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TITLE: Pannexin 1 Channel, a Novel Molecular Mediator and Potential Therapeutic Target for Interstitial Cystitis

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14. ABSTRACT

This project focuses on Interstitial Cystitis (IC) and mechanisms whereby stress and urothelial dysfunction lead to development of IC symptoms. The study premise is that stress disrupts the urothelial barrier function, which results in chronic infiltration of urinary K^+ into the bladder wall that generates an environment for abnormal Panx1 channel activation and expression that ultimately lead to IC and its symptoms by augmenting proinflammatory responses, intercellular signaling and stimulation of bladder sensory fibers. In this reporting period of the project, the new data obtained from studies with Panx1-null mice demonstrated that Panx1 absence reduced the stress-induced IC-like urinary symptoms observed in the animal model and precluded the symptoms persistence, which in wildtype mice is observed long after stress cessation. New data from *in vitro* studies demonstrated that exposure to urine-like K^+ levels induced significant urothelial ATP release that was greatly amplified when combined with mechanical stimulation (mimicking bladder distension) and was inhibited by pharmacological Panx1 blockade. These findings are in line with our overarching hypothesis of the key roles played by Panx1 channels and exposure to urinary K^+ in triggering the IC symptoms in the context of stress-induced disruption of the urothelial barrier.

15. SUBJECT TERMS
Interstitial Cystitis; stress-induced IC symptoms; urothelial dysfunction; ATP signaling; pannexin 1 (Panx1) channels.

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TABLE OF CONTENTS

1. INTRODUCTION:

This research project focuses on Interstitial Cystitis (IC), a chronic and debilitating bladder condition characterized by symptoms that include urinary urgency, frequency, and pelvic pain. The IC etiology is still unclear, but there is accumulating evidence that an increase in permeability of the bladder urothelium contributes to the onset and perpetuation of IC symptoms. Current treatment approaches focused on repairing the urothelial barrier are not always effective, indicating that the deleterious effects of a "leaky" urothelium may persist long after recovery of the barrier function. The studies proposed in this project aim at better understanding mechanisms triggering IC in the context of urothelial barrier dysfunction and identifying novel molecular targets to advance therapeutic approaches for IC patients.

Studies will test the overarching hypothesis that urothelial Pannexin 1 (Panx1) channels play a key role in events leading to bladder sensitization, micturition dysfunction and pelvic pain in IC by amplifying ATP signaling and activating the bladder inflammasome. Specifically, we propose that chronic diffusion of urinary K^+ through a leaky urothelium generates an environment within the bladder wall that results in abnormally high Panx1 channel activation and expression that augment pro-inflammatory responses, intercellular signaling and stimulation of bladder sensory fibers, which ultimately lead to IC and its characteristic urinary and pelvic pain symptoms. These studies are expected to significantly impact the field of basic and clinical research in IC by bringing forth new mechanisms and disclosing Panx1 channels as novel molecular mediators and potential therapeutic targets for IC.

2. KEYWORDS:

Interstitial cystitis (IC); IC/Bladder Pain Syndrome (IC/BPS); Chronic pelvic pain; Stress; Bladder urothelium; Pannexin 1; ATP signaling; Inflammasome.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This project has two major goals:

1 – Investigate the participation of pannexin 1 (Panx1) channels in mechanisms that lead to bladder sensitization, micturition dysfunction and pelvic pain in a stress-induced IC model with disruption of the urothelial barrier.

- Timeline: months 4 to 40 of the award.
- Percentage of completion: 50%

2 – Determine the potential of Panx1 as a novel therapeutic target for interstitial cystitis associated with urothelial barrier dysfunction.

- Timeline: months 13 to 42 of the award.
- Percentage of completion: 15%.

What was accomplished under these goals?

In Year 2 of the award, we continued to make significant progress towards achieving the goals and completing the tasks set for the studies proposed in Specific Aim 1 of this project.

Specific Aim 1 – *Investigate the participation of pannexin 1 (Panx1) channels in mechanisms that lead to bladder sensitization, micturition dysfunction and pelvic pain in a stress-induced IC model with disruption of the urothelial barrier.*

We focused on continuing the performance of the activities planned in Major Task 1 of the project's Statement of Work (SOW) and have successfully resumed the *in vitro* studies planned in Major Task 2 that were momentarily placed on hold, as discussed in Year 1 Report.

Major Task 1: *Characterize the time course of changes in Panx1 expression and function in the bladder of mice submitted to CIS and determine the extent to which these changes correlate with the course of development and the persistence of IC-like symptoms in this animal model*. (Timeline: months 4 – 40).

In this project, we are using a stress-induced animal model of IC. Stress is a factor that is not only clinically relevant but also of major relevance in modern society and particularly in the lives of military personnel and their families. Among the most well-established stress models, we are using the prolonged constant illumination stress (CIS) model that results in a leaky urothelium due to disruption of tight junctions and enhanced desquamation of superficial urothelial cells, similar to what has been observed in histological examination of bladder biopsies from IC patients. (Note: *Subtasks 1 and 2* completed in Year 1 - IACUC and ACURO approval obtained for the studies with this animal model; Year 1 Report).

Subtask 3 – Generate the CIS model and perform the assessment of bladder function in CIS and control mice.

In the CIS model, the mice are individually housed and maintained for 96 hours under constant illumination (regular housing illumination levels). Animals in the control group are kept under conventional illumination (12hrs day/night cycle). At the end of the 96hrs of CIS, the animals are returned to regular housing conditions (3 mice per cage, 12hrs day/night cycle).

The development of IC-like urinary symptoms in CIS mice is then assessed non-invasively using the voided stain on paper (VSOP) method to evaluate changes in voiding behavior.

In the VSOP method, the animals are housed individually in cages with a bottom grid and a filter paper beneath it, and with food and water *ad libitum*. Mice are allowed to acclimate to the VSOP chambers overnight and spontaneous voiding behavior is then continuously assessed for two days. Urine-stained spots are then examined under UV light, drawn on paper, counted, and voiding frequency calculated for both daytime (mice sleep phase) and night-time (mice active phase).

In Year 1 of the project, we started the longitudinal VSOP assessment of bladder function of wildtype (WT) female mice starting at baseline (before CIS initiation), then at the end of the CIS (96hrs CIS), and at 7-days and at 14-days after stress cessation. Bladders from these mice were then harvested at 21-days post-CIS to perform molecular analysis (see *Subtask 4*).

In Year 2 of the project, we continued with these studies and have now:

1) Completed the dataset and tissues collection from CIS and Control WT females at the 14-day post-CIS time point (3 rounds of independent experiments, each round = 3 CIS and 3 control mice; total = 18 mice).

2) Started and are currently running the last of the three rounds of VSOP experiments to complete the dataset for the WT females at the 7-day post-CIS time point.

3) Started the studies with the Panx1-deficient female mice (Panx1-null). We have already completed the first round of longitudinal VSOP assessment of bladder function of CIS and Control Panx1-null females up to 14-days post-CIS. Bladders from these mice were harvested at 21-days post-CIS and used for molecular analysis (see *Subtask 4*).

In Figure 1, we present and compare the VSOP data obtained from the studies with WT females (combined Year 1 and 2) and Panx1-null females (Year 2).

As shown in Figure 1A, exposure to 96hrs of CIS induced a significant increase in the diurnal urinary frequency of WT females that remained at levels higher than those of non-stressed WT controls, even after stress cessation, i.e., at 7-days and 14-days post-CIS.

