THE RECEPTOR P75NTR IN THE ONSET OF INTERSTITIAL CYSTITIS/PAINFUL BLADDER SYNDROME

Benjamin Désormeau
Department of Experimental Surgery
McGill University, Montreal

April 2021

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science

Abstract

Recurrent bacterial cystitis by the uropathogen *E. coli* is a contributing factor to the progression of Interstitial Cystitis/Painful Bladder Syndrome (IC/PBS) – a chronic inflammatory disorder of the bladder without clear etiology or treatment. The symptomatology of these conditions is similar to one another, ranging from severe pelvic pain, increased urinary frequency, urgency, and nocturia, which suggests identical mechanisms might be engaged. At the molecular scale, the receptor TLR4 of *E. coli* lipopolysaccharide (LPS) display higher inflammatory responses in IC/PBS patients. Also, diabetic and spinal-cord injured bladder were found to feature higher activity of the pro-nerve growth factor (proNGF) receptor, p75NTR. In the body, p75NTR and TLR4 both participate in inflammation processes leading to bladder dysfunction. However, the blockage of p75NTR by a small-molecule specific antagonist, THX-B, has shown to restore normal bladder weight and voiding function in type 1 diabetic mice. Most importantly, the anti-inflammatory potency of p75NTR antagonism was demonstrated in the brain of mice with severe sepsis, supporting a molecular interplay between the p75NTR and TLR4 signaling axes in innerved tissues. Therefore, we here hypothesized that the onset of IC/PBS is shaped by a similar interaction in the bladder and be reduced by p75NTR antagonism. To validate that, this study was performed in two parts:

(1) In our first work, the contribution of p75NTR in bladder cell response to TLR4 activity was assessed *in vitro*. Following THX-B treatment, primary urothelial and detrusor smooth muscle cells cultures were either exposed or not to LPS. Data suggests that p75NTR and TLR4 signaling axes form together cell-specific interplays in the bladder. In urothelial cells, TNF-α expression by LPS was blocked with THX-B. The expression of ZO-1 and occludin remained steady with THX-

B, while activation of ERK signaling by LPS was considerably higher with THX-B. Conversely, smooth muscle cells did not display any variation in ERK activation with THX-B but had significantly lower TLR4/TRAF6/NF-κB/JNK signaling activity.

(2) Secondly, the role of p75NTR on bladder pathophysiology was assessed *in vivo* by testing variations in voiding function, morphology and pelvic skin sensitivity after TLR4-induced cystitis. Mice instilled with LPS were given either saline or intraperitoneal injection of THX-B. Our data support previous findings that p75NTR dysregulates bladder morphology and voiding dysfunction, especially by interplaying with TLR4 signaling in infection. Here, p75NTR antagonism decreased phosphorylation of JNK by LPS and prevented an increase in bladder thickness. There was no change in detrusor muscle strips contractility observed to KCl, carbachol or EFS with THX-B, and the EC50 and maximal contractile force values remained steady.

Résumé

La cystite interstitielle (CI), également appelée syndrome de la vessie douloureuse, est une condition inflammatoire chronique de la vessie dont l'étiologie et le traitement demeurent inconnus. Elle ressemble à la cystite bactérienne causée par l'uropathogène *E. coli* et inclut des symptoms de douleur pelvienne, une fréquence de miction excessive, des envies urgentes d'uriner et de la nycturie. Des données suggèrent que l'inflammation systémique causée par le récepteur TLR4 de lipopolysaccharide (LPS) d'*E. coli* est plus sévère chez les patients atteints de CI. Il est aussi démontré que le récepteur du pro-facteur de croissance nerveuse, p75NTR, devient suractivé chez les patients diabétiques ou présentant une lésion de la moelle épinière. Dans le corps, p75NTR est impliqué dans les processus d'inflammation et le développement de troubles urinaires. Cependant, son blocage par un antagoniste spécifique, THX-B, semble pouvoir renverser les effets du diabète de type I sur les fonctions urinaires et le poids de la vessie de souris. Une étude a également révélé le role inhibiteur de THX-B sur la réponse inflammatoire de TLR4 dans l'encéphale de souris avec une sepsis. Ceci suggère que p75NTR et TLR4 peuvent interagir ensemble par des mécanismes identiques. Enc e sens, nous suggérons que l'activation de p75NTR par proNGF peut contribuer à la progression de symptômes de CI et que leur développement peut être ralenti par un antagoniste de p75NTR. Pour verifier cela, l'étude a été réalisée en deux parties:

(1) Nous avons d'abord étudié *in vitro* le rôle de p75NTR dans l'activation de la réponse inflammatoire des cellules de la vessie. Les mécanismes moléculaires et cellulaires des voies de signalisation de TLR4 et de p75NTR ont été étudiés après traitement avec l'antagoniste de p75NTR. Nous avons stimulé in vitro des cellules urothéliales et musculaires lisses du muscle

détrusor avec du LPS en présence ou non de THX-B. Les résultats obtenus démontrent que les voies de signalisation de TLR4 et p75NTR interagissent d'une manière spécifique à chaque type de cellules. L'antagoniste affecte la signalisation de l'antagoniste de p75NTR and neutralisant l'axe inflammatoire TRAF6/NF-κB/JNK commun aux deux récepteurs dans les cellules musculaires lisses. De plus, elle inhibe la production de la cytokine TNF-α par les cellules urothéliales exposées au LPS. L'expression des protéines des jonctions serrées (occludin et ZO-1) est elle aussi partiellement restaurée.

(2) Deuxièmement, nous avons testé *in vivo* la contribution de p75NTR sur la sensibilité pelvienne à la douleur, sur l'activité contractile de la vessie et les changements de sa morphologie. THX-B a été injecté dans le péritoine abdominale d'un modèle murin de cystite aiguë induit avec du LPS par cathéterisation transuréthrale. L'étude démontre que p75NTR et TLR4 interagissent ensemble dans les fonctions de la vessie. L'activation de la signalisation de JNK par le LPS dans les tissus est diminuée avec l'antagoniste de p75NTR. D'autre part, les stimulations du muscle détrusor avec du KCl ou du carbachol sont identiques entre les différents groupes. Cependant, la contraction du muscle détrusor avec l'EFS est diminuée chez les souris saines injectées avec THX-B seulement, mais est similaire avec l'injection de LPS, avec ou sans THX-B. Enfin, l'injection de l'antagoniste p75NTR prévient l'épaississement de la couche muqueuse par le LPS

Acknowledgements

I owe so much thanks and respect to my thesis supervisor Dr. Lysanne Campeau, the patience and expertise of whom I will always be grateful for. Doing research in a global pandemic has brought considerable strain on the workflow, the instability of which also had me thinking of dropping out of the degree many times. Yet, Dr. Campeau's resilience from being a physician-scientist, a wife and a mother of a newborn has been inspiring and kept on bouncing me back always on my feet until the successful submission of this thesis. I quit this journey more than immensely grown and stronger as a person and will forever remember my passage at her lab as a privileged opportunity.

A part of my acknowledgement and appreciation also goes to Dr. Philippe Cammisotto, whose assistance saved me on repeated times from doing mistakes, forgetting deadlines and preparing for meetings. Very early in my degree, I came on understanding that Philippe's goodwill and constant friendship were helping me cope with major lab failures. Hence, I believe that none of this work could have been done without his help and will keep in my memory the figure of the kind, dedicate and passionate scientist he was. Thank you for backing me up over and over, Philippe.

Many credits also go to my colleague and friend, Dr. Abubakr H. Mossa, the years of contribution of whom are found throughout this work. It is also imperative that he be equally acknowledges for the time that he invested in me. I am thankful for his continuous reliability and all the advancing advice and discussions shared over the years. Further thanks are also extended to other students as well as they paved this experience with lifelong memories and contributions – Dr. Aalya Hamouda, Dr. Stephanie Sirmakesyan, Dr. Maude Crevier, Dr. Bryan Luu, Dr. Laura R. Yan, Dr. Samer

Shamout. This applies equally to other researchers at the Lady Davis Institute – Dr. Maria Petropavlovskaya and Dr. Stephanie Lehoux – and McGill University Health Centre – Dr. Jacques Lapointe and Dr. Simone Chevalier –, as well as employees from the LDI Animal Quarters and Cell Imaging Facility – Yvhans Cherry, Kathy Forner, Veronique Michaud, Darleen Element. All of them surpass themselves at providing the best animal care and graduate training every day.

Lastly, I should recognize all the love and support that I received unconditionally from my parents, extended family and friends, in my best and hardest times even.

Table of Contents

Preface

The present thesis was designed in a traditional monograph format. Authors are listed in decreasing order of contribution. The thesis, including its English and French abstracts, was written and prepared by Benjamin Desormeau, while revision was done by Dr. Lysanne Campeau. The content presented is organized into 6 main chapters. Chapters 1, 2 and 3 are the introduction, the literature review, and the research hypothesis with its objectives and experimental design. first chapters, along with Chapter 6, were all redacted by Benjamin Desormeau, while contributors to Chapter 4 and 5 are as follows:

- Chapter 4 is an *in vitro* study on urothelial and detrusor smooth muscle cells of the bladder. The study design was proposed by Dr. Lysanne Campeau and Dr. Abubakr H. Mossa. Experiments and data statistical analyses were equally performed by Benjamin Desormeau, Dr. Laura R. Yan, Dr. Abubakr H. Mossa and Dr. Philippe Cammisotto. Figures were prepared by Dr. Philippe Cammisotto. This chapter was written by Benjamin Desormeau, Dr. Abubakr H. Mossa, Dr. Philippe Cammisotto and Dr. Lysanne Campeau.

- Chapter 5 is an *in vivo* study on the effect of p75NTR on bladder morphology and function. The study was designed by Benjamin Desormeau, Dr. Philippe Cammisotto and Dr. Lysanne Campeau. All experiments were performed by Benjamin Desormeau, whereas data statistical analysis was done by Benjamin Desormeau and Dr. Philippe Cammisotto. The chapter and figures were written and prepared by Benjamin Desormeau.

List of Figures

Abbreviations

WHO: World Health Organization

NCD: non-communicable diseases

IC/PBS: interstitial cystitis/painful bladder syndrome

GAG: glycosaminoglycan

UTC: urothelial cells

TJs: tight junction

SP: substance P

5-HT: serotonin

ATP: adenosine triphosphate

CNS: central nervous system

PNS: peripheral nervous system

NGF: nerve growth factor

TNF-α: tumor necrosis factor-α

UPEC: uropathogenic *Escherichia coli*

CUA: Canadian Urological Association

LPS: lipopolysaccharide

UTI: urinary tract infection

CFU: colony-forming unit

proNGF: pro-nerve growth factor

MMPs: matrix metalloproteinases

SMC: smooth muscle cells

ECD: extracellular ligand-binding domain

p75NTR: p75 neurotrophin receptor

TNFR: tumor necrosis factor receptor

Vps10: vacuolar protein sortin domain/

ICD: intracellular domain

RhoA: Rho kinase A

TRAF6: TNF receptor-associated factor 6

NF-ʁB: nuclear factor kappa-B

JNK: c-Jun N-terminal kinases

TLR: toll-like receptor

NO: nitric oxide

iNOS: inducible nitric oxide synthase

DAMPs: damage-associated molecular patterns

PAMPs: pathogen-associated molecular patterns

TIR: Toll/interleukin 1 receptor domain

THX-B: 1,3-diisopropyl-1-[2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl) acetyl]-urea

SD: Sprague Dawley

CCAC: Canadian Council for Animal Care

DMEM: Dubelco's modified Eagle medium

NEDD: sulfaminalide/1-naphthylethylenediamine dihydrochloride

iNOS: inducible nitric oxide synthasVFT: Von Frey test

VSA: voiding-spot assay

EFS: electrical field stimulation

Chapter I. Introduction

1.1. Interstitial Cystitis/Painful Bladder Syndrome

The rate at which the world's elderly population grows makes aging the next public health challenge of the upcoming decades. In 2019, the World Health Organization (WHO) estimated that 703 million people were aged 65 years and over, a proportion estimated to 1.5 billion by 2050 (United Nations 2019). In Canada, data predict approximately 23.4% of the population to be more than 65 years in 2030 (Chagnon et al. 2019). Fast shift in the age distribution of people nationwide pose severe implications on public health, especially since aging increases the occurrence of non-communicable diseases (NCDs). The costs related to the management of NCDs are burdensome for healthcare systems, and their impact on socioeconomic cannot be avoided. In clinical practice, NCDs cover a range of chronic health disorders, including age-related diseases such as interstitial cystitis/painful bladder syndrome (IC/PBS) (Chang et al. 2019).

1.1.1. Epidemiology

IC/PBS is a chronic bladder inflammatory condition of unknown etiology and without treatment. It has a global prevalence of between 0.1% – 2% (Davis, Gnanappiragasam, and Thornhill 2015). In a literature review published in 2017, women made up 4 times more IC/PBS patients than men in the United States, accounting for a pool of 8 million American women. It was further highlighted in that study that white American women median-aged forty living in low economic settings are the most prevalent female case profiles of IC/PBS (Patnaik et al. 2017).

Another study conducted by *Lee and al*. on a cohort of 2,122 patients with IC/PBS also found that women represent the most afflicted group. Between 2002 to 2013, about 78.8% of IC/PBS patients who claimed insurance health reimbursement in Taiwan were women. Furthermore, the prevalence of IC/PBS in these women varied between 35.4 –73.4% in a 2-years period. (Lee, Chang, and Tsai 2018)

Substantial variations exist in the prevalence of IC/PBS across studies as a result of historical bias, ranging from little understanding of the disease to very strict clinical guidelines. This is particularly problematic as it decreases the quality of IC/PBS diagnosis used in systemic reviews and meta-analyses. Variation based on the type of studies (e.g. quantitative or qualitative) and methodology such as auto-administered questionnaires or self-report also likely contribute to an underestimation of IC/PBS prevalence worldwide (Patnaik et al. 2017). While advances were made to reduce errors in the prevalence of IC/PBS, we still know that 90% of IC/PBS women are not diagnosed. Moreover, those who received a diagnosis claimed waiting up to 7 years until it was correctly provided, a delay that is highly problematic for patients and clinicians (Chancellor 2007).

1.1.2. Diagnosis and Clinical Gaps

Diagnostic delays are common with IC/PBS mainly because it remains a diagnosis of exclusion. This means it lacks testing and objective biomarkers that would otherwise serve in excluding diseases with similar symptomatology. While efforts were made to address these gaps, advances have been poor and continue to puzzle physicians who need to decide on the best course of treatment at the best of their abilities. (Kim 2016; Cox et al. 2016; Patnaik et al. 2017).

IC/PBS symptoms include severe pelvic pain, increased urinary frequency – between 17 – 25 time a day –, voiding urgency, and nocturia (Patnaik et al. 2017). Clinical reports show that pelvic pain occurs mostly with bladder filling, though evidence support the contribution of other risk factors such as stress, sexual intercourse, diet or menses (McKernan et al. 2018; Cox et al. 2016). Saying that IC/PBS patients are at greater risk of mental health condition is also noteworthy. As their condition significantly reduce their quality of life, sign of depression, anxiety and suicidal ideation that ultimately worsen IC/PBS patient's condition are common (McKernan et al. 2020).

1.1.3. Pathophysiology

Studies shows that bladder inflammation, which is absent in as many as 43% patients who present symptoms, is not exclusive to IC/PBS. In clinical settings, this means that biochemical markers or even tissues injury have little to no value in providing an accurate diagnosis and screening the disease early. Our little understanding of IC/PBS pathophysiology also add strain on these clinical processes and the development of therapeutics (Kim 2016; Cox et al. 2016; Patnaik et al. 2017).

On the other hand, when cystitis is clear, clinicians also note that degree of inflammation in the bladder are not correlated with symptoms severity in IC/PBS patients. However, categories of bladder anomalies were found associated with apparition of specific symptoms (Cox et al. 2016). For instance, it was shown that increased urothelium permeability is linked to an increase in voiding frequency and pelvic pain (Grundy, Caldwell, and Brierley 2018; Birder and de Groat 2007). The mechanisms underlying increased urothelium permeability in IC/PBS remain unclear,

nonetheless. Alterations to the glycosaminoglycan (GAG) layer that covers the urothelium is one theory to explain it. As a group of polysaccharides found on urothelial cells (UTC) surface, change in GAG expression have been found to cause pain and increase frequency. (Grundy, Caldwell, and Brierley 2018; Hurst et al. 2015; Patnaik et al. 2017). One reason for that is that, in a healthy bladder, GAG acts against the compressive forces of urine and stop soluble metabolic products and potassium from accessing innermost tissues. It is also known to block pathogen adhesion on UTC (Wyndaele et al. 2019). Increased potassium levels in tissues depolarizes afferents nerve fibers and cause pain and bladder overactivity. The primary objective of therapies such as intravesical instillation of hyaluronic acid or chondroitin sulfate is, therefore, to reverse urothelium permeability, though the benefits of to this approach has shown to provide poor therapeutic relief over time (Cox et al. 2016; Hurst et al. 2015; Wyndaele et al. 2019).

Another theory suggests a loss of expression of tight-junctions (TJs) in the urothelium, notably occludin and ZO-1, as seen in bladder overactivity (Birder and de Groat 2007; Lee and Lee 2014; Jhang and Kuo 2016). TJs are crucial cell-to-cell paracellular protein structures that regulate fluid and soluble products movement between bladder cells. The reduction of TJ has been shown to increase bladder permeability and afferent nerves activity (Birder and de Groat 2007; Hurst et al. 2015; Montalbetti et al. 2017). Increased afferents activation engage bladder inflammatory and pain mechanisms through neuronal releases of inflammatory peptides such as substance P (SP), and serotonin (5-HT). Furthermore, chemical insults were reported to promote purinergic control of the bladder through an increased release of adenosine triphosphate (ATP), leading to bladder overactivity (Montalbetti et al. 2017; Grover et al. 2011).

