W3: ICS Methodology in Basic Science

Workshop Chair: Naoki Yoshimura, United States
13 September 2016 09:00 - 10:30

Start	End	Topic	Speakers
09:00	09:15	Introduction and overview	Karl-Erik Andersson
09:15	09:35	Methods and terminology of in vivo experiments	Yasuhiko Igawa
09:35	09:55	Methods and terminology of in vitro experiments	Maryrose Sullivan
09:55	10:15	Animal modeling of lower urinary tract dysfunction	Naoki Yoshimura
10:15	10:30	Discussion	All

Aims of course/workshop

This workshop entitled "ICS Methodology in Basic Science" is designed to provide the audience with basic and advanced knowledge of methodologies and terminology for data assessment of basic research experiments that explore the pathophysiological mechanisms underlying Neuro-Urological diseases such as overactive bladder, bladder pain and stress urinary incontinence. We also discuss the advantages and limitations of the use of animal models of lower urinary tract dysfunction when clinical translation is considered. Target audience includes urologists, gynecologists, basic scientists and those interested in basic research on Neuro-urology. This course will especially benefit the beginners who have limited previous research experience.

Learning Objectives

After this workshop participants should be able to:

1. To understand the current status of basic research in Neuro-Urology
2. To understand the basics of in-vivo & in vitro experiments
3. To understand the animal modeling of human Neuro-urological diseases with its advantages and limitations

Learning Outcomes

After the course, the student will be able to:

1. Gain the basic knowledge of methodologies and terminology for data assessment of basic research experiments and
2. Understand the usefulness and limitations of basic research when translating the results to clinical conditions of Neuro-urological disorders.

Target Audience

Urologists, gynaecologists and basic scientists interested in basic research on Neuro-urology

Advanced/Basic

Basic

Suggested Reading

- Fry CH, Daneshgari F, Thor K, Drake M, Eccles R, Kanai AJ, Birder LA. Animal models and their use in understanding lower urinary tract dysfunction. Neurourol Urodyn. 2010 Apr;29(4):603-8.
- Andersson KE, Soler R, Füllhase C. Rodent models for urodynamic investigation. Neurourol Urodyn. 2011 Jun;30(5):636-46.
- McMurray G, Casey JH, Naylor AM. Animal models in urological disease and sexual dysfunction. Br J Pharmacol. 2006;147 Suppl 2:S62-79.
- Shea VK, Cai R, Crepps B, Mason JL, Perl ER. Sensory fibers of the pelvic nerve innervating the Rat's urinary bladder. J Neurophysiol. 2000;84(4):1924-33.
- Cristofaro V, Peters CA, Yalla SV, Sullivan MP. Smooth muscle caveolae differentially regulate specific agonist induced bladder contractions. Neurourol Urodyn. 2007;26(1):71-80.
- Chaudhury A, Cristofaro V, Carew JA, Goyal RK, Sullivan MP. Myosin Va plays a role in nitrergic smooth muscle relaxation in gastric fundus and corpora cavernosa of penis. PLoS One. 2014;9(2):e86778.
- Kanai A, Zabbarova I, Ikeda Y, Yoshimura N, Birder L, Hanna-Mitchell A, de Groat W. Sophisticated models and methods for studying neurogenic bladder dysfunction. Neurourol Urodyn. 2011 Jun;30(5):658-67
- de Groat WC, Griffiths D, Yoshimura N. Neural control of the lower urinary tract. Compr Physiol. 2015 Jan;5(1):327-96.
- Yoshimura N, Miyazato M. Neurophysiology and therapeutic receptor targets for stress urinary incontinence. Int J Urol. 2012 Jun;19(6):524-37.

Karl-Erick Andersson

Dr Andersson will give an introductory talk to overview the current status of basic research in Neuro-Urology, which includes both usefulness and limitations of the use of animals when translating the results to clinical conditions of the diseases.

Yasuhiko Igawa

Dr Igawa will discuss about in vivo experiments. In vivo experiments are essential for evaluating lower urinary tract (LUT) function, which include voiding and nociceptive behaviour measurements, urodynamic studies, and bladder afferent fiber activity measurements. This lecture focuses on these methods mainly applied to rodents.

1. Voiding behaviour (Frequency-volume; FV) measurement

FV measurement is a good method for evaluating 24 h voiding behaviour naturally. Animals are individually placed in a metabolic cage, which is enable to measure water intake, voided volume per micturition, total voided volume during the light or dark cycle, and for 24 h. This method allows various parameters to be monitored continuously in a stress-free and physiologically relevant environment in the absence of anesthesia, tethering or restraint.

2. Nociceptive behaviour measurements

Two types of nociceptive behaviour, licking (lower abdominal licking) and freezing (motionless head-turning to the lower abdomen), can be used at least in rats. Licking is predominantly induced by urethral pain sensation carried through the pudendal nerve, whereas freezing is related to pelvic nerve-mediated bladder pain.

3. Urodynamic studies

1) Cystometry (CMG)
   Regardless of the species or model, CMG is the most commonly utilized means of exploring bladder function. Intravesical pressure and voided volume are monitored during intravesical instillation of saline at a constant filling rate, via a bladder dome or urethral catheter, until the point of fullness in order to elicit a micturition response. This can be performed in either anesthetized (usually with urethane) or conscious animals. The effect of drugs, administered systemically or intravesically on bladder function can thus be assessed.

2) Urethral sphincter electromyography (EMG)
   A widely utilized method for indirectly measuring external urethral sphincter (EUS) function is the measurement of EMG activity. In rats, the EUS is active with bursting occurring during voiding. These oscillations may milk urine through the urethra or aid with urine marking of territories.

3) Leak point pressure measurement
   Leak point pressure measurement is taken as the peak bladder pressure at which urine starts to leak, measured using a suprapubic bladder cannula. This measurement has the advantage of being a dynamic test that directly evaluates the ability of the urethra to protect against leakage caused by increases in abdominal pressure and the potential of drugs to improve this ability.

4. Bladder afferent fiber activity measurements

Mechanosensitive properties of the pelvic nerve afferent fibers innervating the urinary bladder can be electrophysiological classified by their conduction velocity (CV) as fibers having high CV (myelinated, A□-fibers) and those having low CV (unmyelinated, C-fibers). The rat is the most intensively utilised animal in this field, and the cat is the second. The cut-off value of the CV between A□- and C-fibers proposed most frequently in the literature is 2.5 m/s, but the value varies from 1.7 to 2.5 m/s.

Finally, progress in the working group of ICS Standardisation on Basic Science Terminology will be introduced in this lecture.

Take Home Message: In vivo experiments are an essential part of functional evaluation of LUT.

Maryrose Sullivan

Dr Sullivan will discuss about in vitro experiments.

- The in vitro tissue bath assay is a classical experimental approach to pharmacologic evaluation of smooth muscle systems. However, this versatile and reliable methodology can be tailored to meet a variety of experimental needs, not only allowing pharmacological assessment of contractile or relaxation responses of bladder tissue, but the evaluation of its biomechanical properties, neurotransmission processes and signal transduction events. This important methodology offers significant advantages over more reductionist approaches by maintaining the two dimensional arrangement of bladder tissue or the three dimensional structure of the whole organ, and preserving the complex physiologic interactions among various cell types in the bladder that contribute to generation of isometric force. Moreover, confounders that can complicate interpretation of intact animal preparations, including systemic factors and anesthesia effects, are omitted with this methodology. Thus isolated tissue or whole organ functional assays are invaluable investigational tools that can be included in researchers' armamentarium to advance our understanding of the physiology and biomechanics of the bladder and urethra, as well as the pathophysiology of lower urinary tract disease.