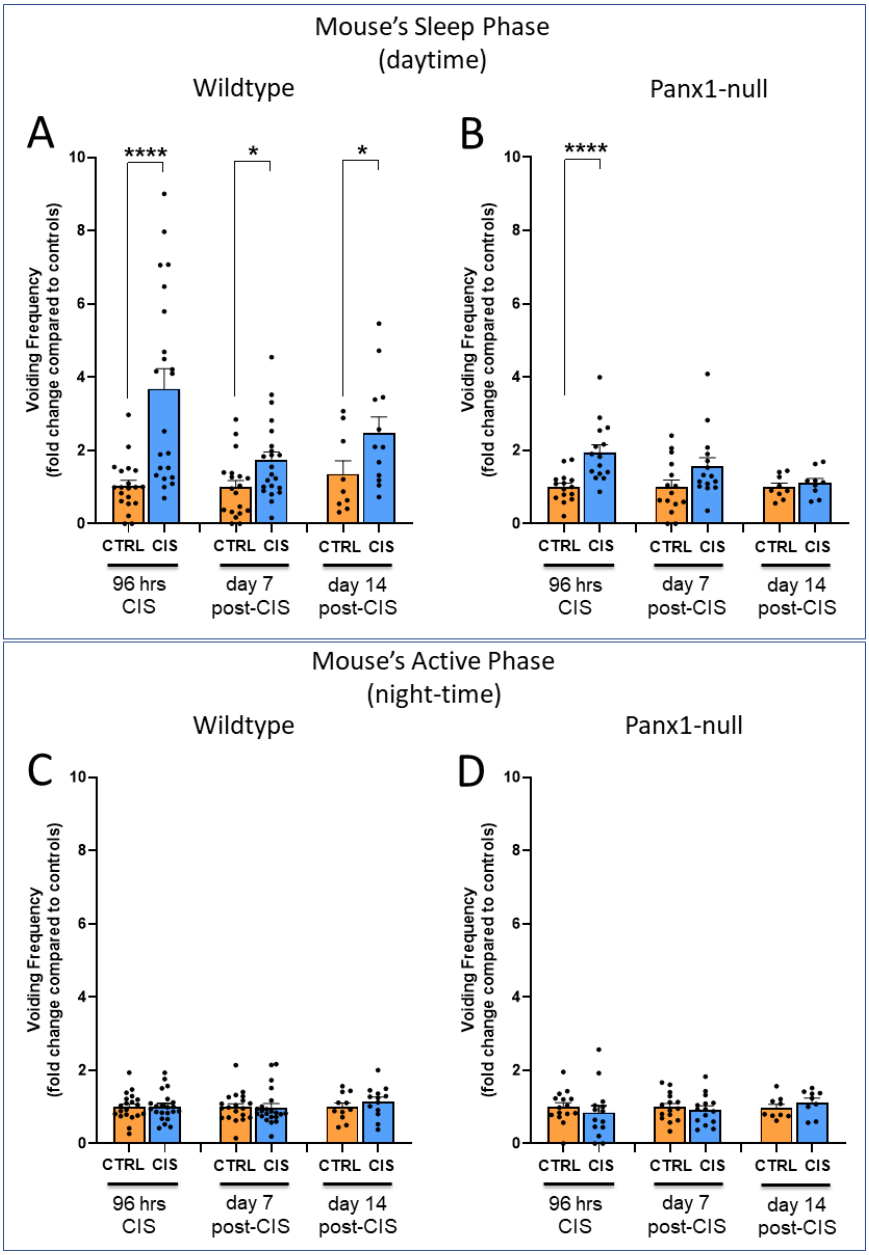

Figure 1 – *Exposure to 96hrs of constant illumination stress (CIS) triggers the development of characteristic IC-like symptoms of urinary frequency in wildtype and Panx1-null female mice that are evident during the animals' sleep phase, indicating the development of nocturia-like symptoms similar to observed in IC patients.* Voiding behavior was assessed at baseline and animals were then divided into CIS and control (CTRL) groups. The CIS mice were

then exposed to 96hrs of CIS and CTRL mice were kept under regular housing conditions (12hrs light/dark cycle). Changes in voiding behavior were then assessed continuously for 2 days at 96hrs-CIS, and then at 7- and 14-days after stress cessation. Voiding frequency was normalized by each baseline line value and then by control values at each time point. Data is represented as Mean±SEM, n = 9-22 WT mice and n = 9-15 Panx1-null mice per group and experimental time point. The Mann-Whitney test was used to determine statistical differences between CIS and CTRL at each time point (*$p<0.05$; ****$p<0.0001$).

Exposure to 96hrs of CIS stress also induced an increase in the diurnal urinary frequency of Panx1-null females (Fig. 1B), but to a level that was in average 2-fold lower than observed in WT females ($p<0.01$). In addition, this effect of stress on voiding function of Panx1-null females subsided soon after stress cessation, while it persisted in WT females for up to 14-days post-CIS.

It is also interesting to notice that similar to observed for WT females, effects of stress on voiding function of Panx1-null mice are observed during the animals' sleep phase (daytime) but not during their active phase (night-time) (Figs 1C and D). As we discussed in Year 1 report, this finding indicates that stress induces nocturia in the CIS animal model, which is also one of the cardinal symptoms observed in patients with IC. Our findings now show that absence of Panx1 ameliorates stress-induced symptoms of nocturia.

Overall, we are pleased that the VSOP studies are progressing as originally planned and we are excited with the new findings obtained from Panx1-null mice. Comparison of WT and Panx1-null data clearly indicates that absence of Panx1 expression not only attenuates the acute effect of stress on voiding function, but also "protects" against the long-lasting effects seeing after stress cessation. These findings support our overarching hypothesis by providing evidence that Panx1 channels participate in mechanisms of stress-induced urinary symptoms and may thereby provide a therapeutic target to manage IC symptoms.

Subtask 4: *Molecular analyses of the bladder tissues*.

In Year 1 of the project, following the longitudinal evaluation of bladder function at 96hrs of CIS and then at 7- and 14-days post-CIS, we euthanized the WT mice and harvested the bladder tissues at 21-days post-CIS. In Year 2, we generated new sets of CIS and CTRL animals and harvested the bladder tissues from WT females at 14-days post-CIS and from Panx1-null mice at 21-days post-CIS. The urothelium was separated from the underlying lamina propria and detrusor muscle under a dissecting microscope and processed to quantify expression levels of Panx1 channels and of other molecular targets.

As mentioned in Year 1 report, we observed in pilot studies that exposure to 96hrs of CIS induced a significant increase in Panx1 expression in the bladder mucosa at levels that were 2.5-fold higher than in non-stressed controls ($p<0.001$). Such upregulation of Panx1 is consistent with the observed significant increase in voiding frequency in CIS mice (see Fig. 1) and is in line with the proposed role played by this channel in the emergence of the IC-like symptoms in this animal model.

Data obtained in Year 1 from bladders harvested 21-days after stress cessation demonstrated that Panx1 protein expression in the urothelium of CIS mice was downregulated and reduced to levels that were significantly lower than those observed in non-stressed controls (Fig. 2A; day 21 data). As we discussed in Year 1 report, this finding was consistent with the progressive reduction on stress-induced IC-like urinary symptoms observed in the CIS mice after stress cessation. However, this level of Panx1 downregulation was somewhat at odds with the functional data, considering that at 14 days post-CIS the voiding frequency of CIS mice was reduced when compared to that observed at 96hrs of CIS, but remained higher than that of non-stressed controls ($p<0.05$). Based in this finding, we advanced the hypothesis that this apparent discrepancy could be explained by changes in expression levels of urothelial TRPV4 channels and P2X7 receptors.

