-tubulin expression 8 days after injury appeared to occur with BDNF treatment compared to sham injury, but was not statistically significant.

**Microscopic Imaging:** Sham-injured animals demonstrated normal appearing EUS histology (Fig 4). Less EUS atrophy, disruption, and fibrosis 4 and 8 days after injury were observed with BDNF compared to saline treatment. The EUS in BDNF-treated animals resembled those of sham-injured animals 12 days after injury, while those in injured animals treated with saline did not. Immunofluorescence demonstrated a consistent pattern 4 and 8 days after injury with sham-injured rats expressing more EUS BDNF than injured animals treated with saline, which expressed more EUS BDNF than injured animals treated with BDNF (Fig 4). Injured animals treated with saline expressed the
most EUS BDNF 12 days after injury, while sham-injured animals expressed the least. Immunofluorescence demonstrated more PN βII-tubulin 4 days after injury with BDNF treatment than either saline treatment or sham injury (Fig 5). BDNF-treated animals demonstrated the most βII-tubulin 8 days after injury, while saline-treated animals expressed the least. BDNF-treated animals expressed less βII-tubulin than saline-treated animals 12 day after injury, but more than to sham-injured animals (Fig 5).
Discussion

SUI was estimated to cost the US over $19.5 billion in 2000, with the burden expected to increase as the population ages. Since postpartum incontinence is associated with a 2.4-fold greater likelihood of chronic SUI development, effectively treating as well as preventing this condition could provide substantial benefits. Mechanical SUI etiologies, including urethral hypermobility and mild pelvic organ prolapse, are amenable to surgically correction. However, sphincteric deficiency, whether from direct injury to the sphincter or its innervation, is managed sub-optimally with surgery, bulking injections, or physiotherapy. Regenerative treatments, such as stem cells, have the potential to repair sphincteric injury. However, in the presence of PN injury, targeting the nerve for regeneration is likely more beneficial than improving bulk in a denervated sphincter. Therefore, this investigation aimed to improve PN recovery to facilitate the restoration of EUS function after simulated childbirth injury.

Neurotrophins, including BDNF, are necessary for the maintenance of innervation, normal neuromuscular function, and nerve regrowth. They are produced by myelinating cells and innervated target organs after nerve injury. Neurotrophin treatment improved functional and anatomic recovery in peripheral nerve injury models. The same rodent model used in this study demonstrated EUS neurotrophin upregulation following PN crush injury. Therefore, it is likely that BDNF treatment could improve functional recovery of the neuromuscular continence system after PN injury. To date, localized, continuous neurotrophin treatment has been the most effective means of treatment compared to other dosing.
Despite facilitating regeneration of peripheral nerves, BDNF is detrimental to muscular recovery and development since it impairs neuromuscular junction formation as well as myogenic myoblast differentiation and maturation.\textsuperscript{11, 12} Childbirth causes injury to both the PN and EUS, as well as their NMJ, all of which comprise the neuromuscular continence mechanism.\textsuperscript{5, 14, 15} In this animal model, BDNF downregulation occurs after EUS injury, supporting the hypothesis it may hinder muscular recovery.\textsuperscript{12, 14} Therefore, it is imperative that BDNF treatment intended to improve PN neuroregeneration neither impairs EUS nor NMJ recovery, providing further rationale for the targeted delivery implemented in this study.

The same combined PNC-VD model used in this study demonstrated LPP recovery 3 weeks after injury, similar to saline-treated animals in this study.\textsuperscript{14} In comparison, LPP recovered 10 days after VD and 14 days after PNC in prior work, suggesting that the synergistic effect of combined EUS and PN injuries slows recovery.\textsuperscript{14} Other work using the same animal model and a more severe PN crush with and without VD identified persistent LPP deficits and impairments in different EUS EMG measures 3 weeks after injury.\textsuperscript{13} The current study demonstrated that BDNF treatment accelerated recovery from 3 to 2 weeks, likely as a result of concurrent PN and EUS improvement, as measured by improved LPP and EUS EMG. Histological findings of less EUS atrophy and fibrosis with BDNF treatment compared to saline treatment supported the functional findings, with the latter resembling previous EUS changes after untreated VD-PNC.\textsuperscript{14}

A 2\(\mu\)g/day dose of localized, continuous BDNF treatment, higher than that used in nerve tubes, was given to account for diffusion from the catheter site.\textsuperscript{22} Both PCR and
immunofluorescence detected lower EUS BDNF levels 4 days after PNC-VD than after sham injury, similar to previously observed EUS BDNF patterns 1 day after injury.\textsuperscript{14} This pattern persisted 8 and 12 days after injury, suggesting local PN BDNF treatment may prevent EUS BDNF upregulation after PN injury, which would facilitate both EUS muscle recovery and NMJ restoration.

The PN neuroregenerative response, indicated by expression of $\beta_{II}$-tubulin, a cytoskeletal protein upregulated in axonal growth and repair, was observed previously to increase 2.49-fold 7 days after isolated PN crush injury, which is similar, despite the lack of statistical significance, to the 2.29-fold upregulation 8 days after untreated sham injury.\textsuperscript{16} This likely stems from PN trauma during dissection and isolation in the sham injury. The documented upregulation following PN crush was no longer significant 14 days after injury, which was similarly observed 12 days after sham injury in this study.\textsuperscript{16} Patterns of PN $\beta_{II}$-tubulin mirrored those of EUS BDNF expression after sham injury, suggesting EUS BDNF levels and PN neuroregenerative response may be related.

Similar trends between EUS BDNF expression and PN neuroregenerative response were evident after injury treated with saline. Specifically, saline treatment appeared to delay both EUS BDNF upregulation and PN neuroregenerative response, with no statistically significant difference in either EUS BDNF or PN $\beta_{II}$-tubulin expression 4 or 8 days after injury. Thus, it appears that unlike sham injury, which likely has a component of isolated PN trauma and demonstrates an appreciable EUS BDNF upregulation, a combined PN and EUS injury appears to impair stimulation of the neuroregenerative response by delaying the EUS BDNF increase.
PN BDNF treatment appeared to overcome the lack of EUS BDNF upregulation associated with simulated childbirth injury involving both the PN and EUS. Treating the PN with BDNF was related to a 4.2-fold, but non-significant neuroregenerative response 8 days after injury, which was no longer present 12 days after injury. Furthermore, a decrease in EUS BDNF occurs concurrently with the increased neuroregenerative response, suggesting another source of stimulus for the PN recovery. Therefore, it is likely that BDNF treatment provided the drive for neuroregeneration.

A limitation of the current study is the use of simulated delivery in a quadruped animal model to investigate post-partum SUI. However, since no other animal undergoes as traumatic a delivery as humans, simulated delivery must be implemented for the preclinical testing of potential therapies. Additionally, the involvement of PN trauma in the sham injury model challenged analyses in the study. While the sham injury was intended to reproduce other physiologic effects of the model, it was not intended to produce nerve trauma, which likely limited the significant differences detected in various analyses. Nonetheless, the study design and multi-modal analysis of BDNF treatment compared to saline treatment provided insight into the potential beneficial effects of neurotrophin therapy for maternal childbirth injuries.

With the growing interest in preventative medicine, postpartum treatments aimed at facilitating recovery of the neuromuscular continence system may help reduce the societal burdens of SUI. Degradable neurotrophin releasing beads have been shown to improve neuroregeneration and could feasibly be injected near the PN. Degradable neurotrophin releasing beads have been shown to improve neuroregeneration and could feasibly be injected near the PN. PN electrical stimulation resulted in increased neurotrophin expression and PN neuroregenerative
response in a rat model of childbirth injury.24 Lastly, adipose-derived stem cells have been found to secrete BDNF and promote nerve healing.25 Therefore, treating the neuromuscular continence mechanism and optimizing recovery of both PN and EUS may be a feasible postpartum intervention.
Conclusions

Following simulated childbirth injury, localized and continuous PN treatment with BDNF accelerated recovery of the neuromuscular continence mechanism, as evidenced by functional, electrophysiological, anatomic, and molecular data. Since PN and EUS injury result in stimuli that oppose recovery of the other structure, overcoming this phenomenon therapeutically may provide a means of facilitating recovery from these complex neuromuscular injuries. Further investigation into means of increasing neuromuscular continence recovery is needed.
**Figure Legends**

**Figure 1:** Leak point pressure in all injury-treatment groups between 2 and 3 weeks as indicated in the legend. Results shown are the means and standard errors of data from 6 to 11 animals. Each symbol represents statistical significance within the same time point as follows: \( x \) indicates a difference from the sham injury group, while + designates a difference from BDNF treatment cohort.

**Figure 2:** External urethral sphincter electrophysiological data in all injury-treatment groups between 2 and 3 weeks as indicated in the legend: amplitude at baseline (A) and during leak point pressure (B) and firing rate at baseline (C) and during leak point pressure (D). Within the same time point, \( x \) designates statistical significance of a difference from the sham injury group. Results shown are the means and standard errors of data from 6 to 11 animals.