- Technical details related to implementation of the in vitro functional assay will be addressed, including equipment requirements, various muscle bath configurations, calibration of force transducers, preparation and optimal adjustment of tissue, methods of stimulation and data acquisition considerations. Proper identification of potential artifacts is critical to data interpretation. Thus examples of common pitfalls that may arise during in vitro experiments and methods to correct or reduce their impact on experimental data will be discussed.

- This methodological approach allows evaluation of physiologic responses to a variety of experimental conditions, including hypoxia, pH, chemicals, mechanical load or temperature, in human or animal samples. Force or pressure measurements can be combined with simultaneous measurements of electrical activity, endpoint assays of second messengers, detection of released substances, and monitoring of intracellular ion activity or free radical production using fluorescent probes or bioluminescent assays. Unlike in vivo approaches, this technique provides a means to assess the contributions of separate tissue components to bladder function, including the mucosa, smooth muscle, interstitial cells, and nerves. Importantly, various aspects of the functional response to pathologic conditions can be addressed using bladder tissue obtained from appropriate animal models of bladder outlet obstruction, spinal cord injury, diabetes, interstitial cystitis, or pelvic ischemia.

- Successful in vitro tissue bath experiments are followed by the critical processes of data reduction, quantification, analysis and interpretation. A variety of meaningful parameters can be captured or derived from the acquired data generated under resting or stimulated conditions. Strategies for normalization of parameters and presentation of data, which depend on the experimental design and goals of the investigation, will be discussed.

Take Home Message: In vitro functional experiments remain a powerful method of evaluating physiologic and pathophysiologic processes at work in the bladder under well controlled conditions that can easily be manipulated to meet the demands of the experimental protocol and to measure a variety of relevant functional responses that may be of interest to the investigator.

Naoki Yoshimura

Dr Yoshimura will discuss about the current animal models of Neuro-urological diseases.

- Animal models are essential for understanding the pathophysiology of human diseases and developing new and effective therapeutic modalities for the human diseases. This is also true for the research of lower urinary tract (LUT) dysfunction including overactive bladder (OAB), stress urinary incontinence (SUI) and bladder pain syndrome/interstitial cystitis (BPS/IC).

- However, as the etiology of these LUT diseases is multifactorial, it is not an easy task to develop an animal model that fits all aspects of disease conditions although animal models allow us to perform the study in controlled conditions (e.g., duration and/or severity of insults) and to utilize invasive experimental methods. Thus, it is important to understand the biochemical, physiological, and pathophysiological mechanisms, either validated or postulated, of the human diseases, and which mechanisms(s) are reproduced in each of animal models.

- Also the symptoms of OAB or BPS/IC such as urgency or pain are often subjective ones in humans while the findings in animal models (e.g., non-voiding contractions or enhanced reflex voiding in OAB models) are objective, especially when performed under anesthesia. Therefore, it is imperative to know what are the appropriate, surrogate marker(s) in animal models for human LUTS including urgency, frequency, incontinence and pain although it is often difficult.

- In addition, while animal models induced by acute bladder irritation or injury (e.g. acute cystitis models for OAB/BPS or a vaginal distension model for SUI) have often been used, the majority of LUT dysfunctions in humans are chronic conditions. Thus the development of chronic animal models would be desired although acute animal models are suitable for screening of new drugs or testing of new ideas.

- Other factors such as species differences in drug effects and effects of anesthesia should also be considered to interpret the animal data and extrapolate the human disease mechanisms. Then, ultimately the pharmacological targets or new therapeutic modalities identified in animal models have to be validated in testing in humans.

Take Home Message: Animal modeling is essential for understandings of the pathophysiology of LUT dysfunction in various diseases. However, it is also essential to understand the limitation in clinical application of experimental data.

Why and Where Do We Need Animal Models in Neuro-urology?

Karl-Erik Andersson

WFIRM, Wake Forest University, Winston Salem, NC, and Institute for Clinical Medicine, Aarhus University, Aarhus, Denmark

K-E Andersson: Disclosures

Consultant/Advisory board:

Allergan
Astellas
Ferring
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Why and Where Do We Need Animal Models in Neuro-urology?

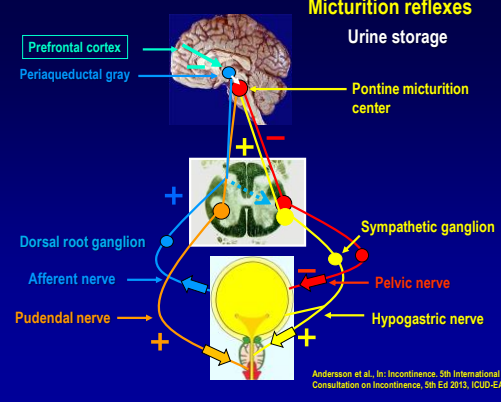
Why use animal models?
The lower urinary tract and the micturition reflex in animals and man have many similarities (the general components are basically the same)

Why perform animal experiments?
To get insights in physiological and pathophysiological mechanisms of bladder dysfunction possibly applicable to the human situation

Where to perform experiments?
In the laboratory it is possible under controlled conditions to recreate dysfunctions found in humans and to exclude confounding factors

Micturition reflexes

Urine storage



Andersson et al., In: Incontinence. 5th International Consultation on Incontinence, 5th Ed 2013, ICUD-EAU 2013

Type of Animal Experiments

In vivo
the effects of various biological entities are tested on whole, living organisms

Ex vivo
experimentation or measurements done in or on tissue from an organism in an external environment with minimal alteration of natural conditions

In vitro
studies are performed with microorganisms, cells or biological molecules outside their normal biological context

Why Use Animal Models?

What animal for what purpose?

Small animals, e.g.;
rodents

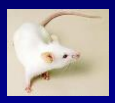
Large animals, e.g.:
dogs,
pigs
non-human primates

Translational impact?

Most Often Used Animals

Rat

Mouse



Guinea pig

"Large Animals"

Dog

Pig

Monkey

Why Use Animal Models?

Type of Models

Normal animals

Disease models, e.g.:

- *Outflow obstruction*
- *Spinal cord injury*
- *Diabetes*

Translational impact?

Why Use Animal Models?

Tools for in vivo study, e.g.:

Urodynamics

Voiding behavior

Other

Translational impact?

Why Use Animal Models?

Tools for ex vivo study, e.g.:

Isolated, perfused bladder

Recording of afferent activity

Other

Translational impact?

Why Use Animal Models?

Tools for in vitro study, e.g.:

Organ bath experiments

Histology, immunohistochemistry

Molecular biology

Translational impact?

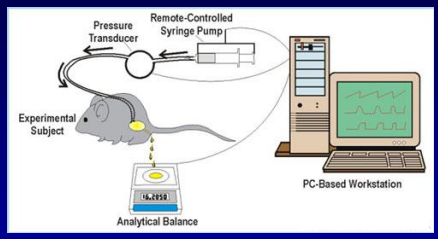
Cystometry

"Filling cystometry is the method by which the pressure/volume relationship of the bladder is measured during bladder filling"

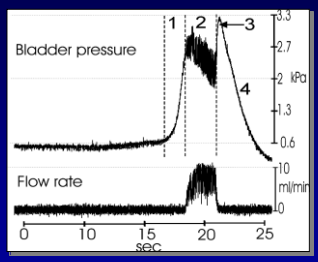
In humans, "the filling starts when filling commences and ends when the patient and urodynmicist decide that 'permission to void' has been given"

In rodents filling is most often continuous and ends when the micturition reflex is elicited

Rat Cystometry



Rat Cystometry (Anesthesia): The Phases of Bladder Pressure During One Micturition Cycle



Streng et al., BJU Int. 2004 Oct;94(6):910-4.