Increased neuronal plasticity of sympathetic and nociceptive nerves is also a detrimental feature in IC/PBS patients (Liu et al. 2010; Chen et al. 2016). In the central nervous system (CNS) and peripheral nervous system (PNS), neural remodelling is tightly regulated by neurotrophic factors such as the nerve growth factor (NGF) for which higher levels are found in IC/PBS patients (Liu et al. 2010; Chen et al. 2016). NGF also help activates inflammatory mast cells that release granules of pro-inflammatory, vasoactive and nociceptive mediators such as histamine involved in pain and inflammation (Yoshimura et al. 2014; Patnaik et al. 2017; Sant et al. 2007). Mast cells are found in high number in IC/BPS bladders, releasing histamine, leukotrienes, prostaglandins, tumor necrosis factor-alpha (TNF-α) and NGF (Yoshimura et al. 2014; Patnaik et al. 2017; Sant et al. 2007). Other inflammatory factors such as SP can also be secreted by mast cells, ultimately forming along with neurons, a closed loop of reactions built around afferent activity and sustained inflammatory cell response (Patnaik et al. 2017; Sant et al. 2007).

1.1.4. Research Models of IC/PBS

Understanding by which way bladder damages connect to IC/PBS symptoms has conveyed the development of models of cystitis through chemical, mechanical and biological methods. So far, models have helped pinpoint an overwhelming list of potential mechanisms underlying IC/PBS, yielding over 180 potential targets of interventions (Mullins et al. 2015; Cox et al. 2016). Yet, one issue is that the majority of these proved to have poor therapeuitcs effects in humans as most models are based on potential aetiologies (Moutzouris and Falagas 2009; Cox et al. 2016). For that reason, their relevance was called into question, and browsing the history of IC/PBS patients has shown to provide more translational value (Mullins et al. 2015).

1.2. Recurrent Bacterial Cystitis

Data suggest for a long time a potential association between the recurrences of bacterial cystitis by extraintestinal uropathogenic strains of *Escherichia coli* (UPEC), the gram-negative bacteria, and symptoms of IC/PBS (Warren et al. 2008; Peters, Killinger, and Ibrahim 2009). For instance, the Canadian Urological Association (CUA) reported in 2016 that 50% patients with IC/PBS have had previous history of UTI (Cox et al. 2016). Furthermore, the systemic inflammatory response of IC/PBS patients to lipopolysaccharide (LPS) was found to be considerably higher than healthy patients (Schrepf et al. 2015). To understand the potential implication of bacterial cystitis in onset of IC/PBS symptoms, a better grasp of its clinical presentation is necessary.

1.2.1. Diagnosis

Known as lower urinary tract infection (UTI), acute bacterial cystitis is an infection that causes inflammation of the bladder. It is the commonest form of bacterial infection worldwide and has the highest prevalence among women lifelong. In fact, over 25% women claim having at least one episode of UTI by the age of 24 years, 50% by age of 32 years, and the lifetime risk goes up 60% in women of all ages (Geerlings 2016; Öztürk and Murt 2020; Flores-Mireles et al. 2015; Chung 2016). The clinical presentations of UTI include acute-onset symptoms of dysuria with severe pelvic pain, increased urgency, voiding frequency and nocturia. Laboratory-based evidence of hematuria and bacteriuria measured as $>10^5$ colony forming units (CFU)/mL on urine culture are diagnostic mainstays of patient with UTI. Common isolated agents of UTI are extraintestinal pathogenic strains of UPEC, which are detected in 75-95% of cases. However, other bacterial or

viral species can also be at the origin of the condition, though their occurrence is rare (Anger et al. 2019; Flores-Mireles et al. 2015; Öztürk and Murt 2020).

1.2.2. Epidemiology of Recurrences

Physicians rely on short term regimen of antibiotic as first-line therapies to address symptoms of UTI (Anger et al. 2019). However, with the rise of antimicrobial resistance patterns that makes UTI harder to treat, non-antibiotic antimicrobial alternatives need to progressively replace our current approach. Also, some studies have put into question the use of antibiotherapy in patients with strains of UPEC that are well-known to cause recurrences. This area deserves research as reinfections by the same strain of UPEC concern 80% of case of recurrence (Flores-Mireles et al. 2015). In fact, 20 – 30% of women have another infection within 6 months after treatment, 70% within one year, and 5% experience a third episode even (Geerlings 2016; Chung 2016). Typical risk factors of UTI span from frequent sexual intercourses, metabolic syndrome, poor personal hygiene to lower urinary tract anomalies (Anger et al. 2019).

1.3. Bacterial Cystitis in the Onset of IC/PBS

The high prevalence of UTI in women and its similarity with IC/PBS makes both diseases likely to share pathophysiological mechanisms similar to one another. This theory still awaits clear demonstration, nonetheless. Molecular pathways that drive chronic bladder disorders have been studied in different pathological contexts (spinal-cord injury, ischemia, diabetes mellitus and UTI). This has helped understand by which course of action most bladder disorders and symptoms develop at varying extents.

One common finding across studies shows that NGF levels are high in bladder biopsies and urine specimen of patients with diabetes mellitus and spinal-cord injury (Ochodnický et al. 2011; Nomiya et al. 2012; Giannantoni et al. 2006; Bjorling et al. 2001; Gonzalez et al. 2005; Chuang, Liu, and Kuo 2016). Overexpression of NGF is common in other conditions beyond bladder disorders as well, including sepsis and neurodegenerative diseases (Monteleone et al. 2018; Schreiber, Nikolaus, and Hampe 1998; Skaper 2017; Fields et al. 2014). During embryonic development and human aging, NGF regulates immune cell responses and neuronal cell plasticity (Mufson et al. 2019; Cuello, Pentz, and Hall 2019). It is expressed as a proprotein form, pro-nerve growth factor (proNGF), and is converted by matrix metalloproteinases (MMPs) into its mature NGF.

The effects of proNGF relay distinct physiologic function from mature NGF, which regulates synaptic plasticity, neurogenesis, and neuron survival (Mullins et al. 2015; Vilar and Mira 2016). In the body, tissues damages interfere with proNGF maturation, which leads to cell death and neurodegeneration (Vilar and Mira 2016; Ioannou and Fahnestock 2017; Fahnestock and Shekari 2019; Platón-Corchado et al. 2017). For instance, in diabetes and spinal-cord injury, loss of urothelium integrity occurs following overexpression of proNGF by bladder UTC and detrusor smooth muscle cells (SMC). In diabetic bladder cells, proNGF activates inflammatory and degenerative responses that progress into voiding dysfunction (Mossa et al. 2021; Ryu et al. 2018; Mossa, Galan, et al. 2020a). However, the role proNGF in IC/PBS is not elucidated.

Chapter II. Literature Review

2.1.The proNGF/p75NTR Axis

2.1.1. The Neuroinflammatory Cascades

Most neuroinflammatory disorders such as Alzheimer's disease and diabetic retinopathy feature a disbalance in the ratio of proNGF/NGF levels (Tiveron et al. 2013; Cuello, Pentz, and Hall 2019; Mohamed and El-Remessy 2015; Platón-Corchado et al. 2017). Oxidative stress mediated by injury, ischemia, infection and metabolic syndrome interferes with proNGF maturating process, leading to its accumulation in neurons and non-neuronal cells (Nomiya et al. 2012; Wang, Zuo, and Chan 2012; Mossa, Cammisotto, et al. 2020; Ji et al. 2018). To relay signal, proNGF must binds exclusively the ligand-binding domain (ECD) of his high-affinity p75 neurotrophin receptor (p75NTR). A member of the tumor necrosis factor receptor (TNFR) superfamily, p75NTR interacts with the vacuolar protein sortin domain (Vps10) of the co-receptor, sortilin, to expose its intracellular domain (ICD) that acts as a death domain (Houlton et al. 2019; Wislet, Vandervelden, and Rogister 2018; Skeldal et al. 2012; Elshaer et al. 2019).

Downstream p75NTR signals are relayed by cytoplasmic effectors such as the GTPase Rho kinase A (RhoA) and TNFR-associated factor 6 (TRAF6). As an E3 ubiquitin ligase assisting in the assemblage of signaling complexes, TRAF6 binds p75NTR ICD and relay signals associated with inflammation and cell death (Kisiswa et al. 2018; Elshaer et al. 2019; Mossa et al. 2021; Sycheva et al. 2019). Specifically, the recruitment of TRAF6 by p75NTR ICD helps activate nuclear factor

kappa-B (NF-ϰB) and AP-1/c-Jun N-terminal kinases (JNK) signaling (Khursigara, Orlinick, and Chao 1999; Dhillon et al. 2019; Schellino, Boido, and Vercelli 2019). It is further worth noting that TRAF6 can associate to variety of TNFR, as well as members of the Toll-like receptor (TLR) family (Dou et al. 2018; Gentry et al. 2004; Dhillon et al. 2019). Therefore, the versatility of TRAF6 to switch one receptor to another ensures that similar detrimental pathways be activated in response to more than one ligand (Shanab et al. 2015; Ioannou and Fahnestock 2017).

2.1.2. Bladder Dysfunction

Playing tumor suppressor functions, overactivity of p75NTR brings a pool of detrimental change on bladder function, nonetheless. This is notably the case in diabetes mellitus and spinal-cord injury (Ryu et al. 2018; Zabbarova et al. 2018; Kashyap et al. 2016). Studies show that ligand activation of p75NTR signaling increases in either pathologies and is linked to higher loss of UTC viability, and lower tight junction expression of occludin (Sycheva et al. 2019; Mossa et al. 2021). Recently, it was further demonstrated that active RhoA in umbrella cells alters mitochondrial function, thereby triggering cell death via intrinsic caspase-3 activation (Mossa et al. 2021).

Stimulation of p75NTR in UTC was further found to affect transcriptional activity of pro-inflammatory such as TNF-α and nitric oxide (NO), the product of inducible nitric oxide synthase (iNOS) (Ryu et al. 2018; Mossa et al. 2021). Otherwise, its effects on SMC are largely different, driving shifts from contractile to migrative and proliferative phenotype through NF-ϰB and JNK activity in cells (Mossa et al. 2021). In addition to that, a number of studies support that activation

of JNK signaling mounts the deposition of type I collagen in SMC, ultimately causing bladder hypertrophy (Mossa, Galan, et al. 2020b; Kushida et al. 2001; Dugan et al. 2014).

2.2.LPS/TLR4

In the body, TLR members such as TLR4 provide protection against microbial invasion. In that matter, they can be widely found expressed in many tissues throughout the body, notably in the bladder, and recognizes a range of pathogens over which damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) such as LPS are found (Behzadi and Behzadi 2016; Birder and Andersson 2013). Stimulation of TLR4 expose an intracellular cytoplasmic Toll/interleukin 1 receptor domain (TIR) within TLR4 that recruits signaling factors such as MyD88 and TIR adaptors (Kawasaki and Kawai 2014; Kuzmich et al. 2017). Interestingly, TIR adaptors have a death domain within their structure, which is therefore capable of binding protein effectors, including TRAF6. In fact, it was found that TRAF6 is implicated in TLR4 signal transduction as such to mediate NF-ϰB and JNK signals in neuronal and non-neuronal cells (Dou et al. 2018; Dhillon et al. 2019).

2.2.1. Bladder Dysfunction

LPS found on the outermost membrane of gram-negative bacteria such as UPEC not only triggers inflammation in tissues but also sets off molecular mechanisms of nociception with TLR4 as well (Rudick et al. 2010; Liu et al. 2015). In fact, stimulation of TLR4 was found to play a considerable role in the pathophysiology of pain in bladder disorders (Cui et al. 2019; Ma et al. 2017). Studies

show that LPS in the bladder increases pain-related behaviours in mice at low mechanical pressure applied the pelvis (Kamei et al. 2018; Kamei et al. ; Kogan et al. 2018; Rudick et al. 2010). On the opposite, deleting TLR4 in the bladder was found to provide the analgesic effect in mice with cyclophosphamide-induced cystitis (Cui et al. 2019).

Furthermore, other reports show that the LPS–TLR4 has detrimental impact on detrusor function. In studies where mice were instilled with LPS, the contractile capacity of SMC to acetylcholine was increased after LPS exposure (Weng et al. 2009)·(Tambaro et al. 2014). Another study also demonstrated that, in type 1 diabetic bladder, blockage of TLR4 reduces hypertrophy and hypercontractility of the detrusor muscle, supporting the role TLR4 in detrusor muscle dysfunction (Szasz et al. 2016).

2.3. Small Molecule p75NTR Antagonist (THX-B)

In their study in 2010, *Bai and al*. demonstrated for the first time that the small molecule p75NTR antagonist (THX-B) could provide neuroprotection against p75NTR detrimental signals in retinal ganglion cells (Bai et al. 2010). Then, other studies that follow also succeed showing similar finding in neuronal cells of mice with diabetic retinopathy (Galan et al. 2017; Barcelona et al. 2016). Furthermore, in the bladder, *Zabarova and al*. showed that blocking p75NTR improved bladder compliance in mice affected with detrusor overactivity (Zabbarova et al. 2018). Similar results were also reported in a study on spinal-cord injured patients in which levels of proNGF in urine were significantly higher (Ryu et al. 2018)

Interestingly, the two previous studies also revealed that urothelial cell death by proNGF overexpression was effectively abolished with p75NTR blockage or knockout even, an evidence likely suggesting that p75NTR antagonism might have therapeutic value against cystitis. Extensive work done in our lab confirmed this hypothesis in type 1 diabetic mice with voiding dysfunction. By treating mice with THX-B, Mossa and al. observed that mice displayed a significant reduction in bladder weight as compared to untreated diabetic mice. Moreover, it was noted that cytokinesis of TNF-a was considerably reduced (Mossa, Galan, et al. 2020b).

Note that, while the therapeutic effect of p75NTR antagonism on innerved tissues is demonstrated in diabetic and spinal-cord injured bladder (Mossa, Galan, et al. 2020b; Ryu et al. 2018) and diabetic retinopathy (Galan et al. 2017), its properties still await to be studied in bacterial cystitis. Based on the evidence that p75NTR antagonism blocks degenerative pathways attributed to TLR4 in the brain of septic mice (Ji et al. 2018), we have reason to believe its anti-inflammatory potency might be translatable into infected bladders.

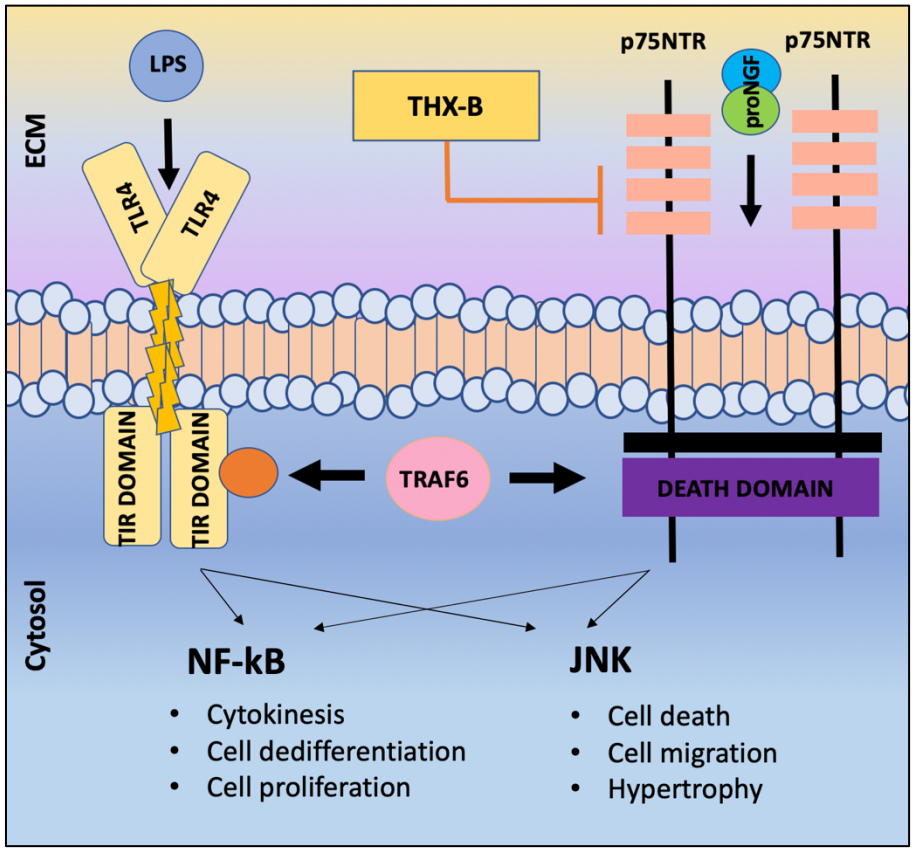

Figure 1. Basis of signal transduction by p75NTR and TLR4 in neurons.

Neurons express receptors p75NTR and TLR4 on their surface. Injuries cause accumulation of proNGF binding the p75NTR ligand binding domain. This exposes a death domain located in the p75NTR intracellular domain, which allows recruitment of effectors. One of them, TRAF6, assists NF-kB and JNK complexes assembly. Examples of NF-kB and JNK signals effects include gene transcription of pro-inflammatory factors or cells entry into apoptosis, respectively. Note that LPS on UPEC also trigger these pathways, as TRAF6 binds with equal affinity the TIR domain of adaptors located on TLR4 in the cytosol. Adding THX-B prevents p75NTR activation.

Chapter III. Hypothesis and Objectives

3.1. Study Hypothesis

To goal of this project was to identify the contribution of the proNGF/p75NTR inflammatory axis in the onset of IC/PBS. Based on previous data in our lab, we hypothesized that blocking specifically p75NTR would dampen features of cystitis. To answer that, we tested the effects of LPS and p75NTR antagonism on the bladder at the molecular, cellular and physiological levels.

3.2. Objectives

This project had two majors aims:

Aim 1. To identify an interplay between the p75NTR and TLR4 signal transduction.