**Figure 3:** Relative gene expression levels, as assessed by quantitative PCR in all injury-treatment groups 4, 8, and 12 days after injury and treatment initiation as indicated in the legend: external urethral sphincter BDNF expression (A) and pudendal nerve \( \beta_{II} \)-tubulin expression (B). Results shown are the means and standard errors of data from 3 to 5 animals. Irrespective of time point, \( x \) designates statistical significance of a difference from the sham injury group 12 days after injury.
**Figure 4:** Representative immunofluorescence (upper half) and histology images (lower half) of the external urethral sphincter, indicated by the star, from all injury-treatment groups 4, 8, and 12 days after injury and treatment initiation. Immunofluorescence highlights BDNF (green) protein, while Masson’s trichrome histology illustrates muscle (red) and fibrosis (blue) within the sphincter.

**Figure 5:** Representative immunofluorescence of pudendal nerve motor nuclei from all injury-treatment groups 4, 8, and 12 days after injury and treatment initiation. Immunofluorescence highlights βII-tubulin (red) protein within and neurofilament (green) comprising the cell bodies.
Figure 1

![Graph showing pressure in cm H2O for different conditions: Injury + BDNF, Injury + Saline, and Untreated Sham. The graph compares 2-Week and 3-Week conditions.](image-url)
Figure 2
Figure 3

(A) Graph showing relative expression levels for Injury + BDNF, Injury + Saline, and Sham + None groups.

(B) Graph showing relative expression levels over Day 4, Day 8, and Day 12.

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Figure 4

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Figure 5

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Appendix 1: Supplemental Background

Overview:

Urinary incontinence is a common condition, affecting 1 of every 3 - 4 women, and accounts for over 19.5 billion dollars in the US each year. While many treatments exist for addressing incontinence, none specifically target its underlying pathophysiology – failure of the neuromuscular continence mechanism. Pudendal nerve (PN) injury, associated with vaginal birth trauma, has been implicated as a major mechanism in the pathogenesis of stress urinary incontinence (SUI). Animal models of PN injury, such as PN crush (PNC), reduce urinary leak point pressure (LPP), mimicking SUI in humans. Furthermore, external urethral sphincter (EUS) electromyography (EMG) and PN electroneurography (ENG) reveal relationships between reduced as well as abnormal electrical activity and impaired LPP, again echoing clinical findings in SUI and PN dysfunction.

Prior work has revealed that PNC and muscle injury from vaginal distention (VD) produce opposing responses of EUS neurotrophins, which are cytokines involved in the maintenance and regeneration of nerve function. Specifically, PN injury induced upregulation of brain-derived neurotrophic factor (BDNF) in the EUS, while muscle injury reduced BDNF expression. It is hypothesized that this downregulation of BDNF impairs PN recovery following childbirth, and may be a substantial contributor to SUI in women. As such, a novel intervention consisting of a localized and continuous infusion of BDNF to the injured PN was developed and investigated with the hypothesis that it would
improve PN regeneration and possibly be further developed as a treatment as well as preventative measure for SUI in women.

**Urinary Incontinence:**

SUI is the undesired leakage of urine caused by abdominal pressure increases with sneezing, laughing, coughing, or other activity. Population studies estimate between 25% and 46% of women suffer this condition. SUI presents not only a substantial economic burden, accounting for over 19.5 billion dollars annually in the US, but also inflicts a significant emotional burden by detracting from the quality of life of those it affects.

Incontinence has long been anecdotally related to childbirth. Vaginal childbirth is associated clinically with damage to the tissues of the pelvic floor. While estimates of incontinence rates vary, the prevalence of SUI is 250% greater amongst women who delivered vaginally than those who did not. Furthermore, a strong association between antenatal SUI and postpartum SUI exists in primiparae. Women who developed SUI during pregnancy were 5.79 times more likely to have SUI 1 year postpartum. While both antenatal and immediate post-partum SUI were predictive of chronic SUI, with 73%-92% of women who had SUI 3 months post-partum being incontinent 5-6 years after giving birth. Clearly, a link exists between vaginal delivery and SUI, both immediately postpartum and later in life.

Conceptually, urinary continence is afforded by a mechanism consisting of 2 major components. The first is structural and comprised of the pelvic floor musculature
and connective tissue while the second is a neuromuscular system consisting of the PN and EUS. Presently, SUI is largely treated with surgical procedures and symptom management techniques, while less commonly behavioral interventions, physical therapy or exercises, and pharmacologic treatments may be pursued. Most surgical treatments focus upon restoring the structural integrity of the pelvic floor or bulking the EUS to coapt the urethra – neither of which benefit recovery of a non-functioning sphincter muscle that may be denervated.

Kegel exercises, a conservative SUI intervention, do target the neuromuscular continence mechanism, but are heavily relatively reliant upon an intact PN-EUS neuromuscular circuit for benefit. This highlights their approximately 53% success rate, which is largely attributed to behavioral adaptations and not neuromuscular improvement. While stem cell injections also target a defunct EUS and may provide additional muscle bulk through differentiation, the benefit this could provide a denervated sphincter remains unclear. Thus, no method effectively targets the underlying pathophysiology of SUI, a compromised neuromuscular continence mechanism.

Childbirth Injury:

In vaginal deliveries, SUI most likely results from injury to both structural and neuromuscular contributors to continence. Damage to pelvic floor structures, such as the pubovisceral portion of the levator ani, can occur during vaginal delivery and results in tissue defects that are associated with SUI. Injury to the PN, which innervates the
EUS and the deep perineal space, also occurs during vaginal delivery.\textsuperscript{6, 7} Antenatal and postpartum neurophysiological recordings identified PN injury after vaginal delivery, but not Cesarean section or pregnancy itself.\textsuperscript{8, 35, 43-45} Clinically, it has also been shown that direct urethral injury occurs at higher rates in women delivering vaginally than in Cesarean section.\textsuperscript{46} Thus, injuries to both structural and neuromuscular components of the urinary continence mechanism occur with vaginal delivery. Furthermore, persistent PN damage has been noted 7 years following delivery, as evidenced by increased pudendal nerve terminal motor latency (PNTML) and prolonged motor unit potential durations.\textsuperscript{8, 35} Therefore, it appears the neuroregeneration occurring after childbirth-mediated PN injury is insufficient to adequately restore the neuromuscular function necessary for maintaining urinary continence.

**Animal Models:**

Rats are the most common model used to investigate postpartum SUI.\textsuperscript{47-49} The lower urinary tract anatomy and physiology of Sprague-Dawley rats have long been well characterized.\textsuperscript{50-53} Furthermore, the organization and role of the PN in urinary function and dysfunction following injury has also been well studied.\textsuperscript{54, 55} The PN crush model demonstrates urodynamic changes that parallel those clinically observed after vaginal delivery.\textsuperscript{52, 56-59} These include a rapidly decreasing urethral resistance to leakage and significantly reduced urinary leak point pressure.\textsuperscript{59-64} Furthermore, rat pup delivery has also been shown to have a deleterious effect on urinary function by impacting urethral sphincter innervation.\textsuperscript{65} Nonetheless, rodents differ substantially from humans on
account of their quadruped status, which results in the levator ani not cradling the bladder neck and bolstering continence, as is the case in humans.\textsuperscript{66} However, this benefits the use of this model in research because it causes the EUS-PN neuromuscular continence mechanism to serve as the key facilitator of urinary control.\textsuperscript{67} Thus, rodent models of simulated childbirth injury are a sound platform for studying SUI.

\textit{Vaginal Distention:}

Most rodent-based models of SUI utilize catheter-induced VD to simulate passage of newborns through the birth canal.\textsuperscript{52, 57, 59, 68} This is accomplished by inserting a modified-tip Foley catheter into the vagina with diameter and balloon volume adjusted according to rat size. Expansion of the balloon in the vagina simulates the second stage of labor, and damages the EUS, PN, and tissues of the pelvic floor.\textsuperscript{14, 69, 70} Specifically, VD has been found to cause hypoxic injury to the EUS, incite inflammation, and also cause disruption and fragmentation of the striated muscle.\textsuperscript{5, 57, 71} Because the urethra courses along the anterior vaginal wall, posterior to the pubic symphysis in humans, it is vulnerable to trauma during the second stage of labor and has been found to be injured at higher rates with vaginal delivery compared to Cesarean section.\textsuperscript{46, 72} This supports the use of VD as an animal model, however, unlike humans, postpartum incontinence induced with the VD model is consistently recoverable.\textsuperscript{57, 69}