As shown in Figure 2, at 21-days post-CIS the expression levels of P2X7R are not different, but TRPV4 levels are higher in the urothelium of CIS mice when compared to those in non-stressed controls. P2X7R and TRPV4, like Panx1, are components of the urothelial mechanotransduction system and play a key role in urothelial ATP signaling. Most importantly, they have been shown to also activate Panx1 channels. In this regard, an upregulated urothelial TRPV4 expression and activity could significantly contribute to enhance Panx1 activation and overall function of these channels and consequently result in the observed persistence of IC-like symptoms long after stress cessation.

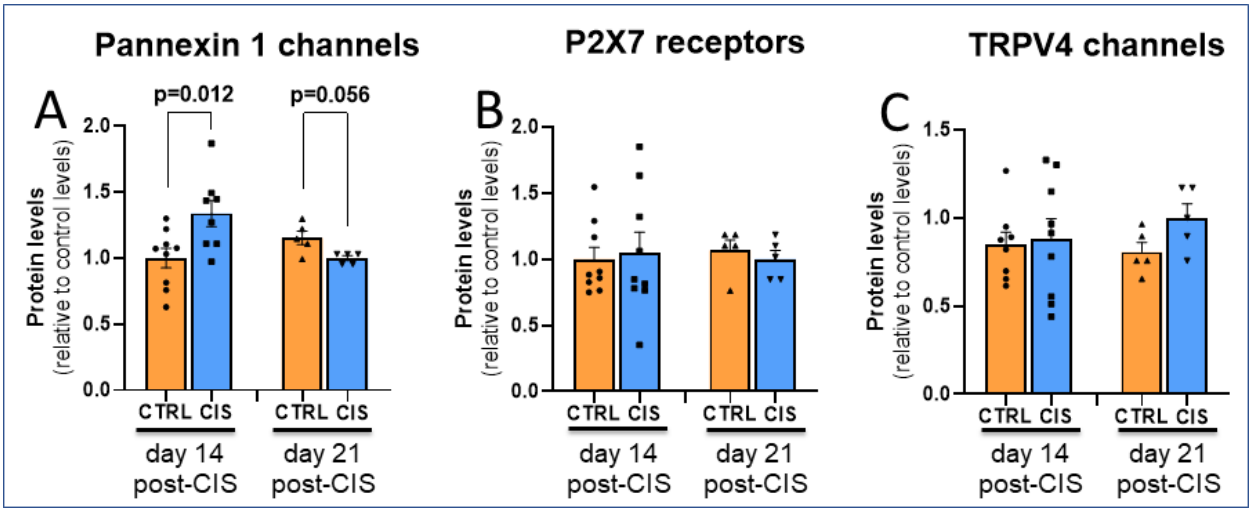

Figure 2 – Protein expression levels of Pannexin 1 channels (A), P2X7 receptors (B) and TRPV4 channels (C) in the bladder urothelium of WT control (CTRL) and CIS female mice at 14-days and 21-days after stress cessation. Protein levels normalized to loading control and expressed relative to average control levels. Data is represented as Mean±SEM, n = 5-9 mice per group. The Mann-Whitney test was used to determine statistical differences.

Data that we have now obtained in Year 2 from bladders harvested from WT females at 14-days post-CIS (Fig. 2; day 14 data) and from Panx1-null mice at 21-days post-CIS (Fig. 3) support this hypothesis of the proposed involvement of TRPV4 channels in stress-induced voiding dysfunction. As shown in Figure 2A, urothelial Panx1 expression is significantly upregulated in CIS mice at 14-days post-CIS, consistently with the higher voiding frequency observed in CIS when compared to control mice (Fig.1A). At this time point, both P2X7R (Fig. 2B) and TRPV4 (Fig. 2C) expression is not different between CIS and Control mice. However, from day 14 to day 21, when Panx1 levels transition from upregulated to downregulated, TRPV4 expression is upregulated. Interestingly, in Panx1-null mice at 21-days post-CIS, TRPV4 expression is also elevated and at levels significantly higher when compared to controls (Fig. 3B).

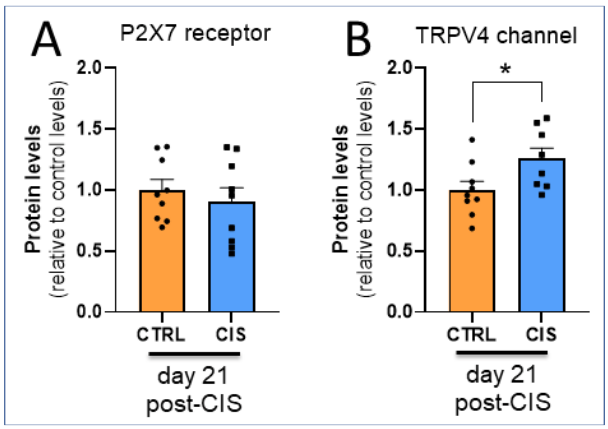

Figure 3 – Protein expression levels of P2X7 receptors (A) and TRPV4 channels (B) in the bladder urothelium of Panx1-null control (CTRL) and CIS female mice at 21-days after stress cessation. Protein levels normalized to loading control and expressed relative to average control levels. Data is represented as Mean±SEM, n= 8-9 mice per group. The Mann-Whitney test was used to determine statistical differences.

It remains to be determined if the time course and pattern of changes in TRPV4 expression in WT and Panx1-null from day 14 to day 21 are similar (we are currently running the 3rd and last

round of experiments to collect the tissues from Panx1-null mice at 14 days post-CIS). However, the fact that upregulation of TRVP4 in Panx1-null CIS mice did not concur with signs of post-CIS voiding dysfunction, as observed in WT CIS mice, indicates that TRPV4 channels are likely acting through their functional interplay with Panx1 channels.

Subtask 5: *In vivo bladder dye-uptake to assess changes in Panx1 function.*

The studies planned as part of the activities under this subtask will provide further insights into the potential Panx1-TRPV4 functional interplay and its role in the emergence and persistence of IC-like urinary symptoms in the CIS mouse model. We are currently processing the bladders and imaging Yo-Pro dye-uptake in the bladder sections obtained from CSI and controls at 21-days post-CSI. Findings will be correlated with those already obtained from the molecular analyses (see *Subtask 4*).

Subtask 6: *Assessment of urothelial ATP signaling.*

Activities in this subtask involve immediate collection and freezing of spontaneously voided urine from CIS and control mice at baseline and then at 96hrs of CIS, and at 7-, 14- and 21-days after stress cessation. ATP levels in the voided urine were then quantified using the luciferin-luciferase chemiluminescence assay. Quantification of voided ATP levels is routinely used as a non-invasive approach to assess changes in urothelial ATP release and indirectly evaluate changes in bladder sensory function. Urothelial ATP release is increased in animal models of bladder overactivity. ATP levels in the urine of IC patients has also been shown to be higher than those in healthy individuals, in line with the proposed central role that ATP signaling plays in the regulation of bladder sensation and detrusor contractility.

