Published in final edited form as:

Neurol Urodyn. 2011 June ; 30(5): 658–667. doi:10.1002/nau.21120.

Sophisticated models and methods for studying neurogenic bladder dysfunction

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INTRODUCTION

Common methods for inducing neurogenic bladder overactivity include: (1) spinal cord transection or contusion injury, (2) supraspinal injury (decerebration, local lesions, middle cerebral artery occlusion and (3) systemic (*e.g.*, cyclophosphamide) or intravesical administration of irritant or inflammatory agents (*e.g.*, acrolein, acid, lipopolysaccharide). These insults can cause peripheral, spinal and supraspinal effects which are difficult to resolve using traditional cystometry alone. To address this problem, we have developed new methods as well as new highly reproducible models to study neurogenic bladder dysfunction in the mouse. These models include direct sensitization of the bladder using ionizing radiation or indirect sensitization through colonic irradiation (figure 1). The classic model for neurogenic bladder overactivity is spinal cord injury which results in non-voiding contractions during the filling phase. However, there is also an intrinsic myogenic component that is present when studied *in vitro*. Our radiation models are purely neurogenic and do exhibit overactivity in isolated tissues.

New *in vitro* studies utilize bladder-urethra sheets, spinal cord slices and in-line cultured cells. Bladder-urethra sheets can be isolated with the hypogastric (T₁₁-L₂) and pelvic (L₆-S₂) nerves and imaged for the propagation of optical activity along the bladder wall simultaneously with afferent nerve and tension recordings. Alternatively, bladder sheets can be mounted vertically as cross-sections for measuring the spread of activity across the wall from mucosa to serosa. Spinal cord slices are sectioned 0.5 to 1 mm thick to include the dorsal and ventral roots which can be stimulated to optically map pathologically-induced changes in spinal cord circuitry. In-line cultured cells, on the other hand, allow for different linear arrangements of urothelial, interstitial, smooth muscle and dorsal root ganglion cells

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APPROVAL TO SHOW LIVE ANIMALS

Approval to show images of live animals (Figures 1 and 11) has been granted by the University of Pittsburgh's Institutional Animal Care and Use Committee.

to map alterations in the flow of information in cell-to-cell communication. *In vivo* studies utilize cystometry which can be combined with cerebro-cortical imaging of optical activity during bladder filling and emptying. The new models of neurogenic overactivity are induced by irradiation of the bladder (direct sensitization) or colon (indirect cross-sensitization) both of which result in inflammation and urothelial damage leading to bladder overactivity. The changes induced by these models have been compared to those induced by the well established models of chronic spinal cord transection at the thoracic (T₈-T₉) and lumbar (L₄-L₅) levels.

NEUROLOGIC ANIMAL MODELS

Direct and Indirect Bladder Irradiation Models

The adverse effects of ionizing radiation on tissues, including the bladder and colon, are mediated through the generation of reactive oxygen and nitrogen species¹. Mitochondria are the principal source of these species in the cell and play an important role in irradiation-induced apoptosis and necrosis of the linings of the bladder and gut. The three cell types most sensitive to irradiation-induced damage are hemopoietic cells, urothelial cells lining the bladder lumen and endothelial cells lining the capillaries in the villi of the gut²⁻⁴. The use of free radical scavengers and nitric oxide synthase (NOS) inhibitors have proven to be radioprotective in normal tissues and in some cases radiosensitizing in tumors⁵. We have demonstrated that the presence of a NOS inhibitor in the bladder lumen during irradiation prevents the development of radiation cystitis and the accompanying bladder overactivity more effectively than free radical scavengers or the overexpression of mitochondrial manganese superoxide dismutase (MnSOD) using transgene therapy^{6, 7}. Since systemic administration of NOS antagonists can have adverse effects, we have developed a conjugate peptide dragging strategy based on the amino acid sequence of the naturally occurring membrane-active antibiotic gramicidin S, to mitochondrial-target new potent NOS inhibitors which enhance effectiveness and decreases adverse side effects⁸.

To generate the direct bladder irritation model, the bladder is briefly withdrawn through a small abdominal incision so that only the bladder is irradiated (figure 1A). Cystometry performed one week following irradiation (10 gray; 1 Gy = 100 rads) shows enhanced reflex contractions (figure 1C-ii) that are absent when bladders are excised and tension measured in sheet preparations (figure 1D-ii). This demonstrates that intrinsic bladder contractions are absent and that this is purely a neurogenic model. To generate the indirect irritation model, the descending colon is briefly withdrawn through a small abdominal incision and irradiated (10 Gy; figure 1B). Like the direct model, there are enhanced reflex bladder contractions during cystometry (figure 1C-iv) but no intrinsic activity in isolated sheet preparations (figure 1D-iv). One notable difference with this model, however, is that the bladder is not protected by intravesical administration of NOS antagonists. This is presumably because the colon which is not protected and develops colitis resulting in the prolonged release of inflammatory neuropeptides in the bladder from dicotimizing primary (1st order) afferents that co-innervate both organs (figure 2A-1 or 2A-2), or *via* sensitization of central neural pathways leading to enhanced efferent outflow to the bladder (figure 2B3).