\textit{Pudendal Nerve Crush:}
Injury to the PN, which innervates the EUS and the deep perineal space, occurs during vaginal delivery and also during simulated childbirth.\textsuperscript{6,7} Similar to humans, the PN innervates and controls the EUS in rats.\textsuperscript{50,51} Clinical findings suggest PN injury contributes significantly to the development of SUI.\textsuperscript{6,7,73} Specifically, electrophysiologically detected PN dysfunction is associated with SUI.\textsuperscript{8,35,43-45} Mechanistically, the PN as it traverses Alcock’s canal in the obturator internus fascia, is susceptible to injury during childbirth.\textsuperscript{43} To simulate this, a surgical model employing PN crush within the ischiorectal fossa was developed in Sprague-Dawley rats.\textsuperscript{56,74} Since both the left and right PN innervate the EUS, bilateral PN injuries are required to consistently reduce urethral resistance and simulate SUI while unilateral injury does not.\textsuperscript{56,75,76} Like VD, PN crush is a recoverable model of childbirth injury and neurogenic postpartum incontinence in rats.\textsuperscript{16,58}

\textit{Combined Vaginal Distention and Pudendal Nerve Crush:}

Combined childbirth injuries, consisting of both EUS and PN damage have also been developed.\textsuperscript{47-49} These incorporate both aforementioned models, as childbirth produces a double insult to the neuromuscular urinary continence mechanism – damage to muscles that act to prevent leakage and interruption of the nerve supply that provides their activation for urinary control.\textsuperscript{70,77} Combined VD and PN crush models in Sprague-Dawley rats shed valuable insight into the effects of simultaneous muscle and nerve injury, demonstrating that together the injuries produce a detriment in LPP as
well as PN and EUS function that lasts longer than either injury alone.\textsuperscript{14, 70, 77} Specifically, detriments in EMG and ENG amplitudes mirrored impairments in firing rates.\textsuperscript{70, 77}

Mechanistically, this is thought to occur through opposing responses to injury and stimuli for recovery produced in the PN and EUS.\textsuperscript{14} In particular, the largest upregulation of BDNF in the EUS occurred following PN crush, while downregulation of BDNF was noted after VD, and when combined VD and PN crush were performed.\textsuperscript{14} Furthermore, histological analyses 10 days post-injury showed fewer normal nerve fasicles after combined PN crush and VD followed next by PN crush and then VD.\textsuperscript{14} As such, it appears there is a relationship between BDNF levels and nerve regeneration or neuron survival. As a result, it is thought that PN damage contributes greatly to the pathogenesis of SUI not only via directly impairing muscular function acutely but also through limiting recovery of the EUS on account of persistent denervation while EUS damage is felt to not only impair urethral coaptation and resistance to leakage but also neuroregeneration of the PN on account of impaired retrograde stimulation.

**Neuroregeneration:**

Neurotrophins are cytokines released by innervated target organs and myelinating cells, which function to both maintain normal innervation and neural function as well as stimulate axonal regeneration and neuronal growth. Several neurotrophic factors have been implicated in the maintenance and regeneration of mature neurons.\textsuperscript{19} The dominant one is BDNF, which functions through activation of the type-B tyrosine kinase receptor (\textit{trkB}).\textsuperscript{78} In the rat pelvic ganglion, \textit{trkB} activation
initiates JAK/STAT signaling that mediates neurite outgrowth, an important component of neuroregeneration.\textsuperscript{79} BDNF-\textit{trkB} signaling is crucial for neuroregeneration, as heterozygous mice carrying a null \textit{trkB} allele demonstrate only approximately 50\% as many regenerated motoneurons following nerve transection as wild type mice.\textsuperscript{80} The terminals of spinal motoneurons contain \textit{trkB} receptors while their supporting Schwann cells and innervated tissues express BDNF.\textsuperscript{81, 82} This distribution suggests retrograde BDNF signaling is involved in the maintenance of innervation in addition to neuroregeneration.\textsuperscript{19} Further support for this is found following sciatic nerve injury when BDNF expression increases in the gastrocnemius muscle, reaching maximal levels 7-14 days after injury.\textsuperscript{9, 83} Upregulation of BDNF in target tissues and the Schwann cells of distal nerve stumps begins soon after axonal injury and lasts 3-4 weeks.\textsuperscript{84} BDNF is increased in the EUS 1 day after PNC in rats.\textsuperscript{14} BDNF expression is required for neuroregeneration to occur and local administration to sites of injury reduces motoneuron death following transection.\textsuperscript{10, 85} The importance of BDNF signaling is demonstrated by the administration of anti-BDNF antibody, which significantly impairs nerve regeneration following sciatic nerve crush.\textsuperscript{10} Additionally, infusion of \textit{trkB}-IgG into the gastrocnemius muscle in the absence of nerve injury results in decreased motoneuron conduction velocity.\textsuperscript{86} Thus, BDNF-\textit{trkB} signalling is not only important for neuroregeneration following injury but also for the maintenance of normal neuromuscular function. In contrast, BDNF expression is inhibitory to and reduced during neuromuscular junction (NMJ) formation and restoration as well as myogenic myoblast differentiation.\textsuperscript{11, 12, 87}
As such, the concurrent injury of the PN and pelvic floor musculature, specifically the EUS, during childbirth likely impairs the neuroregenerative response via downregulation of BDNF to facilitate EUS muscle repair. Consistent with this hypothesis, reduced expression of BDNF has been observed in the EUS following simulated childbirth with VD and PNC compared to isolated PNC alone.\textsuperscript{14} In addition, it was also shown that PN function, as assessed by LPP as well as EUS EMG and PN ENG, recovered more slowly when the EUS and PN were simultaneously injured compared to either injury alone.\textsuperscript{14, 70, 77} Therefore, supplemental BDNF may be able to overcome the muscle injury-induced downregulation of the neurotrophin and improve PN regeneration and hasten LPP recovery.

**Competing Injuries:**

Damage to the urethral striated muscle during vaginal delivery likely involves the loss of both NMJ integrity and entire myocytes.\textsuperscript{5, 15} The restoration of NMJs beneath synaptic end plates is facilitated by agrin, which is required for acetylcholine receptor (AChR) clustering and prevents neuronal sprouting while promoting NMJ formation.\textsuperscript{87} BDNF, which is upregulated in target tissues following nerve injury, demonstrates unchanged expression levels following NMJ damage.\textsuperscript{88} Furthermore, administration of exogenous BDNF inhibits agrin-mediated AChR clustering.\textsuperscript{11} Therefore, it appears that damage to the PN, which would upregulate EUS BDNF levels, would resultantly hinder NMJ recovery despite their loss of integrity following simulated childbirth injury in the Sprague-Dawley rat model.
In addition to its effects on NMJ recovery, reduced BDNF expression has been observed during the natural myogenic differentiation of precursor cells with further suppression of BDNF expression by exogenous siRNA enhancing muscular differentiation. As such, it is likely that EUS striated muscle injury causes a downregulation of BDNF in the EUS to facilitate restoration of the myocyte population in addition to recovery NMJ integrity. The observed downregulation of EUS BDNF with muscular damage induced by VD and impaired recovery of PN function compared to PNC alone support this. Therefore, it is likely that this reduction in BDNF level may impair BNDF-mediated PN regeneration and maintenance of PN-EUS function as the neuromuscular continence mechanism. As such, it is possible that targeted BDNF treatment can overcome this inherent downregulation and facilitate improved PN recovery, which in return may facilitate maintenance of the EUS musculature by limiting atrophy and denervation.

Neuroregeneration can be assessed by molecular methods, in addition to functional assessments. In particular, βII-Tubulin, a cytoskeletal protein found in neuronal sprouts of regenerating nerves and has been observed in the sciatic nerve crush model to undergo increased synthesis and transport from the centrally-located nerve cell bodies to distal sites of axonal injury. As such, expression of the protein has been used reliably as a measure of peripheral nerve regeneration. Previously, quantification of βII-Tubulin levels in the PN has been performed to assess the response of the nerve to injury and been found to correlate with functional recovery as evidenced by voiding patterns.
Assessment of the neuroregenerative response has been accomplished using \textit{in situ} hybridization with radiolabeled $\beta_{II}$-Tubulin cDNA to detect mRNA in the PN cell bodies, which comprise Onuf's nucleus in the L6 dorsolateral nuclei.\textsuperscript{16} Expression of $\beta_{II}$-Tubulin was significantly higher 7 days after PN crush and normalized by 14 days. Likewise, voiding behavior 6 days after PNC was abnormal and normalized by 13 days, suggesting neuroregeneration occurs prior to voiding pattern normalization.\textsuperscript{16} Further support of this comes from the observed recovery of LPP 14-21 days after nerve injury.\textsuperscript{14, 70, 74} Functional electrophysiological analyses similarly show recovery of PN electrical activity by 21 days after PN crush.\textsuperscript{70} Thus, it appears neuroregeneration precedes, and likely facilitates, functional recovery of the PN and EUS neuromuscular continence mechanism.