There are similarities in the mechanisms of inflammation between proNGF/p75NTR and TLR4 signal transduction in neuronal and non-neuronal cells. Evidence shows that the transcription of inflammatory cytokines via NF-κB signaling activity, as well as JNK-induced apoptosis and loss of tight junctions are common outcomes of the p75NTR and TLR4 axes. Interestingly, blocking proNGF/p75NTR via a specific p75NTR antagonism has demonstrated to reduce inflammation in neurons of infected mice, supporting a molecular interplay between these two receptors.(Ji et al. 2018) Given that, we hereby hypothesized that the proNGF/p75NTR and TLR4 axes are at interplay in bladder cells, therefore having implications on the progression of chronic bladder inflammation.

Experimental design

Primary urothelial and smooth muscle cells will be cultured out from bladders of female Sprague-Dawley rats. These cells will be initially validated for cell-surface expression of the p75NTR receptor, then subsequently tested for the effects of LPS on cellular activation of inflammatory pathways, survival signals and apoptotic signaling cascades. The secretion of inflammatory cytokines and mediators and loss of tight junctions will also be evaluated before repeating the experiments with pharmacological treatment by a specific p75NTR antagonist. This section of the study will therefore allow us to draw potential conclusions on the molecular and cellular contribution of proNGF/p75NTR in the bladder when exposed to LPS.

Aim 2. To establish the contribution of p75NTR in bladder dysfunction in a mouse model of LPS-induced cystitis.

Accumulation of proNGF in bladder tissues is common across models of voiding dysfunction, including acute bacterial cystitis. More specifically, mice intravesically instilled with LPS show with higher levels of inflammation in bladder tissues, altered voiding function, and lower pelvic pain threshold. However, silencing TLR4 in LPS-treated mice has been reported to relieve pelvic pain without affecting bladder inflammation and function. On the other hand, the antagonist alleviated pain in mice with metabolic syndrome. We therefore hypothesized that p75NTR contributes to the onset of inflammation in the bladder after LPS insults without affecting pain.

Experimental design

Young C56BL/6 female mice were intravesically instilled with saline or LPS for 1 hour. Voiding patterns and pelvic pain sensitivity were evaluated at baseline and after 24-hour recovery by voiding spot assay and Von Frey test, respectively. In order to validate the onset of acute cystitis by LPS, alteration in detrusor muscle contractility were assessed by organ bath and changes in bladder morphology by histology. These experiments were repeated in mice injected intraperitoneally with the specific p75NTR antagonist during the infection. This will allow us to compare the therapeutic effects of the p75NTR antagonism treatment on bladder dysfunction.

Chapter IV. Contribution of p75NTR Signaling in Urothelial and Smooth Muscle Cells Inflammatory Responses

4.1. Introduction

In this chapter, we focus on identifying the contribution of the receptor p75NTR on mediating inflammatory responses in urothelial and smooth muscle cells after stimulation of TLR4 with LPS. We begin by establishing the presence of p75NTR on urothelial and smooth muscle cells surface in primary cell cultures. Then, guided by previous studies in neurons, we confirmed whether TLR4 interferes with p75NTR expression in bladder cells. Confirming the absence of interaction at this level had us next moved on looking at the effect of p75NTR blockage on activation of TLR4 signaling outcomes associated with molecular cascades and cellular structures.

4.2. Materials and Methods

4.2.1. Cell culture

Female Sprague-Dawley (SD) rats aged 3 months were housed and handled in accordance with the Canadian Council for Animal Care (CCAC). All protocols were approved by the Animal Ethics Committee of McGill University (Montreal, Canada). Animals were fed with standard Purina chow and had free access to water. After euthanasia by exsanguination under anesthesia with isoflurane (3%), bladders of mice were excised and placed in cold sterile phosphate-buffered saline (PBS) (pH 7.4), then cut longitudinally. Cells were isolated from bladder using collagenase type IV digestion as previously reported (Mossa et al. 2018; Mossa et al. 2021) The urothelium layer was carefully scraped and left for 15-20 minutes in Dubelco's modified Eagle medium (DMEM) containing 100 U/ml of collagenase IV. Then, urothelial cells where washed twice in DMEM containing 10% fetal bovine serum (FBS) and cultured at 37˚C in a humidified incubator with 5% CO2 atmosphere in Dulbecco's DMEM low glucose/keratinocyte (50/50) medium containing FBS (10%), GlutaMAX (X1), a mix of factors (insulin 5μg/ml, dihydrocortisone 0.5μg/ml, adenine 15μg/ml, ethanolamine 0.1 mM), Rho inhibitor 4-[(1R)-1-aminoethyl]-N-pyridin-4-ylcyclohexane-1-carboxamide (Y27623) (10μM), and 1% penicillin/streptomycin (100U/ml, 100μg/ml). The medium was changed every 2 to 3 days until cell confluency, and cells were starved 24 hours prior to use. In parallel, the detrusor muscle of the bladders was finely minced and incubated for 45 minutes with intense shaking in DMEM medium containing 250 U/ml of collagenase IV. Undigested tissues were removed with a strainer (40 μm mesh) and smooth muscle cells were then washed twice in DMEM/FBS and cultured in similar conditions to urothelial cells

on a Petri dish with DMEM medium supplemented with FBS (10%), GlutaMAX (X1), high glucose (27 mM) and penicillin/streptomycin (100U/ml, 100μg/ml). Prior to use, cells were starved for 72 hours in normoglycemic condition.

4.2.2. Immunoblotting

Cells were lysed in ice-cold RIPA buffer containing antiprotease mix (Roche Diagnostics, Montreal, Canada) for 10 minutes. Then, lysates were collected, and protein concentrations were measured with a MicroBCA assay kit (Boster Biological Technology). Equal amounts of proteins (15-30μg) were loaded on 6-8% SDS-PAGE polyacrylamide gel. After electrophoresis, proteins were transferred on PVDF membranes, then blocked in TBS-Tween (0.1%) with non-fat milk (5%) for 1 hour. Overnight incubation with a primary antibody at 4˚C was carried out using the following dilutions: ERK (1:1000), pERK (1:1000), JNK (1:1000), pJNK (1:1000), NF-$\varkappa$B (1:1000), TNF-α (1:2000), Lamin (1:2000), ß-actin (1:5000), TRAF-6 (1:600), Occludin (1:8000), E-cadherin (1:2000), ZO-1 (1:2000), Smoothelin (1:2000), p75NTR (1:8000), iNOS (1/3000). After thorough washings, membranes were incubated with a secondary HRP-bound antibody (1:3000) (Millipore, CA, USA) for 1 hour. The signal was mediated with Luminata Crescendo HRP substrate (Millipore, Billerica, MA) and quantified using ImageStudioLite.

4.2.3. RT-qPCR

The extraction of cell RNAs was performed using a classic phenol-chloroform protocol. Primers were obtained from Integrated DNA Technologies (IDT, Coralville, IOWA, USA): p75NTR

forward, p75NTR reverse, GAPDH forward, GAPDH reverse. Sensifast Probe Low-ROX kit containing SYBR-green was used to amplify the samples on an Applied Bioscience 7500 Fast Real-Time PCR. The following conditions were used: 95 °C 10 min then 45 cycles of 95 °C 15 seconds and 57 °C 40 seconds, followed by melt curve analysis. All samples were done in triplicates. Each primer was assessed for specificity and efficiency (90-110%). Controls were done using purified RNA without reverse transcription. Data were analyzed using the 2-ΔΔCT method.31

4.2.4. Extraction of nuclear NF-κB

Nuclear and cytoplasmic NF-κB were isolated by the method of Schreiber.32 After 30 minutes of treatment with LPS, cells were lysed with ice-cold Buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, pH 7.9) containing antiproteases (Roche Diagnostics, Montreal, Canada) for 10 minutes. The lysate was subsequently homogenized and centrifuged (3000 rpm) for 10 minutes at 4˚C. The resulting supernatant (cytoplasmic fraction) was then removed and kept at 4˚C, and the pellet was resuspended in another lysing buffer (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9) containing 300 mM NaCl before being homogenized, incubated on ice for 30 minutes and centrifuged (24000 x g) 20 minutes at 4˚C. The final supernatant corresponding to the nuclear extract fraction was saved and its pellet discarded.

4.2.5. Immunoprecipitation

Following treatment for 5 minutes, cells were lysed with ice-cold Cell Lysis Buffer for 10 minutes. An equal amount of proteins (200-400 μg) was immunoprecipitated overnight with an ICD anti-p75NTR rabbit antibody (1:600) at 4˚C with gentle rocking. The next day, immunocomplexes were incubated for 3 hours with 20μl of protein G agarose beads (Thermofisher, Montreal, Canada) at 4˚C, then centrifuged. The resulting supernatant was discarded, and the pellet washed 5 times with ice-cold 1X cell lysis buffer before being resuspended in 20 μl of loading buffer and proceeded to electrophoresis on 8% SDS-PAGE gel.

4.2.6. Immunofluorescence

Cells were seeded on glass coverslips until confluency, then fixed 30 minutes in paraformaldehyde 4% in PBS pH 7.4. Coverslips were washed with PBS and cells permeabilized with Triton X100 (0.1% in PBS, pH 8.0). After washing in PBS, blocking was performed with BSA 1% in PBS for 1 hour. Cells were incubated with primary antibodies overnight at 4˚C. Incubation with the secondary antibody conjugated to dy-488 (Abcam, Toronto, Canada) for 1 hour was followed by thorough washing with 1X Tris-buffered saline containing 0.1% Tween-20 (TBST). Slides were finally mounted on DAPI for examination under fluorescence microscopy (Leica Microsystem).

4.2.7. Nitric oxide assay

Levels of NO were diffused in cell culture media were detected using Griess' colorimetric method involving sulfaminalide/1-naphthylethylenediamine dihydrochloride (NEDD) (Sun et al. 2003).

4.2.8. Data analysis

Values are expressed as mean ± S.E.M. Statistical significance was established as $P < 0.05$, 0.01 and 0.001. GraphPad Software was used to compare differences between control, LPS, THX-B LPS+THX-B groups by performing unpaired Student's t test and One-way ANOVA (Bonferroni post-hoc test).

4.3. Results

Expression of p75NTR in urothelial and detrusor smooth muscle cells with LPS: Expression of p75NTR on UTC and SMC has previously been reported throughout the bladder *in vitro* and *in vivo* (Mossa et al. 2021; Mossa, Galan, et al. 2020a). Here, immunostaining reiterated this finding and found that p75NTR is expressed on cell membrane, in the cytoplasm, and within vesicles of UTC and SMC (Fig. 1A). Expression of p75NTR is reported to be higher in the detrusor muscle than the bladder urothelium. Our RT-qPCR confirmed significantly higher levels of p75NTR mRNA in SMC compared to UTC. (Fig. 1B). Levels of p75NTR are found to vary according to the degree of cell injury in neurons. To validate that in bladder cells, we exposed UTC and SMC to increasing concentrations of LPS (0.001 to 10 μg/ml). Protein levels of p75NTR ICD (32 kDa) and ECD (50 kDa) domains were not different of control in UTC and SMC at any concentrations (results not shown).

Cytokinesis and diffusion of TNF-α and NO in response to LPS and THX-B: During sepsis, endothelial cells stress by LPS secrete inflammatory cytokines and activate inducible nitric oxide synthase (iNOS) activity. The iNOS product, NO, causes vasodilatation and severe drop in blood pressure. In the bladder, UTC showed to significantly increased cytokinesis (membrane-bound form) and secretion (free form) of TNF-α in contact with LPS (1 μg/ml) for 24h. When pre-treated with THX-B (5 μg/ml) up to 30 minutes, levels of cytokinesis and secretion of TNF-α were much lower in these cells (Fig. 2A). On the other hand, incubation with LPS (100 μg/mL) for 24 hours increased iNOS expression and NO levels in UTC (Fig. 2B). However, there was no difference in iNOS and NO levels after pre-treatment of THX-B (5 μg/mL) for 30 minutes (Fig. 2C).

Modulation of intracellular pathways in the bladder: In neurons, p75NTR and TLR4 induces similar degenerative and inflammatory outcomes. It is reported that both receptors rely on similar signaling effectors such as TRAF6 to mediates their effects(Dou et al. 2018; Khursigara, Orlinick, and Chao 1999). Because *Muhuo and al.* also showed in their study that p75NTR antagonism reduces neuroinflammation in mice with sepsis (Ji et al. 2018), it is likely that both receptors form together molecular interplay in body cells, including in the bladder.

Here, after 2 minutes, we found that the ratio of pERK/ERK increased significantly in UTC with LPS (100 µg/ml). Furthermore, levels of pERK were not any different with THX-B pre-treatment (5 µg/ml) compared to LPS (Fig. 3A). Activation of ERK is associated with urothelial cells proliferation and survival in the bladder, notably in bladder cancer. Accordingly, our data confirmed our expectations, that is no difference in levels of JNK and NF-kB activity with LPS after 10 and 30 minutes, respectively, compared to control (Fig 3B, 3C). The ratio of active TRAF6/total TRAF6 was also not change after 5 minutes with LPS compared to control (Fig 3D).

On the other hand, our data revealed opposite reactivity patterns in SMC. LPS (100 µg/ml) did not engage different levels of ERK signaling after 2 minutes compared to control (Fig.4A). However, there was a significantly increased in activation of JNK and NF-kB signaling after 10 and 30 minutes, respectively. Therefore, we also found that pre-treatment of THX-B (5 µg/ml) reversed the ratio of pJNK/JNK, and reduced levels of NF-kB activation (Fig. 4B, 4C). Most importantly, the amount of active TRAF-6 bound to p75NTR was significantly increased with LPS and blocked after 5 minutes with THX-B (Fig. 4D).

Regulation of tight junction and cytoskeleton integrity: LPS was added individually or in combination with THX-B (5 µg/mL) to culture media of UTC or SMC for 24 hours. After 24h, E-cadherin, occludin and ZO-1 were detected in UTC by immunostaining. Location of all three tight junctions was observed on the cytoplasmic membrane, in the cytoplasm (Fig. 5A, B and C). E-cadherin expression was not affected by LPS (100 µg/mL) (Fig 5A), whereas levels of Occludin (Fig. 5B) and ZO-1 (Fig. 5C) decreased significantly. THX-B showed to restore occludin expression but had no effect on loss of ZO-1. In SMC, expression of E-cadherin, ZO-1 and Smoothelin were all unaffected by LPS (Fig. 6A, B and C).

4.4.Discussion

Examining the degree of expression of p75NTR in UTC and SMC when exposed to LPS revealed higher levels of the receptor in SMCs. This is in line with a previous study that has demonstrated higher detection of p75NTR in the detrusor muscle.(Zabbarova et al. 2018) Some studies suggest that TLR4 modulates the expression of p75NTR in neural and non-neural cells.(Jiang et al. 2008; Freria et al. 2016) For instance, by using a model of peripheral axotomy, one study showed that expression of p75NTR on nerve distal stumps of mice who had undergone sciatic nerve transection was increased in TLR4 mutant mice compared to wild-type mice.(Freria et al. 2016) While this suggests that TLR4 can control the expression of p75NTR in some cells, our data support that it is not the case in bladder cells. We found that expression of the ICD and ECD domains of p75NTR was not changed in UTC and SMC exposed to LPS. Therefore, we have reason to believe that sensitization to proNGF/p75NTR activity in bladder cells occurs independently of TLR4 signal transduction.

The urothelium of the bladder is not only an important barrier, but also an essential sensor and communicator to neural and immune cells.(Winder et al. 2014; Birder and Andersson 2013) By recognizing pathogens in urine, it releases a cocktail of immunomodulator cytokines and neurotransmitters that stimulates active response of immune cells and afferents from the bladder.(Rudick et al. 2010; de Groat and Yoshimura 2009) For example, one study clearly showed that afferent inputs and leukocyte infiltration increase after secretion of ATP by UTC exposed to LPS.(Ueda et al. 2020) Here, we found that UTC released significantly more TNF-α and NO in contact with LPS. In the bladder, their acute expression constitutes a defence mechanism against bacterial invasion.(Lacerda Mariano and Ingersoll 2020; Yu et al. 2019) However, chronic

overexpression of TNF-α in bladder tissues, as seen in IC/PBS patients, induces chronic pelvic allodynia, increased urinary frequency, decreased voided volume, and urothelium damage.(Yang et al. 2018) It was further propose to increase the propensity to reinfections.(Yu et al. 2019) Therefore, we think that, in our study, UTC were in a defensive pro-inflammatory state with LPS. As blocking p75NTR prevented the cytokinesis TNF-α without interfering with iNOS and NO expression, we support that p75NTR and TLR4 signaling form together an interplay in UTC by relaying chronic release of TNF-α.

While TNF-α increased with LPS, we did not observe activation of the TLR4/Traf6/NF-ϰB axis in UTC. Instead, we found higher ERK survival activity in absence of apoptosis, which opposes several studies.(Weng et al. 2009; Rackley et al. 1999; Song and Abraham 2008) However, it must be recalled that UTC have innate immune abilities to regulate the degree of inflammatory response through non-canonical mechanisms. For example, LPS can enter the cytosol of cells, non-canonical pathways of lower inflammatory potency are known to be initiated. For instance, caspase-11 is known to bind directly LPS in the cytoplasm of urothelial cells, thereby promoting the expression of pro-inflammatory cytokines without initiating apoptotic pathways.(Huang et al. 2019) Another example is that of the secretion of NO, which is known as an indicator of low reactive oxygen species (ROS) levels and the non-canonical caspase-1/inflammasome pathways. While these findings seem to indicate that LPS alone is insufficient to activate TLR4/TRAF6/NF-ϰB, it yet remains clear that it still holds potency to sensitize cells to pro-inflammatory responses.

Loss of ZO-1 and occludin expression has been previously reported in chronic inflammatory conditions such as ulcerative colitis and interstitial cystitis. In the past few years, the role of these

TJ has also been extended to that of molecular effector in apoptotic signalling. ZO-1 deletion was found to induce JNK-activated caspase-3-dependent apoptosis.(Herrero et al. 2019; Kuo et al. 2019) Here, our findings supported by the existing literature suggests that activation of TLR4 downregulates ZO-1 and occludin. However, we found that antagonism of p75NTR partially reintroduced occludin expression without changing caspases activity caused by ZO-1 downregulation. Taken together, we suggest that p75NTR antagonism does not affect urothelial cells pro-survival signals in response to a sudden loss of ZO-1 by TLR4 activity.