UML and LML Models

Transection of the spinal cord at different segmental levels has different effects on the lower urinary tract. Following a supralumbosacral lesion (*e.g.*, an upper motor neuron lesion (UML) above T₁₁; figure 3), descending neural control/modulation of the bladder and urethral function from higher centers is disrupted, rendering the bladder areflexic until emergence of a spinal cord-to-bladder reflex at approximately two weeks after transection in the mouse⁹. This areflexia in combination with detrusor-sphincter-dyssynergia (DSD) results in urinary retention and hypertrophy which can damage the bladder and kidneys. After partial recovery of voiding function due to remodeling of the neural connections within the spinal cord, this model exhibits neurogenic (figure 4A–ii) and myogenic (figure 4B–ii) bladder overactivity^{10, 11}. The intrinsic component manifests itself as large-amplitude, spontaneous bladder contractions that can directly stimulate afferent nerves. The role of urinary retention in developing intrinsic overactivity is evidenced by its absence if the ureters are diverted (to the uterus in females or the gut in males) prior to spinal cord transection (figure 4B–iii). In contrast, after a lower motor neuron lesion (LML) (below L₃ or at the level of the cauda equina; figure 3) the bladder and sphincter are decentralized and areflexic resulting in overflow incontinence¹² and thus no hypertrophy or intrinsic bladder overactivity occurs. This is demonstrated in the cystometrogram in figure 4A–iii.

PERIPHERAL, SPINAL AND SUPRASPINAL PREPARATIONS

Bladder Sheets with Afferent Nerves

We evaluated changes in the properties of afferent nerves using an *in vitro* method which involves isolating the bladder with the associated spinal roots that make up the pelvic and hypogastric nerves. This method was expanded to include optical mapping and tension measurements from the bladder as depicted in figure 5. Using this preparation, we have demonstrated that there is direct correlation between afferent firing and spontaneous contractile activity in the bladders of UML mice (figure 5D)¹³. This suggests that stimulation of sensory nerves by spontaneous contractions contributes to afferent sensitization. This approach also allows for evaluation of treatments on afferent function using agents such as botulinum toxin-A¹⁴ or antibodies/antisense to nerve growth factor^{15, 16}. Since these therapeutic approaches are long lasting, these agents can be injected into half the bladder (figure 6A) determined by injecting them along with dyes of comparable molecular weight (*e.g.*, 150 kDa blue dextran for botulinum toxin-A). Using this approach, the firing or density of afferent nerves in the treated and untreated sides can be compared in the same bladder.

Bladder Wall Cross-sections

It is hypothesized that there is communication between the different cell layers in the bladder wall during filling, with transduction of signals from the urothelium to interstitial cells, afferent nerves and smooth muscle¹⁷. Alterations in this signaling pathway are believed to be an underlying cause of detrusor overactivity. In order to study this communication, we optically mapped Ca²⁺ transients from cross-sections of mouse and rat bladder walls (figure 7). In control bladders, application of low concentrations of a muscarinic agonist (carbachol, 50 nM) to the mucosal surface evoked Ca²⁺ transients (white