\textbf{Neuroregenerative Treatment:}

Administration of exogenous BDNF to the site of nerve transection enhanced functional recovery and reduced neuronal death in vivo.\textsuperscript{21, 85} Early studies analyzing various methods of BDNF administration following nerve injury showed increased choline acetyltransferase (ChAT) activity and protein levels following intrathecal, intravascular, intraneuronal, subcutaneous, and impregnated matrix administration, indicating cholinergic nerve activity.\textsuperscript{92, 93} Studies using repeated subcutaneous or single nerve tube injections demonstrated no significant benefits to histological or functional nerve regeneration.\textsuperscript{94, 95} Subsequently, administration route and duration have been identified as key factors influencing the effectiveness of neurotrophin treatment.\textsuperscript{19, 96}
This point was recently articulated by highlighting the effectiveness of local administration to the injury site compared to systemic routes, which can be generally complicated by side effects, such as seizures and difficulty controlling concentration at the injury site.\textsuperscript{97, 98} Thus, localized and targeted administration methods appear most effective.

A number of methods of prolonged, local BDNF administration following nerve transection have been investigated.\textsuperscript{20-22} Outcome measures in this work include improved functional recovery, increased axon diameter, and a greater recovery of muscle fiber size. The use of silastic nerve tubes infused continuously with differing levels of BDNF showed no change in short term tibial nerve regeneration but did demonstrate an 83\% increase in the number of long term regenerated motoneurons in a dose-dependent response to BDNF.\textsuperscript{22} Maximal regeneration, as assessed by the number of regenerated axons 2 months after injury, was observed with a dose of 0.5 \(\mu\)g/day over a 28 day period. However, the utilization of a nerve-tube requires surgical intervention and is suited only for transection injuries, where a physical discontinuity in the nerve exists.

Other means of providing continuous and targeted BDNF treatment have been investigated. Prolonged-release capsules of calcium-alginate hydrogel microbeads impregnated with BDNF were synthesized, tested, and used within an autologous fascia nerve tube for BDNF administration following nerve transection.\textsuperscript{23} The microspheres provided a consistent release of neurotrophin for 4 weeks and generated faster regeneration at all time points, compared to placebo, according to the length of
regenerated nerve fibers. Despite the use of a nerve tube and transection model, these results further highlight that continuous and targeted treatment with BDNF is effective at improving neuroregeneration. Additional support of this comes from recent work with adipose-derived stem cells that have been shown to secrete BDNF and improve nerve healing and axon growth.\textsuperscript{25} As such, investigation of local, continuous BDNF treatment near the site of PN crush injury is warranted. In the long term, the injection of similar sustained release capsules near the PN after childbirth could be pursued clinically as a method of local BNDF administration.

**Assessment Methods:**

SUI is defined as “the complaint of involuntary leakage on effort or exertion, or on sneezing or coughing” and thus requires patient identification of the leakage as being involuntary.\textsuperscript{26} Therefore, animal models cannot recreate SUI per say, but can facilitate its study by reproducing the physiologic and mechanistic aspects of the condition. Specifically, urethral resistance can be reproducibly decreased by injuries in animal models and assessed with methods similar to clinical urodynamics that identify the particular effects of various injuries and treatments. Additionally, electrophysiologic recordings, generally used as adjuncts to clinical pressure measurements, can provide further insight into the function of specific components of the continence mechanism in animal models.

*Urinary Leak Point Pressure:*
A commonly used pressure measurement is LPP, the bladder pressure at which urine leakage occurs, which serves as an estimate of urethral resistance to urine flow. In the absence of a bladder contraction, a significantly reduced LPP is associated with impaired urethral resistance and simulate SUI in animal models much like low bladder pressures during urine leakage, in the absence of a detrusor contraction, are clinically associated with SUI. Many techniques exist for assessing LPP in animals, which is a continuous measurement that can provide insight into the degree of incontinence as well as response to therapy. Direct bladder compression is a straightforward means of assessing urethral resistance. Methods utilizing this technique may incorporate a closed abdominal approach or an open abdomen with direct bladder visualization. Pressure within the bladder can be measured using either suprapubic or urethral catheters. Most LPP studies are performed under anesthesia to minimize distress on the rodent and facilitate repeatable procedures, as such, urethane anesthesia is generally used since animals retain voiding reflexes when anesthetized with it. The open abdominal approach with direct bladder visualization and compression is well suited for use in studies where concurrent electrophysiologic measurements, requiring the dissection and exposure of neuromuscular structures are necessary.

*Electrophysiological Recordings:*

Electrophysiological recordings of PN and EUS activity provide insight into the functionality of each structure, which are both associated with urinary incontinence if dysfunctional. Recording the PN motor branch can be accomplished with careful
dissection and isolation from the pudendal neurovascular bundle.\textsuperscript{70} Similarly, the EUS muscle fibers can be recorded following exposure with a midline abdominal incision and pubic symphysectomy but can also be analyzed using transcutaneous electrodes and a closed abdomen.\textsuperscript{56, 70, 76} Changes in electrophysiological recordings have been quantified and the levels of neuromuscular function associated SUI and continence identified with concurrent LPP recordings.\textsuperscript{70, 76} Thus, electrophysiological and functional studies provide insightful information about the status of the neuromuscular mechanism and urinary control.

\textit{Tissue Analyses:}

Histological analyses also provide insight into the continence mechanisms in rats. An example is periurethral fibrosis, which has been associated with LPP and improved continence \[55\]. Conversely, structural disruption of the EUS and PN after simulated childbirth injury have been associated with reduced LPP, mimicking SUI.\textsuperscript{14, 69} Electrophysiological changes in EUS and PN activity have also been associated with histological changes.\textsuperscript{70, 77} Furthermore, histology can supplemented with immunohistochemistry or immunofluorescence to facilitate the detection of protein levels indicative of hypoxia, tissue injury, inflammation, and repair or regeneration. Thus, an array of analysis methods exist for gaining detailed insights into the functional, structural, and neuromuscular aspects of continence in the rat model.
Appendix 2: Detailed Methods

Pudendal Nerve Crush (PN Crush): Ketamine and xylazine anesthesia was used for animals undergoing functional analyses while isofluorane anesthesia was used for animals utilized for molecular and histological assessments. While sedated under anesthesia, the rat’s posterior was clipped free of long hair and antiseptically prepared using betadine. Using sterile tip technique, a single midline skin incision was made cranially, approximately 2 cm long, starting 0.5 cm above the base of the tail. The skin was then retracted laterally to expose the lumbodorsal fascia. Starting on the left side, a longitudinal incision through the lumbodorsal fascia was made, approximately 1cm long, and roughly 0.5 cm lateral to midline. The incision was then retracted and the posterior iliac crest lateralized to expose the ischiorectal fossa. At this point, a self-stabilizing retractor was placed and the pudendal neurovascular bundle was visualized. Using blunt dissection, the pudendal neurovascular bundle was freed from surrounding connective tissue with the aid of an ophthalmologic surgical microscope. Upon isolating the bundle, both the PN motor and sensory branches as well as nerve and vein were crushed twice, consecutively, with a Castro-Viejo needle holder for 30 seconds. This was repeated on the contralateral side. Pump implantation was then performed, as described below. Following this, the lumbodorsal fascia was reapproximated with simple, interrupted 5-0 silk sutures, bilaterally. Skin was closed using simple, interrupted, 3-0 vicryl sutures. Post-surgical analgesia consisted of buprenorphine every 12 hours for 2 days after recovery from surgical anesthesia.
**Sham Pudendal Nerve Crush:** Sham nerve crush animals underwent all steps of PN crush except closure of the Castro-Viejo needle holder. No treatment, and thus, no pump implantation was performed. Post-surgical analgesia consisted of buprenorphine every 12 hours for 2 days after recovery from surgical anesthesia.

**Vaginal Distention (VD):** As in PN crush, rats were anesthetized with ketamine and xylazine or isofluorane if used for functional or tissue-related analyses, respectively. All vaginal distention was performed after anesthesia induction and prior to PN crush. The vagina was first accommodated to a larger capacity by inserting and removing increasing sizes of urethral dilators (24Fr. to 32Fr.), coated with surgilube (E. Fougera & Co., New York). A modified 10 French Foley catheter was then dipped in surgilube and inserted into the vagina. The tip of the catheter was first cut off to facilitate complete insertion of the balloon into the rat’s vagina. Next, a single 3-0 silk suture was placed through the labia majora to secure the catheter in place. The balloon was then distended, inside the vagina, with 3 ml of sterile water, which has been determined to be a suitable volume for 200-250g rats. After 4 hours, the suture was cut and removed, the catheter deflated and taken out of the vagina, and PN crush performed as described. Post-operative analgesia consisted of buprenorphine given every 12 hours for 2 days after recovery from surgical anesthesia.
Combined Simulated Childbirth Injury (PNC and VD): Animals that received both VD and PN crush underwent VD first and then PN crush, both as described above. The procedures were performed with the animal is fully anesthetized and not permitted to wake between or during either component of the procedure. As noted, buprenorphrine was given for post-operative analgesia every 12 hours for 2 days after recovery from surgical anesthesia.

