Effects of acute selective pudendal nerve electrical stimulation after simulated childbirth injury

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1Department of Biomedical Engineering, Cleveland Clinic, Cleveland, Ohio; 2Glickman Urological and Kidney Institute, Cleveland Clinic, Cleveland, Ohio; 3Department of Colorectal Surgery, Cleveland Clinic, Cleveland, Ohio; and 4Advanced Platform Technology Center, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio

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Jiang H-H, Gill BC, Dissaranan C, Zutshi M, Balog BM, Lin D, Damaser MS. Effects of acute selective pudendal nerve electrical stimulation after simulated childbirth injury. Am J Physiol Renal Physiol 304: F239–F247, 2013. First published November 14, 2012; doi:10.1152/ajprenal.00235.2012.—During childbirth, a combinatorial injury occurs and can result in stress urinary incontinence (SUI). Simulated childbirth injury, consisting of vaginal distension (VD) and pudendal nerve crush (PNC), results in slowed recovery of continence, as well as decreased expression of brain-derived neurotrophic factor (BDNF), a regenerative cytokine. Electrical stimulation has been shown to upregulate BDNF in motor neurons and facilitate axon regrowth through the increase of βIII-tubulin expression after injury. In this study, female rats underwent selective pudendal nerve motor branch (PNMB) stimulation after simulated childbirth injury or sham injury to determine whether such stimulation affects bladder and anal function after injury and whether the stimulation increases BDNF expression in Onuf’s nucleus after injury. Rats received 4 h of VD followed by bilateral PNC and 1 h of subthreshold electrical stimulation of the left PNMB and sham stimulation of the right PNMB. Rats underwent filling cystometry and anal pressure recording before, during, and after the stimulation. Bladder and anal contractile function were partially disrupted after injury. PNMB stimulation temporarily inhibited bladder contraction after injury. Two days and 1 wk after injury, BDNF expression in Onuf’s nucleus of the stimulated side was significantly increased compared with the sham-stimulated side, whereas βIII-tubulin expression in Onuf’s nucleus of the stimulated side was significantly increased only 1 wk after injury. Acute electrical stimulation of the pudendal nerve proximal to the crush site upregulates BDNF and βIII-tubulin in Onuf’s nucleus after simulated childbirth injury, which could be a potential preventive option for SUI after childbirth injury.

Neurotrophins in general, and brain-derived neurotrophic factor (BDNF) in particular, are highly expressed in striated muscle after injury to facilitate peripheral nerve regeneration (14, 58). After muscle injury, expression of neurotrophins decreases, and BDNF becomes undetectable (46). Decreased neurotrophin expression in the EUS and delayed functional recovery of the urethra and pudendal nerve have been demonstrated in a simulated childbirth injury model, consisting of both vaginal distension (VD) and pudendal nerve crush (PNC) (9, 27, 40). In addition, treatment with BDNF accelerates recovery from simulated childbirth injury (17). Therefore, EUS injury in vaginal childbirth may inhibit recovery of pudendal nerve injury via insufficient upregulation of neurotrophins in the EUS.

Electrical stimulation has been demonstrated to promote neuroregeneration after injury and to increase intrinsic BDNF expression in injured neurons (1, 2, 15, 19). Stimulation could therefore provide a method for facilitation of recovery by promoting neuroregeneration and reinnervation if the muscle is damaged simultaneously, such as in childbirth injury. In this study, we applied selective pudendal nerve motor branch (PNMB) electrical stimulation immediately after simulated childbirth injury in rats. We aimed to determine whether pudendal nerve stimulation affects bladder and anal function after simulated childbirth injury and whether it increases BDNF expression in Onuf’s nucleus to stimulate recovery.

MATERIALS AND METHODS

The animal protocol was reviewed and approved by the local Institutional Animal Care and Use Committee. Of the 53 female adult virgin Sprague-Dawley rats (250–300 g) used in this study, 42 rats underwent either simulated childbirth injury (n = 21) or sham-simulated childbirth injury (n = 21). These animals received unilateral pudendal nerve stimulation and bladder and anal function testing immediately after the injury (Fig. IA). Two days or 1 wk after injury, Onuf’s nuclei were harvested and analyzed for BDNF and βIII-tubulin expression by real-time quantitative RT-PCR. Five rats were used as controls for real-time RT-PCR. Six rats were used to determine the intensity threshold of pudendal nerve stimulation.