areas) that spread within the mucosal region but not to the detrusor and did not elicit spontaneous contractions (figure 7C). This concentration of carbachol does not directly stimulate detrusor smooth muscle. In contrast, neonatal or UML adult animals respond readily to low dose muscarinic stimulation with Ca^{2+} transients traveling from the mucosa into the detrusor increasing the amplitude and frequency of spontaneous contractions (figure 7D)¹⁸. This approach has allowed us to examine the chemical mediators and mechanisms involved in this intrinsic communication. For example, addition of an antimuscarinic agent (atropine, 1 μM) blocked the effects of exogenous carbachol and prevented the initiation of mucosal Ca^{2+} transients seen in figures 7C and D at the red asterisks (data not shown). This suggests that carbachol initiates these transients by acting on muscarinic receptors, most likely located in the urothelium. In the presence of a purinergic antagonist (PPADS, 1 μM), carbachol evokes mucosal Ca^{2+} transients, but the waves of activity do not spread into the detrusor (as is seen in figure 7D) and the images obtained from an overactive bladder mimic the ones seen in the control bladder in figure 7C. Thus, carbachol may release ATP from the urothelium which acts submucosally to evoke intrinsic detrusor contractions. Like PPADS, the *c-kit* antagonist, Gleevec (30 μM)¹⁹, or the P2Y_6 antagonist, (MRS2578, 10 μM)²⁰, prevent carbachol evoked mucosal Ca^{2+} transients from reaching the detrusor. *c-kit* and P2Y_6 receptors are localized to interstitial cells in the lamina propria suggesting that these cells are transducers of information between the urothelium and smooth muscle. Communication between interstitial cells may be mediated through gap junctions, as connexin 43 expression increases in these cells in pathology^{21–23}. We hypothesize that in UML animals, interstitial cells in the lamina propria form a syncytium, transducing urothelial signals and coordinating micromotion into large amplitude spontaneous contractions. These large amplitude contractions have been shown to directly stimulate afferent nerves¹³ and so may contribute to incontinence. We refer to this as the urotheliogenic hypothesis²⁴, which combines aspects of the myogenic²⁵ and neurogenic²⁶ theories.

Bladder-urethral Sheets

Spinal cord lesions can result in dramatic alterations in the function of bladder afferents. By using our optical mapping approach with bladder-urethra preparations (figure 8A), we have determined that there is particular plasticity in the trigone of bladders from UML mice. In these experiments, bladders with urethras ($n > 6$) were excised and placed in a recording chamber with oxygenated Krebs solution. Organs were cut from outlet to dome along the midline ventral aspect to form sheets. The sheets were pinned across the dome to a fixed platform with the mucosal surface facing up and the bladder base and urethra connected to a tension transducer (figure 8A). Preparations were stretched to optimal resting length and allowed to equilibrate for 30 min. They were stained with voltage-sensitive (di-4-ANEPPS; 5 μM) and/or Ca^{2+} -sensitive dyes (Rhod-2-AM; 5 μM) for 20 min each. Longitudinal stretch was applied *via* the tension transducer connected to a programmable stepper motor. In control bladders, bipolar electrical stimulation of the bladder dome caused the spread of action potentials and intracellular Ca^{2+} transients throughout the bladder and urethra (not shown). In contrast, stimulation of the superficial trigone caused a limited spread of activity restricted to that region (figure 8B, top traces), suggesting that the trigone is electrically isolated from the underlying detrusor in normal bladders. However, in UML bladders,

electrical stimulation of the trigone resulted in the spread of activity beyond the trigone into the surrounding detrusor (figure 8C, top traces). Following desensitization with capsaicin, the spread of this activity was attenuated in both normal and pathological bladders; indicating the activity is driven by capsaicin-sensitive nerves (*i.e.*, C-fibers, figures 8B and C, bottom traces). Figure 8D shows bladder tension following two consecutive doses of capsaicin (1 μ M) to an UML bladder, demonstrating desensitization and the lack of excitatory peptide release after the first dose. We hypothesize that after spinal cord injury, activity evoked in the trigone region spreads to adjacent regions of the bladder as a result of neural remodeling and sprouting of afferent terminals, making the trigone a particularly desirable therapeutic target.

In-line Cultured Cells

The mechanisms underlying cell-cell interactions within the bladder mucosa are still largely unclear because they are difficult to study in intact tissue. Therefore, it is necessary to investigate these mechanisms at the cellular level. We have developed a cell culture-based optical mapping approach where different bladder cell types are discretely cultured on coverslips to study the communication between cells of the bladder wall in greater detail (figure 9A)²⁷. For example, by arranging in-line coverslips with urothelial, interstitial and smooth muscle cells, and selectively stimulating one cell type (*e.g.*, urothelial cells with hypo-osmotic solution), we can determine whether there is communication with the downstream cell types (figure 9B). By removing one of the cell types (*e.g.*, interstitial cells) we can determine their importance in cell-cell communication (figure 9C). Studies have demonstrated that cultured mouse lamina propria interstitial cells have rhythmic Ca^{2+} transients (figure 9D), providing further evidence for their potential role as pacemakers and promoters of intrinsic bladder overactivity.