Miniature Osmotic Pump Implantation: Using a sterile technique, osmotic pumps were filled to capacity with the specified treatment or placebo solution. Vinyl catheters were then attached to flow regulators and connected to the pumps. An overnight incubation in sterile 37°C physiologic saline was used to prime the pumps and initiate and stabilize their constant 0.5 μl/hour effusion rate. Based upon the dose-response relationship observed in nerve tube administrations, a larger, but still maximally beneficial dose of 2 μg/day will be used to account for diffusion from the injury site.22

Animals then received subcutaneous miniature osmotic pumps during the PN survival surgery. Specifically, while still under anesthesia from the PN injury procedure, the midline incision used for PN crush was utilized for pump implantation. With the incision open, prior to reapproximating the lumbodorsal fascia, a pocket for the pump was made by placing a hemostat subcutaneously in a cranial orientation through the end of the incision and spreading the tips. The pocket was approximately 2 mm wider than the implant on each side and 5 mm longer than the implant in the cranial direction. The miniature osmotic pump, with catheter attached, was then removed from the
sterile 37°C physiologic saline and incubated overnight and inserted in a sterile fashion through the incision and into the subcutaneous pocket. The vinyl catheter was then trimmed to length, such that its end rested near the site of PN injury when it was held against paraspinal musculature halfway along its length. The catheter was then secured to the paraspinal musculature and surrounding fascia using a 4-0 prolene (polypropylene) suture. Following this, the lumbodorsal fascia was rejoined with silk suture, as described in the PN crush procedure. The pump implantation procedure was then repeated on the contralateral side. Finally, the skin was closed using vicryl suture as described in the PN crush procedure. This means of treatment provides a targeted, local, and continuous supply of neurotrophins, which has been shown to be the most effective means of improving neuroregeneration.

**Animal Boarding:** Prior to interventions, rodents were kept together with at least 2 and no more than 3 rats per holding container. Free access to standard rodent chow and water was provided and corn cob bedding was used. Following interventions, rodents were placed together again in groups of 2 or 3 per container. All rats received a combination of fruit-flavored and chocolate treats following surgical interventions. Within the animal facility, rats were removed individually from their containers for post-operative analgesia and given subcutaneous buprenorphine in order to minimize disruption and reduce any additional stress that would be incurred in moving them to the operating facility.
**Leak Point Pressure (LPP):** Rats were anesthetized with 1.2 g/kg of intraperitoneal urethane. The urinary bladder was then exposed through a short midline abdominal incision made cranially 1 cm from the pubic symphysis. This was performed following symphasectomy used to expose the EUS, as described later. A polyethylene catheter constructed from PE50 tubing was inserted transurethrally into bladder for bladder filling and intravescical pressure measurement. The bladder was then drained and filled with room temperature saline at 5 ml/hr via the catheter. After a minimum of 3 filling and voiding cycles were observed, LPP testing was pursued. When the bladder reached half-capacity, at approximately 0.4 ml, gentle pressure was applied directly to the bladder with a cotton-tipped applicator. Bladder compression was increased steadily while intravescical pressure was continually measured via the transurethral catheter. Pressure increase was continued until the rat either leaked or a void was induced, with the latter being excluded from analyses. At the instant urinary leakage was observed at the urethral meatus, all externally applied bladder compression was rapidly removed. The peak pressure at this timepoint is taken as LPP. Following a successful LPP acquisition, the bladder was drained, refilled at the same rate, and the procedure was repeated again until a minimum of 3 smooth LPP curves were generated. Prior to data acquisition, a water column was used to calibrate the pressure measurement system with the zero level set at the pubic symphysis of the rat. This is an end stage procedure on account of the urethane anesthesia. Thus, following the simultaneous LPP, EMG, and ENG recording, rats were euthanized via intracardiac pentobarbital injection.
External Urethral Sphincter Electromyography (EUS EMG): Electromyography was performed in conjunction with LPP testing, thus the female Sprague-Dawley rats were anesthetized intraperitoneally with urethane prior to a craniocaudal midline incision being made above the pubic symphasis. Following this, pubic symphasectomy was performed and a hemostat was used to spread the pubis laterally and expose the full length of the urethra. The incision was extended cranially to expose the bladder and caudally to skin level by the urethral meatus. Two platinum monopolar electrodes, in a bipolar electrode configuration, were then rested ventrally upon the EUS to record EMG with the assistance of an adjustable arm. The polyethylene transurethral catheter was inserted into the bladder prior to EUS electrode placement. Data was recorded continuously during LPP and at rest between trials, as well as during filling and voiding. Prior to data recording, the amplifier system was calibrated with standardized waveforms. All rats were euthanized at the end of the recordings using intracardiac pentobarbital as noted above.

Spinal Cord and Urethral Procurement: Rats undergoing molecular analyses were anesthetized with ketamine and xylazine prior to tissue collection and euthanized via intracardiac perfusion with heprinated, phosphate-buffered saline until the liver was pale. This cleared blood from tissues to facilitate optimal histological and immunofluorescence imaging. Immediately following perfusion, rats were placed in a prone position and a dorsal midline incision made from the midback to tail base. A lumbar laminectomy was then performed from L2-S3 to expose the lower spinal cord.
Upon exposure, the spinal cord was frozen *in situ* with a copious amount of liquid nitrogen. Following this, transection at L3 and S2 was performed and the portion of spinal cord between removed *en bloc* and placed in a pre-cooled and labeled cryovial, which was subsequently stored in liquid nitrogen. The rat was then placed in a supine position and the pubic symphysis exposed through a midline incision. A pubic symphysectomy was then performed and the pubis spread laterally with hemostats to expose the complete length of the urethra. Next, an incision extending from the introitus to the level of the bladder neck was made approximately 1-2 mm lateral to the urethra. The bladder neck was then transected and the portion of tissue comprised of the anterior vaginal wall and urethra removed. The skin at the urethral meatus was then removed and the tissue placed in a tissue cassette filled with TissueTek OCT such that it was fully submerged. Next, the cassette was placed in liquid nitrogen to flash-freeze the tissue. Following this, specimens were stored at -80°C until sectioned.

**Spinal Cord and Urethral Sectioning:** A cryostat set at -20°C was used for tissue sectioning following cleaning with 100% ethanol and nuclease inhibiting solution prior to sectioning and between each specimen. Spinal cord specimens were mounted with OCT (Tissue Tek, Optimal Cutting Temperature) on the sectioning platform, such that transverse sections were cut. Spinal cords were sectioned to 10 µm thickness with spinal cord levels determined by anatomical identification based upon occasional sections captured on a glass slide, stained with Thionin solution, and analyzed by light microscopy. Once the L5/L6 level containing Onuf’s Nucleus and the PN cell bodies was
reached, 7 sections were mounted on each of 2 lasermicrodissection (LMD) PET membrane slides and 3 plain glass slides in an alternating fashion. All slides were then stored at -80°C in a slide box until analyzed. Urethral specimens were mounted in an identical fashion and sectioned at a thickness of 10 μm with occasional specimens similarly captured on a glass slide and stained with Thionin to localize the EUS. Once the EUS was identified, 5 sections were mounted on each of 3 plain glass slides and stored at -80°C in a slide box until analyzed.

**Laser Microscope Dissection and Quantitative PCR:** Prior to dissection, slides were thawed for 30 seconds, rehydrated with ultrapure nuclease free water, stained with Thionin, rinsed, dehydrated with ethanol, and fixed with xylene. Following this, 5 Onuf’s Nucleus regions from each slide or 5 EUS regions from one slide were collected together. Dissected cells were captured in a microcentrifuge tube containing cell lysis solution, which inhibited nuclease activity. Following cell capture, the tube was placed on dry ice. RNA isolation was then performed using a commercially available kit and was followed directly by reverse transcription (RT) using a commercially available kit. Due to the very small sample size, after RT the cDNA was pre-amplified using commercially available, non-biasing, pre-amplification reagents and primers specific for βII-Tubulin in the PN and BDNF in the EUS. Following this, the pre-amplified cDNA from one animal in each experimental group was diluted and loaded in quadruplicate into a 6 x 8 PCR reaction tray placed on ice. PCR assays for βII-Tubulin in the PN and BDNF in the EUS as well as 18S, an endogenous control to account for sample size, were added to each well.
along with standard quantitative PCR reagents. A 2 hour, 40 cycle quantitative PCR reaction was then run. Data was analyzed by the comparative (delta-delta) CT method, providing the expression of βII-Tubulin mRNA in each sample relative to the control specimen and adjusted for sample size by 18S expression. Automatic reaction thresholding provided by the PCR software system was utilized for all samples.

Histology and Immunofluorescence: Standard Masson’s Trichrome staining was performed on EUS specimens collected on glass slides after thawing, rehydrating, and tissue fixation in Bouin’s solution. Histological images were obtained via standard light microscopy. Immunofluorescence preparation began with thawing PN and EUS samples on glass slides and fixing the tissue with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. Following this, 3 PBS washes were performed and then tissues were blocked with a standard universal blocking buffer for 30 minutes. After which, samples were exposed to primary antibodies at 4°C overnight. EUS samples were placed in a 1:50 Rabbit Polyclonal BDNF antibody solution while PN samples were placed in a 1:50 Mouse Monoclonal βII-Tubulin solution that also contained a 1:50 Rabbit Polyclonal Neurofilament antibody. After this initial exposure, 3 additional PBS washes were performed. Slides were then exposed to secondary antibodies, which consisted of a 1:400 AlexaFluro 488 Donkey anti-Rabbit solution for EUS specimens. A combined 1:400 AlexaFluro 488 Donkey anti-Mouse and 1:1,200 DyLight 594 Goat Anti-Rabbit solution was used for PN samples. After 3 more PBS washes slides underwent anti-fade
treatment and then images were subsequently acquired using standard ultraviolet immunofluorescence.