Detrusor muscle hypertrophy and fibrosis are common features of spinal-cord injury, diabetes mellitus.(Hu, Granger, and Jeffery 2016; Mossa, Galan, et al. 2020b) Reports have demonstrated the expression of proNGF increases in detrusor smooth muscle cells after bladder damage in contexts of spinal-cord injury or metabolic syndrome. Moreover, the proNGF/p75NTR axis was found to initiate NF-κB activity, subsequently promoting the transition from contractile smooth muscle cells to a non-contractile proliferative phenotype.(Mossa et al. 2021) Proliferation can be caused by NF-κB while smooth muscle enlargement and collagen expression are conversely attributed to JNK activity.(Roux 2002) Here, we identified the activation of TLR4 led to an increase in the association of TRAF6 to the p75NTR intracellular domain. This was further joined by overactivation of NF-κB and JNK, the activation of which, along with TRAF6, were prevented by p75NTR antagonism. In cells, TRAF6 plays a pivotal function between many receptors, eliciting NF-κB and JNK signaling.(Dou et al. 2018; Dhillon et al. 2019) Our observations therefore support that TRAF6 acts an intermediate between TLR4 and p75NTR inflammatory signals in detrusor smooth muscle cells, causing significant loss of contractile function through a potential TLR4/TRAF6/NF-κB myogenic dedifferentiation axe.

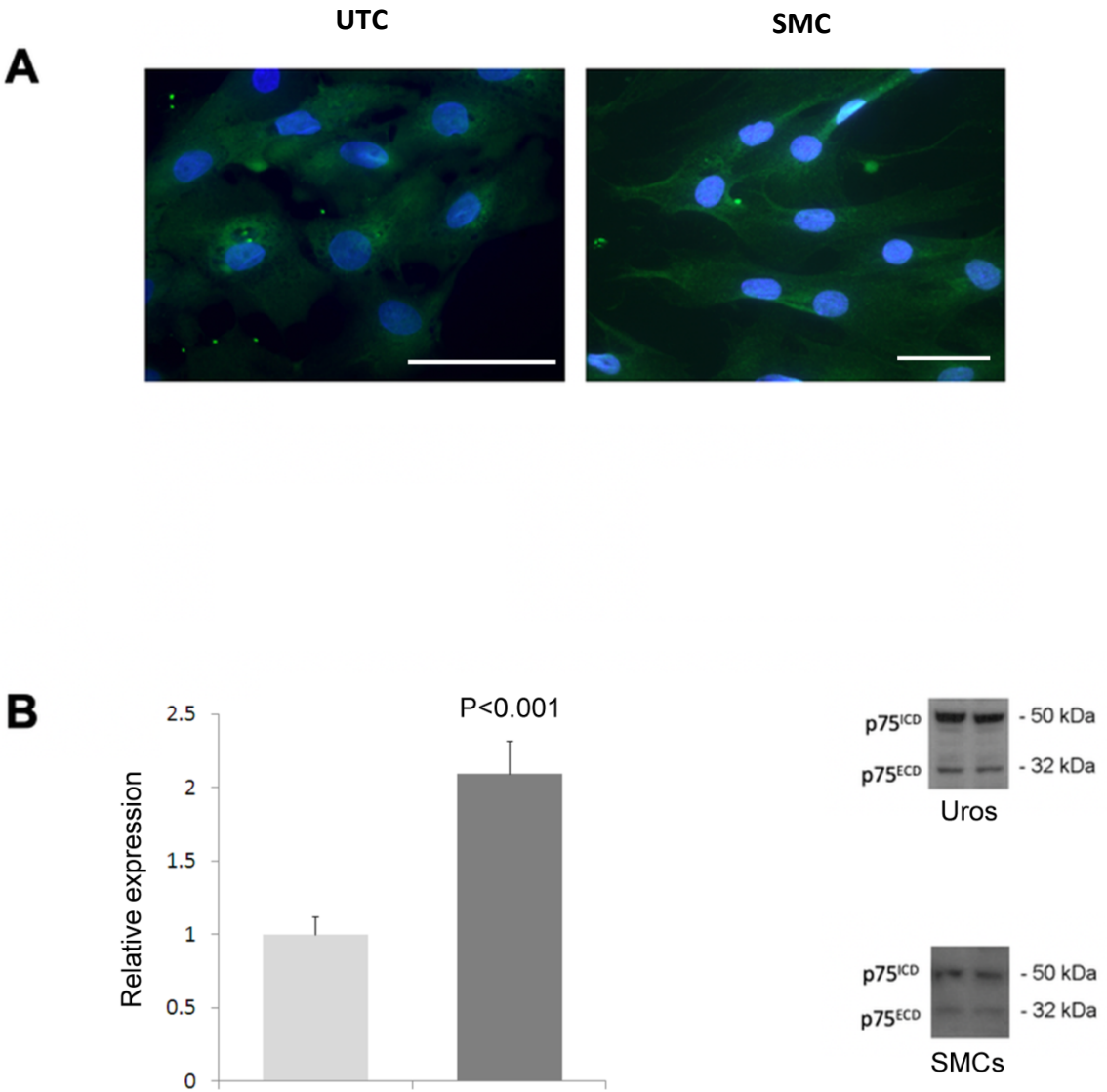
A
UTC
SMC
B
P<0.001
Relative expression
2.5
2
1.5
1
0.5
0
Uros
SMCs
p75ICD
p75ECD
- 50 kDa
- 32 kDa
Uros
p75ICD
p75ECD
- 50 kDa
- 32 kDa
SMCs

Figure 2. Detection of p75NTR in UTC and SMC.

(A) Immunochemistryneuron detected p75NTR in the cytoplasm of UTC and SMC exposed to LPS. Bars = 25 μm. (N=4) (B) RT-qPCR measured p75NTR mRNA relative amount between UTC and SMC with GAPDH as a reference. Immunoblotting detected the cleaved extracellular (32 kDa) and intrallular (50 kDa) domains of p75NTR in UTCs (upper Western blot) and SMCs (lower Western blot) (N=6). One-way ANOVA, *P <0.05, **P <0.01.

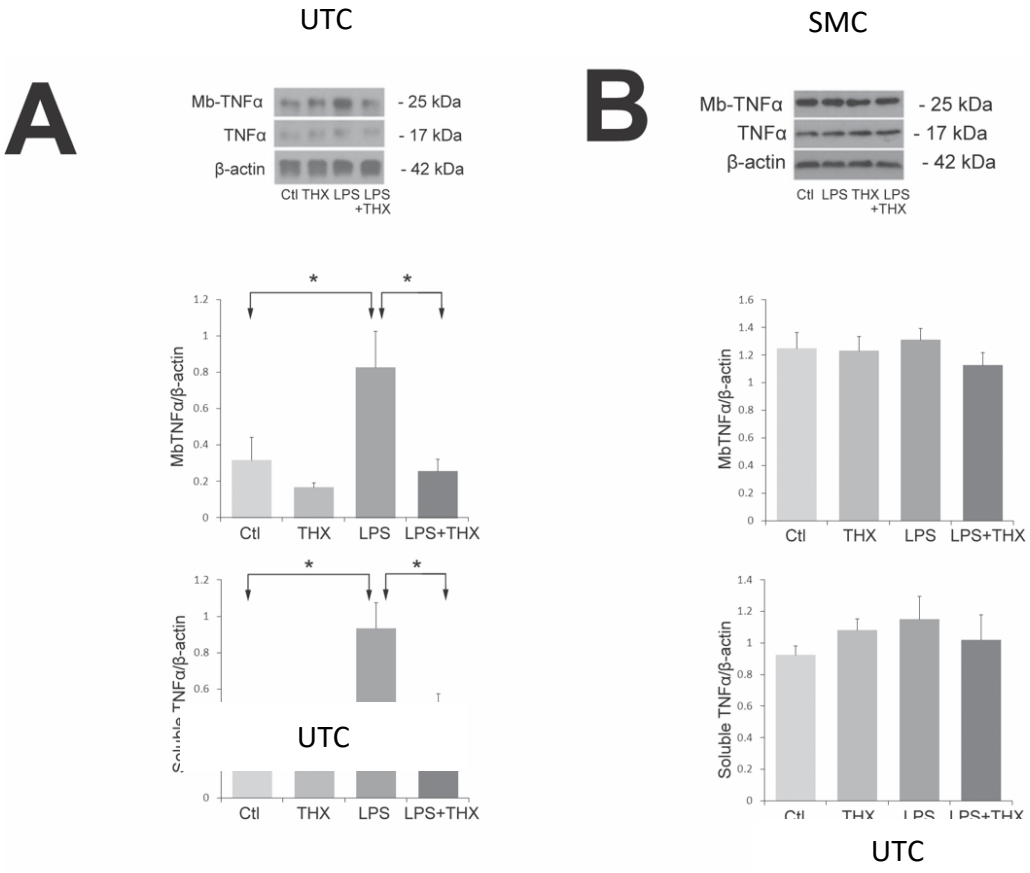
UTC
SMC
A
B
Mb-TNFα
TNFα
β-actin
- 25 kDa
- 17 kDa
- 42 kDa
Ctl THX LPS LPS +THX
MbTNFα/β-actin
Soluble TNFα/β-actin
Ctl
THX
LPS
LPS+THX
UTC
C
iNOS
- 125 kDa
β-actin
- 42 kDa
Urothelial cells
Smooth muscle cells
NO/protein (pmol/μg)
iNOS/β-actin
NS

Figure 3. Variations in TNF-α and NO levels in presence of LPS and THX-B.

Bladder cells were incubated with LPS (100 μg/mL) alone or combined with THX-B (5 μg/mL) for 24 hours. (A) UTC: membrane-bound (Mb) and soluble forms of TNF-α detected by immunoblotting with B-Actin as reference, then semi-quantified in graphs (N=4). (B) SMC: membrane-bound (Mb) and soluble forms of TNF-α detected by immunoblotting with B-Actin as reference, then semi-quantified in graphs (N=4). (C) NO measured in both UTC and SMC and iNOS was measured in UTC only (N=7). One-way ANOVA, *P <0.05, **P <0.01.

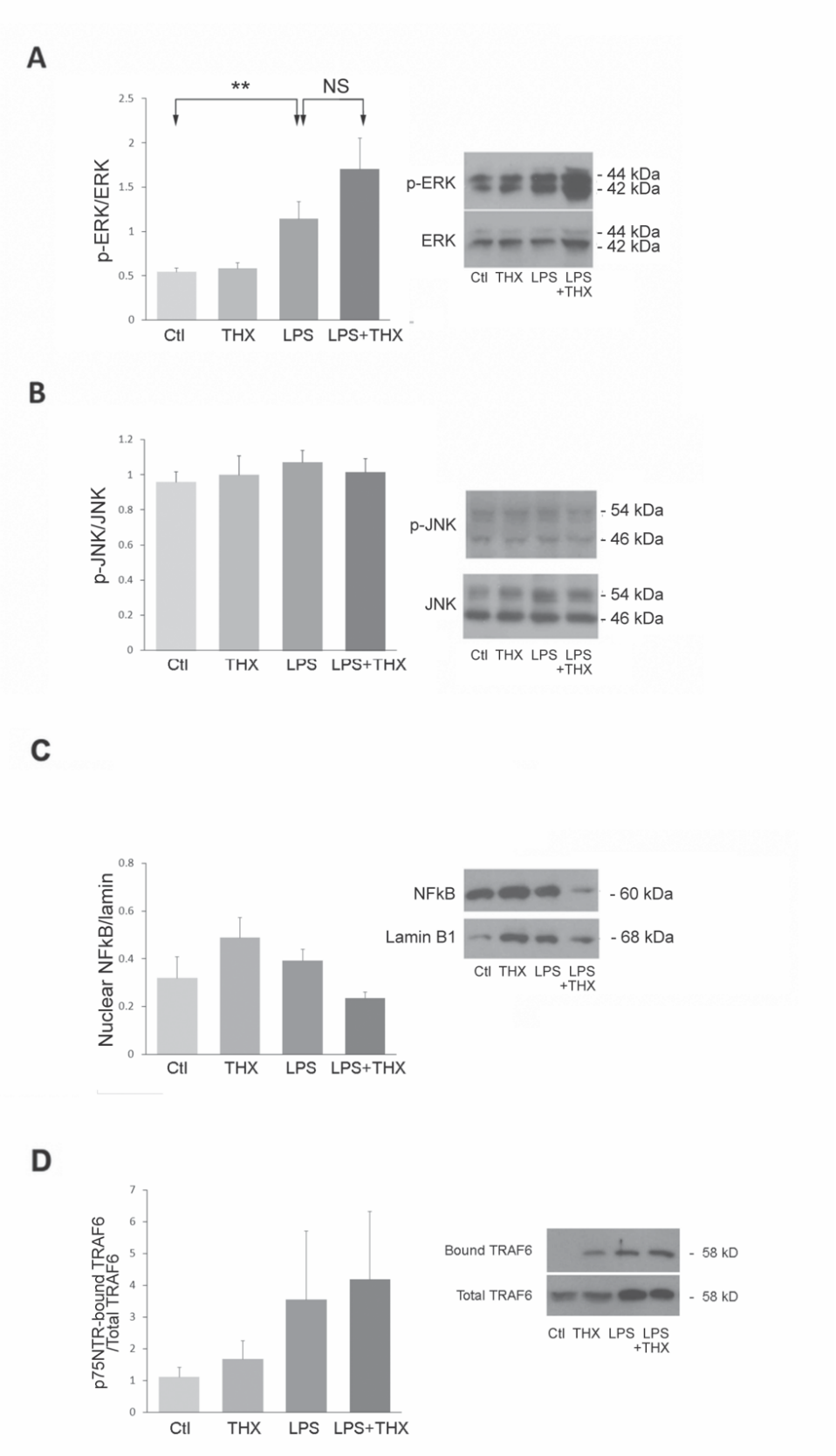
A
**
NS
p-ERK/ERK
2.5
2
1.5
1
0.5
0
Ctl
THX
LPS
LPS+THX
p-ERK
ERK
- 44 kDa
- 42 kDa
- 44 kDa
- 42 kDa
Ctl THX LPS LPS +THX
B
p-JNK/JNK
1.2
1
0.8
0.6
0.4
0.2
0
Ctl
THX
LPS
LPS+THX
p-JNK
JNK
- 54 kDa
- 46 kDa
- 54 kDa
- 46 kDa
Ctl THX LPS LPS +THX
C
Nuclear NFkB/lamin
0.8
0.6
0.4
0.2
0
Ctl
THX
LPS
LPS+THX
NFkB
Lamin B1
- 60 kDa
- 68 kDa
Ctl THX LPS LPS +THX
D
p75NTR-bound TRAF6 /Total TRAF6
7
6
5
4
3
2
1
0
Ctl
THX
LPS
LPS+THX
Bound TRAF6
Total TRAF6
- 58 kD
- 58 kD
Ctl THX LPS LPS +THX

Figure 4 Effects of LPS and THX-B on UTC signaling responses.

Cells were exposed to LPS (100 μg/mL) with or without THX-B (5 μg/mL) preincubation (A) for 2 min for assessment of ERK activation (N=6), (B) 10 min for JNK phosphorylation (N=6), (C) 5 min for NF-$\varkappa$B translocation into the nucleus (N=9) and (D) association of Traf6 to p75NTR (N=5). One-way ANOVA, **P <0.01, *P <0.05.

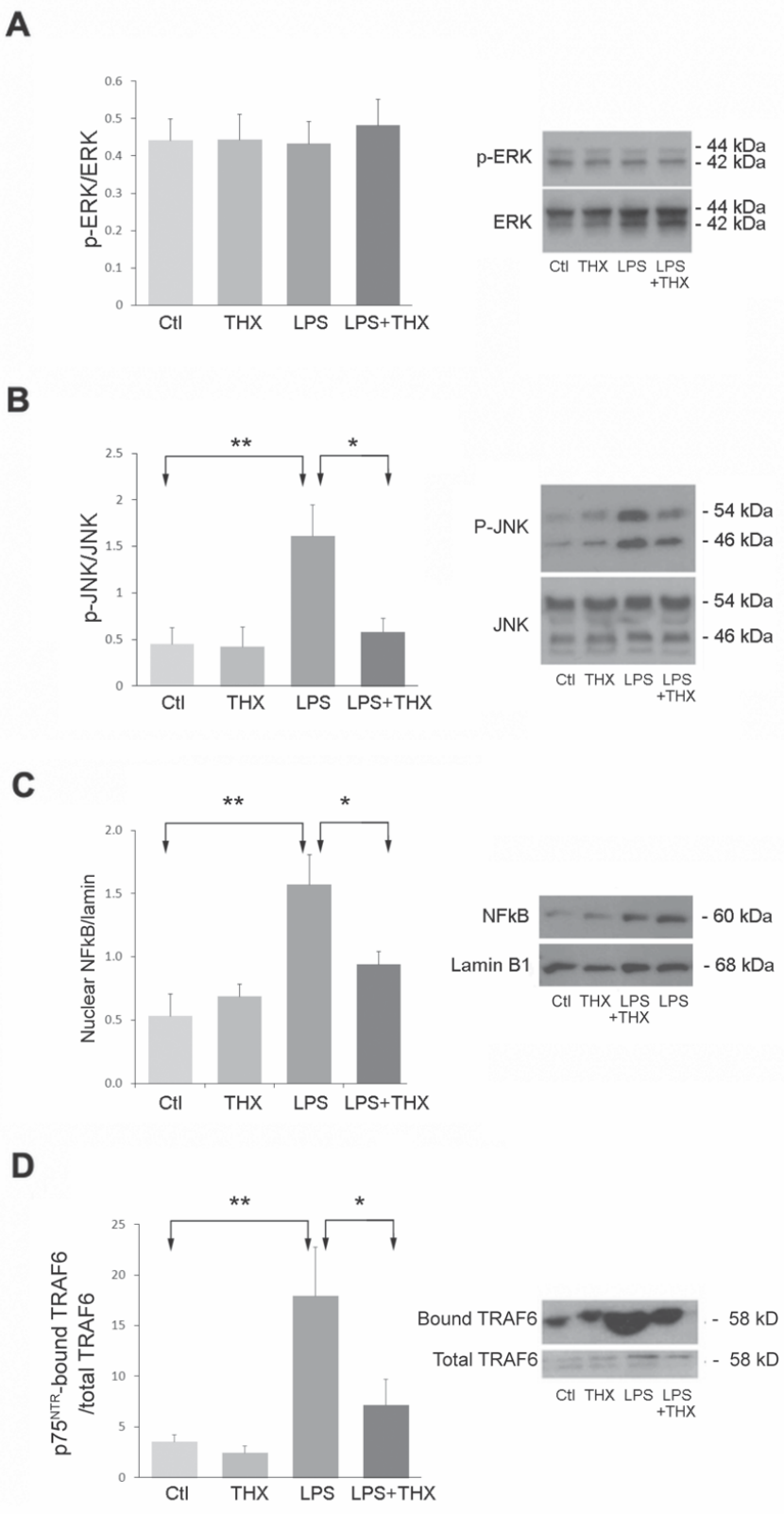
A
p-ERK/ERK
0.6
0.5
0.4
0.3
0.2
0.1
0
Ctl
THX
LPS
LPS+THX
p-ERK
ERK
- 44 kDa
- 42 kDa
- 44 kDa
- 42 kDa
Ctl THX LPS LPS +THX
B
p-JNK/JNK
**
*
2.5
2
1.5
1
0.5
0
Ctl
THX
LPS
LPS+THX
P-JNK
JNK
- 54 kDa
- 46 kDa
- 54 kDa
- 46 kDa
Ctl THX LPS LPS +THX
C
Nuclear NFkB/lamin
**
*
2.0
1.5
1.0
0.5
0.0
Ctl
THX
LPS
LPS+THX
NFkB
Lamin B1
- 60 kDa
- 68 kDa
Ctl THX LPS LPS +THX
D
p75NTR-bound TRAF6 /total TRAF6
**
*
25
20
15
10
5
0
Ctl
THX
LPS
LPS+THX
Bound TRAF6
Total TRAF6
- 58 kD
- 58 kD
Ctl THX LPS LPS +THX

Figure 5. Effects of LPS and THX-B on SMC signaling responses.