Simulated childbirth injury. Childbirth injury was simulated in female rats using a dual-injury model involving both VD and bilateral PNC, as done previously (26). The procedure was not timed to a specific point in the estrus cycle of each rat. In brief, under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia, a modified 10-Fr Foley catheter was inserted and stitched into the vagina. The catheter balloon was distended with water to 3 ml for 4 h. Immediately after VD, the pudendal canal (containing the pudendal nerve) was approached dorsally and crushed bilaterally with a Castroviejo needle holder twice for 30 s each. Sham-simulated childbirth injury followed the same procedure but without balloon distension and nerve crush.
All animals were given subcutaneous buprenorphine (0.1 mg/kg sc) for postoperative analgesia. Filling cystometry and anal pressure testing. Immediately after simulated childbirth injury or sham injury and while maintaining anesthesia, a saline-filled polyethylene catheter (PE-50) was inserted into the bladder via the urethral orifice. The other end was connected to both a pressure transducer (model P122; Astro-Med, West Warwick, RI) and a syringe pump (model 200; KD Scientific, New Hope, PA). Air pressure was calibrated as zero at the level of the bladder. The bladder was filled with saline at 5 ml/h continuously during pudendal nerve stimulation (1 h). Simultaneously, resting anal pressure was recorded with a small water-filled latex balloon (size 4, Kent Scientific, Torrington, CT), as done previously (60). The two pressure transducers (Grass PT300, Astro-Med) were connected to amplifiers (P511 AC Amplifier, Astro-Med) and a multiple channel recording system (DASH 8X, Astro-Med).

Pudendal nerve stimulation. Thirty minutes after simulated childbirth injury or sham injury and while maintaining anesthesia, the PNMB proximal to the injury was exposed bilaterally for 1 h of subthreshold stimulation (20 Hz, 0.3 mA, 0.1-ms pulse) on the left side and sham stimulation on the right side. Under a surgical microscope (Fig. 1A), each PNMB was suspended by a bent-end bipolar parallel platinum electrode (0.8-mm distance between poles, PB AD08100; FHC, Bowdoin, ME). The electrode was fixed and leads were connected to the stimulator (Grass model S88, Astro-Med). Stimulation was timed to be performed between spontaneous anal contractions (ACs). The sham-stimulated side (right) underwent electrode placement but not stimulation. After stimulation, the electrodes were removed, and the dorsal incision was closed.

Before the above experiments were performed, six unmanipulated control rats underwent unilateral pudendal nerve stimulation to determine the response threshold via the AC response. Electrical stimulation (20 Hz, 0.1-ms pulse width, square wave, bipolar) was tested at amplitudes from 0.1 to 1 mA. Anal pressure was monitored with a water-filled balloon placed in the anal canal, as noted above. A subthreshold stimulation intensity not causing an anal sphincter contractile response was selected for use after simulated childbirth injury (Fig. 2).

Onuf’s nuclei specimen harvest. Two days or 1 wk after simulated childbirth injury or sham injury and electrical stimulation, the lumbar-sacral spinal cord was frozen and harvested from each animal following ketamine and xylazine anesthesia and intracardiac perfusion of heparinized saline. The L6-S1 spinal cord was then sectioned (12-μm thickness) using a cryostat such that 18 sections at the level of Onuf’s nucleus were placed on three laser microdissection (LMD) membrane slides (PET Membrane LMD Slides, Leica Microsystems, Wetzlar, Germany). A clean sectioning procedure was used to minimize cross-contamination between specimens and sections. Consistent spinal cord orientation was maintained while sectioning so that Onuf’s nucleus on the right and left side could be identified on the slide. Slides from each animal then underwent LMD (ASLMD, Leica Microsystems). In rats, motoneurons of Onuf’s nucleus innervating EUS and external anal sphincter (EAS) are separated into the dorsal clitoris and EAS. Pudendal nerve separation (dorsal approach) and electrical stimulation. VD, vaginal distension; RT-PCR, reverse transcription-polymerase chain reaction; EAS, external anal sphincter; EUS, external urethral sphincter.
lateral nucleus and dorsomedial nucleus, respectively (54). In this study, the dorsolateral motoneurons of Onuf’s nucleus were identified by their shape and the location after thionin staining. The dorsolateral nucleus was isolated and collected with right-side specimens (sham stimulation) in one container and left-side specimens (stimulation) in another, for each animal and slide. The specimens were immediately frozen on dry ice and stored at −80°C until analyzed.