Rodent Cystometry

What do we measure?

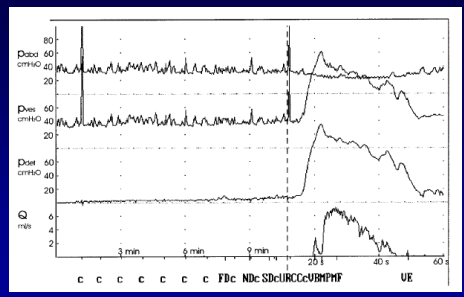
- **Micturition pressure**: *MP = maximum bladder pressure during micturition*
- **Threshold pressure**: *TP = bladder pressure at onset of micturition*
- **Basal pressure**: *BP = minimum bladder pressure between two micturitions*
- **Intermicturition pressure**: *IMP = mean bladder pressure between two micturitions*

Rodent Cystometry

What do we measure?

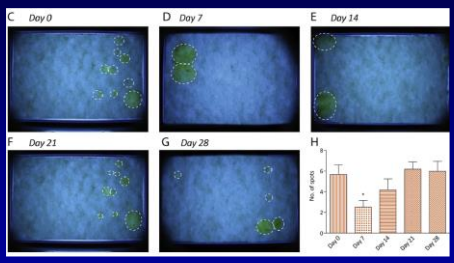
- **Spontaneous activity**: *(SA = IMP minus BP)*
- **Non-voiding contractions** *(NVC; amplitude, frequency)*
- **Micturition frequency**: *(MF)*
- **Bladder capacity**: *(BCap = infusion rate divided by MF)*
- **Micturition volume**: *(MV)*
- **Residual volume**: *(RV = BCap minus MV)*
- **Bladder compliance**: *(BCom = BCap/TP minus BP).*

Normal Human Cystometry



Schaefer et al, Neurourol Urodyn 21: 261, 2002

Voiding Behavior in Rats Before and After Oxybutynin Administration



Uvin et al., Eur Urol.2013 Sep;64(3):502-10.

ICS Definitions: Lower Urinary Tract Symptoms

Urgency "is the complaint of a sudden compelling desire to pass urine which is difficult to defer"

Incontinence "is the complaint of any leakage of urine"

Urgency incontinence "is the complaint of involuntary leakage accompanied by or immediately preceded by urgency"

Abrams et al., Neurourol Urodyn, 21:167, 2002

Detrusor Overactivity: ICS Definition

"a urodynamic observation characterized by involuntary detrusor contractions during the filling phase which may be spontaneous or provoked"

Abrams et al., Neurourol Urodyn, 21:167, 2002

Detrusor Overactivity: ICS Definition

Cystometric characterization

- Phasic detrusor overactivity *"is defined by a characteristic waveform and may or may not lead to urinary incontinence*
- Terminal detrusor overactivity *"is defined as a single, involuntary detrusor contraction, occurring at cystometric capacity, which cannot be suppressed and results in incontinence usually resulting in bladder emptying (voiding)"*

Abrams et al., Neurourol Urodyn, 21:167, 2002

Overactive Bladder (OAB) Syndrome: ICS Definition

"Urgency, with or without urge incontinence, usually with frequency and nocturia"

Also called:

- Urge syndrome
- Urgency-frequency syndrome

Abrams et al., Neurourol Urodyn, 21:167, 2002

Why and Where Do We Need Animal Models in Neuro-urology?

Summary

- Micturition in rodents and humans differs significantly
- Cystometric parameters in rodents are poorly defined and do not correspond to what is used in humans
- Available models have limited translational value
- *Despite many limitations, current animal models may give relevant information on bladder functions*

Why and Where Do We Need Animal Models in Neuro-urology?

What is needed

- Careful standardization of terminology
- Improved characterization of models use
- Well characterized *new models*
- General awareness of translational limitations

Methods and terminology of *in vivo* experiments

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Department of continence Medicine
The University of Tokyo

Affiliations to disclose†:

None

† All financial ties (over the last year) that you may have with any business organisation with respect to the subjects mentioned during your presentation

Funding for speaker to attend:

- [ ] Self-funded
- [x] Institution (non-industry) funded
- [ ] Sponsored by:

Methods and terminology of *in vivo* experiments

In vivo experiments:

Essential for evaluating lower urinary tract (LUT) function

1. Voiding behaviour *(Frequency-volume; FV)* measurements
2. Nociceptive behaviour measurements
3. Urodynamic studies
   - ① Cystometry (CMG)
   - ② Sphincter EMG
   - ③ Leak point pressure (LPP) measurement
4. Bladder afferent fiber activity measurements

This lecture focuses on these methods and terminology mainly applied to rodents.

Methods and terminology of *in vivo* experiments

Clinical (in humans) and relevant animal tests for LUT function

	Clinical (Humans)	Animals (Rodents)
Symptom score	Various questionnaires (IPSS, QOL questions)	not applicable (Pain: Nociceptive behavior test)
Bladder diary	Frequency volume chart (FVC)	FV measurement in metabolic cage
Urodynamic tests	Filling CMG (storage phase)	CMG (storage phase) no information on filling sensation
	Pressure flow study (voiding phase)	CMG (voiding phase)
	uroflowmetry	not applicable
	PVR	PVR
	sphincter EMG	sphincter EMG
Special tests	not applicable	afferent activity measurements

Methods and terminology of in vivo experiments

1. Voiding behaviour *(Frequency-volume; FV)* measurements in a metabolic cage

A good method for evaluating **24 h voiding behaviour naturally**

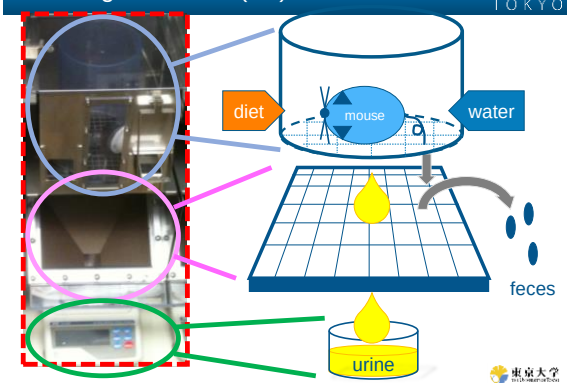
Animals are individually placed in a metabolic cage, which is enable to measure

voiding frequency, voided volume per micturition, total voided volume , and water intake

during the light or dark cycle, and for 24 hrs.

monitor continuously in a stress-free and physiologically relevant environment in the absence of anesthesia, tethering or restraint.

Voiding behaviour (FV) measurements



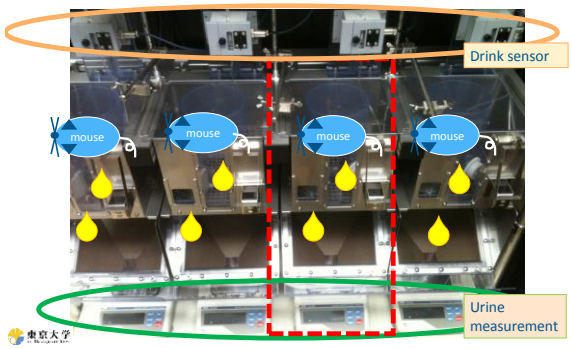
TIPs for FV measurement

Animals are susceptible to environmental changes

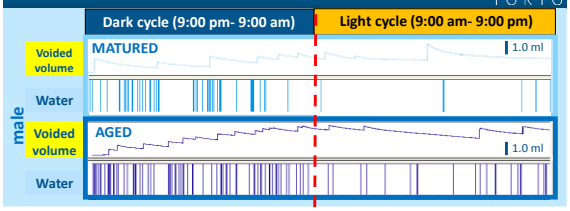
- Take an accommodation time at least 24 h after placing the animal in the metabolic cage before investigation.
- Keep quiet and stress-free environment
- Measure FV simultaneously with control animals to minimize environmental influence.