Exposure of cells to LPS (100 μg/mL) with or without pre-incubation with THX-B (5 μg/mL) was performed for (A) 2 min to detect activation ERK (N=6), (B) 10 min for JNK phosphorylation (N=6), and (C) 5 min for NF-κB translocation into the nucleus (N=9) and (D) association of Traf6 to p75NTR (N=5). One-way ANOVA, **P <0.01, *P <0.05.

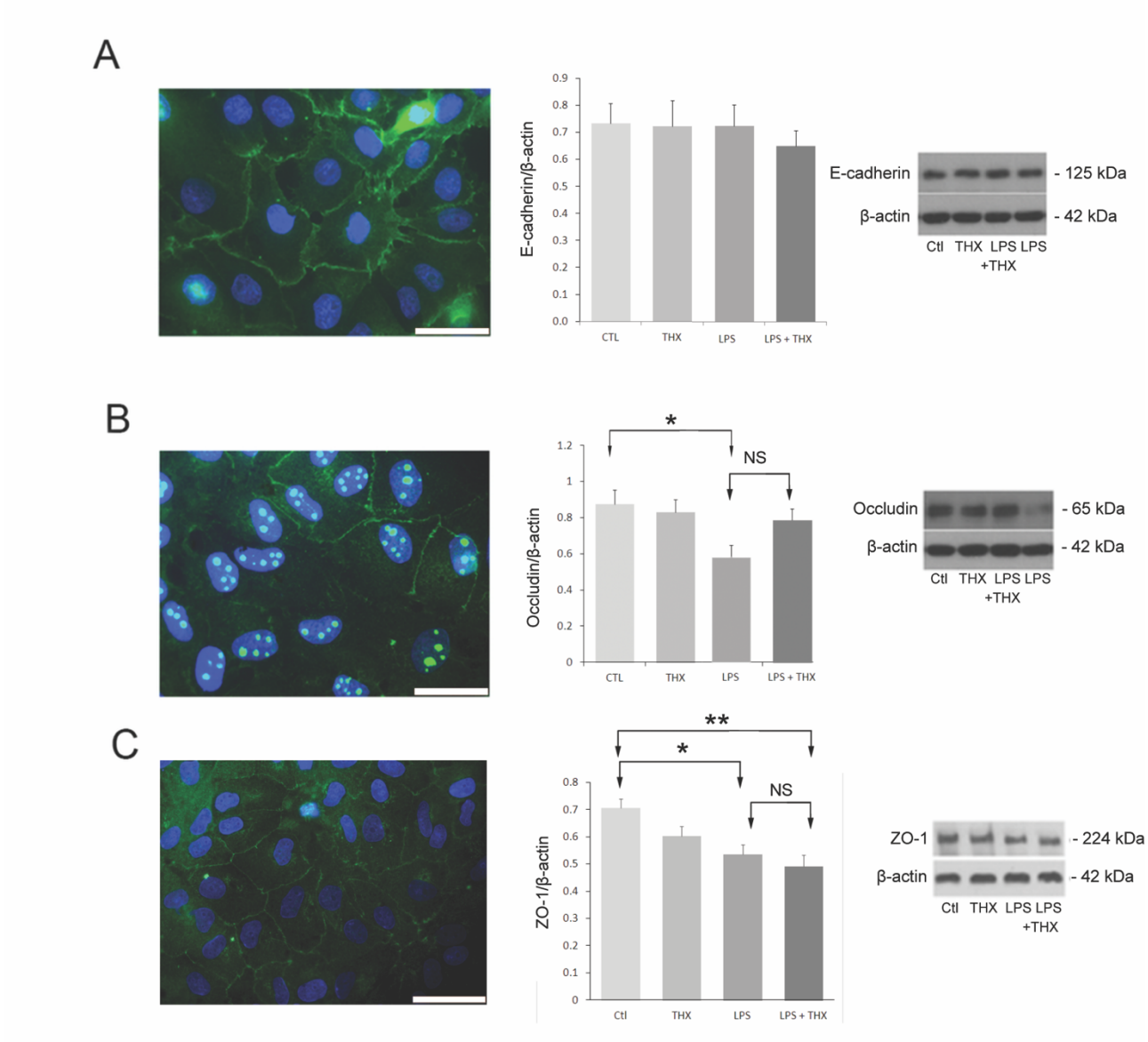
A
E-cadherin/β-actin
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CTL
THX
LPS
LPS + THX
E-cadherin
- 125 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX
B
*
NS
Occludin/β-actin
1.2
1
0.8
0.6
0.4
0.2
0
CTL
THX
LPS
LPS + THX
Occludin
- 65 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX
C
**
*
NS
ZO-1/β-actin
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ctl
THX
LPS
LPS + THX
ZO-1
- 224 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX

Figure 6. Expression of tight junctions in urothelial cells in response to LPS and THX-B.

Immunofluorescence detected (A) E-Cadherin, (B) Occludin and (C) ZO-1 expression on UTC membrane and in the cytoplasm. Immunoblotting of these proteins was performed after 24 hours exposure to LPS [100μg/ml] with or without THX-B [5μg/ml]. E-Cadherin (N=12), Occludin (N=15) and ZO-1 (N=12). One-way ANOVA, *P <0.05, **P <0.01. Bars = 50 μm.

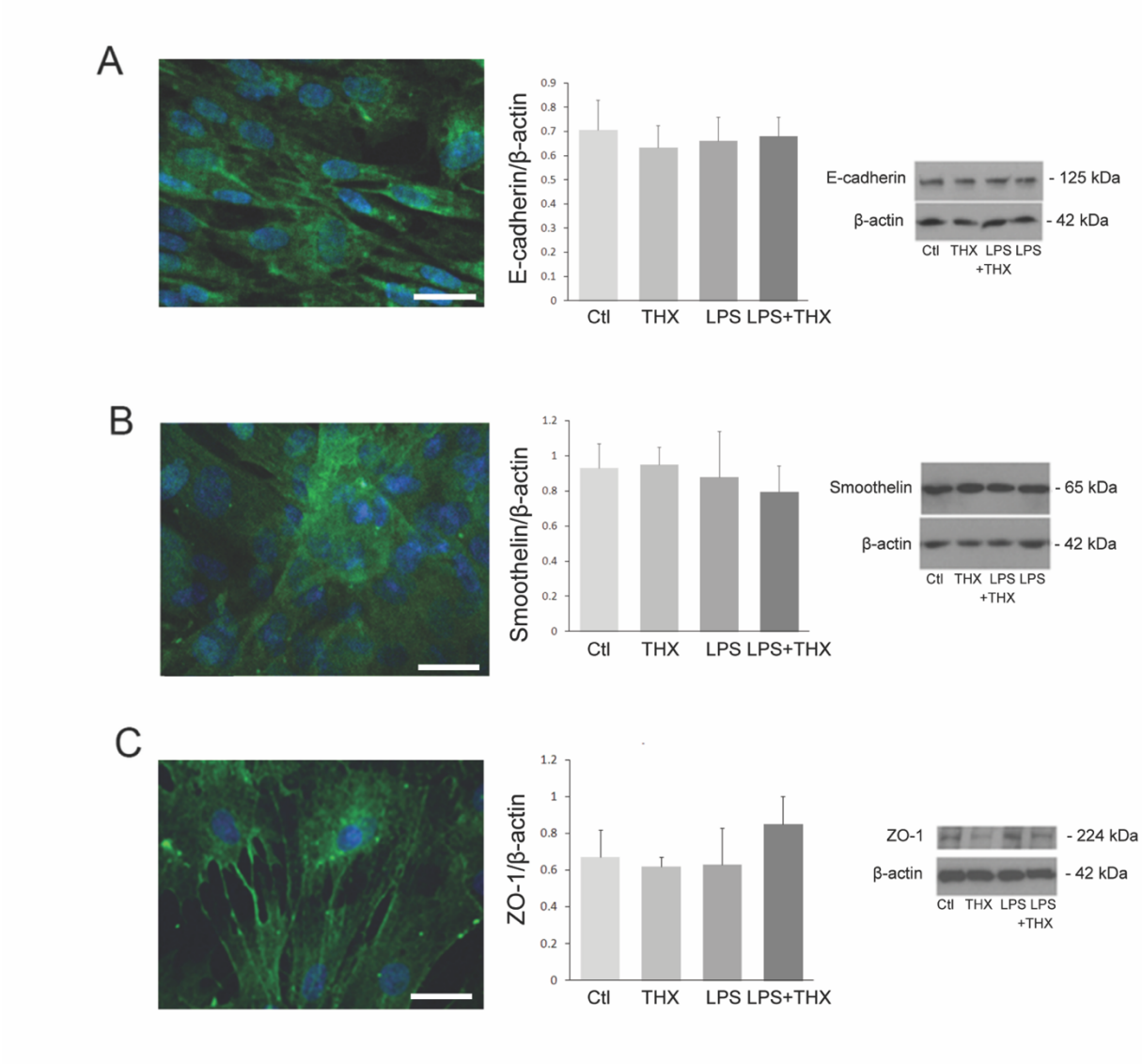
A
E-cadherin/β-actin
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ctl
THX
LPS
LPS+THX
E-cadherin
- 125 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX
B
Smoothelin/β-actin
1.2
1
0.8
0.6
0.4
0.2
0
Ctl
THX
LPS
LPS+THX
Smoothelin
- 65 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX
C
ZO-1/β-actin
1.2
1
0.8
0.6
0.4
0.2
0
Ctl
THX
LPS
LPS+THX
ZO-1
- 224 kDa
β-actin
- 42 kDa
Ctl THX LPS LPS +THX

Figure 7 Effect of LPS and THX-B on tight junction proteins expression in SMC.

Immunofluorescence revealed (A) Smoothelin, (B) E-Cadherin and (C) ZO-1 on the membrane and the cytoplasm of SMC. Immunoblotting showed unchanged expression after 24 hours incubation with LPS or THX-B for all proteins (N=6). Bars = 25 μm.

Chapter V. Role of proNGF/p75NTR on LPS-Induced Cystitis

5.1. Introduction

In the previous chapter, we clarified the role of p75NTR in cells of the bladder following short exposure to LPS. We saw that p75NTR triggered cell-specific inflammatory responses that interplay with TLR4 signal transduction. Not only this finding helps clarify the contribution of bacterial cystitis in chronic bladder inflammation, but also help us select an appropriate model mimicking the mechanisms involved in the pathogenesis of IC/PBS.

For that reason, in this chapter, we next move on to study the physiological effects of p75NTR on bladder function, tissues morphology and pain, using a mouse model of LPS-induced acute cystitis. Many authors have made use of that model in the past as such to study pelvic hypersensitivity, bladder inflammation and overactivity in mice. These features closely reflect the symptomatology of bacterial cystitis and IC/PBS symptoms which, in addition to being simple to reproduce with instillation of LPS, makes this animal model worth working with.

In this chapter, our goal is to demonstrate the physiological contribution of p75NTR in bladder inflammation in tissues, contractile function and pelvic pain after LPS-induced cystitis.

5.2. Materials and Methods

5.2.1. Ethics Statement

All animal experiments were approved by the McGill University Animal Ethics Committee (Montreal, Qc, Canada) and followed standards of the Canadian Council for Animal Care (CACC).

5.2.2. Preoperative Procedure

25 young adults female C57BL/6 mice aged 6-10-week-old (Charles River Laboratories, QC, Canada) were used. Mice were housed in groups of 3-5, had access to water and food (standard Purina chow, Teklad Global, WI, USA) *ad libitum,* and kept on a 12 h light/dark cycle. At least 1 week elapsed from their reception at the Lady Davis Institute (Montreal, QC, Canada) to the start of the preoperative procedure. Hair was removed from the pelvic area at the vicinity of the bladder with hair removal lotion (Nair, Church&Dwight, ON, Canada) under 2-2.5% isoflurane anesthesia. Sterile gauze pads were used to remove hair and lotion, and the skin was water-moistened with lubricant. Mice were at least 4 days to ensure the absence of skin irritation during testing.

5.2.3. Preparation of Catheters

Before surgery, a 30 cm long microtube (0.011" I.D x 0.025" O.D, Scientific Commodities Inc. AZ, USA) was bathed in a Cidex OPA bactericidal solution (Johnson&Johnson, ON, Canada) for 30 minutes. Soluble LPS was reconstituted by adding 1 mL PBS in 1 mg LPS powder (O55:B5

E. coli, Sigma-Aldrich, MO, USA). In a laminar flood hood, insulin syringes of 0.5 mL (Becton Dickinson and Company, NJ, USA), forceps, scissors, 50 mL sterile tubes, and a bag of 0.9% NaCl were sterilized with 70% ethanol and one ultraviolet (U.V.) cycle, typically 25-30 min. Syringes were filled with 150 μL sterile saline and the microtube was inserted on the syringe needle with forceps. The microtube loose end was cut 10 mm away from the tip of the needle and the absence of leakage was validated by evacuating 50 μL saline. The catheter was put in a tube and sterilized once again with one U.V. cycle prior to use.

5.2.4. Induction of Cystitis

Mice were assigned in 4 treatment groups for each experiment: (1) i.p. saline (control, n=6), (2) i.p. saline, intravesical LPS (LPS mice, n=6), (3) i.p. THX-B (THX-B, n=7), (4) i.p. THX-B, intravesical LPS (LPS+THX-B, n=6). Animals were anesthetised with 2-2.5% isoflurane and i.p. injected with 100 μL saline or THX-B at a dose of 50 μg/mouse in 100 μL PBS. The bladder was emptied using the method previously described by *Chew J. and Chua K*. Care was taken to confirm bladder emptying by applying medium abdominal pressure. The urethral meatus was cleaned with sterile gauze and chlorhexidine gluconate (Baxedin, Omega Laboratories, Montreal, Canada) and the catheter was l

ubricated with a water-based lubricant (Vaseline, Unilever Canada, Toronto, Canada). Transurethral catheterization was performed using the method of Zychlinsky Scharff A. and al, the method of which allowed to reach bladder lumen within 10 mm insertion. Bladders were slowly instilled with 100 μL saline or LPS at a dose of 100 μg/mouse. Catheters were secured with adhesive and maintained for 1 h. Mice were kept 24 h post-surgery prior to further testing.

5.2.5. Behavioral Testing

Von Frey test (VFT) was performed in mice in the light cycle (09:00 am) by a male experimenter at baseline and 24h post-surgery. The up-and-down method adapted from *Rudick and al*. was used to evaluate tactile allodynia and hyperalgesia at the pelvis using 6 von Frey filaments of ascending forces (0.04–4 g) (Exacta™, CA, USA) (Rudick et al. 2008). Mice were placed in individual metabolic cages and accustomed for at least 30 min prior to testing. Each filament was applied perpendicularly to different areas of the pelvis a total of 10 times for 1-2 s with an interstimulus interval of 5 s. Positive nociception behavior were defined as 1) sharp retraction of the abdomen, 2) immediate licking or scratching of the stimulated area, and 3) jumping. All responses were recorded.

5.2.6. Voiding-Spot Assay

Mice were placed in individual cages with free access to food without water for 4 h. Cages were fitted with chromatography paper, grade 3 mm CHR (Whatman, GE Healthcare, UK). Voiding-Spot Assay (VSA) was performed at baseline and 24 h post-surgery. The urine spot pattern was assessed by imaging the chromatographic paper with U.V. light using the SynGene Bioimaging system (USA) connected the GeneSnap software. Images were analyzed with the ImageJ software using a calibration curve.