**Electrophysiological Data Recording and Analysis:** An Astro-Med DASH 8X electrophysiological recordings system was used to capture EMG and ENG data at 10,000 Hz along with LPP at 100 Hz. Prior to recording, pressure and electrophysiological channels underwent hardware calibration in triplicate using standard stimuli produced by a fluid column or charge generator. Using AstroviewX proprietary software, a minimum of 3 baseline segments and 3 LPP segments of 1 second duration were exported from the recordings of each animal. Baseline segments were taken early in the bladder filling phase before increased intravesical pressure on account of detrusor compliance was appreciated. Segments acquired at LPP were the 1 second immediately preceding peak pressure. In addition, voltage calibration waveforms produced with fixed electrical stimuli and water column placement were also exported for EMG and ENG, as well as LPP data, respectively.

After exporting data to a standard format, low frequency or baseline variation was removed using Chart5 software. A 151 point moving average, or window of 15.1 ms duration, was computed using each electrophysiological recording, representing baseline variability in the data. This was then subtracted from the original recording, providing a smooth baseline.

Next, the smoothed data was processed in Myosotic SignaPoint 2009 to remove noise and quantify signal characteristics. Based upon qualitative analysis of EMG and
ENG recordings in the frequency domain, line noise appeared to be most prevalent between 59 and 61 Hz as well as between 119 and 121 Hz or the first harmonic. As such, a 4th order Butterworth band stop filter was applied to frequencies between 59 and 61 Hz with an attenuation amplitude of 60 dB and ripple amplitude of 0.1 dB. Next, an identical filter was applied between 119 and 121 Hz to remove the first harmonic. Upon line noise removal, the signal mean was calculated and subtracted from the data in order to center the waveform on the 0 line.

The first analysis performed was calculation of the mean signal amplitude. This was performed by rectifying the signal and finding the mean of all data points comprising the 1 s segment. Next, using the unrectified signal, firing rate was calculated with a zero crossing method utilizing a 0.2 mV threshold, which was determined from the qualitative analysis of multiple EMG and ENG recordings. To account for rise and falls being detected as separate threshold crossings, the number of overall count was divided by 2 to give the total number of firings in the 1 s signal segment.

Final data processing consisted of calculating the mean of all EMG and ENG amplitude measurements from each animal. Likewise, the mean EMG and ENG firing rates for all measurements in each animal were calculated. Lastly, the means of both baseline and LPP pressures were calculated for each animal. Following this, calibration segments were analyzed, and the mean voltages of the 3 hardware calibration repetitions were determined. These were then used to scale the acquired data appropriately.
**Supplemental Methods:**

*Electrophysiological Power Analysis:* All data acquired from EUS and PN recordings were also analyzed with regard to signal power. Specifically, the area under the 1-second epochs was calculated for each signal. This data was then subject to similar statistical analyses as the amplitude and firing rate data presented.

*LMD-PCR Method Validation:* All methods were completed as described in detail previously. A separate group (N=5) of female, virgin, Sprague-Dawley rats underwent unilateral PN crush. Spinal cord procurement was then performed 7 days after injury. Following this, a total of 5 Onuf’s nucleus regions from each animal were collected separately from the injured and contralateral uninjured side using LMD. Expression of \( \beta_{II} \)-Tubulin was then measured using the aforementioned Q-PCR protocol. Results were compared to previous \( \beta_{II} \)-Tubulin expression measurements 7 days after PN crush injury, which were obtained using *in situ* hybridization of radiolabeled \( \beta_{II} \)-Tubulin cDNA to mRNA of the protein.

**Discontinued Methods:**

*Anti-BDNF Antibody Treatment:* A small group (N=4) of animals with only PNC were given commercially available anti-BDNF antibody through an identical pump system to simulate the downregulation of BDNF induced by muscle damage. The dose was 12 \( \mu \)g per day, relatively larger than that of BDNF, and based upon the dosage of intramuscularly infused anti-trkB antibody that caused slowed conduction velocity in
intact motoneurons.\textsuperscript{86} These experiments were discontinued after this initial group was found to have a large immunologic reaction with granuloma formation at the catheter tip and inflammatory changes throughout the ischiorectal fossa and peri-urethral tissues.

\textit{Routine Histological Analysis:} Rats that underwent functional and electrophysiological analysis were euthanized directly after recordings via intracardiac pentobarbital injection. As a pubic symphysectomy was performed to facilitate recording, only an incision extending from the introitus to the level of the bladder neck was made. This was kept approximately 1-2 mm lateral to the urethra. The bladder neck was transected and the portion of tissue comprised of the anterior vaginal wall and urethra removed. The skin at the urethral meatus was then removed, the tissue placed in a tissue cassette, and then stored in a 10\% formalin solution. Following this, the sections were embedded in paraffin wax, sectioned transversely, placed on glass slides, and stained with Masson’s Trichrome to visualize EUS morphology and muscle fiber structure. These experiments were discontinued after it was observed that the urethral dissection and instrumentation appeared to disrupt the periurethral tissues, challenging an accurate assessment of sphincteric integrity.

\textit{Pudendal Nerve Electroneurogram (PN ENG):} All PN ENG were completed concurrently with EUS EMG, thus all female Sprague-Dawley rats were already anesthetized. As such, a pubic symphysectomy was also previously completed. Therefore, preparation for PN
ENG began with dissecting the right pubis free of musculature and connective tissue. Next, a superficial full-thickness skin incision was made, beginning lateral to the urethral meatus, and continuing inferiorly to the ischial tuberosity. Following this, the right pubic symphysis was bluntly reflected laterally, fracturing both pubic rami and facilitating removal of the bone. This provided wide exposure of the ischiorectal fossa. Beginning at the EUS and moving laterally, the pudendal bundle was identified. This was followed as it coursed to the dorso-medial aspect of the ischiorectal fossa and gently freed from its medial aspect with careful blunt dissection. Following this, the PN motor branch was very delicately separated from the connective tissue, sensory branch, nerves, and veins comprising the pudendal neurovascular bundle. Upon isolation of the PN motor branch, two platinum monopolar electrodes, in a bipolar electrode configuration, were used to suspend and isolate the nerve from the surrounding musculature without stretching the tissue. The nerve was then encased in droplets of mineral oil and recording commenced. All rats were euthanized at the end of the recordings using intracardiac pentobarbital as noted above. This was carried out for all animals in the functional arm of the study, and all data was analyzed, but no significant differences were noted to exist between groups. Further review of the data showed a very low signal to noise ratio, which was felt to preclude analyses from detecting utilizable data, thus leading to the exclusion of these results from the study.
Appendix 3: Additional Results

Isolated PN Crush Injury: Following isolated PN injury and treatment with either BDNF or saline placebo, no significant differences in LPP existed between treatment groups or in comparison to sham PN crush at the 2 week time point. Likewise, no differences in EUS EMG amplitude or firing rate were noted 2 weeks after injury between treatment groups or in comparison to sham PN crush. Identical findings existed 3 weeks after injury, with no differences in LPP as well as EUS EMG amplitude or firing rate existing between injury and treatments or sham injury.

Electrophysiological Power: No significant differences in outcomes were noted to exist between electrophysiological power and either amplitude or firing rate analyses. Specifically, comparisons of the EUS EMG power to either EUS EMG amplitude or firing rate revealed neither any additional nor any fewer significant differences between treatment and injury groups. As such, the results presented were only those of amplitude and firing rate analyses, which provide a more in-depth description of electrophysiological activity than signal power, which is related to the two separate measures combined.

LMD-PCR Method Validation: Following PN injury, 1 rat was euthanized and excluded from analyses due to an adverse anesthetic reaction. The remaining 4 animals were successfully taken through the LMD-PCR validation protocol, as described in Appendix 2 supplemental methods section. An upregulation of $2.36 \pm 0.46$ times or 136% $\beta_{II}$-Tubulin
expression was obtained via the PCR method, which did not significantly (p = 0.508) differ from the 2.49 ± 0.38 fold or 149% increased expression detected via in-situ hybridization in previous work.\textsuperscript{16} Examining the difference between the mean upregulation detected by each method reveals that PCR detects an absolute reduction of 0.13 times or a relative reduction of 8.7% of the upregulation detected by in-situ hybridization. This 0.13 fold absolute difference is less than the 0.38 times standard error noted with in-situ hybridization, which equates to 8.7% being less than 25.5% standard error when expressed relative to the upregulation measured by in-situ hybridization.
Appendix 4: Supplemental Discussion

Comparison to Natural History of Simulated Childbirth Injuries:

*Pudendal Nerve Injury*: Crush injury of the PN induces a recoverable model of postpartum SUI, with LPP decreasing to its lowest 4 days after injury and recovering to near-normal levels 2 weeks after the neurological insult.\(^6^7,\,7^4\) Evidence of PN regeneration supports this on a molecular level, as β\(_{II}\)-tubulin expression, a cytoskeletal protein indicative of neuronal growth and regeneration, is upregulated significantly in the PN motoneuron cell bodies 7 days after injury but normalizes at the 2 week time point.\(^1^6\) Furthermore, levels of BDNF and other regenerative factors have been noted to increase acutely in the EUS after PN injury, suggesting their presence may facilitate early neuroregeneration.