Spinal Cord Slices

In the UML model, bladder function partially recovers due to plasticity of nerves within the bladder and synapses in the spinal cord. It is believed that there are new pathways formed between perineal cutaneous afferents and preganglionic neurons that innervate the bladder, allowing for reflex voiding^{28, 29}. To study the changes in spinal circuitry following injury, we have started optically mapping mouse spinal cord slices with attached L6/S1 ventral and dorsal roots (figure 10A). A mouse spinal cord was isolated along with associated L₆ roots, fixed in agar with the roots extended perpendicular and cut in 500 μ m thick slices. The sections with associated dorsal and ventral roots ($n = 4$) were mounted in a recording chamber and stained with a voltage-sensitive dye (di-4-ANEPPS; 5 μ M; figure 10A). Experiments have demonstrated that we can stimulate activity in cord sections by electrical field stimulation of the slice (10 Hz; 0.1 ms; 3 sec train duration; 10 V) or exogenous application of glutamic acid, both of which can be inhibited by lidocaine (figure 10C–E). Thus, this preparation should allow for the comparison of patterns of synaptic activity, in controls and different rodent models, to determine the extent of neural remodeling.

Cortical Imaging

Supraspinal mechanisms are largely responsible for the ability to void at socially acceptable times and places. However, there are only a few limited ways to study brain activity during the micturition cycle using animal models. We have developed an approach to map action potentials in the mouse cerebral cortex induced by activation of bladder afferents (figure 11A). We expose one or both hemispheres of the cortex and stain it using a voltage-sensitive dye with excitation and emission wavelengths unaffected by hemoglobin absorption so that signal fidelity is unaffected by blood flow³⁰. Mice were anesthetized with urethane and the cranial bone above the left cortex hemisphere carefully removed. The exposed cortex was stained with the dye, RH-1691 (5mg/ml, diluted in 0.05% Pluronic F127) for 1 hour. A catheter (PE-50) was inserted into the bladder dome and connected to a syringe pump and pressure transducer. When the bladder was quickly filled through the catheter, there was a prolonged activation in the cortex that occurred just following the pressure change recorded in the bladder (n = 4; figure 11B). The raw trace data and isochronal maps generated demonstrate that this activity occurred in select areas (figure 11C and D). This method can be applied to the bladder- and colon-irradiated models to determine if sensitization involves the higher centers of the central nervous system.

CONCLUSION

Alterations in bladder afferent mechanisms may contribute to voiding dysfunction in irradiation- and spinal cord injury-induced damage. One mechanism for alteration involves neuronal remodeling and sprouting. Injury can/may induce functional and morphological changes in peripheral and central (SC) afferent terminals in the spinal cord transected model and also supraspinally in the case of direct and indirect irradiation models. It has recently been demonstrated that there is increased sprouting of unmyelinated CGRP containing nerves in the lumbosacral region of the cord of spinal transected rats³¹. This remodeling of afferent nerves is believed to facilitate the emergence of reflex bladder activity as well as contribute to the development of visceral pain^{32, 33}. Established drug therapies such as muscarinic receptor antagonists and emerging therapies such as β_3 -adrenergic agonists³⁴, botulinum toxin type-A injections³⁵ or antibodies to nerve growth factor (NGF)³⁶, may act partly through suppression of afferent activity with the former two treatments or inhibition of remodeling with the latter one. The functional remodeling of afferent nerves may be driven by a variety of neurotrophic factors including NGF^{36, 37}. An increase in the responsiveness of dorsal root neurons to noxious stimuli and changes in gene expression can cause a decrease in afferent thresholds that characterize the phenomenon of sensitization. Stimuli that can cause sensitization include tissue inflammation and nerve injury where there is repeated/sustained intense C-fiber stimulation. Primary afferents excited by a variety of strong noxious stimuli release neurotransmitters (including glutamate, tachykinins, *etc.*) antidromically from the peripheral terminals³⁸ as well as in the spinal cord dorsal horn^{39, 40}. Neurotransmitters released in the latter case can sensitize second or third order sensory neurons leading to the transmission of noxious signals to the brain with an intact cord. In the bladder, nociceptive C-fibers can become sensitive to mechanical stimulation in pathology and contribute to incontinence. The combined use of new and established animal models and

methods may help elucidate these mechanisms and lead to new and improved therapeutic options.

Acknowledgments

We thank Travis Wheeler (Manager), Josh Byler, and Greg Szekeres of the Machine and Electronic Shops, of the Department of Cell Biology and Physiology and Pharmacology and Chemical Biology, University of Pittsburgh, for the fabrication of equipment used in these studies.

GRANTS: This research was supported by grants from: Allergan LLC, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, DK071085), and Pfizer Inc to AK; Pfizer Inc, and Sam & Emma Winters Foundation to IZ; NIDDK (KDK085144) to YI; NIDDK (DK057267 and DK068557 to NY; NIDDK (DK54824 and DK64539) to LB; NIDDK 5K01KD080184-02 to AHM; NIDDK (DK049430 and DK077783) to WdeG.

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