Quantitative expression of BDNF and βII-tubulin mRNA in Onuf’s nuclei. Total RNA was isolated from each Onuf’s nucleus specimen by RNA micro isolation (TaQMan MicroRNA Assays; Applied Biosystems, Foster City, CA) and was followed by cDNA reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Each sample was examined (Nano-Drop ND-1000 spectrophotometer; Thermo Fisher Scientific, Wilmington, DE) to confirm RNA and cDNA quality. After preamplification (TaQMan PreAmp Master Mix, Applied Biosystems), BDNF and βII-tubulin mRNA levels were measured by real-time quantitative PCR (StepOne Real-Time PCR, Applied Biosystems). The BDNF (TaQMan Gene Expression Assay, Rn03928990_g1, Applied Biosystems) and βII-tubulin (TaQMan Gene Expression Assay, Rn01435557_g1, Applied Biosystems) primers and probes consisted of a 20-mix of unlabeled PCR primers and TaQMan minor groove binder probe (FAM dye-labeled reporter). The fold-change in gene expression was determined by the ΔΔCT method (33). Relative mRNA quantities (RQs) were determined by comparison to uninjured controls with sample size variance accounted for by reference gene an S18 (TaQMan Gene Expression Assay, Rn03928990_g1, Applied Biosystems) internal control (VIC dye-labeled reporter).

Data collection and statistical analysis. A one-way repeated-measures ANOVA, followed by a Holm-Sidak multiple comparison test (SigmaPlot 11.0; Systat, Point Richmond, CA) vs. control (sham stimulation, 0 mA), was used to determine the anal sphincter stimulation response threshold after a normal anal pressure increase was confirmed. A Z-test or Chi-square test was used to compare changes in contraction strength after injury or sham injury. AC frequency (number per minute), AC amplitude (cmH2O), and AC duration (in seconds) were obtained from anal sphincter pressure from each animal in 15-min segments immediately before, during, and after pudendal nerve stimulation. Similarly, bladder response to injury and stimulation was characterized by frequency (number/min), amplitude (cmH2O), and duration (s) of nonvoiding contractions and frequency (number/min), peak pressure (cmH2O), duration (s), and interval (s) of voiding contractions in 15-min segments immediately before, during, and after pudendal nerve stimulation. A mean value from each animal was used to calculate a mean and SE for each injury-treatment group. Two-way repeated-measures ANOVA following by Student-Newman-Keuls pairwise comparisons was used to analyze data against the two factors of injury and stimulation. For real-time quantitative RT-PCR, target gene RQs were normalized to the sham stimulation side and compared, using a one-sample t-test to determine whether pudendal nerve stimulation affected the target gene. P < 0.05 was taken to indicate a statistically significant difference between groups for all tests.

RESULTS

Determination of threshold for pudendal nerve stimulation. In the six naive female rats, the anal contractile response to unilateral electrical stimulation increased with stimulation intensity (Fig. 2B). A noticeable increase in anal pressure occurred from 0.3 to 0.4 mA, but only with 0.5 or 1.0 mA was there a statistically significant increase in anal pressure. All rats demonstrated an AC response with stimulation of 0.5 mA and higher. Simultaneous bladder pressure recording demonstrated no increase with stimulation, excluding the possibility that the anal pressure increase was due to abdominal pressure transmission. We therefore chose 0.3 mA as subthreshold stimulation amplitude for use in experiments.
**Anal function after simulated childbirth injury and pudendal nerve stimulation.** Anal pressure demonstrated spontaneous rhythmic contractions, as noted previously (60). Immediately after simulated childbirth injury, no spontaneous AC occurred in 19% of the injured animals \( (P = 0.116; \text{Fig. 3A}) \). In the 81% of injured animals demonstrating AC, frequency and amplitude of the contractions were significantly decreased compared with sham-injured animals (Fig. 3B). These significant differences between groups were maintained both during and after unilateral pudendal nerve stimulation. Within injury or sham injury groups, there were no significant changes before, during, or after stimulation aside from AC amplitude, which significantly increased after stimulation compared with both before and during stimulation.