Device for FV measurements

(001-006 metMCM/TOA-UFabolic cage (Mitsubishi Chemical Medience, Tokyo, Japan)



Representative charts of FV measurements



Evaluable parameters

Voided volume per micturition, Voiding frequency, Total voided volume, and Water intake

2. Nociceptive behaviour measurements

Two types of nociceptive behaviour, licking and freezing, can be used at least in rats.

1. **Licking behavior:** lower abdominal licking
   - predominantly induced by urethral pain sensation carried through the pudendal nerve

2. **Freezing behavior:** Immobility with the nose pointing toward the lower abdomen without licking
   - related to pelvic nerve-mediated bladder pain

Saitoh C, et al., J Urol, 2008;
Funahashi Y, et al., J Urol, 2013

2. Nociceptive behaviour measurements

- Licking behavior

Methods and terminology of in vivo experiments

3. Urodynamic studies
   - ① Cystometry (CMG)
   - ② Sphincter EMG
   - ③ Leak point pressure (LPP) measurement

Conditions during measurements

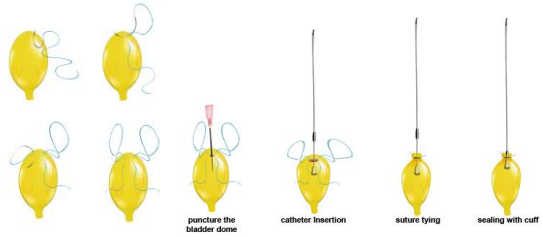
- Conscious free-moving
- Conscious restraint
- Urethane-anesthetized
- Decerebrated un-anesthetized

Urodynamic studies

① Cystometry (CMG)

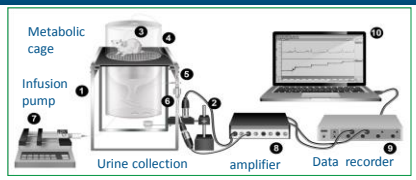
- **the most commonly utilized means of exploring bladder function**.
- Intravesical pressure and voided volume are monitored during intravesical instillation of saline at a constant filling rate.
- In either anaesthetized (usually with urethane) or conscious animals
- **To evaluate the effects of drugs**, administered systemically or intravesically, on bladder function
- **To assess the differences in bladder function between normal and pathological model**

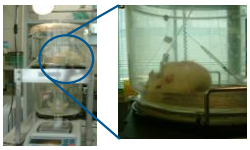
Bladder catheterization for Conscious CMG



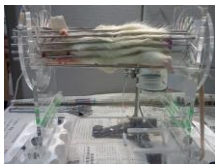
To avoid artifacts by catheter-implantation, CMG investigation is carried out at least 3 days after catheter-implantation.

Conscious CMG measurement (RAT)



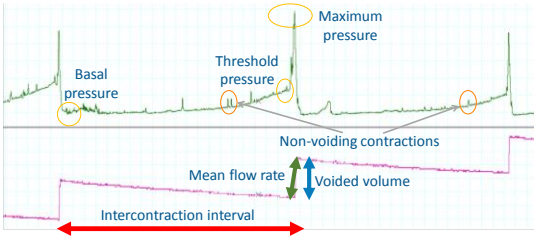


Conscious free-moving condition



Conscious restraint condition

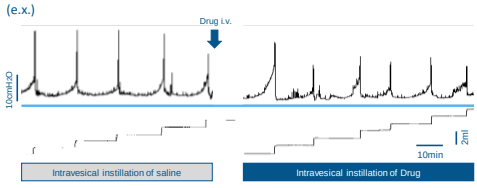
Evaluable parameters on the CMG measurements



Bladder capacity (BC) [Intercontraction interval x infusion rate]

Bladder compliance [BC/(Th. P. − B.P.)]

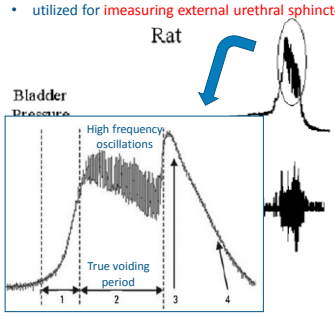
Experimental protocol (e.x.)



Urodynamic studies

② Sphincter EMG

- utilized for imeasuring external urethral sphincter (EUS) function



In rats, during voiding, EUS EMG activity increases with high-frequency bladder pressure

The Max P is not always the Max P during voiding.

Mc Murray G, et al, BJP, 2006
Streng t, et al, BJU Int, 2004

Urodynamic studies

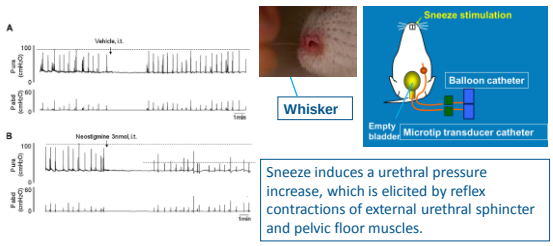
③ Leak point pressure (LPP) measurement

- defined as **the peak bladder pressure at which urine starts to leak**, measured using a suprapubic bladder cannula
- can directly **evaluate the ability of the urethra to protect against leakage caused by increases in abdominal pressure** and the potential of drugs to improve this ability

Urodynamic studies

Sneeze-induced urethral continence reflex measurement

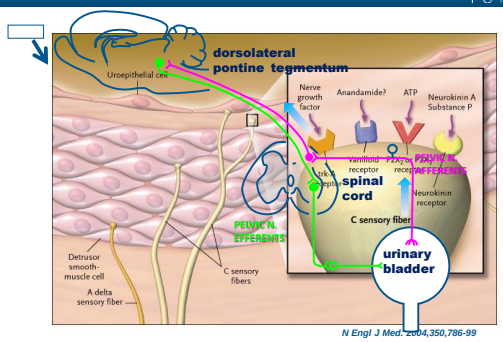
- The sneeze reflex is induced by a rat's whisker cut and inserted gently into the nostril under urethane anesthesia.



Sneeze induces a urethral pressure increase, which is elicited by reflex contractions of external urethral sphincter and pelvic floor muscles.

Yoshikawa S, Yoshimura N, et al, NAU, 2014

Afferent innervation of the bladder



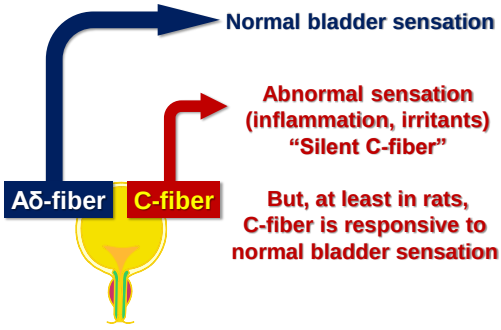
N Engl J Med. 2004,350,786-99

Methods and terminology of in vivo experiments

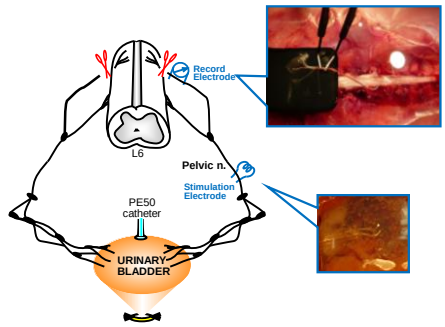
4. Bladder afferent fiber activity measurements

- Mechanosensitive and nociceptive
  - **Mechanosensitive:** responds to bladder filling & distention
  - **Nociceptive:** responds to nociceptive stimuli (irritants, inflammation)
- electrophysiologically classified by their conduction velocity (CV)
  - **High CV: Myelinated, Aδ-fibers**
  - **Low CV: Unmyelinated, C-fibers**
- The cut-off value of the CV between Aδ- and C-fibers proposed most frequently in the literature is 2.5 m/s, but the value varies from 1.7 to 2.5 m/s.
- The rat is the most intensively utilized animal in this field, and the cat is the second.