5.2.7. Tissue and Protein Extraction

Following 2-2.5% isoflurane anaesthesia and cardiac exsanguination, the urinary bladder was removed and sliced into two equal halves. For protein extraction, one half was immediately snapped frozen on dry ice and kept at -80˚C until homogenization in RIPA buffer containing an antiprotease cocktail (Complete Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN, USA). The second half was fixed in 4% formaldehyde and successively immersed in 10, 20 and 30% sucrose in PBS for 24 h. Fixed tissues were covered with OCT (Leica Biosystems, IL, USA) and left for acclimatization a few minutes before being frozen on dry ice. Tissue sections were prepared at 7 μm thickness using a Leica CM3050S cryostat.

5.2.8. Contractile Assay: Organ Bath

The urinary bladder was removed following euthanasia, emptied of urine and weighted. Under a microscope, the bladder dome and base were removed, and two longitudinal strips of 2 x 5 mm were cut from the bladder body surface without removing the urothelium. The remaining tissues were kept for protein extraction. Strips were mounted in a 4-channel Tissue Bath System – 720 MO (DMT Inc., Ann Arbor, MI) with 6 mL of Krebs-Ringer solution (95% O_2, 5% CO_2, 37˚C). Stabilization was ensured at a passive tension of 0.5 g, changing the Krebs-Ringer solution every 15 min for a total of 1 h. Strips were then stimulated with 60 mM KCl twice, carbachol (3 nM–100 μM), and electrical field stimulation (EFS; 1–32 Hz) using a Grass Technologies S88 Stimulator (West Warwick, RI, USA), adequately washing strips between stimulations. Tension

values were normalized to the respective weight of strips and analysed with the LabChart 7 software (ADInstrument, CO, USA).

5.2.9. Histology

Tissues sections were staining following a standard protocol for Hematoxylin and Eosin. Bladder wall thickness was measured using a LSM800 – Airyscan microscope.

5.2.10. Immunoblotting

Protein concentration in bladder homogenates was measured by Micro BCA assay kit (Boster Biological Technology, CA, USA). A total amount of 20 μg protein was loaded on a 8% polyacrylamide gel, then transferred on PVDF membranes. Blockage was performed for 1 h at room temperature with 5% non-fat skimmed milk in TBST. Membranes were incubated overnight at 4°C with primary antibodies against pJNK (1:1000) and JNK (1:2000). Incubation with secondary anti-rabbit and anti-mouse antibodies (1:2000) was done for 1 h at room temperature. Bands were revealed with the Immobilon Crescendo Western horseradish peroxidase (HRP) substrate (Millipore Corporation, MA, USA), then quantified by ImageJ.

5.2.11. ELISA assay for TNF-α

An amount of 50 μg total protein was taken from bladder homogenates to measure the content of TNF-a in bladder tissues. The protocol and data analysis were both performed as outlined by the manufacturer (TNF-α, Catalog# EK0527, Boster Bio, CA, USA).

5.2.12. Statistical Analysis

Values are indicated as the mean ± SEM. Statistical analyzes were performed using the GraphPad Prism 9 software. Multiple comparisons between the treatment groups were done by One-way ANOVA with post-hoc Bonferroni. For behavioral testing, two-way ANOVA followed by post-hoc Bonferroni was used to account for both treatment effects and time points (Baseline vs Day 1). Maximal tensions and EC_{50} values were determined with the dose-response curve to carbachol and comparisons were carried out with the Extra sum-of-square F Test. Significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

5.3. Results

Pelvic sensitivity after treatments of THX and LPS: Bladder urothelial and detrusor smooth muscle cells express receptors p75NTR and TLR4 on their surface. TLR4 has been demonstrated by several studies to have nociceptive function in animals and humans with IC/PBS. Of most interest, it has been noted to induce pelvic pain in different mouse models of cystitis. To verify if p75NTR antagonism could help reduce pain, we first tested pelvic sensitivity in mice before and after treatment. At baseline, a significant effect of forces was observed on the frequency of pelvic response (F=170.2, P=0.001), which increased with increasing filament forces. Furthermore, there was no significant effect of the preoperative procedure on the number of pelvic responses (F=1.20, P=0.344). We observed no significant difference in pelvic response to increasing forces across mice at baseline at each filament force (Fig. 7A). Thus, this indicates that all mice equally displayed force-dependent pelvic responses before surgery.

At 24 h post-surgery, there was a significant force x treatment interaction on the frequency of pelvic responses across the treatment groups (F=2.22, P=0.008). A significant force x treatment interaction was observed on the frequency of pelvic responses in THX-B mice (F=2.22, P=0.008). THX-B injection significantly increased the frequency of pelvic responses to low filament forces (0.04–0.16 g) compared to baseline. On the other hand, we found no force x treatment interaction in LPS mice (F=1.73, P=0.150). There was a significant effect of force in LPS mice (F=27.60, P=0.001), as seen at baseline. LPS also increased the pelvic response frequency to low filament forces (0.04–0.16 g) compared to baseline, as a result of a treatment effect (F=2.22, P=0.008). Administration of THX-B in LPS-mice generated a significant force x treatment interaction

(F=4.97, P=0.001). LPS+THX-B mice showed significantly more frequent pelvic responses at low filament forces (0.04–0.4 g) compared to baseline (Fig. 7B). Furthermore, the frequency of pelvic responses in LPS+THX-B mice at low filament forces (0.04–0.16 g) was not decreased compared to LPS. In fact, both treatment groups showed no significant difference in their frequency at these forces. (Fig. 7B)

Effects of THX-B and LPS on bladder strips contractile response to carbachol, KCl and EFS: Intravesical instillation of LPS has been shown to decrease post-residual volumes in mice, suggesting that mice had bladder overactivity(Takezawa et al. 2014). To determine if blockage of p75NTR could help improve contractions, we examined the response of bladder strips with intact urothelium to specific stimulation of purinergic receptors with carbachol. Our data showed no difference in contractions between THX-B and control with increasing carbachol concentrations. (Fig. 8A) On the other hand, LPS strips presented significantly higher contractile potency compared to control at high carbachol concentrations (10–100 μM). (Fig. 8A) Their half maximal effective concentration (EC_{50}) was also significantly increased compared to control. (Fig. 8B) Similar contractile response patterns to LPS were found in LPS+THX-B strips with carbachol. (Fig. 8A) To confirm these changes were only purinergic and muscarinergic receptor-mediated, bladder strips were voltage-gate depolarized with KCl. There was no difference observed in contractile response between all treatment groups. (Fig. 8D) Acute instillation of LPS in the bladder has also shown to enhance mouse detrusor contractions during nerve stimulation by EFS (Weng et al. 2009). We found that stimulation of LPS and LPS+THX-B strips with increasing electrical field frequencies induced no difference in contractions compared to control. (Fig. 8C)

On the other hand, contractile potency was reduced in THX-B strips at 32 Hz compared control. (Fig. 8C)

Voiding patterns of mice with or without cystitis and THX treatment: To understand how change in contractility affects bladder compliance in mice after treatment of p75NTR antagonism with or without cystitis, mice were tested before and 24h after treatment for voiding pattern during 4h. Our results show that there was no difference in the number of urine spot (Fig. 9A) and total volume of urine (Fig. 9C) across the treatment groups at baseline or 24h post-treatment. Nonetheless, LPS+THX mice showed a significantly higher mean urine volumes per spot at baseline compared to control. (Fig. 9B) After 24h post-treatment, these same mice showed a significant decrease in their mean urine volumes per spot compared to baseline. However, this change was not significantly different from that of control at 24h post-treatment. (Fig. 9B)

Bladder inflammatory characteristics in mice with or without cystitis and THX-B treatment: At 24h after treatment in mice, we next assessed molecular and histologic characteristics attributed to features of inflammation in the bladder. These included accumulation of TNF-α in bladder tissues, activation of the JNK signaling pathways, as well as change in bladder thickness. We found that mice instilled with LPS had significantly increased activation of JNK signaling compared to control. (Fig. 10A) By injecting of THX-B in LPS mice, the ratio of pJNK/JNK was significantly returned to control levels. (Fig. 10A) On the other hand, there was no difference in the expression of TNF-α levels in bladder tissues across the groups (Fig. 10B). We next observed that the ratio of mucosal/detrusor layer length of LPS mice was significantly higher compared to control. Concomitant addition of THX-B in these mice then partially decreased this ratio.

Discussion

In this chapter, we were successful in showing that p75NTR plays a physiological function in inflammation of the bladder. Furthermore, our findings indicate that this receptor also directly interplay at the molecular level with TLR4 signal transduction to have deleterious impact on bladder morphology.

We have found that blockage of p75NTR effectively prevents the activation of JNK signaling in bladder tissues after acute exposition to LPS. Activation of JNK in response to damaging stimulation is in line with previous reports that have shown similar finding using different models of cystitis (Klinger and Vizzard 2008; Zhao et al. 2016). Our experiment further reveals that the onset of this molecular cascade emerges from an interaction between the receptor of proNGF and LPS. Assuming that this reflects our previous finding *in vitro*, where SMC have increased JNK activity in response to LPS, the activation of p75NTR may be involved in differentiation of SMC from a contractile phenotype to a migratory and proliferative phenotype. Some studies have indicated that the JNK effector also enhances collagen production and proliferation of SMC after tissues injuries, the outcomes of which is associated with hypertrophy and hyperplasia of the bladder (Xiao et al. 2012; Mossa, Galan, et al. 2020b). Until now, it is difficult to validate this concept, but more work investigating the effect of p75-induced JNK signaling on detrusor muscle function could address this avenue.

We also detected that detrusor muscle strips having intact mucosal layer had partially decreased contraction efficacy to EFS stimulation after blockage of p75NTR without cystitis. EFS is used to

depolarizes cholinergic and purinergic neurons secreting acetylcholine (ACh) and ATP(White et al. 2015). Given there was no change in contractions with KCl and carbachol, a pharmacological analog of ACh, we believe that desensitization of the detrusor muscle to purinergic stimulation is a function that p75NTR modulates in the bladder. A potential mechanism could involve the inhibition of P2X3, a purinergic receptor expressed on sensory nerves and urothelial cell in mice and rats.(Ferguson et al. 2015) One study has shown that antagonism of P2X3 reduces purinergic response of SMC to EFS stimulation, while not changing contraction to Ach (Ferguson et al. 2015). Future work should focus on examining change in purinergic regulation of p75NTR to clarify its underlying mechanisms in contractile function.

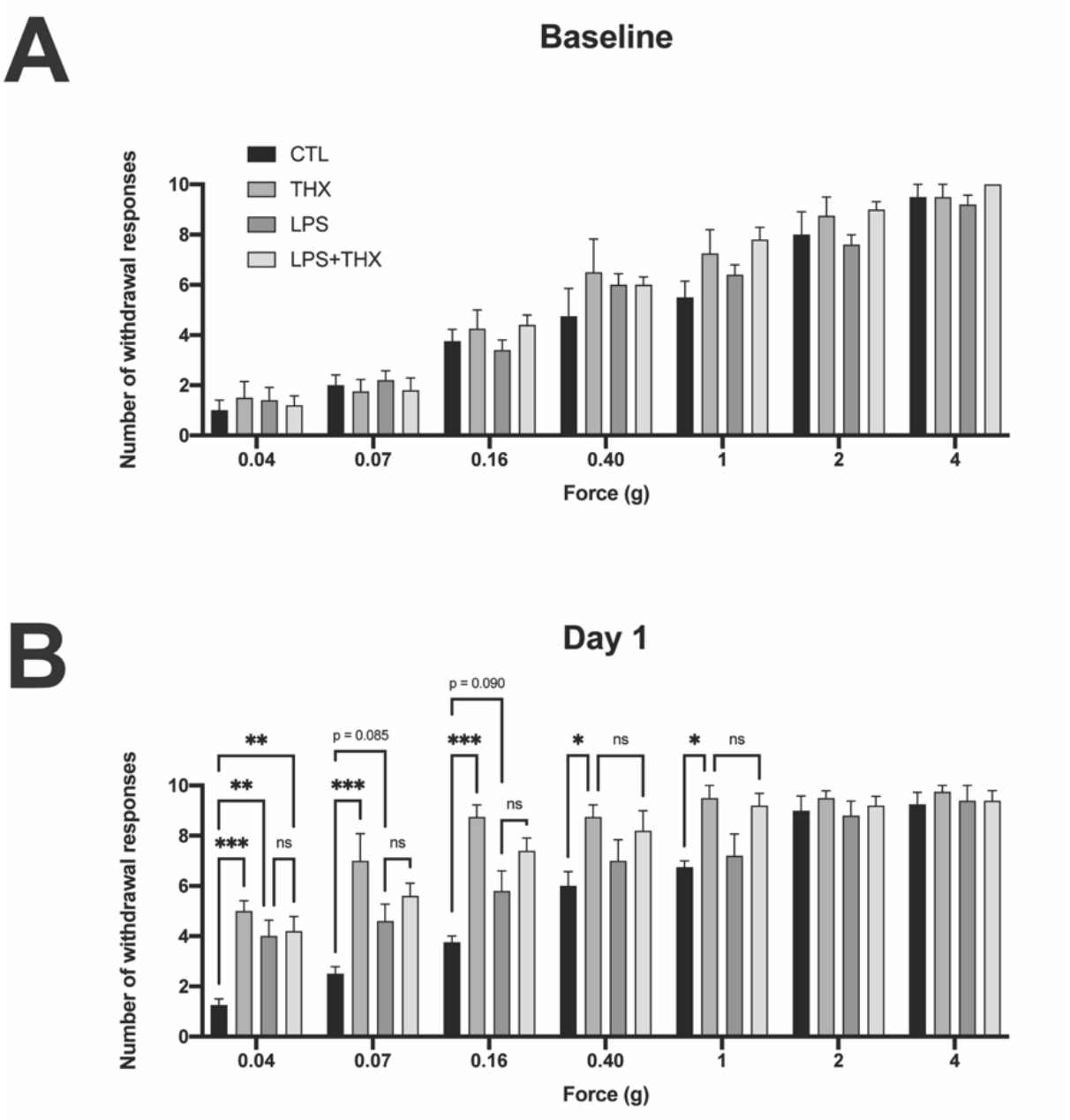
A
Baseline
CTL
THX
LPS
LPS+THX
Number of withdrawal responses
0
2
4
6
8
10
0.04
0.07
0.16
0.40
1
2
4
Force (g)
B
Day 1
p = 0.090
p = 0.085
**
**
***
ns
***
ns
***
ns
*
ns
*
ns
Number of withdrawal responses
0
2
4
6
8
10
0.04
0.07
0.16
0.40
1
2
4
Force (g)

Figure 8. Pelvic pain response of mice to von Frey filaments of increasing forces.

Frequency of pain response observed in mice at baseline (A) and after 24h (B) post-instillation of 100µL LPS (1mg/mL) with or without i.p. injection of 100 µL THX (0.5mg/mL). Mice were stimulated 10 times per filament forces between 0.04–4g. Mean ± SEM, N=5/group, two-way ANOVA with post-hoc Bonferonni (*P < 0.05, **P < 0.01, ***P < 0.001).

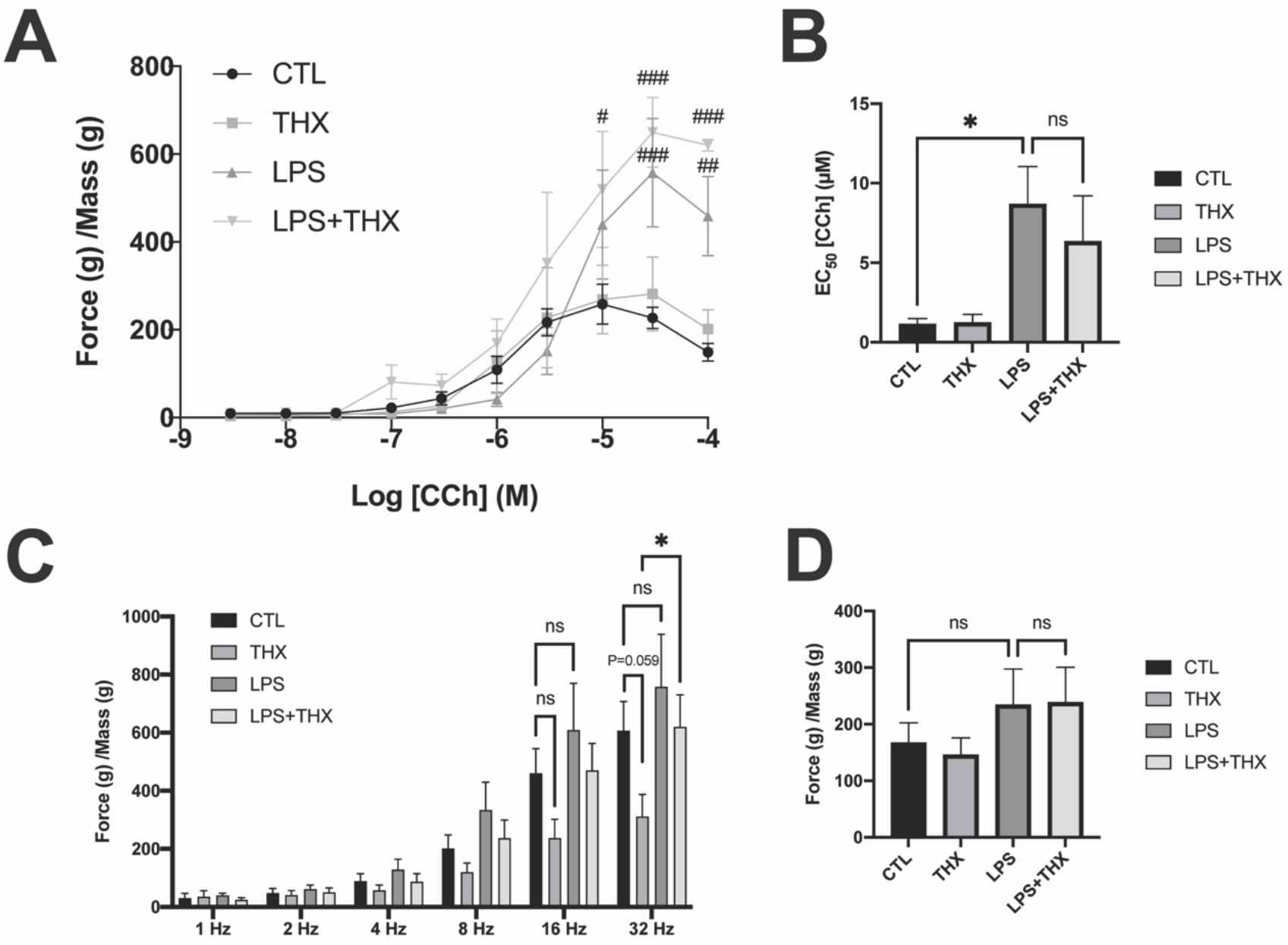
A
800
600
400
200
0
Force (g) /Mass (g)
CTL
THX
LPS
LPS+THX
###
#
###
###
##
-9
-8
-7
-6
-5
-4
Log [CCh] (M)
B
15
10
5
0
EC50 [CCh] (µM)
*
ns
CTL
THX
LPS
LPS+THX
C
1000
800
600
400
200
0
Force (g) /Mass (g)
CTL
THX
LPS
LPS+THX
ns
ns
P=0.059
ns
*
1 Hz
2 Hz
4 Hz
8 Hz
16 Hz
32 Hz
D
400
300
200
100
0
Force (g) /Mass (g)
ns
ns
CTL
THX
LPS
LPS+THX

Figure 9 Contractile response of mouse bladder strips to carbachol, KCl and EFS stimulation.