**Bladder function after simulated childbirth injury and pudendal nerve stimulation.** Immediately after injury, 19% of animals had only voiding contractions, 57% had voiding contractions and nonvoiding contractions, 14% had only nonvoiding contractions, and 10% had no contractions at all. Compared with sham injury, the percentage of rats with voiding contractions decreased significantly only after injury. In contrast, the number of rats with nonvoiding contractions, regardless of voiding contractions, significantly increased after injury (Fig. 4).

After injury, frequency of nonvoiding contractions was significantly increased compared with sham injury (Fig. 5). This trend persisted during and after stimulation. Within the sham injury group, baseline pressure and duration of nonvoiding contractions decreased significantly during stimulation compared with both before and after stimulation. Within the injury group, there were no statistically significant changes in nonvoiding contractions before, during, or after stimulation (Fig. 5).

Simulated childbirth injury significantly increased voiding contraction frequency. In contrast, it significantly decreased peak pressure, duration, and interval of voiding contractions (Fig. 5). This difference continued during and after stimulation. After sham injury, the frequency, peak pressure, and interval of voiding contractions were significantly changed during stimulation compared with either before or after stimulation. After injury, voiding contraction frequencies during and after stimulation were lower than before stimulation (Fig. 5).

**BDNF and βIV-tubulin expression in Onuf's nucleus.** Both 2 days and 1 wk after injury and unilateral pudendal nerve stimu-
lation, BDNF in the left Onuf’s nucleus, of which the pudendal nerve was stimulated, was significantly increased compared with the right side, which received sham stimulation (Fig. 6). βII-Tubulin was also increased both 2 days and 1 wk after injury and stimulation, but only significantly so 1 wk after injury. Neither BDNF nor βII-tubulin levels changed significantly with pudendal nerve stimulation compared with sham stimulation after sham injury (Fig. 6).

DISCUSSION

SUI, a common condition affecting at least 35% of women over age 40, is expected to increase 250–300% in prevalence by the year 2030 in the United States (11, 24). During vaginal childbirth, the muscles, organs, nerves, and ligaments of the pelvic floor can be damaged as the baby passes through the pelvic outlet, leading to postpartum voiding dysfunction and the development of SUI (16, 22, 51). Electrical stimulation can promote peripheral axon regeneration by increasing BDNF in neurons (2, 15), providing a possible method of facilitating SUI recovery by promoting EUS reinnervation in light of simultaneous muscle injury during childbirth. The goal of this study was to determine the effects of pudendal nerve electrical stimulation on the neuroregenerative response, as well as on bladder and anal function immediately after simulated childbirth injury.

Simulated childbirth injury disrupts both anal and bladder function. Simulated childbirth injury in rats is one of the most common animal models for investigating the mechanisms of stress incontinence (18, 32). The urethra and the pudendal nerve have been well-studied both anatomically and functionally after simulated childbirth injury (9, 27, 40). However, anal and bladder function have not been well-studied in these injury models (25). Nonetheless, these organs may develop dysfunction immediately postpartum, as observed clinically (16, 48).