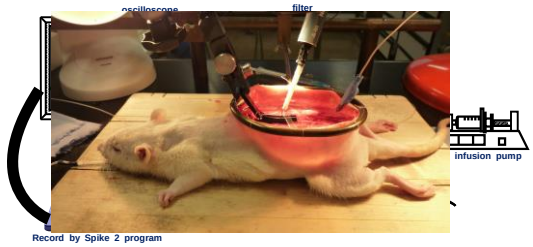
Two subtypes of bladder afferent fibers

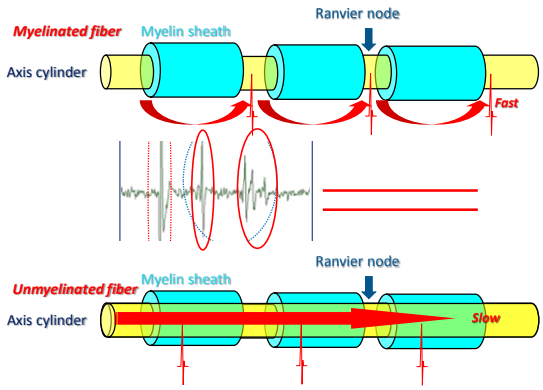


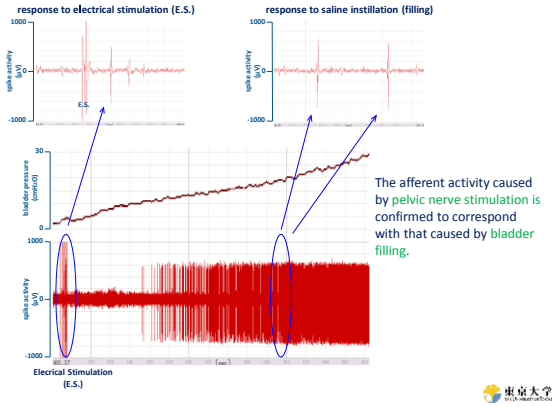
4. Bladder afferent fiber activity measurements



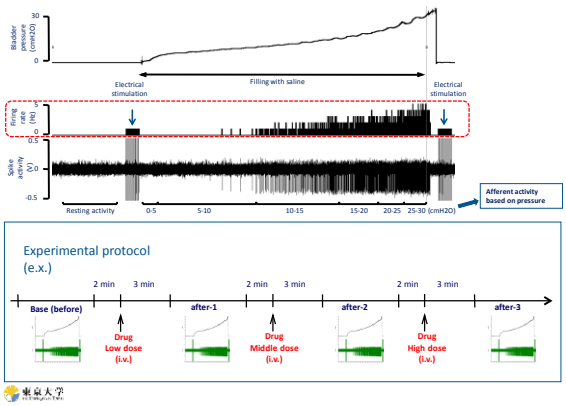
4. Bladder afferent fiber activity measurements







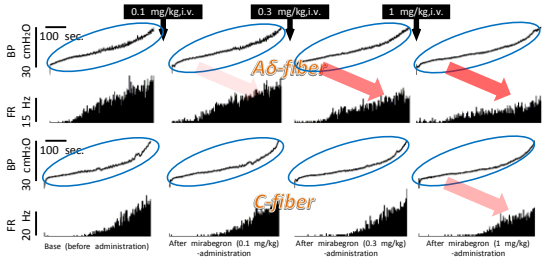
The afferent activity caused by pelvic nerve stimulation is confirmed to correspond with that caused by bladder filling.



Effects of Mirabegron

During Filling

No significant changes in the bladder compliance after mirabegron-treatment.



Aizawa N, et al, Eur Urol, 2012;62(6):1165-73

ICS Standardisation on Basic Science Terminology

Six categories:

1. **Cell:** interstitial cell, mucosa, lamina propria, urothelium, etc.
2. **Neuro:** neural remodelling, silent neurons, sensation, etc.
3. **Integrative:** micromotion, contractility, decompensation, etc.
4. **Urodynamic:** compliance, volume, partial BOO, detrusor underactivity, detrusor overactivity, non-voiding contraction, etc.
5. **Strategic:** therapeutic target, urgency/OAB, etc.
6. **Brain:** Standards for specifying brain regions and white matter tracts, etc.

TAKE HOME MESSAGES

- ***In vivo* experiments are essential for evaluating LUT function.**
- **FV measurements and CMG are the most commonly used methods.**
- **Afferent fiber activity measurements give us direct information on bladder afferent activities.**
- **A working group on ICS standardisation on basic science terminology was organised and this standardisation will be opened in the public shortly.**

Methods and terminology of *in vitro* experiments

Maryrose Sullivan, Ph.D.
VA Boston Healthcare System
Harvard Medical School

Maryrose Sullivan

Affiliations to disclose†:

None

† All financial ties (over the last year) that you may have with any business organisation with respect to the subjects mentioned during your presentation

Funding for speaker to attend:

- [X] Self-funded
- [ ] Institution (non-industry) funded
- [ ] Sponsored by:

Advantages of *In Vitro* tissue bath assay

- Classical experimental approach to pharmacologic evaluation of SM systems
- Tissue retains 2-D structure
- Contraction in system with multiple cell types
  - neural responses
  - contractile or relaxation responses
  - Biomechanical properties
- Well controlled environment
- Systemic factors, anesthesia effects that can complicate interpretation of intact animal preparations are avoided
- Multiple samples, relatively high-throughput, paired experimental design
- Small quantities of drugs or expensive reagents

Tissue bath Systems

Optimal Conditions for *in vitro* assay

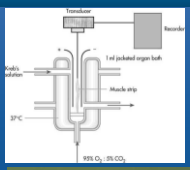
- Temperature
  - Water jacketed bath
- Physiologic so
  - M

Circulating pumps

Physiologic Buffer Solutions

- Artificial solution to maintain viability, chemical composition similar to plasma
- Ringer's, Tyrode's , Krebs, Krebs-Henseleit
- water purity, analytical grade reagents
- Metabolic substrate
  - Glucose (5.5 -11 mM), pyruvate, lactate
- carbonates, bicarbonates and phosphates
  - Maintain normal anion homeostasis, increase buffering capacity
  - Equilibrium with CO_2
  - precipitation of calcium phosphate

Force Transducers



- Isotonic, Isometric transducers
  - Displacement, Force

Data Acquisition Systems

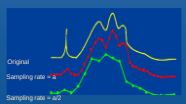
Amplifiers/Signal Conditioners

DAQ

Sensors

Data Acquisition

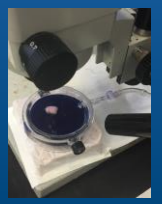
- Sampling rate determined by Nyquist theorem:
  2X > highest f component of signal.