Data obtained in Year 1 of the project showed that ATP levels in the urine of WT female mice were significantly elevated at 96hrs of CIS and that this effect of stress on urothelial ATP signaling persisted at 7- and 14-days after stress cessation. In Year 2, we confirmed and expanded this finding by including data from urine collected from additional sets of animals and from animals before sacrifice, at 21-days post-CIS. As shown in Figure 4A, at 7-, 14- and 21-days post-CIS the ATP levels in the voided urine of WT CIS mice were 3-, 6- and 1.5-fold higher than those of non-stressed controls. We have now also obtained initial data of voided ATP levels from our first sets of Panx1-null CIS and control mice. As shown in Figure 4B, the ATP levels detected in the voided urine of Panx1-null CIS mice were not different from those of Panx1-null controls. This data indicates that Panx1 channels play a critical role in the stress-induced changes in urothelial ATP signaling.

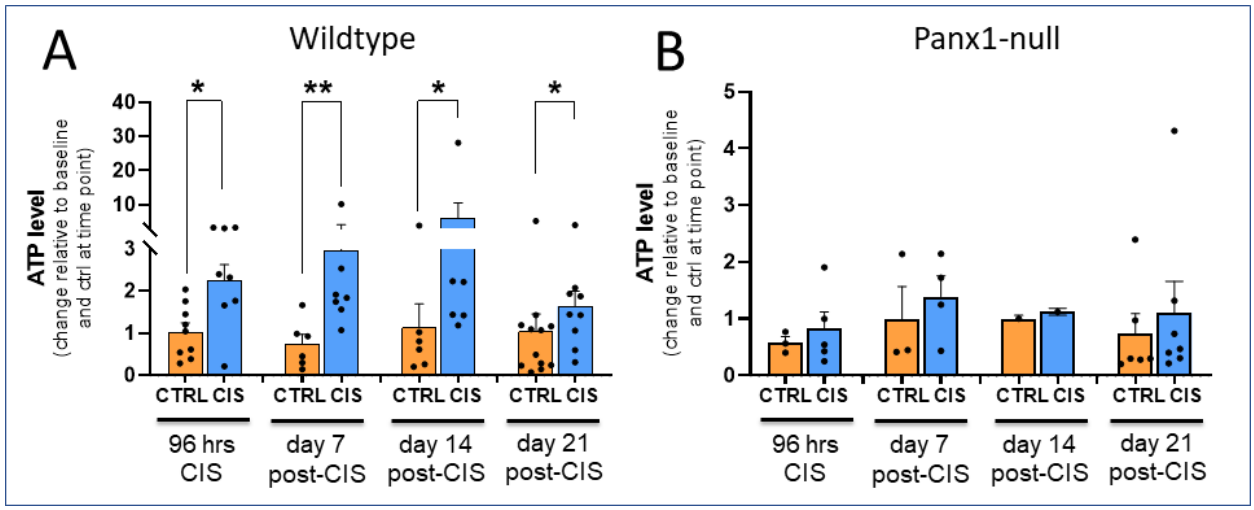

Figure 4 – *Exposure to 96hrs of constant illumination stress (CIS) induces a persistent increase in urothelium ATP release in wildtype female mice but not in Panx1-null female mice, which indicates that Panx1 expression is required for stress-induced amplification of urothelial mechanosensory responses and its persistence long after stress cessation.* Voided ATP levels were quantified at baseline and animals were then divided into CIS and control (CTRL) groups. The CIS mice were then exposed to 96hrs of continuous room illumination and CTRL mice were kept under regular housing conditions (12hrs light/dark cycle). Voided ATP levels were then quantified at 96hrs-CIS, and at 7- and 14- and 21-days after stress cessation and normalized to baseline levels and to respective controls at each time point. Data is represented as Mean±SEM, WT cohorts: n = 6-12 CTRL and n = 6-9 CIS. Panx1-null cohorts: n = 1-6 CTRL and n = 1-7. The Mann-Whitney test was used to determine statistical differences between CIS and CTRL at each time point.

Overall, findings obtained from both WT and Panx1-null support our proposal that stress alters the mechanosensory function of the bladder urothelium, and that dysregulation of Panx1 channels and consequent dysregulation of urothelial ATP release and signaling are among the main factors driving the higher activation and sensitization of the bladder sensory afferents, which ultimately lead to the development and persistence of IC-like urinary symptoms in the CIS mouse model.

Major Task 2: *Determine the role played by exposure of bladder tissues to high K^+ as the trigger of events leading to changes in Panx1 channel activation and expression, and downstream local inflammatory responses involving Panx1-mediated activation of the NLRP3 inflammasome.*
(Original Timeline: months 8 – 14).

Activities planned under this Major Task 2 of the SOW involve use of primary bladder cell cultures prepared from wildtype and Panx1-null mice (*Subtask 1*), and quantification of mRNA and protein levels of Panx1, inflammasome components and associated cytokines (*Subtask 2*). As detailed in Year 1 report, we started the experiments as scheduled but soon after we placed them on hold. The reason was to optimize our urothelial cell culture protocol by supplementing the media with a Rho Kinase 1 inhibitor (Y027632) that had been shown to markedly increase the long-term proliferation of human keratinocytes (PMID:20516646). We observed that treatment of freshly isolated urothelial cells with Y-27632 also supported the long-term proliferation of urothelial cells, particularly of basal and intermediate cells, which under regular culture conditions are rapidly lost. Most importantly, prolonged treatment with Y-27632 did not alter urothelial Panx1 expression, which would preclude its use in our studies. Moreover, no significant changes in cell morphology and viability nor on Panx1 expression were observed by removing Y-27632 from the media one week after plating the cells. This optimization of our protocol enabled us in Year 1 to successfully generate an hTERT-immortalized mouse urothelial cell line (mUC2-hTERT), which is something that we had been trying for years. Generation of these cells significantly benefited our research efforts, and we have since started using the mUC2 cells in the studies proposed in this project and in other ongoing studies in our lab.

In Year 2 of the project, we made significant progress in the performance of the *in vitro* studies planned to investigate the extent to which exposure to high extracellular K^+ triggers activation of Panx1 channels and ATP release from urothelial cells. These studies involved bathing the urothelial mU2C cells in high K^+ (10 mM) or control (2.5 mM) culture media in the absence or presence of mechanical stimulation to mimic the conditions experienced by these cells in the bladder during a normal voiding cycle and in the context of a disrupted or intact urothelial barrier.

We used two modalities of mechanical stimulation: a) laminar fluid flow shear (LFFS), imposed by placing the urothelial culture dishes in a rocker shaker for 5 min, and (b) cell membrane distension, imposed by exposure to a 25% or 50% hypotonic solution for 30 min.

Aliquots (50µL) of the cells bathing media were collected at baseline (before stimulation) and then at 5, 15 and 30 mins after mechanical stimulation or after the addition of high K^+ or hypotonic solutions. ATP levels were quantified using the luciferin-luciferase chemiluminescence assay.