In rats, spontaneous rhythmic ACs with small multiple peaks in each pressure wave are considered to be the summative effect of the EAS and the internal anal sphincter, which are regulated by both autonomic and somatic nerves (47, 60). In

Fig. 4. Bladder pressure during filling cystometry characterized by voiding contractions (VC), nonvoiding contractions (nVC), or the absence of contractions immediately after simulated childbirth injury. *Significant difference compared with sham injury (P < 0.05). Each bar represents the number of rats with the specific contraction pattern, with percentages of injury group denoted parenthetically (n = 21 in all cases).
In this study, injury disrupted ACs since ~20% of rats had no contractions and the remaining injured rats demonstrated significantly decreased contractile activity compared with sham-injured animals. Thus, anal function is disrupted after a dual injury comprised of VD and PNC. Anal dysfunction is likely due to the combined effects of nerve and muscle injury, as has been demonstrated in bladder function after the same simulated childbirth injury (26, 27, 40).

The bladder was also dysfunctional after simulated childbirth injury, which likely resulted from hypoxia due to the catheter balloon compression of tissues against the pubic symphysis and overdistension due to the bladder outlet obstruction caused by balloon inflation (10). Moreover, the prolonged distension caused compression from surrounding tissues and consisted of stretching the nerves (31, 40, 42), which could also contribute to bladder dysfunction. Common clinically observed bladder dysfunction during and immediately after pregnancy includes urinary frequency, stress incontinence, and retention (48). Both overactive bladder and SUI are more prevalent during pregnancy or immediately postpartum than before pregnancy or 3 mo postpartum (56). Moreover, women may have bladder neck descent and hypermobility postpartum (13, 49). Urethral injury and pudendal nerve damage can also contribute to urinary problems via an altered urethra-to-bladder connection.

Fig. 5. Bladder pressure during filling cystometry characterized by VC and nVC before, during, and after electrical stimulation immediately after sham or simulated childbirth injury. The example bladder pressure trace is during stimulation. Each bar represents means ± SE of data from 21 rats. *Significant difference compared with sham injury at the same time point relative to stimulation (P < 0.05). Paired Greek letters indicate a significant difference between 2 groups (P < 0.05).
reflex (12). In this study, although some sham-injured rats had nonvoiding contractions, the frequency of these nonvoiding contractions was significantly lower than that observed after injury. The contributing factors may include anesthesia, transurethral filling cystometry, and pudendal nerve exposure (7, 59). Clinically, nonvoiding contractions may present idiosyncratically, as is the case in many instances of overactive bladder in young nulliparous women (53).

Selective pudendal nerve stimulation inhibits bladder contraction with impaired efficacy after simulated childbirth injury. Although pudendal nerve stimulation has various beneficial effects on many pelvic floor functions (21, 41, 43, 50, 57), it may produce unintended effects on the bladder or the anal sphincter, depending on stimulation parameters, delivery method, and spinal cord condition (41, 43, 50). Clinically, pudendal nerve stimulation can be utilized to inhibit overactive bladder contractions. Although the exact mechanism of pudendal neuro-modulation is unknown, it may occur via the pudendal-to-bladder reflex since the nerve contains both afferent and efferent innervation (21, 57). During childbirth injury, both afferent and efferent in pudendal nerve can be significantly injured (26, 40, 54, 55). This effect was demonstrated by disrupted bladder and anal function in our study. In contrast to ACs, which changed little due to stimulation, we demonstrated that pudendal nerve stimulation in female rats inhibits nonvoiding bladder contractions in sham-injured animals. In contrast, stimulation appeared to increase pressure, duration, and interval of voiding contractions in sham-injured animals, possibly by altering continence reflexes. In our study, the PNMB was isolated for electrode placement to prevent sensory branch stimulation. Therefore, the mechanism of bladder contraction inhibition may be different from other studies that have investigated more proximal pudendal nerve stimulation, stimulation of the dorsal clitoral nerve, or stimulation of the perineal skin (5, 8, 38, 39).