Comparisons of PN injury models shows that the degree of PN injury directly determines the severity of both functional impairments as well as the duration of functional recovery.\(^4^8\) This is highlighted by two studies using a similar PN crush model and outcomes measures, where a more severe PN crush in one on account of the use of pediatric needle drivers instead of Castro-Viejo needle drivers produced a longer lasting LPP deficit.\(^1^4,\,7^0\) The findings in the current study showed the recovery of LPP and EMG activity by 2 weeks in animals given only PN crush, which are in agreement with previous work.\(^1^4,\,7^4\) These findings are challenging to translate clinically, but electrophysiological studies of the PN, reflex loops, and EUS muscle may facilitate such comparisons since associations between neurophysiological findings and SUI suggest PN damage may be a factor in SUI development.\(^7,\,8,\,4^4,\,4^5,\,10^4-10^7\)
The findings of direct electrophysiological studies of PN activity following injury in rats support the functional results from studies measuring LPP. Previously, EUS EMG amplitude and firing rate, measures of overall muscle activity, were noted to be lowest 4 days after PN crush injury. Three weeks following PN crush, EMG activity remained significantly decreased, however, not to the degree observed acutely. This finding echoes the trend in LPP which shows recovery 2 weeks after PN crush. Yet, these electrophysiological findings differ from observations in the current study, likely on account of that PN crush being done with pediatric needle drivers instead of a Castro-Viejo needle holder, as in the current work. Nonetheless, EUS EMG activity recovered at 2 weeks in the current study with isolated PN crush, which parallels the differences in time course of LPP recovery across the discrepant injury severities.

Although ENG was attempted in this study and ultimately not utilized on account of a poor signal-to-noise ratio and data not suitable for analysis, such findings provide further insight into the neuromuscular continence mechanism and its recovery form injury. Direct recordings of PN electrical activity, or ENG, revealed findings similar to EMG with ENG activity significantly lower 4 days after PN crush and recovered completely 3 weeks after injury. These findings link changes in PN activity to those observed in the EUS, and with comparison to PN transection, further highlight the role injury severity plays in neuromuscular continence deficits. This provides valuable support to previous clinical studies associating abnormal electrophysiological findings with SUI and suggesting childbirth-related PN damage as an etiologic factor.
**Vaginal Distension:**

Vaginal distension (VD) simulates the second phase of labor where the fetus passes through the birth canal, and the injury has been shown to incite both EUS and distal PN branch damage.\(^5,15,52,58,107\) Like PN crush, VD is also a recoverable simulated childbirth injury where severity is related to functional deficits, as increased VD has been noted to prolonging recovery.\(^5,52,69\) However, histology showed EUS tissue damage was similar across differing VD durations, but this has been refuted recently.\(^5\) In addition to duration, the extent of VD, as controlled by balloon size in rodents, is related to the severity of LPP deficit and a loss of nerves in the EUS.\(^108\) Thus, VD both functionally recreates SUI and produces injury to the EUS. Clinically, a prolonged second stage of labor is associated with postpartum incontinence, but increasing baby weight or macrosomia is.\(^46,107,109,110\)

Functional studies further highlight EUS trauma from VD and differentiate it from potential concurrent PN injury. While the EMG amplitude of the EUS response 4 days after VD was significantly reduced, it does not differentiate the two.\(^77,111\) However, ENG recorded from the proximal PN showed no differences in firing rate or amplitude after VD.\(^70\) Furthermore, LPP was decreased significantly after VD and took as long as 3 weeks to recover, while PN crush deficits in LPP had recovered 2 weeks after injury in this and prior studies with similar PN crush models.\(^14,70\) Thus, it appears that compared to PN crush, VD produces an independent and different SUI model. As such, the possibility of isolated birth-induced EUS injury occurring independently of PN trauma and dysfunction...
is appreciable. This may have implications clinically in situations where no electrophysiological PN dysfunction is noted but sphincteric insufficiency exists.

In addition to functional studies, VD has also been shown to significantly reduce blood flow to the bladder, urethra, and vagina during balloon expansion, which rapidly returns with deflation, but induces hypoxia in the smooth and striated EUS muscle in addition to the vaginal and bladder epithelium. Furthermore, hypoxia-inducible factor alpha (HIF-α) is upregulated increasingly in the urethra with longer VD duration. These studies specifically highlight the susceptibility of the urethra, including the EUS, to hypoxic and/or reperfusion injury during delivery. Furthermore, disruption of the EUS muscle has been well characterized as well. Thus, it is likely that these insults may impair not only EUS function but also the ability of the EUS to stimulate recovery, as evidenced by the observed downregulation of BDNF and other regenerative factors in the EUS following VD. However, with hypoxia being a known stimulus of stem cell homing factor expression, the potential for utilizing targeted therapies based upon such changes may be promising.

**Combined Vaginal Distension and Pudendal Nerve Injury:** Considering the effects of both isolated PN and VD injury, as well as the physiology of childbirth, a more clinically relevant model of childbirth injury is the combined VD and PN crush. Functional results demonstrate mixed results with some studies showing a significantly reduced LPP persisting beyond 3 weeks and another showing recovery by this time point. As with individual injuries, this is likely the result of a differing severity of neuromuscular insult,
specifically a discrepancy in PN crush model as a result of pediatric needle holders and Castro-Viejo needle holders being used. The current study demonstrates that LPP recovers by the 3 week time point with dual injury regardless of BDNF or saline placebo treatment. This is in agreement with prior work utilizing the Castro-Viejo needle holder to perform the PN crush in the dual injury. However, unlike previous work, it appeared that BDNF treatment of the PN injury accelerated the recovery of LPP to normal values by at least 1 week.

Electrophysiological analyses of EUS EMG and PN ENG activity suggest neuroregeneration is impeded by the dual PN-EUS injury compared to either single injury alone. Specifically, EUS EMG amplitude and firing rate were significantly reduced after PN crush, VD, and dual PN-EUS injury, but the deficit was notably more severe with dual injury than either single injury 3 weeks later. This work utilized a pediatric needle holder for PN crush, unlike the current study that utilized a Castro-Viejo needle holder and demonstrated the recovery of both EUS EMG amplitude and firing rate 3 weeks after injury regardless of treatment. However, as with LPP, it appeared that BDNF treatment eliminated EUS electrophysiological deficits by 2 weeks after injury in comparison to saline placebo, which did not. Prior results from PN ENG were similar to EUS EMG findings, with dual injury significantly reducing PN ENG firing rate and amplitude 3 weeks after injury compared to either isolated PN or EUS injury, which both had recovered by this time. Therefore, compared to either PN or EUS injury alone, dual PN-EUS injury appears to more severely impair PN function, which coincides with EUS functional deficits.
Regeneration of the PN has been well studied, particularly regarding neurotrophins, which are cytokines that promote neuroregeneration. Brain-derived neurotrophic factor (BDNF), a specific neurotrophin, is upregulated in innervated target organs and myelinating cells following peripheral nerve injury in order to facilitate regeneration by retrograde signaling through neurons.\textsuperscript{19, 22, 80, 96, 114} The prolonged recovery time observed after dual injury compared to either PN or EUS injury alone, irrespective of severity, is thought to be result from opposing effects of PN crush and VD on BDNF production in the EUS.

Specifically, BDNF levels in the EUS decrease following VD, which imparts injury to the EUS muscle, but increase after PN crush, injury of the innervation to the EUS muscle.\textsuperscript{14} Therefore, simultaneous VD is likely detrimental to PN recovery on account of the EUS injury it produces and impairment of BDNF production since the neurotrophin is necessary for neuroregeneration.\textsuperscript{19, 22, 80, 96, 114} Immunohistochemistry of the EUS following isolated PN crush, isolated VD, and combined PN-EUS injury revealed that dual injury does not demonstrate the BDNF upregulation observed after PN crush, and in fact, appears to demonstrate a lower BDNF expression than uninjured control animals.