- Signal distortion can be avoided by
  - Sampling signal at high rate, digitally filter high f
  - Limit bandwidth of signal < $\frac{1}{2}F_s$ with LP filter in front of A/D converter, provided input signal is sampled at ≥2f

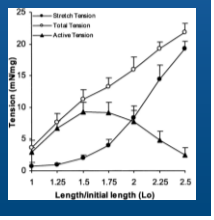
Tips for *in vitro* tissue assays

- Remove organ quickly after euthanasia
- Minimal handling
- Transport in physiologic solution on ice
  - Decrease metabolic processes, reduce requirements for oxygen/nutrients, decrease enzyme activity
- Dissecting bath under stereo-microscope or magnifier
  - Sylgard + blue pig
  - Insect pins



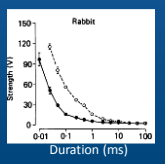
Tips for *in vitro* tissue assays

- Attach tissue using non-compliant material
- Avoid contact with walls, aeration
- Stretch to optimum length, defined force (~ 1 g)
- Avoid overstretching
- Equilibration >50-60 min



Evoked Responses - EFS

- Electrical Field stimulation
  - Electrodes within walls of bath
  - Attached to tissue supports
  - Field strength of 10-50 V/cm
  - Depends on distance between and area of electrodes
  - Frequency 0.5 – 64hz, duration 3-10s, pulse width 0.05-0.5 ms, 10-40V
  - Verify neurotransmission with TTX



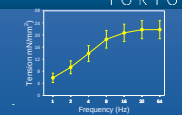
Stimulators

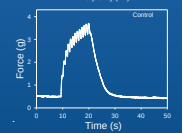


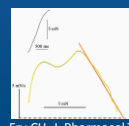
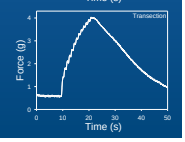
Power output of stimulator at high voltage or current

Analysis of EFS responses

- Frequency-Response curves
  - Peak Force
  - Area under the curve
- Rate of tension generation
  - Phase plots
  - Multiple transmitters with different rates of tension development
  - Plot dT/dt as function of T



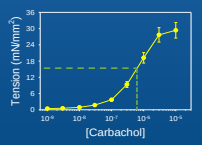


Fry CH, J. Pharmacol.Toxicol. Methods. 49, 2004

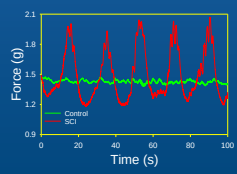
Evoked Responses - Agonists

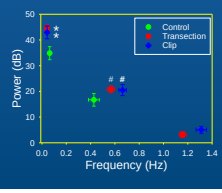
- Agonists added directly to bath
  - Wide range of concentrations, 10^{-9} – 10^{-5}M
    - Add agents at peak of contraction
    - Single dose
  - In presence or absence of antagonist
- Dose-Response curves
  - EC_{50} – measure of drug potency
    - concentration inducing a response halfway between baseline and maximum
    - four-parameter logistic function: $y = D + \frac{A-D}{1+10^{(x-logC)B}}$
  - Emax – measure of drug efficacy



Analysis of Spontaneous Activity

- Presumed to be myogenic
- Incidence/amplitude/frequency altered by disease, presence of mucosa
- Measure amplitude, frequency, AUC
- FFT



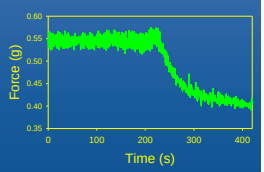


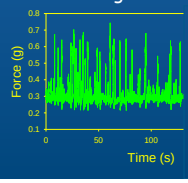
Standardization of Force

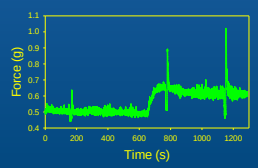
- Normalize by KCl,
- % Maximum Response
- Tension (mN/mm^2) = (force (g) x 0.0098 N/g)/CA
  - CA = tissue weight (g)/ρ (g/cm^3)/tissue length (mm)

Artifacts in *In vitro* tissue bath assay

- Environmental Artifacts
  - Loss of carbogen
  - Change in temperature
- Mechanical Noise
  - noise from aerator
  - mechanical artifacts during dose response







Artifacts in *In vitro* tissue bath assay

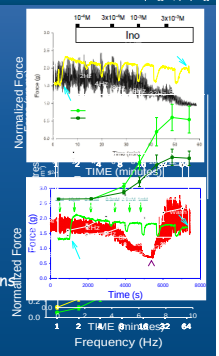
- Electrical Noise
  - 50-60 Hz
  - Cable failure
  - Transducer failure
  - Amplifier failure
- Structural
  HVAC
  elevators

Experimental Controls

- Time controls
  - Responsiveness may change throughout the day
- Vehicle controls
  - DMSO affects nerve and SM responses
- Drug controls

Uses of *In Vitro* assays

- Spontaneous activity
- Assessing SM vs neural components
- Assessing purinergic vs. cholinergic contributions to neurotransmission
- Effect of mucosa
- Pre-clinical drug testing
- Transgenic phenotyping
- Comparison of drug effects or gene knockout across visceral organs
- Species/gender/age differences
- Effect of surgical/chemical interventions
  - Scaffolds, pBOO, denervation, IC



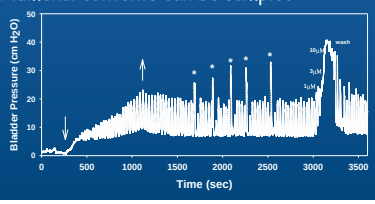
Versatility of In Vitro Tissue Assay

Modifications for measuring additional parameters

- Chemicals released by tissue
- Ion selective electrodes
  - pH, anions/cations, NO, CO_2, O_2
- Hypoxic, hyperglycemic conditions
- Calcium activity with fluorescent indicators
- Free radical production using bioluminescent assays
- Potentiometric electrodes - catecholamines
- Bioassay

Ex Vivo Cystometry

- Whole organ preparation
  - Devoid of extrinsic neural innervation with intramural nerves present
  - Structural relationships maintained
  - Drug concentrations can be determined
  - Effects of intra-vesical delivery
  - Intra-lumenal contents can be sampled



Summary

- *In vitro* tissue bath assay
  - Powerful, versatile method of evaluating physiologic and pathophysiologic processes
  - Well-controlled conditions that can be manipulated
  - Experimental set-up can be modified to measure other variables

Thank you

ICS 2016 TOKYO

Animal Modeling of Lower Urinary Tract Dysfunction

Naoki Yoshimura, M.D., Ph.D.
Professor of Urology
Chair of Neuro-urological Research
University of Pittsburgh

ICS 2016 TOKYO

Affiliations to disclose[+]:

There are no conflicts of interest for this presentation.