Contraction of bladder strips from mice after 24h instillation of 100 µL LPS (1mg/mL) for 1h, with or without i.p. injection of 100 µL THX-B (0.5 mg/mL). (A) Kill curve of the dose-response to carbachol 3 nM–100 µM. (B) Graph bars of the EC_{50} values to carbachol. (C) Graph bars of the contractile response to electrical field stimulation 1–32 Hz. (D) Graph bars of the contractile response to 60 mM KCl. Force is represented as the ratio of the tension in gram to the bladder strip weight in gram. Mean ± SEM, N=7/group, EC_{50} values of each experiment was determined with the F-test for non-linear regression. one-way ANOVA and two-way ANOVA with post-hoc Bonferonni (*P < 0.05, **P < 0.01, ***P< 0.001; #P < 0.05, ##P < 0.01, #P < 0.001 vs CTL)

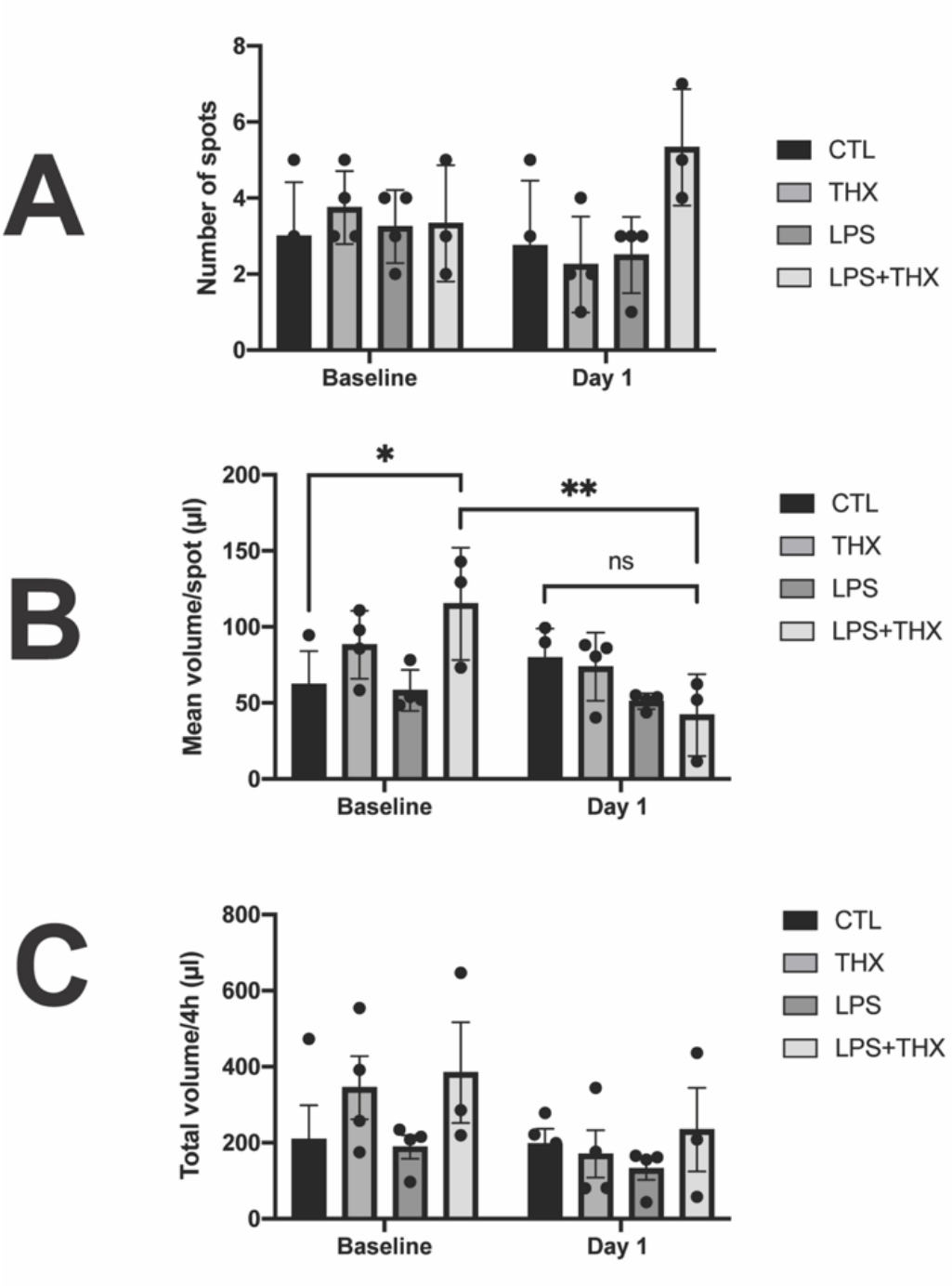
A
Number of spots
8
6
4
2
0
Baseline
Day 1
CTL
THX
LPS
LPS+THX
B
Mean volume/spot (µl)
200
150
100
50
0
*
**
ns
Baseline
Day 1
CTL
THX
LPS
LPS+THX
C
Total volume/4h (µl)
800
600
400
200
0
Baseline
Day 1
CTL
THX
LPS
LPS+THX

Figure 10. Voiding patterns in mice with or without cystitis and THX-B treatment.

(A) Number of urine spots, (B) mean volume per spot and (C) total voided urine volume in mice performing voiding-spot assay for 4h before and 24h after surgery. Mean ± SEM, N=4/group. One-way ANOVA with post-hoc Bonferonni (*P < 0.05, **P < 0.01, ***P< 0.001)

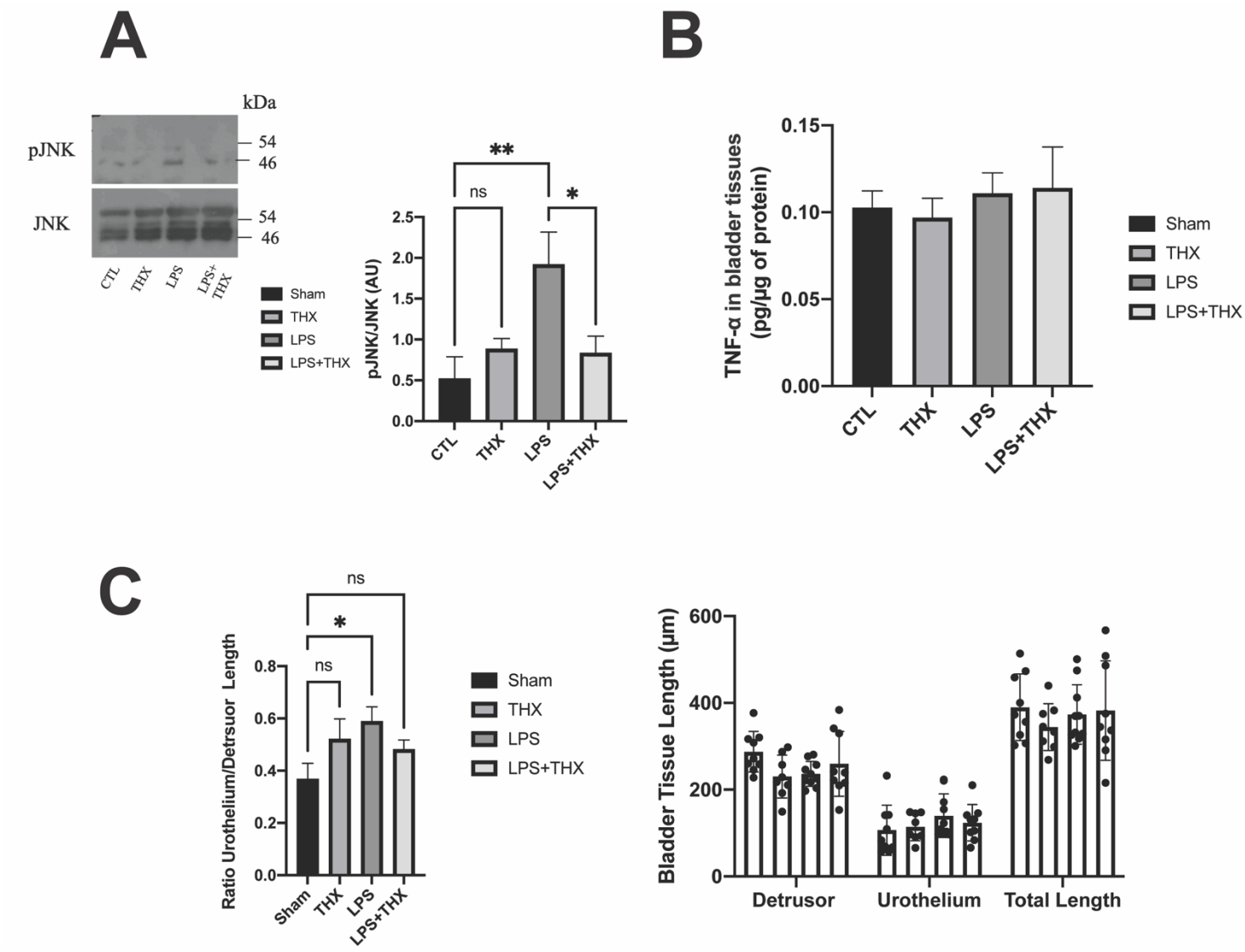
A
kDa
pJNK
54
46
JNK
54
46
CTL
THX
LPS
LPS+THX
Sham
THX
LPS
LPS+THX
pJNK/JNK (AU)
**
ns
*
2.5
2.0
1.5
1.0
0.5
0.0
CTL
THX
LPS
LPS+THX
B
TNF-α in bladder tissues (pg/μg of protein)
0.15
0.10
0.05
0.00
CTL
THX
LPS
LPS+THX
Sham
THX
LPS
LPS+THX
C
Ratio Urothelium/Detrsuor Length
ns
*
ns
0.8
0.6
0.4
0.2
0.0
Sham
THX
LPS
LPS+THX
Sham
THX
LPS
LPS+THX
Bladder Tissue Length (μm)
600
400
200
0
Detrusor
Urothelium
Total Length

Figure 11. Bladder characteristics with or without cystitis and THX-B treatment.

(A) Quantification of the bands density observed by Western blotting for JNK and pJNK in bladder tissues at 24h post-treatment (N=6-8 mice/group). (B) Quantification of the concentration of TNF-a detected by ELISA kit assay in bladder tissues at 24h post-treatment. (N=6-8 mice/group) (C) Quantification of the length of the mucosal and detrusor muscle layers and total bladder section at 24h post-treatment (N=1-2 bladders/group). One-way ANOVA with post-hoc Bonferonni (*$P < 0.05$, **$P < 0.01$)

Chapter VI. Conclusion

In conclusion, adopting an aetiological-based research perspective opens the path to new potential mechanisms by which previous conditions such as acute bacterial cystitis activate onset of IC/PBS symptoms that would otherwise be screened and treated more efficiently early. Potential aetiologies found in the epidemiological literature deserve more attention for the molecular effects they have on bladder physiology over time and on therapy responses. Here, an association between bacterial cystitis and IC/PBS is strongly suggested through an interplay between the receptor p75NTR found in many bladder disorders and early activation of receptors TLR4. This connection is worth further investigations as an indicator of sustained inflammatory response potentially leading to IC/PBS symptoms.

The detrimental role of p75NTR on bladder dysfunction has been previously demonstrated in diabetes mellitus and spinal-cord injury. It engages TNF-a production in urothelial cells and proliferation of smooth muscle cells through NF-kB after several weeks in mice with diabetes mellitus. The increased of TNF-a levels expected here as well with insult of LPS was found *in vitro* but not *in vivo*, a difference that might be attributable to a mechanism that develop over a longer period of time and for which our acute model of cystitis was unsuited for. Indeed, mice used here were sacrificed within a week and induced with acute cystitis only. Therefore, an interplay between p75NTR and TLR4 on TNF-a expression over time should be investigated.

We found a TLR4/TRAF6/JNK/NF-kB signaling interplay *in vitro* that was formed in smooth muscle cells and translated *in vivo* through increased activation of JNK signaling. Early activation

of TLR4 seem to activate p75NTR through the TRAF6 adaptor binding its death domain, leading to phosphorylation of JNK in detrusor muscle cells and early increase in bladder thickness. The individual role that each component of the TLR4/TRAF6/JNK/NF-kB axis plays in detrusor muscle dysfunction, especially in relation to IC/PBS symptoms, is unclear but should be investigated specifically. Yet, it is clear from our study that p75NTR antagonism holds sufficient potency to block its deteriorating effects on bladder function and structure.

References

Anger, J., U. Lee, A. L. Ackerman, R. Chou, B. Chughtai, J. Q. Clemens, D. Hickling, A. Kapoor, K. S. Kenton, M. R. Kaufman, M. A. Rondanina, A. Stapleton, L. Stothers, and T. C. Chai. 2019. 'Recurrent Uncomplicated Urinary Tract Infections in Women: AUA/CUA/SUFU Guideline', *J Urol*, 202: 282-89.

Bai, Yujing, Pauline Dergham, Hinyu Nedev, Jing Xu, Alba Galan, Jose Carlos Rivera, Shi Zhihua, Hrishikesh M. Mehta, Sang B. Woo, Marinko V. Sarunic, Kenneth E. Neet, and H. Uri Saragovi. 2010. 'Chronic and Acute Models of Retinal Neurodegeneration TrkA Activity Are Neuroprotective whereas p75NTR Activity Is Neurotoxic through a Paracrine Mechanism', *Journal of Biological Chemistry*, 285: 39392-400.

Barcelona, P. F., N. Sitaras, A. Galan, G. Esquiva, S. Jmaeff, Y. Jian, M. V. Sarunic, N. Cuenca, P. Sapieha, and H. U. Saragovi. 2016. 'p75NTR and Its Ligand ProNGF Activate Paracrine Mechanisms Etiological to the Vascular, Inflammatory, and Neurodegenerative Pathologies of Diabetic Retinopathy', 36: 8826-41.

Behzadi, Elham, and Payam Behzadi. 2016. 'The role of toll-like receptors (TLRs) in urinary tract infections (UTIs)', *Central European journal of urology*, 69: 404-10.

Birder, Lori A., and William C. de Groat. 2007. 'Mechanisms of disease: involvement of the urothelium in bladder dysfunction', *Nature clinical practice. Urology*, 4: 46-54.

Birder, Lori, and Karl-Erik Andersson. 2013. 'Urothelial signaling', *Physiological reviews*, 93: 653-80.

Bjorling, D. E., H. E. Jacobsen, J. R. Blum, A. Shih, M. Beckman, Z. Y. Wang, and D. T. Uehling. 2001. 'Intravesical Escherichia coli lipopolysaccharide stimulates an increase in bladder nerve growth factor', *BJU International*, 87: 697-702.

Chagnon, Jonathan, Patrice Dion, Nora Galbraith, Elham Sirag, and Yuxianxian Zhang. 2019. "Population Projections for Canada (2018 to 2068), Provinces and Territories (2018 to 2043):

Technical Report on Methodology and Assumptions." In, edited by Statistics Canada. Statistics Canada.

Chancellor, Michael B. 2007. 'A Multidisciplinary Consensus Meeting on IC/PBS: Outcome of the Consensus Meeting on Interstitial Cystitis/Painful Bladder Syndrome, February 10, 2007, Washington, DC', *Reviews in urology*, 9: 81-83.

Chang, Angela Y., Vegard F. Skirbekk, Stefanos Tyrovolas, Nicholas J. Kassebaum, and Joseph L. Dieleman. 2019. 'Measuring population ageing: an analysis of the Global Burden of Disease Study 2017', *The Lancet Public Health*, 4: e159-e67.

Chen, Wei, Da-Yong Ye, Deng-Jun Han, Guang-Qing Fu, Xiang Zeng, Wei Lin, and Yong Liang. 2016. 'Elevated level of nerve growth factor in the bladder pain syndrome/interstitial cystitis: a meta-analysis', *SpringerPlus*, 5.

Chuang, Fei-Chi, Hsin-Tzu Liu, and Hann-Chorng Kuo. 2016. 'Lower Levels of Urinary Nerve Growth Factor Might Predict Recurrent Urinary Tract Infections in Women', *International Neurourology Journal*, 20: 33-39.

Chung, Hong. 2016. 'The Association between Chronic Inflammation and Recurrent Cystitis in Women', *Urogenital Tract Infection*, 11: 86.

Cox, Ashley, Nicole Golda, Genevieve Nadeau, J. Curtis Nickel, Lesley Carr, Jacques Corcos, and Joel Teichman. 2016. 'CUA guideline: Diagnosis and treatment of interstitial cystitis/bladder pain syndrome', *Canadian Urological Association journal = Journal de l'Association des urologues du Canada*, 10: E136-E55.