Supplementing the PN with BDNF to facilitate neuroregeneration appears to be successful based upon the accelerated recovery of LPP as well as EUS EMG activity at 2 weeks compared to that observed at 3 weeks with saline placebo treatment. With that, analysis of BDNF levels in the EUS demonstrate that supplemental PN BDNF treatment after combined PN-EUS injury appears to reduce EUS BDNF levels compared to saline placebo. This is beneficial, as BDNF has been shown to be detrimental to myocyte
maturation as well as neuromuscular junction formation and organization, which are both likely components of EUS recovery that occurs after VD injury to the structure.\textsuperscript{11, 12, 87} Thus, since both PN and EUS injury likely occur during vaginal delivery, treatments that can increase neurotrophin supply to the PN nerve may facilitate recovery from SUI and even prevent its development following childbirth if used in a preventative approach.

**LMD-PCR Method Validation:** Assessment of the neuroregenerative response to axonal injury has been well studied previously.\textsuperscript{115} Axonal growth, during development or nerve regeneration, is facilitated by neuronal sprouting and outgrowth, which is facilitated by the production and extension of new cytoskeletal proteins.\textsuperscript{90, 116} Thus, expression of the genes for these structural proteins provides a means of assessing for the presence and intensity of the neuroregenerative response.\textsuperscript{117, 118} The tubulin proteins, particularly the $\beta_{II}$ subset, has been utilized previously in assessments of the peripheral nerve neuroregenerative response to injury and regenerative treatments.\textsuperscript{16, 119, 120} Thus, $\beta_{II}$-Tubulin expression levels can be utilized to measure the neuroregenerative response of the PN.

Previous measurement of the PN neuroregenerative response was based upon \textit{in-situ} hybridization of cDNA radioisotopes to $\beta_{II}$-Tubulin mRNA, which provided a means of quantifying its expression.\textsuperscript{16} This method utilized a 4-week film exposure for the radioisotope labeled samples to be detectable. Furthermore, the exposed “grains” produced by the radioisotopes that were superimposed on PN cell bodies were counted
microscopically to quantify βII-Tubulin expression. Relying upon human judgment for both grain counts and the identification of PN cell bodies subjected these outcome measures to appreciable variability on account of human error.

Nonetheless, *in situ* hybridization was the standard method for assessing the neuroregenerative response, despite its relatively high labor and time costs, especially if the 4-week exposure needed to be repeated. Additionally, the need for radioisotopes to label mRNA by exposing the film resulted in the requirement for special authorization and waste disposal on account of the radioactive reagents. These further increased the labor and time costs associated with this method. However, despite these drawbacks, this methodology is relatively resistant to contamination.

The use of PCR-based methods for assessing gene expression and identifying genotypes, in addition to a number of other applications, has become commonplace in basic science research. The use of such a technique to assess neuroregeneration is one that can be readily adopted by many laboratories based upon the continually increasing ease of access to PCR equipment. Additionally, such a method is an excellent option if the use of radioactive reagents is not feasible or is prohibited. Collecting the entire urinary region of Onuf’s nucleus and utilizing automated Q-PCR measurements substantially reduces the influence of human error has on measurements. However most beneficial is that PCR-based methodology can assess the neuroregenerative response within 1 day compared to 4-weeks with *in-situ* hybridization. Numerous ready-made reagents and mixes, from a variety of vendors, facilitate this and greatly simplify and streamline the process. However, despite these benefits, PCR is extraordinarily
sensitive to sample contamination while in-situ hybridization is not. Thus, extraordinary care must be taken when using PCR-based techniques.

Overall, PCR, compared to in-situ hybridization, provides many advantages in assessing the PN neuroregenerative response. It is more cost effective with regard to time and labor intensity, it can be performed in nearly any laboratory, and the reagents needed are safe and readily available. The new PCR-based technique also successfully replicated findings obtained with previous in situ hybridization methods. Thus, the using PCR to assess the PN neuroregenerative response can provide a useful adjunct to studies analyzing regenerative treatments and their applications in incontinence and urology.

**Limitations:** Limitations of this study included the use of newly developed and validated laboratory techniques, including PCR-based analyses and BDNF treatments. Also, limited sample size for some analyses challenged the ability to detect statistically significant differences in some comparisons. The lack of an anti-BDNF treated group in functional and molecular studies as well as isolated PN injury group in molecular analyses subsequently differed from the initial study design and would have strengthened the interpretation of results, but did not detract from those made.

The BDNF treatment in this study appeared to benefit neuroregeneration according to functional and electrophysiological measures. While non-significant statistically, molecular outcomes also appeared to support these findings. However, the dose used in this study was pursued to assess feasibility. Thus, further research is
required to determine the optimal dosing of BDNF to PN injury that is required to maximize neuroregeneration and functional recovery. Additionally, investigation of the side-effects of BDNF is warranted as well. Insight into this was confounded in the current study on account of a Ketamine recall that occurred partway through the first arm of the study assessing function.

The PCR method utilized in this study was developed prior to undertaking this project and validated before beginning molecular analyses. As a result of this relatively novelty, some samples were ultimately unusable while others produced no results. This unfortunately decreased sample size for the molecular studies, which limited statistical power and the ability to detect significant differences in these outcomes. Nonetheless, informative results were still obtained from both EUS BDNF and PN βII-Tubulin. Furthermore, these results were supported by immunofluorescence.

Some animals were eliminated from functional analyses due to complications requiring euthanization. These stemmed from a Ketamine recall, which in a post-hoc analysis appeared to be associated with a brief series of self mutilations and digital auto-amputations observed in the front paws of the rats. Interestingly, these were not in the lower extremities, which are innervated by the sciatic nerve and could possibly have been attributed to coincidental nerve irritation or injury. Otherwise, a series of corneal ulcerations also occurred during this time and appeared to be related to more prominent eye bulging from the anesthetic, requiring some animals to be euthanized and others treated with antibiotic eye ointment. Nonetheless, these issues resolved and
were not encountered after the recalled batch of Ketamine was discarded. Otherwise, no difficulties related to pump implantation, erosion, or migrations were encountered.

Elimination of two experimental groups from the study occurred after initial surgeries and review of the functional data. After the initial animals treated with Anti-BDNF Antibody were anesthetized and prepared for functional analyses, it was determined the treatment was ineffective and appeared to result in detrimental tissue changes. Specifically, upon inspecting the catheter tips bilaterally, large granulomas were observed encasing the catheters and blocking outflow of the antibody solution. In addition, the walls of the ischiorectal fossa were densely adhered, which complicated PN dissection for recording and subjected the structure to notable injury based upon electrophysiological recordings. Furthermore, these inflammatory changes appeared to extend to the peri-urethral tissues and urethra itself, which both appeared stiffened. This caused transurethral catheter insertion to be difficult and resulted in urethral puncture in half of these cases. As such, following these initial antibody-treated animals, this experimental group was eliminated from the study. Future work could utilize a different anti-BDNF antibody or an anti-trkB antibody as done previously in other work.10, 86

The isolated PN crush group was not carried from the functional arm to the molecular arm of the study. As functional studies were completed, review of the data demonstrated a lack of significant differences between isolated PN crush treated with BDNF or saline. Based upon this lack of difference in functional recovery, the pursuit of molecular studies of the neuroregenerative response between the treatment groups
was terminated. Had this group been included in the molecular arm of the study, the
differential effects of isolated PN injury and combined PN and EUS injury on the
neuroregenerative response could have been analyzed. This would have provided
insight into the difference in BDNF response of the EUS to PN crush with and without
concomitant EUS injury. Furthermore, observations of the strength of
neuroregenerative response with placebo treatment of PN injury with and without
concurrent EUS injury could have been compared. As such, future work analyzing the
differential EUS BDNF and PN neuroregenerative responses to untreated injuries should
be pursued.
Future Directions: The field of regenerative medicine holds a number of possibilities for incontinence and voiding dysfunction. Already, stem cell treatments have been studied for use in restoring the structural integrity of the urogenital organs and functional bulk of the urethral sphincter. Early investigation of neuroregenerative treatments, to restore PN function and EUS innervation have also shown promise. Specifically, both electrical stimulation as well as neurotrophins administration have improved functional aspects of the continence mechanism in models of post-partum incontinence.

Clinical Extension: With the growing focus on regenerative and preventative medicine, a means of not only treating SUI and repairing the neuromuscular continence mechanism, but also preventing SUI development is an enticing concept. Treatment aimed at improving neurotrophin levels to facilitate PN recovery may accomplish this. As observed in this study, direct neurotrophin treatment at the PN injury site, provided in a continuous and targeted fashion, improved functional recovery. Similar results have been noted in prior studies of neurotrophin treatment, and in particular with one using degradable hydrogel beads that release BDNF over time. As such, a depot injection that provides a timed release of BDNF to the PN, administered transvaginally similar to a PN block, is an exciting possibility. Otherwise, transvaginal electrical stimulation of the PN, which has been shown in animal models to stimulate the neuroregenerative response and increase BDNF levels, may also be an option. Interestingly, postpartum transvaginal electrical stimulation has been anecdotally associated with improved continence by physical therapists in some European countries. Nonetheless, the
development of regenerative and preventive treatments for SUI pose an exciting challenge for ongoing and future research.
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