As shown in Figure 5, exposure to both modalities of mechanical stimulation induced significant ATP release from urothelial cells, but with different kinetics. LFFS induced a fast ATP release with levels peaking immediately after stimulation (5 min), which then progressively declined after stimulation, mostly due to ATP metabolization by the ectonucleotidases. Exposure to 25% and to 50% hypotonic solutions induced a slower, progressive, and more sustained ATP release, which mirrors the slower and progressive cell swelling/distension induced by the hypotonic shock and concurrent activation of Panx1 and other mechanosensitive channels. Exposure to high K^+ also induced a slower and sustained urothelial ATP release that is also in line with a progressive activation of ATP release mechanisms. Noteworthy, the responses to mechanical stimulation and to high K^+ were amplified when these two stimuli were combined (Fig. 5A; LFSS+K^+ data), which is a condition observed in the context of urothelial barrier dysfunction, when cells are stimulated not only by urine flow and bladder distension, but also by high urinary K^+ that diffuses through the leaky urothelium.

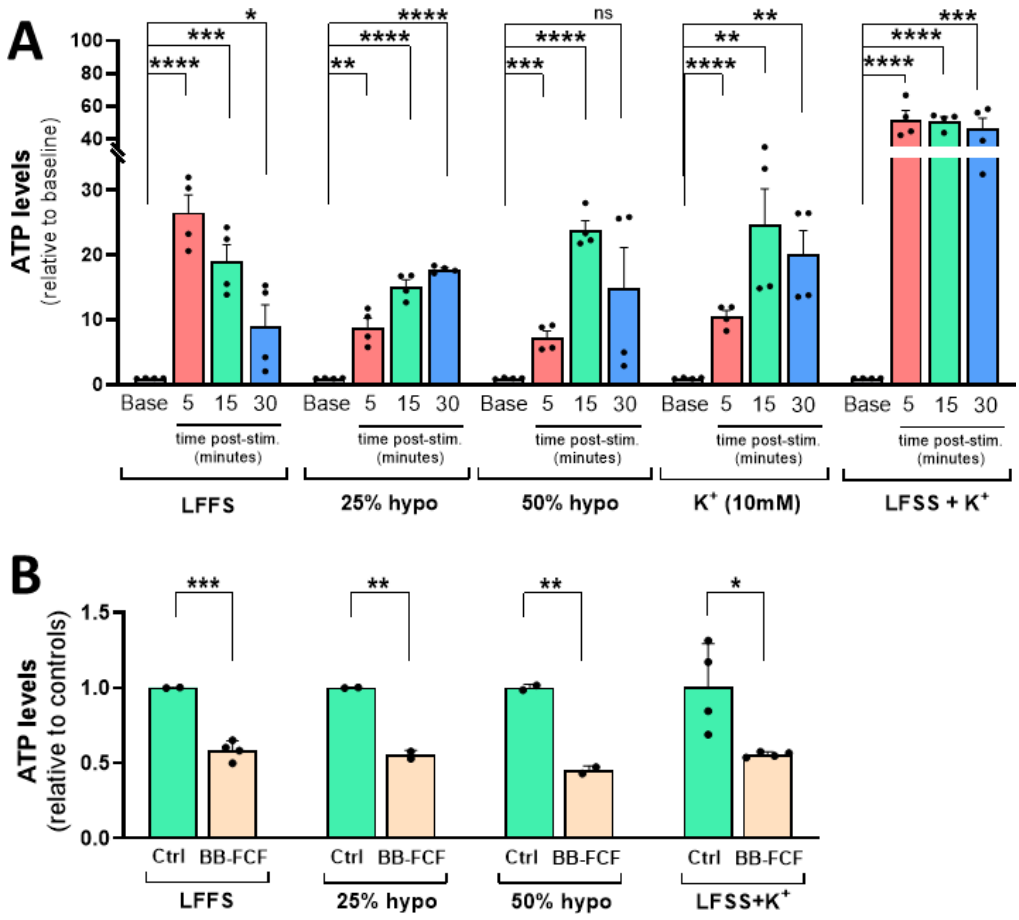

Figure 5 – (A) Mechanical stimulation and exposure to high extracellular K^+ induces ATP release from urothelial cells that is markedly amplified when cells are simultaneously exposed to both stimuli and (B) are blunted by pharmacological blockade of Panx1 channels with BB-FCF. Urothelial cells (mU2C cells) grown in 35 mm dishes were exposed to two modalities of mechanical stimulation [Laminar fluid flow shear (LFFS) or cell membrane distension induced by exposure to a 25% or 50% hypotonic solution], or were exposed to a high extracellular K^+ solution (10 mM) that mimics the K^+ levels found in normal urine, or to both LFFS and K^+. Studies were conducted in the absence (A) or in the presence (B) of the Panx1 channel blocker BB-FCF (10µM). ATP released amounts were quantified from the cells bathing solutions at baseline (before stimulation) and at 5-, 15- and 30-min after

stimulation. ATP levels are expressed relative to baseline levels (A) or also relative to control values at 15-min post-stimulation (B). Data correspond to Mean±SEM from 2 to 4 independent experiments per condition. Statistical differences were determined by Student's t-test: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001. Note: Statistical difference was not computed in (B) for 25% and 50% hypotonic control and BB-FCF groups as the n=2. We are currently increasing the number of experiments under these conditions.

To evaluate the extent to which Panx1 channels are involved in mechanisms of ATP release induced in response to high K^+, to mechanical stimulation and to combined high K^+ and mechanical stimulation, we pre-treated the urothelial cell cultures for 30 min with the Panx1 channel blocker BB-FCF (10μM) and conducted the experiments in the presence of this blocker.

As shown in Figure 5B, ATP release induced by LFFS, 25% and 50% hypotonic solution, and by combined LFFS+ K^+ was reduced by over 50% in the presence of BB-FCF when compared to controls. This finding demonstrates that Panx1 channels participate in these responses and suggests that an abnormal increase in Panx1 activation is one of the early factors driving the amplification of urothelial ATP release observed in the context of urothelial barrier disruption.

Overall, findings from these *in vitro* studies support our hypothesis that activation of urothelial Panx1 channels by urinary K^+ provide one of the mechanisms whereby breach of the urothelial barrier can significantly amplify urothelial ATP release and signaling and lead to emergence of urinary IC/BPS symptoms.

Specific Aim 2 – *Determine the potential of Panx1 as a novel therapeutic target for interstitial cystitis associated with urothelial barrier dysfunction.*

In Year 2 of the award, we started both the *in vivo* pharmacological and genetic manipulations planned in Major Tasks 1 and 2 of the project's SOW.

Major Task 1: *Pharmacological manipulation of Panx1channels.*
(Timeline: months 21 – 48).

Studies in this Major Task 1 aim at evaluating the efficacy of FDA approved Panx1 blockers (i.e., probenecid and BB-FCF) in ameliorating the stress-induced IC-like symptoms in CIS mice.

In Year 2 of the project, we focused on the activities planned in *Subtask 1*, which involve scale up/down pilot studies with WT mice submitted to 96hrs of CIS to determine the best dose range and regimen for intravesical (*Subtask 2*) and for systemic (*Subtask 3*) treatment with the Panx1 blockers probenecid and BB-FCF.

We started the studies with BB-FCF at a 5 mg/kg dose administered systemically by i.p. injection every other day for 5 days, starting the day after stress cessation. Treatment efficacy was evaluated by comparing the data obtained from VSOP assessment of voiding function of BB-FCF treated CIS and vehicle-treated CIS mice. At this treatment dose and regimen, we did not observe significant differences between the BB-FCF treated and vehicle-treated groups at 2, 4 or 6 days after treatment, when comparisons were made relative to their VSOP values at 96hrs of CIS.