[+] All financial ties (over the last year) that you may have with any business organisation with respect to the subjects mentioned during your presentation

Funding for speaker to attend:

- [ ] Self-funded
- [X] Institution (non-industry) funded
- [ ] Sponsored by:

Basic Research -Why is it needed?-

- The etiology of lower urinary tract symptoms (LUTS) is still not known, but is likely to be multifactorial
- We need to understand the biochemical, physiological, and pathophysiological mechanisms, either validated or postulated, of the disease
- Basic research using animal models permits a controlled analysis of some aspects of the chronic syndrome

Etiology of LUTS/OAB

- Neurogenic LUTS/OAB
  - Supraspinal level
    - » Aging
    - » Cerebrovascular disease
    - » Parkinson's disease
    - » Depression
    - » Multiple system atrophy
  - Spinal level
    - » Spinal cord injury
    - » Multiple sclerosis
  - Peripheral level
    - » Diabetes mellitus
- Idiopathic LUTS/OAB
  - Bladder outlet obstruction (BPH)
  - Mixed incontinence (SUI patients)
  - Unknown

Animal models of LUTS

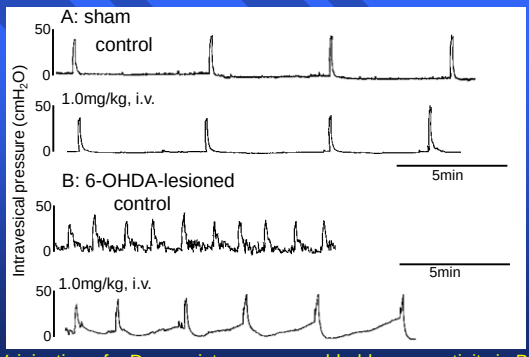
Direct disease models of human neurological disorders

- **Supraspinal level**
  - **Cerebrovascular disease-** Middle cerebral artery occlusion
  - **Parkinson's disease-** 6-OHDA /MPTP lesion of nigro-striatal pathways
- **Spinal level**
  - **Spinal cord injury-** Spinal cord transection or contusion
  - **Multiple sclerosis-** Experimental autoimmune encephalomyelitis (EAE)
- **Peripheral level**
  - **Diabetes mellitus-** Type 1 & Type 2 Diabetes

Parkinson's Disease (PD)

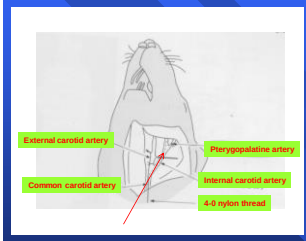
- Degeneration of dopamine (DA) neurons in the substantia nigra changes motor function (tremor, rigidity, akinesia), and also induces detrusor overactivity (50-70%)
- Bladder-sphincter coordination in PD is normal, but initiation of voiding is slowed
- Treatment with l-dopa reduces symptoms

Cystometry in sham and Parkinson's disease models-rat



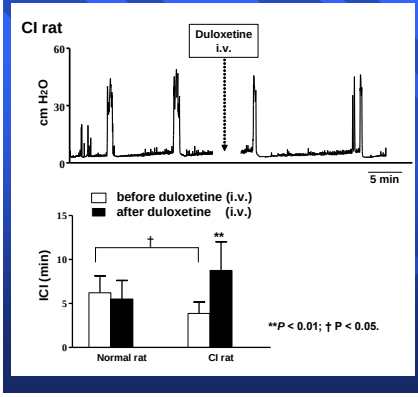
I.V. injection of a D_1 agonist suppresses bladder overactivity in B

Cerebral infarction model



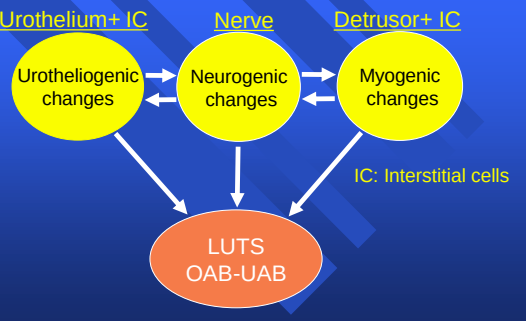
- A cerebral infarction (CI) model was created by occlusion of the left middle cerebral artery was using a 4-0 nylon
- Three days after creation of CI, evaluation was performed before and after duloxetine injection (1 mg/kg i.v.)

Continuous cystometry- CI rats



Duloxetine prolonged intercontraction intervals in CI rats, but not in normal rats.

Mechanisms inducing LUTS (OAB+UAB)



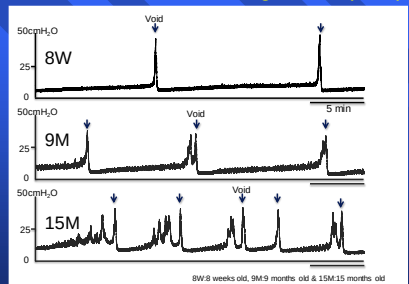
Animal models of LUTS/OAB

Disease models of various etiologic factors of OAB

- **Aging (OAB & UAB)**
  - Aged animals
- **Tissue ischemia/oxidative stress (OAB to UAB)**
  - Arterial balloon endothelial injury (AI) of the iliac arteries
  - Iliac vein ligation-pelvic congestion
  - Spontaneously hypertensive rats (SHR)
- **Afferent sensitization**
  - Intravesical chemical irritation (acute & chronic [~2 wks])
  - Spinal cord injury
- **Obesity**
  - High fat diet or transgenic
- **Bladder outlet obstruction (BOO)**
  - Partial urethral ligation (Model of BPH-associated LUTD)

LUTS pathophysiology- Aging

OAB+UAB in middle aged rats (15M)



Non-voiding contraction with reduced voiding contraction

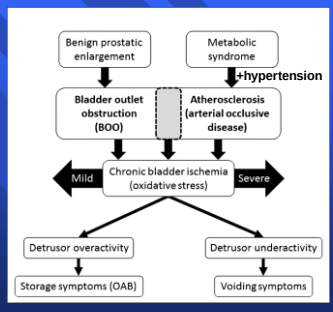
Mori et al, Neurourol Urodyn, 2016

LUTS pathophysiology

Ischemia➔Oxidative stress

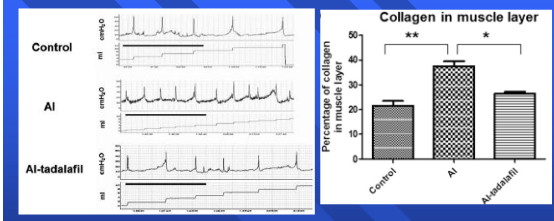
Male & Female
Atherosclerosis
Diabetes

Male
Bladder outlet obstruction (BPH)



Yamaguchi et al, Neurourol Urodyn, 2014

A rat model of chronic ischemia



- Arterial balloon endothelial injury (AI) of the iliac arteries with high-cholesterol diet induces bladder overactivity, which is reduced by tadalafil treatment
- Tadalafil treatment reduced AI-induced fibrosis (collagen) in the bladder

(Nomiya et al, J Urol, 2013)

LUTS/OAB pathophysiology

Afferent Sensitization

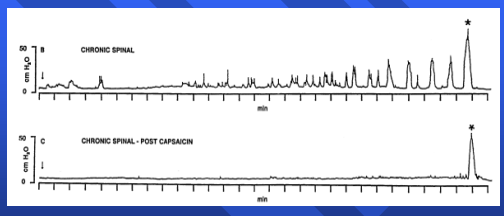
- Intradetrusor Botox treatment suppresses urgency in patients with idiopathic and neurogenic DO and reduces TRPV1 and P2X3-ir in bladder nerves (Apostlidis et al., 2005)
- Intravesical RTX treatment reduced LUTS in unobstructed patients with idiopathic DO (Silva et al., 2002)
- Neurokinin-1 receptor antagonists suppresses urgency and urgency incontinence episodes in OAB women (Green et al., 2006, 2009)

Commonly used animal models -Afferent sensitization-

- Acute chemical irritation of the bladder
  - Acetic acid
  - Capsaicin, RTX
  - Cyclophosphamide (CYP), etc.
- Chronic injury
  - Spinal cord injury
  - Bladder outlet obstruction? (More suitable for myogenic overactivity?)