It is possible that the PNMB may also contain afferent fibers that inhibit bladder contractions via the pudendal-to-bladder reflex mechanism (43, 50); however, it lacks small γ-motoneuron axons that provide sensory information from muscle spindles (44). In addition, Onuf’s nucleus is different than limb motor neuron nuclei, which are tightly grouped with bundled dendrites (4, 36, 54, 55). Amyotrophic lateral sclerosis highlights this, as the disease results in degenerated motoneurons but spares Onuf’s nucleus even at the terminal stage (28, 35). Nevertheless, Onuf’s nucleus contains bundled transverse dendrites with one of the major projections dorsally toward the sacral parasympathetic nucleus (54). As such, electrical stimulation in this study likely affected Onuf’s nucleus retrogradely, which subsequently transmits to the parasympathetic neurons via the transverse dendrites and/or a related interneuron network. It is also possible that afferent fibers were stimulated, likely affecting related parasympathetic neurons. However, determination of the mechanism of action requires further investigation. The effects of unilateral pudendal nerve stimulation on both bladder and anal sphincter contractions were significantly reduced after simulated childbirth injury, suggesting that nerve and target organ condition may modulate stimulation efficacy. This could explain why pudendal neuromodulation has little or no clinical effect in some patients.

In the anal and bladder function tests, we did not record electromyography simultaneously because it is very difficult to record electromyography in these small animals during electrical stimulation. In addition, anal sphincter pressure, a bilateral outcome, may provide a more global measure of the unilateral stimu-
ulation we utilized in this study. Our primary outcome was to determine whether such a stimulation protocol can improve recovery and whether this stimulation affects bladder and anal function significantly. Therefore, many outcomes in this study with no significant change during or after stimulation imply that the stimulation has no significant side effects on bladder and anal function.

Pudendal nerve stimulation increases neurotrophin expression in Onuf’s nucleus. Neurotrophins, and BDNF in particular, are upregulated in innervated target muscle after nerve injury, which facilitates axonal regrowth and promotes peripheral nerve reinnervation of muscle (20, 29, 40). However, vaginal distension causes a significant decrease of BDNF and other neurotrophins in the EUS (40) since the distension liking childbirth during delivery damages the EUS and its neuromuscular junctions, such as striated muscle fiber disruption and atrophy and acetylcholine receptors and neurofilament in a precisely overlapping distribution with no associated proximal axon (30, 40). Generally, BDNF downregulation after muscle injury enables neuromuscular junction repair (46), but in childbirth injury is associated with delayed pudendal nerve regeneration after VD and PNC, both functionally and anatomically (27, 40). Exogenous BDNF has been shown to improve urinary continence and EUS recovery after simulated birth injury (17).

Neurotrophins can also be produced by motoneurons in amounts required for axonal regeneration (14). Electrical stimulation has been demonstrated in rat femoral nerves to increase intrinsic BDNF and NT-4 expression in injured motoneurons and promote neuroregeneration after injury (2, 15). In this study, a statistically significant upregulation of BDNF mRNA in the stimulated Onuf’s nucleus was noted both 2 days and 1 wk after injury and stimulation compared with the sham-stimulated side. βIII-Tubulin mRNA was also significantly upregulated 1 wk after injury and stimulation compared with sham stimulation. Our results indicate the upregulation of BDNF occurs earlier than the upregulation of βIII-tubulin. BDNF upregulation likely promotes axonal regrowth since βIII-tubulin, which constitutes microtubules for neurofilaments for axon, when upregulated, indicates the regenerative effort of the neuron in response to injury (34). Moreover, expression of βIII-tubulin, as measured by mRNA levels, has been used to assess neurotrophin treatments (6, 17, 52). Therefore, βIII-tubulin upregulation is evidence of pudendal nerve regeneration, whereas BDNF upregulation suggests it is promoted by the electrical stimulation. Since childbirth injury and delayed recovery of urethral function are correlated with later SUI development (37, 45), subthreshold pudendal nerve electrical stimulation has potential to be utilized as a preventive therapy immediately after childbirth if validated further by functional and clinical investigation.

In this study, we did not assess estrous cycle status. Although estrus cycle status could affect the results from physiological tests, the relatively large number of animals per group could minimize such an effect. Furthermore, in most related experimental and clinical studies on bladder or anal function, estrus cycle status has not been regularly investigated, although onset of menses is usually considered. For the BDNF and βIII tubulin results, we expect a limited effect of estrus cycle status since we compared the injured side with the uninjured side in the same subject. In addition, a recent study on simulated childbirth injury indicated that urinary incontinence does not depend on estrous cycle (23). Nonetheless, it still remained a potentially important factor.

REFERENCES