We have now changed the treatment regimen from a single dose of 5 mg/mL every other day to daily and started testing the efficacy of every other day treatment with a higher dose of 50 mg/mL BB-FCF.

Major Task 2: *Bladder-targeted genetic manipulation of Panx1 channels.*
(Timeline: months 12 – 42).

Studies in this Major Task 2 aim at evaluating the efficacy of targeted urothelial Panx1 knockdown as an intravesical treatment to ameliorate stress-induced IC-like symptoms in CIS mice.

We originally proposed to use liposomes containing Panx1-siRNA in these studies, but recently came across another approach that also uses nanovesicles as delivery systems but in combination with CRISPR/Cas9 technology, which would result in a prolonged, more sustained Panx1 knockdown than observed with Panx1-siRNA.

In Year 2 of the Project, we focused on implementing this technology using the Rosa26 Cre-dependent Cas9 mouse (B6J.129(B6N)-Gt(ROSA)26Sortm1(CAG-cas9*,-EGFP)Fezh) and Cre-gesicles (Takara Bio USA, Inc.), which are cell-derived nanovesicles containing Cre-recombinase protein that allow for controlled Cas9 expression and genetic manipulations in a time and dose-dependent manner.

We completed the *in vitro* studies with primary urothelial cells prepared from Rosa26 mice to determine the required Cre-gesicles concentration and time course of Cas9 expression. As shown in Figure 6, Cas9 protein expression is detected as early as 8-12hrs after treatment with 10µM Cre-gesicles and remains at high levels up to 48hrs post-treatment.

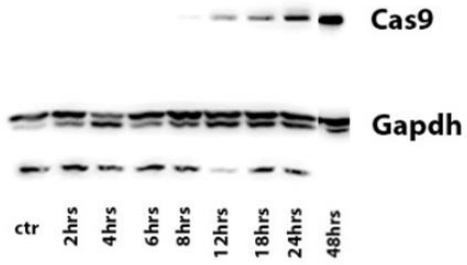

Figure 6 – *Representative western blot of Cas9 protein expression levels in untreated control (ctrl) and in Cre-gesicles treated primary urothelial cell cultures prepared from Rosa26 mice.* Cells grown in 12-well plates were treated with 10µM Cre-gesicles per well or with control vehicle-only (Optifect + protamine). Cells were then collected at 2, 4, 6, 8, 12, 18, 24 and 48hrs post-treatment and processed for western blot quantification of Cas9 and Gapdh (loading control) protein levels.

Based on this data, we started with 10µM gesicles in 100µL Optifect + protamine as the initial dose for intravesical treatment. The *in vivo* Cre-gesicles administration was conducted in Rosa26 female mice under anesthesia. The animals were kept anesthetized for a minimum of 1 hour to increase the time of residence of the nanovesicles in the bladder, as the animals void as soon as they wake up from anesthesia. Bladders were then harvested at 24hrs and 48hrs after treatment and the effectiveness of Cre-gesicles for *in vivo* CRISP/Cas9-mediated targeted manipulation of gene expression in the bladder urothelium was evaluated by quantifying and comparing Cas9 protein expression in the urothelium harvested from Rosa26 Cre-gesicles treated and vehicle (Optifec + protamine) treated mice. Unfortunately, we were unable to detect Cas9 expression in the urothelium of Cre-gesicles treated mice at 24 or 48hrs after treatment, which was rather surprising given the high level of expression that we observed in primary urothelial cells. One possible reason could be a low uptake of Cre-gesicles by the bladder urothelium, as an intact urothelial barrier is notoriously difficult to breach. However, protamine that is used with Optifect to prepare the Cre-gesicles solution should facilitate uptake by the cells, as seen in culture. We discussed this setback with colleagues and most recently, also with Dr. Jonathan Beckel, the leading author in studies that we cited in our grant proposal in which liposomes were used to deliver Panx1-siRNA into the bladder urothelium of rats. He suggested instilling the bladder with a 0.1% solution of *n*-Dodecyl-β-D-maltoside (DDM), a mild detergent, for 1 min before treating with the nanovesicles. This is a routine protocol in his lab but unfortunately, it is not mentioned in the methods section of the published paper that we mentioned above (PMID: 25630792).

Before starting a new round of experiments with Cre-gesicles treated Rosa26 mice, we first tested this change in protocol by instilling the bladders first with DDM or vehicle (saline), and then with Hoechst 33342 dye that stains the nuclei of living or fixed cells. As shown in Figure 7A-B, dye-uptake by the bladder urothelium and also its diffusion into the lamina propria (submucosa) are evident in the bladders pre-treated with the detergent.

In contrast, Hoechst-uptake is virtually absent in the untreated bladders and restricted to only a few umbrella cells that form the outermost layer of the urothelium that faces the bladder lumen (Fig. 7C-D). Based on this new finding, we have now included pre-treatment with DDM in our *in vivo* studies and are currently repeating the experiments to optimize the dosage for intravesical administration of Cre-gesicles for *in vivo* CRISP/Cas9-mediated targeted manipulation of urothelial Panx1 expression.

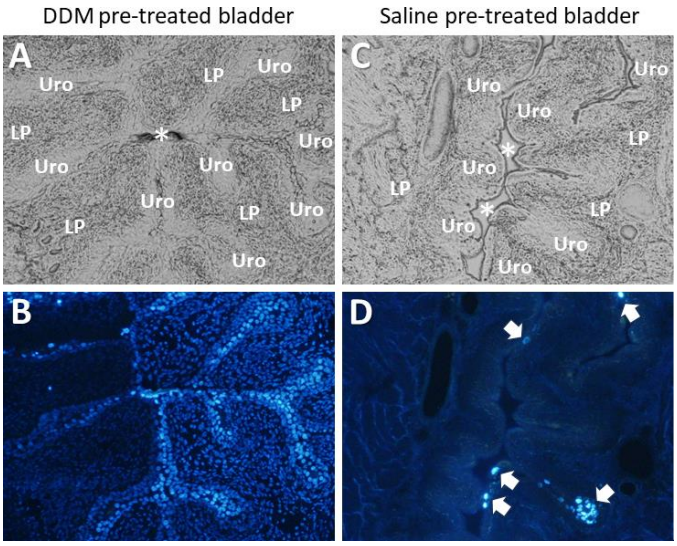

Figure 7 – *Phase contrast (panels A and C) and Hoechst staining (panels B and D) in bladder sections from Rosa26 mice treated with n-Dodecyl-β-D-maltoside (DDM; A and B) or vehicle-control (saline; C and D).* Rosa26 female mice were anesthetized, the bladders instilled with 1% DDM for 1 min, washed with saline and then instilled with Hoechst (0.03mM) for 30 min. Note abundant Hoechst nuclear staining in the urothelial layer (Uro) folds of DDM treated bladder (B) that extends into the underlying lamina propria (LP) /submucosa, whereas only a few of the outermost cells in urothelial layer (umbrella cells) are stained in saline pre-treated mice (D, white arrows) (* = bladder lumen).

What opportunities for training and professional development has the project provided?