Should be aware that they are not the model of OAB, but rather represent afferent hyperexcitability

SCI animals as a model of afferent hyperexcitability

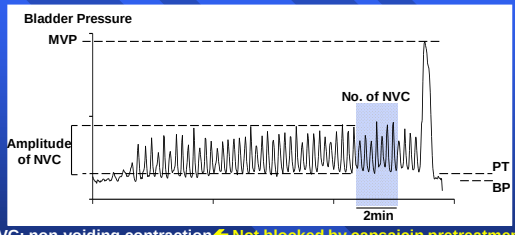


C-fiber desensitization by high-dose capsaicin suppresses bladder overactivity (=NVCs) in SCI

CL Cheng et al, Brain Res 1995

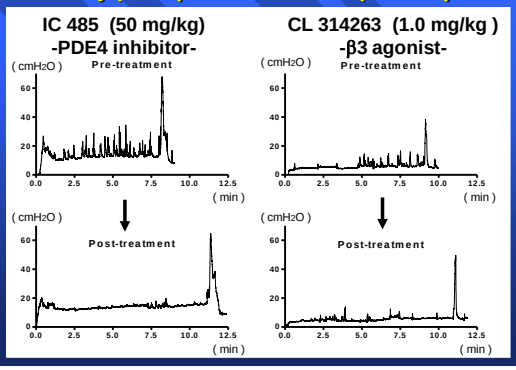
LUTS pathophysiology- BOO

A model of myogenic hyperactivity?

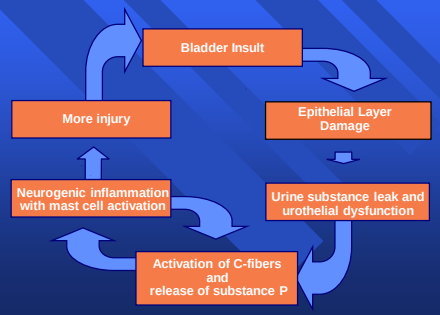


NVC: non-voiding contraction➔ Not blocked by capsaicin pretreatment
MVP: maximal voiding pressure
PT: pressure threshold for voiding
BP: baseline pressure

Effects of PDE4 inhibitor and β3-agonist on detrusor overactivity (NVCs) in rats with BOO (6 weeks)



Proposed pathogenesis of BPS/IC -Inside the Bladder-



Animal models of BPS/IC

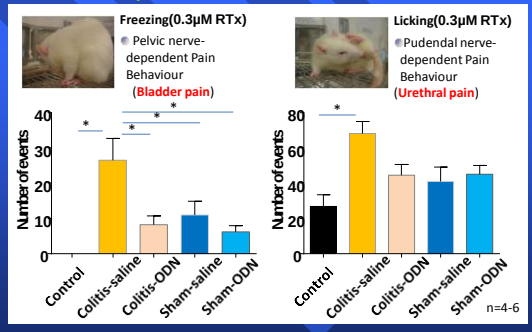
Direct model

- Feline IC
  - Closest model of human BPS/IC with limited availability

Indirect models

- Urothelial injury
  - Intravesical protamine sulfate
  - Intravesical synthetic antiproliferative factor (APF)
  - Uroplakin protein autoimmunity(immunization, transgenic)
- Inflammation
  - Chemical, neurogenic or bacterial (LPS) cystitis
- Organ cross sensitization
  - Experimental colitis, Pudendal nerve ligation
- Environmental stress
  - Water avoidance stress (WAS), Social stress

Bladder pain behavior in colitis rats



Colitis-induced increases in bladder pain behavior are suppressed by NGF antisense treatment (Kawamorita et al., J urol, 2016)

Stress Urinary Incontinence (SUI)

- **Involuntary release of urine during sudden increases of abdominal pressures without bladder contractions**

Problems in urethral closure mechanisms

Animal models of SUI- No direct models

Menopause

- *Deficiency of estrogen after menopause is one of risk factors of SUI*
- *Estrogen replacement therapy can be occasionally effective to patients with SUI in clinical studies*
  - ➔ **Rats with ovariectomy (OVX)**

Childbirth

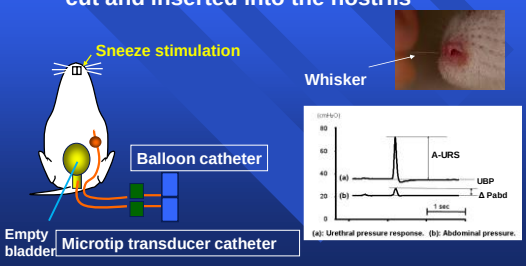
- *Vaginal childbirth induces the damage of pelvic floor nerve, muscle and connective tissues*
  - ➔ **Rats with simulated birth trauma induced by vaginal distention (VD)**

Aging

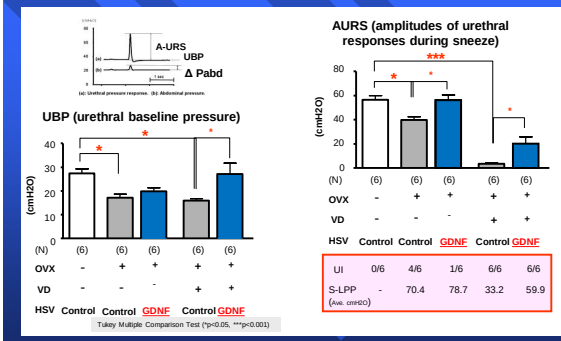
- *Urethral pressure decline with EUS apoptosis*
  - ➔**Aged animals**

A-URS and UBP

- Female rats were used
- Sneezes were induced by a rat's whisker cut and inserted into the nostrils



Effect of GDNF gene therapy on UBP & AURS



HSV-GDNF treatment enhanced sneeze-induced urethral continence reflex, resulting in the prevention of urinary leakage after OVX & VD

Preclinical Animal Models

- Animal models are essential for understanding the pathophysiology of human diseases and developing new and effective therapeutic modalities
- Animal models allow us to perform the study in controlled conditions (e.g., duration and/or severity of insults) and to utilize invasive experimental methods
- However, it is difficult to develop an animal model that fits all aspects of disease conditions

Preclinical Animal Models

- Need to understand the biochemical, physiological, and pathophysiological mechanisms, either validated or postulated, of the human diseases
- Need to understand which mechanism(s) are reproduced in each of animal models

Problems & Concerns

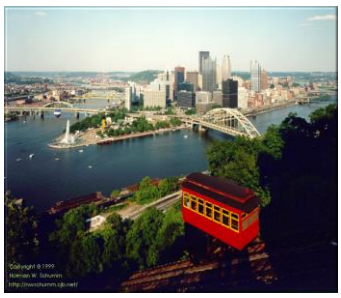
- Effects of anesthesia- Testing in awake condition?
- Species & strain differences (Bjorling et al., Am J Physiol, 2015)
  - Testing in multiple animal models?

Corresponding parameters of animal models for human objective/subjective symptoms

	Human	Animal model
OAB	Urgency	• Frequent urination (awake)??
	Urgency incontinence	• Voiding spot analysis??
	Detrusor overactivity	• Non-voiding contractions during storage phase
BPS/IC	Pelvic (bladder) pain	• Referred cutaneous hyperalgesia (von Frey testing) • Visceromotor response during bladder distention (anesthesia) • Pain behavior (licking/freezing)
SUI	Stress incontinence	• Sneeze test (urine leakage) • Electrical stimulation of abdominal wall (urine leakage)
	MUCP	• UBP (microtranducer catheter)
	Cough or abdominal LPP	• LPP/A-URS during sneeze or abdominal compression

Take home messages

- Animal models are essential for research of LUT dysfunction
- However, there is no animal model that represents all aspects of disease conditions (OAB/LUTS, SUI or pain)
- Therefore, it is important to understand which pathophysiological mechanism(s) are reproduced in each of animal models
- Finally, the basic research findings have to be validated in clinical trials



Thank You