This project was not originally intended to provide training and professional development opportunities. However, with the start of the project, these opportunities came from the role that Dr. Suadicani (PI) plays as the basic science mentor for fellows in the Female Pelvic Medicine and Reconstructive Surgery (FPMRS) fellowship program of the OBGYN Department at Einstein and Montefiore. In this 3-year program, the fellows have protected time to conduct basic and translational research as part of their thesis and requirement for their specialty certification. While discussing ideas for new projects that the fellows could conduct in the lab and talking about ongoing projects, Dr. Whitney Clearwater demonstrated a deep interest for this project, as it directly aligned with her clinical interests. She has since been working closely with Dr. Suadicani in this project, specifically on the activities involving the assessment of changes in voiding function and time course of IC-like urinary symptoms in the CIS model. This opportunity has allowed Dr. Clearwater to look at IC from a basic science perspective, learn the use of pre-clinical models and functional assessments, and to gain a better understanding of IC pathophysiology. Her participation in the project has also been beneficial to the research team by bringing a clinical perspective to our discussions. In Year 2 of this project, Dr. Clearwater's participation in the studies has once again been recognized by her peers and continues to further her professional development through presentation of our new findings in the Society of Urodynamics, Female Pelvic Medicine & Urogenital Reconstruction (SUFU, March 2023). Of note, our study was selected once again for a podium presentation at SUFU, as one of the top 10 basic science abstracts.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In Year 3 of the award, we will continue to work diligently towards accomplishing the goals of this project. Specifically, for the studies planned in:

Specific Aim 1:

Major Task 1, we will generate new cohorts of WT and Panx1-null female CIS and non-stressed controls to continue with the functional assessments and characterization of the time course of IC-like symptoms in the CIS mice (*Subtask 3*), the bladder tissue collections at the various experimental time points for molecular analyses (*Subtask 4*), and the *in vivo* assessments of Panx1 function (*Subtask 5*) and urothelial ATP release (*Subtask 6*).

Major Task 2, we will proceed with the planned *in vitro* studies using the mUC2 urothelial cells and will also run experiments in parallel with primary urothelial cell cultures prepared from WT and Panx1-null mice to demonstrate reproducibility of findings from this new urothelial cell line. We will specifically focus on completing the dataset for studies evaluating the effect of high K^+ on Panx1 activation, will quantify K^+-induced changes in expression of Panx1 and other molecular mediators (i.e., TRPV4 and P2X7R) and evaluate the extent to which exposure to high K^+ activates the bladder inflammasome.

Specific Aim 2:

Major Task 1 – *Pharmacological inhibition of Panx1 channels.* We will continue and complete the planned scale up/down pilot studies with WT female mice submitted to 96hrs of CIS to determine best dose range for intravesical and i.p. treatments with the Panx1 blockers probenecid and BB-FCF (*Subtask 1*). We will start the planned intravesical (*Subtask 2*) and systemic (*Subtask 3*) treatments with Panx1 blockers.

Major Task 2 – *Bladder targeted Panx1 knockdown.* We will complete the optimization of intravesical delivery of nanovesicles for target modulation of urothelial Panx1 expression (*Subtask 1*) and conduct the scale up/down pilot studies to determine the best concentration and exposure time for intravesical delivery (*Subtask 2*). Based on this information, we will then start the planned intravesical treatment regimen and functional assessments to evaluate the extent to which targeted urothelial Panx1 knockdown ameliorates the IC-like symptoms in the CIS animal model.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Findings obtained from studies conducted with the animal model of constant illumination stress (CIS) reemphasized the importance of stress in the etiology and exacerbation of IC symptoms. They also provided support for the premise that dysregulation of the urothelium mechanosensory function plays a central role in the emergence and persistence of IC symptoms. In addition, by further demonstrating that in the CIS model the animals develop symptoms that closely resemble those of IC patients, this project is now not only validating the use of this model by others in future mechanistic and pre-clinical studies but is also providing the field with a model that is simple and does not require specialized equipment to be generated. The optimization that we made in the protocol that is broadly used in the field to culture primary urothelial cells and the generation of the hTERT-immortalized mouse urothelial cells are also expected to be beneficial to the scientific community as they will significantly facilitate the performance of *in vitro* studies that are essential to gain mechanistic understanding of urothelial function and dysfunction. Overall, the additional *in vivo*

findings with the CIS animal model and *in vitro* studies with the generated mU2 urothelial cell line presented in this report further the relevance of this project that continues to make a notable impact in the field in terms of improving both our basic knowledge of factors and mechanisms in IC etiology and the available experimental tools to study IC.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications

Nothing to Report.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Clearwater, W., Wang, Y., Urban-Maldonado, M., Suadicani, S.O. *"*PANNEXIN 1 PLAYS AN ESSENTIAL ROLE IN STRESS-INDUCED CHANGES IN MOUSE VOIDING BEHAVIOR*"*. SUFU 2023 Winter Meeting, March 7 – 11, 2023, Nashville, TN. Poster #4, Podium Presentation in the Top 10 Basic Science Abstract Presentations session. *Neurourology and Urodynamics*, Volume 42, Issue S1. Supplement: SUFU 2023 Abstracts Issue, February 2023, pages S9-S10.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sylvia Suadicani
Project Role:	Principal Investigator (PI)
Researcher Identifier (ORCID ID):	0000-0001-6811-4029
Nearest person month worked:	5
Contribution to Project:	Dr. Suadicani directed the project, supervised the activities of all the involved investigators, participated in the generation of the animal model and performance of functional assessment of bladder function and tissue harvesting, reviewed the data analyses, headed the discussions and interpretation of findings from all the experiments conducted during this report period.
Name:	Whitney Clearwater
Project Role:	Fellow
Researcher Identifier (ORCID ID):	0000-0002-1578-1853
Nearest person month worked:	1.5
Contribution to Project:	Dr. Clearwater worked closed with Dr. Suadicani in the generation of the animal model and performance of functional assessment of bladder function, data analysis, interpretation, and presentation of findings from the *in vivo* studies conducted during this report period.
Funding Support:	Female Pelvic Medicine and Reconstructive Surgery Fellowship Program (FPMRS) of the OBGYN Department at the Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.
Name:	Marcia Maldonado
Project Role:	Senior Research Technician
Researcher Identifier (ORCID ID):	0000-0001-8791-0880
Nearest person month worked:	12
Contribution to Project:	Mrs. Maldonado conducted the *in vitro* studies with urothelial cell cultures, prepared the cultures, assisted in tissue harvesting and molecular studies, ordered supplies and animals, conducted the husbandry and maintenance of the mouse colonies and of the animal model.

Name:	Mia M. Thi
Project Role:	co-Investigator
Researcher Identifier (ORCID ID):	0000-0001-6157-5842
Nearest person month worked:	2.5
Contribution to Project:	Dr. Thi assisted in the supervision, performance, data analysis and interpretation of findings from the urothelial ATP release and signaling studies.

Name:	Kelvin P. Davies
Project Role:	co-Investigator
Researcher Identifier (ORCID ID):	
Nearest person month worked:	1.5
Contribution to Project:	Dr. Davies assisted in the supervision, data analysis and interpretation of the molecular studies conducted with bladder tissues.

Name:	Yi Wang
Project Role:	Research Associate/Instructor
Researcher Identifier (ORCID ID):	0000-0001-6623-3905
Nearest person month worked:	10
Contribution to Project:	Dr. Wang worked closely with Drs. Suadicani and Clearwater on the functional assessment of bladder function of CIS and control mice, assisted with tissue harvesting, and assisted in the performance of the molecular studies and data analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: Attached are two pages of the Supplement Issue of the *Neurology and Urodynamics*, Volume 42, Issue S1, February 2023 with our published abstract (pages S9-S10) that was presented at the *Top 10 Basic Science Abstract Presentations* session at the Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction (SUFU).

- Clearwater, W., Wang, Y., Urban-Maldonado, M., Suadicani, S.O. "PANNEXIN 1 PLAYS AN ESSENTIAL ROLE IN STRESS-INDUCED CHANGES IN MOUSE VOIDING BEHAVIOR". *Neurourology and Urodynamics,* Volume 42, Issue S1. Supplement: SUFU 2023 Abstracts Issue, February 2023, pages S9-S10.

Conclusions: We demonstrate chimeric BoNT/A-B has a faster onset and great potency at lower doses in smooth muscle ex vivo. This novel toxin may provide improved neuromuscular paralysis smooth muscle disorders, such as overactive and neurogenic bladder.

Funding: H.T. is supported by the NIH T32 Grant, AUA Urology Care Foundation Research Scholar Award, and the SUFU Chemodenervation Grant, and the Office of Faculty Development at Harvard Medical School. M.D. is supported by grants from the NIH, and holds the Investigator in the Pathogenesis of Infectious Disease award from the Burroughs Wellcome Fund. M.P.S. received support from the U.S. Department of Veterans Affairs.

BS#4 | PANNEXIN 1 PLAYS AN ESSENTIAL ROLE IN STRESS-INDUCED CHANGES IN MOUSE VOIDING BEHAVIOR

Whitney Clearwater[2], Yi Wang[1], Marcia Urban-Maldonado[1], Sylvia Suadicani[1]

[1]Albert Einstein College of Medicine, [2]Montefiore Medical Center

Presented By: Whitney Clearwater, MD, MPH

Introduction: In previous studies, we demonstrated that stress induced by prolonged exposure to constant illumination (CI) changes the mouse voiding behavior, resulting in increased urinary frequency that persists after stress cessation. We have now investigated the potential

mechanisms underlying this effect of CI stress on bladder function. Pannexin 1 (Panx1) channels mediate urothelial mechanosensory responses and play a key role in bladder sensation. The goal of this study was to determine the effect of CIS on urothelial Panx1 expression and the role of Panx1 on CIS-induced bladder dysfunction.

Methods: Wildtype (WT) and Panx1-knockout (Panx1-KO) female mice (20-weeks old) were randomly assigned to control (submitted to conventional 12 h/day room illumination) or CIS (96 hrs of constant room illumination) groups. Voiding behavior was assessed during the daytime using the voided stain of paper (VSOP) method. VSOP was conducted at baseline (pre-CIS), at the end of the 96 hrs-CIS and at 7 and 14 days after CIS cessation. VSOP data was expressed relative to pre-CIS levels and to non-stressed controls. Urothelial Panx1 expression was quantified by real time qPCR. Statistical differences were determined at $p \leq 0.05$ level using the Mann-Whitney test.

Results: Exposure to 96hrs-CIS induced a 4.0-fold increase in urinary frequency of WT-CIS mice when compared to non-stressed WT controls (4.11 ± 0.68-fold; $n = 15$; $p \leq 0.001$). A 7- and 14-days post-CIS, the urinary frequency of WT-CIS remained 2.0-fold higher than WT controls (D7: 1.95 ± 0.28-fold, $p \leq 0.05$; D14: 2.29 ± 0.44-fold, $p = 0.14$). CIS induced a 2.5-fold increase in urothelial Panx1 expression at 96hr (2.53 ± 0.29-fold; $N = 4$; $p \leq 0.001$). In Panx1KO mice, urinary frequency was only increased at 96hr-CIS (1.90 ± 0.31-fold; $n = 9$; $p = 0.04$). No differences were observed at 7- and 14-days when comparing CIS and control Panx1KO mice (D7: 1.25 ± 0.17-fold; $p = 0.39$; D14: 1.11 ± 0.13; $p = 0.55$). Remarkably, the early and only effect of CIS on Panx1KO mice at 96hrs-CIS was 50% less than was observed in WT mice.

Conclusions: Exposure to constant illumination stress causes persistent changes in voiding function that involves upregulation of urothelial Panx1 and is blunted in Panx1 deficient mice. These findings suggest that CIS alters bladder sensation and that Panx1 plays an essential role in mechanisms underlying the effects of stress on urinary function.

Funding: DoD-CDMRP W81XWH-21-1-0465

BS#5 | TOLL-LIKE RECEPTOR-4 EXPRESSED IN BLADDER SENSORY NEURONS MEDIATES SENSORY SYMPTOMS DURING UTI

Nicolas Montalbetti[4], Marianela Dalghi[1], Sheldon Bastacky[2], Gerard Apodaca[3], Marcelo Carattino[3]

[1]Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, [2]Department of Pathology, University of Pittsburgh, [3]Renal-Electrolyte Division, Department of Medicine, and Department of Cell Biology, University of Pittsburgh, [4]Renal-Electrolyte Division, University of Pittsburgh

Presented By: Nicolas Montalbetti, PhD

Introduction: Urinary tract infections (UTI) are among the most common bacterial infections and the second cause of antibiotic prescription worldwide. Sensory symptoms including urinary frequency, urgency, and pelvic pain are hallmarks of UTI, suggesting that bladder afferents are activated during bacterial cystitis. Yet, there is limited understanding of how bacterial infections cause sensory symptoms. The aim of the present work is to evaluate the role of toll-like receptor-4 (TLR4) in the development of irritative voiding symptoms and pelvic pain during UTI.

Methods: Female mice were inoculated intravesical with uropathogenic E. coli (UPEC) strain UTI89. Histopathological analysis was conducted to characterize the inflammatory process. Somatic sensitivity in the pelvic area was determined with von Frey filaments. Voiding behavior was evaluated in conscious freely moving mice with a video-monitored void-spot assay. Electrical properties of bladder sensory neurons were evaluated with the patch-clamp technique.

Results: Inoculation of mouse bladders with UTI89 caused pelvic allodynia, increased voiding frequency, and prompted an acute inflammatory process marked by edema and leukocytic infiltration of the bladder wall. These effects were correlated with an increase in the excitability (i.e. sensitization) of bladder sensory neurons of both Adelta- and C-fiber origin. Incubation of isolated bladder sensory neurons in vitro for 24 h with 10 μg/mL of ultrapure lipopolysaccharide (LPS), a TLR4 agonist, resulted in the sensitization of bladder sensory neurons with C-fibers, but not those with Adelta. The excitability of bladder Adelta-afferents were only affected when ultrapure LPS was combined with other UPEC-derived virulence factors. We used mutant mice lacking Tlr4 gene specifically in DRG neurons to evaluate the role of this receptor in the development of sensory symptoms during UTI. Gene expression analysis revealed that both peptidergic and non- peptidergic bladder sensory neurons express Tlr4. While bladder inflammation and tissue bacterial loads were similar between mutant mice and control littermates, mice lacking Tlr4 specifically from sensory neurons exhibit attenuated sensory symptoms during UTI.

Conclusions: Activation of TLR4 expressed in bladder afferent neurons plays a key role in the development of sensory symptoms associated with UTI.

Funding: This work was supported by the Urology Care Foundation Research Scholar Award Program and the Samuel and Emma Winters Foundation (to NM), NIDDKR01DK119183 (to MDCand GA), and by the Physiology and Model Systems Core of the Pittsburgh Center for Kidney Research Grant P30DK079307.