Cuello, A. Claudio, Rowan Pentz, and Hélène Hall. 2019. 'The Brain NGF Metabolic Pathway in Health and in Alzheimer's Pathology', *Frontiers in Neuroscience*, 13.

Cui, X., X. Jing, S. K. Lutgendorf, C. S. Bradley, A. Schrepf, B. A. Erickson, V. A. Magnotta, T. J. Ness, K. J. Kreder, M. A. O'Donnell, and Y. Luo. 2019. 'Cystitis-induced bladder pain is Toll-like receptor 4 dependent in a transgenic autoimmune cystitis murine model: a MAPP Research Network animal study', *Am J Physiol Renal Physiol*, 317: F90-f98.

Davis, Niall F., Sanjith Gnanappiragasam, and John A. Thornhill. 2015. 'Interstitial cystitis/painful bladder syndrome: the influence of modern diagnostic criteria on epidemiology and on Internet search activity by the public', *Translational andrology and urology*, 4: 506-11.

de Groat, William C., and Naoki Yoshimura. 2009. 'Afferent nerve regulation of bladder function in health and disease', *Handbook of experimental pharmacology*: 91-138.

Dhillon, Bipandeep, Fatemah Aleithan, Zahi Abdul-Sater, and Ali A. Abdul-Sater. 2019. 'The Evolving Role of TRAFs in Mediating Inflammatory Responses', *Frontiers in Immunology*, 10.

Dou, Yang, Xiaodi Tian, Jian Zhang, Zhong Wang, and Gang Chen. 2018. 'Roles of TRAF6 in central nervous system', *Current Neuropharmacology*, 16.

Dugan, C., S. Malley, L. Arms, V. May, and M. A. Vizzard. 2014. 'Role of c-Jun N-terminal kinase (JNK) activation in micturition reflexes in cyclophosphamide (CYP)-induced cystitis in female rats', *Journal of molecular neuroscience : MN*, 54: 360-69.

Elshaer, Sally L., Abdulrahman Alwhaibi, Riyaz Mohamed, Tahira Lemtalsi, Maha Coucha, Frank M. Longo, and Azza B. El-Remessy. 2019. 'Modulation of the p75 neurotrophin receptor using LM11A-31 prevents diabetes-induced retinal vascular permeability in mice via inhibition of inflammation and the RhoA kinase pathway', *Diabetologia*, 62: 1488-500.

Fahnestock, Margaret, and Arman Shekari. 2019. 'ProNGF and Neurodegeneration in Alzheimer's Disease', *Frontiers in Neuroscience*, 13.

Ferguson, Andrew C., Broderick W. Sutton, Timothy B. Boone, Anthony P. Ford, and Alvaro Munoz. 2015. 'Inhibition of urothelial P2X3 receptors prevents desensitization of purinergic detrusor contractions in the rat bladder', *BJU International*, 116: 293-301.

Fields, Jerel, Wilmar Dumaop, T. D. Langford, Edward Rockenstein, and E. Masliah. 2014. 'Role of neurotrophic factor alterations in the neurodegenerative process in HIV associated neurocognitive disorders', *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, 9: 102-16.

Flores-Mireles, Ana L., Jennifer N. Walker, Michael Caparon, and Scott J. Hultgren. 2015. 'Urinary tract infections: epidemiology, mechanisms of infection and treatment options', *Nature Reviews Microbiology*, 13: 269-84.

Freria, C. M., D. Bernardes, G. L. Almeida, G. F. Simões, G. O. Barbosa, and A. L. R. Oliveira. 2016. 'Impairment of toll-like receptors 2 and 4 leads to compensatory mechanisms after sciatic nerve axotomy', *Journal of Neuroinflammation*, 13.

Galan, Alba, Pablo F. Barcelona, Hinyu Nedev, Marinko V. Sarunic, Yifan Jian, and H. Uri Saragovi. 2017. 'Subconjunctival Delivery of p75NTR Antagonists Reduces the

Inflammatory, Vascular, and Neurodegenerative Pathologies of Diabetic Retinopathy', *Investigative Opthalmology & Visual Science*, 58: 2852.

Geerlings, Suzanne E. 2016. 'Clinical Presentations and Epidemiology of Urinary Tract Infections', *Microbiology Spectrum*, 4.

Gentry, J. J., N. J. Rutkoski, T. L. Burke, and B. D. Carter. 2004. 'A functional interaction between the p75 neurotrophin receptor interacting factors, TRAF6 and NRIF', *J Biol Chem*, 279: 16646-56.

Giannantoni, A., S. M. Di Stasi, V. Nardicchi, A. Zucchi, L. Macchioni, V. Bini, G. Goracci, and M. Porena. 2006. 'Botulinum-A toxin injections into the detrusor muscle decrease nerve growth factor bladder tissue levels in patients with neurogenic detrusor overactivity', *J Urol*, 175: 2341-4.

Gonzalez, R. R., T. Fong, N. Belmar, M. Saban, D. Felsen, and A. Te. 2005. 'Modulating bladder neuro-inflammation: RDP58, a novel anti-inflammatory peptide, decreases inflammation and nerve growth factor production in experimental cystitis', *J Urol*, 173: 630-4.

Grover, S., A. Srivastava, R. Lee, A. K. Tewari, and A. E. Te. 2011. 'Role of inflammation in bladder function and interstitial cystitis', 3: 19-33.

Grundy, Luke, Ashlee Caldwell, and Stuart M. Brierley. 2018. 'Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome', *Frontiers in Neuroscience*, 12: 931-31.

Herrero, Raquel, Lucia Prados, Antonio Ferruelo, Ferranda Puig, Rachele Pandolfi, Raquel Guillamat-Prats, Laura Moreno, Gustavo Matute-Bello, Antonio Artigas, Andres Esteban, and José Ángel Lorente. 2019. 'Fas activation alters tight junction proteins in acute lung injury', *Thorax*, 74: 69-82.

Houlton, Josh, Nashat Abumaria, Simon F. R. Hinkley, and Andrew N. Clarkson. 2019. 'Therapeutic Potential of Neurotrophins for Repair After Brain Injury: A Helping Hand From Biomaterials', *Frontiers in Neuroscience*, 13.

Hu, H. Z., N. Granger, and N. D. Jeffery. 2016. 'Pathophysiology, Clinical Importance, and Management of Neurogenic Lower Urinary Tract Dysfunction Caused by Suprasacral Spinal Cord Injury', *Journal of veterinary internal medicine*, 30: 1575-88.

Huang, Xiaoli, Yang Feng, Guanqing Xiong, Shona Whyte, Jing Duan, Yucen Yang, Kaiyu Wang, Shiyong Yang, Yi Geng, Yangping Ou, and Defang Chen. 2019. 'Caspase-11, a

specific sensor for intracellular lipopolysaccharide recognition, mediates the non-canonical inflammatory pathway of pyroptosis', *Cell & Bioscience*, 9: 31.

Hurst, Robert E, Beverley Greenwood-Van Meerveld, Amy B Wisniewski, Samuel VanGordon, HsuehKung Lin, Bradley P Kropp, and Rheal A Towner. 2015. 'Increased bladder permeability in interstitial cystitis/painful bladder syndrome', *Translational andrology and urology*, 4: 563.

Ioannou, Maria, and Margaret Fahnestock. 2017. 'ProNGF, but Not NGF, Switches from Neurotrophic to Apoptotic Activity in Response to Reductions in TrkA Receptor Levels', *International Journal of Molecular Sciences*, 18: 599.

Jhang, Jia-Fong, and Hann-Chorng Kuo. 2016. 'Pathomechanism of Interstitial Cystitis/Bladder Pain Syndrome and Mapping the Heterogeneity of Disease', *International Neurourology Journal*, 20: S95-S104.

Ji, Muhuo, Hongmei Yuan, Shanwu Yuan, Jiangyan Xia, and Jianjun Yang. 2018. 'The p75 neurotrophin receptor might mediate sepsis-induced synaptic and cognitive impairments', *Behavioural Brain Research*, 347: 339-49.

Jiang, Yingming, Guoyou Chen, Yuanyuan Zheng, Lin Lu, Cong Wu, Yi Zhang, Qiuyan Liu, and Xuetao Cao. 2008. 'TLR4 signaling induces functional nerve growth factor receptor p75NTR on mouse dendritic cells via p38MAPK and NF-κB pathways', 45: 1557-66.

Kamei, Jun, Naoki Aizawa, Takayuki Nakagawa, Shuji Kaneko, Tetsuya Fujimura, Yukio Homma, Haruki Kume, and Yasuhiko Igawa. 'Lacking transient receptor potential melastatin 2 attenuates lipopolysaccharide-induced bladder inflammation and its associated hypersensitivity in mice', *International Journal of Urology*, n/a.

Kamei, Jun, Naoki Aizawa, Takayuki Nakagawa, Shuji Kaneko, Haruki Kume, Yukio Homma, and Yasuhiko Igawa. 2018. 'Attenuated lipopolysaccharide-induced inflammatory bladder hypersensitivity in mice deficient of transient receptor potential ankilin1', *Scientific Reports*, 8: 15622.

Kashyap, Mahendra, Subrata Pore, Naoki Yoshimura, and Pradeep Tyagi. 2016. 'Constitutive expression Of NGF And P75(NTR) affected by bladder distension and NGF antisense treatment', *Life sciences*, 148: 93-98.

Kawasaki, Takumi, and Taro Kawai. 2014. 'Toll-Like Receptor Signaling Pathways', *Frontiers in Immunology*, 5.

Khursigara, G., J. R. Orlinick, and M. V. Chao. 1999. 'Association of the p75 Neurotrophin Receptor with TRAF6', 274: 2597-600.

Kim, Hyun-Jung. 2016. 'Update on the Pathology and Diagnosis of Interstitial Cystitis/Bladder Pain Syndrome: A Review', *International Neurourology Journal*, 20: 13-17.

Kisiswa, L., D. Fernández-Suárez, M. C. Sergaki, and C. F. Ibáñez. 2018. 'RIP2 Gates TRAF6 Interaction with Death Receptor p75(NTR) to Regulate Cerebellar Granule Neuron Survival', *Cell Rep*, 24: 1013-24.

Klinger, Mary Beth, and Margaret A. Vizzard. 2008. 'Role of p75NTR in female rat urinary bladder with cyclophosphamide-induced cystitis', *American Journal of Physiology-Renal Physiology*, 295: F1778-F89.

Kogan, Paul, Suming Xu, Yaoqin Wang, Michael A. O'Donnell, Susan K. Lutgendorf, Catherine S. Bradley, Andrew Schrepf, Karl J. Kreder, and Yi Luo. 2018. 'Sub-noxious Intravesical Lipopolysaccharide Triggers Bladder Inflammation and Symptom Onset in A Transgenic Autoimmune Cystitis Model: A MAPP Network Animal Study', *Scientific Reports*, 8: 6573-73.

Kuo, W. T., L. Shen, L. Zuo, N. Shashikanth, Mldm Ong, L. Wu, J. Zha, K. L. Edelblum, Y. Wang, Y. Wang, S. P. Nilsen, and J. R. Turner. 2019. 'Inflammation-induced Occludin Downregulation Limits Epithelial Apoptosis by Suppressing Caspase-3 Expression', *Gastroenterology*, 157: 1323-37.

Kushida, Nobuhiro, Yukihito Kabuyama, Osamu Yamaguchi, and Yoshimi Homma. 2001. 'Essential role for extracellular Ca2+ in JNK activation by mechanical stretch in bladder smooth muscle cells', *American Journal of Physiology-Cell Physiology*, 281: C1165-C72.

Kuzmich, Nikolay, Konstantin Sivak, Vladimir Chubarev, Yuri Porozov, Tatiana Savateeva-Lyubimova, and Francesco Peri. 2017. 'TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis', *Vaccines*, 5: 34.

Lacerda Mariano, Livia, and Molly A. Ingersoll. 2020. 'The immune response to infection in the bladder', *Nature Reviews Urology*, 17: 439-58.

Lee, Jane-Dar, and Ming-Huei Lee. 2014. 'Decreased expression of zonula occludens-1 and occludin in the bladder urothelium of patients with interstitial cystitis/painful bladder syndrome', 113: 17-22.

Lee, Ming-Huei, Kun-Min Chang, and Wen-Chen Tsai. 2018. 'Morbidity rate and medical utilization in interstitial cystitis/painful bladder syndrome', *International Urogynecology Journal*, 29: 1045-50.

Liu, Hsin-Tzu, Pradeep Tyagi, Michael B. Chancellor, and Hann-Chorng Kuo. 2010. 'Urinary nerve growth factor but not prostaglandin E2 increases in patients with interstitial cystitis/bladder pain syndrome and detrusor overactivity', *BJU International*, 106: 1681-85.

Liu, Yan, Sylvie Mémet, Ricardo Saban, Xiangpeng Kong, Pavel Aprikian, Evgeni Sokurenko, Tung-Tien Sun, and Xue-Ru Wu. 2015. 'Dual ligand/receptor interactions activate urothelial defenses against uropathogenic E. coli', *Scientific Reports*, 5: 16234.

Ma, Fei, Dimitrios E. Kouzoukas, Katherine L. Meyer-Siegler, Karin N. Westlund, David E. Hunt, and Pedro L. Vera. 2017. 'Disulfide high mobility group box-1 causes bladder pain through bladder Toll-like receptor 4', *BMC Physiology*, 17: 6.

McKernan, Lindsey C., Kemberlee R. Bonnet, Michael T. M. Finn, David A. Williams, Stephen Bruehl, W. Stuart Reynolds, Daniel Clauw, Roger R. Dmochowski, David G. Schlundt, and Leslie J. Crofford. 2020. 'Qualitative analysis of treatment needs in interstitial cystitis/bladder pain syndrome: Implications for intervention', *Canadian Journal of Pain*, 4: 181-98.

McKernan, Lindsey C., Colin G. Walsh, William S. Reynolds, Leslie J. Crofford, Roger R. Dmochowski, and David A. Williams. 2018. 'Psychosocial co-morbidities in Interstitial Cystitis/Bladder Pain syndrome (IC/BPS): A systematic review', *Neurourology and Urodynamics*, 37: 926-41.

Mohamed, Riyaz, and Azza El-Remessy. 2015. 'Imbalance of the Nerve Growth Factor and Its Precursor: Implication in Diabetic Retinopathy', *Journal of Clinical & Experimental Ophthalmology*, 06.

Montalbetti, Nicolas, Anna C. Rued, Stefanie N. Taiclet, Lori A. Birder, F. Aura Kullmann, and Marcelo D. Carattino. 2017. 'Urothelial Tight Junction Barrier Dysfunction Sensitizes Bladder Afferents', *eneuro*, 4: ENEURO.0381-16.

Monteleone, F., C. G. Nicoletti, M. Stampanoni Bassi, E. Iezzi, F. Buttari, R. Furlan, A. Finardi, G. A. Marfia, D. Centonze, and F. Mori. 2018. 'Nerve growth factor is elevated in the

CSF of patients with multiple sclerosis and central neuropathic pain', *J Neuroimmunol*, 314: 89-93.

Mossa, A. H., P. G. Cammisotto, S. Shamout, and L. Campeau. 2020. 'Imbalance of nerve growth factor metabolism in aging women with overactive bladder syndrome', *World J Urol*.

Mossa, A. H., A. Galan, P. G. Cammisotto, M. Velasquez Flores, S. Shamout, P. Barcelona, H. U. Saragovi, and L. Campeau. 2020a. 'Antagonism of proNGF or its receptor p75(NTR) reverses remodelling and improves bladder function in a mouse model of diabetic voiding dysfunction', *Diabetologia*, 63: 1932-46.

Mossa, Abubakr H., Jacob Abdaem, Philippe Cammisotto, and Lysanne Campeau. 2021. 'Deleterious impact of nerve growth factor precursor (proNGF) on bladder urothelial and smooth muscle cells', *Cellular Signalling*, 81: 109936.

Mossa, Abubakr H., Alba Galan, Philippe G. Cammisotto, Monica Velasquez Flores, Samer Shamout, Pablo Barcelona, H. Uri Saragovi, and Lysanne Campeau. 2020b. 'Antagonism of proNGF or its receptor p75NTR reverses remodelling and improves bladder function in a mouse model of diabetic voiding dysfunction', *Diabetologia*, 63: 1932-46.

Mossa, Abubakr, Monica Velasquez, Philippe Cammisotto, and Lysanne Campeau. 2018. 'Beta-3 Adrenoceptor Signaling Pathways in Urothelial and Smooth Muscle Cells in the Presence of Succinate', *Journal of Pharmacology and Experimental Therapeutics*, 367: jpet.118.249979.

Moutzouris, Dimitrios-Anestis, and Matthew E. Falagas. 2009. 'Interstitial Cystitis: An Unsolved Enigma', *Clinical Journal of the American Society of Nephrology*, 4: 1844-57.

Mufson, Elliott J., Scott E. Counts, Stephen D. Ginsberg, Laura Mahady, Sylvia E. Perez, Stephen M. Massa, Frank M. Longo, and Milos D. Ikonomovic. 2019. 'Nerve Growth Factor Pathobiology During the Progression of Alzheimer's Disease', *Frontiers in Neuroscience*, 13.

Mullins, C., T. Bavendam, Z. Kirkali, and J. W. Kusek. 2015. 'Novel research approaches for interstitial cystitis/bladder pain syndrome: thinking beyond the bladder', *Translational andrology and urology*, 4: 524-33.

Nomiya, M., K. Sagawa, J. Yazaki, N. Takahashi, N. Kushida, N. Haga, K. Aikawa, T. Matsui, M. Oka, T. Fukui, K. E. Andersson, and O. Yamaguchi. 2012. 'Increased bladder activity

is associated with elevated oxidative stress markers and proinflammatory cytokines in a rat model of atherosclerosis-induced chronic bladder ischemia', *Neurourol Urodyn*, 31: 185-9.

Ochodnický, Peter, Célia D. Cruz, Naoki Yoshimura, and Martin C. Michel. 2011. 'Nerve growth factor in bladder dysfunction: Contributing factor, biomarker, and therapeutic target', *Neurourology and Urodynamics*, 30: 1227-41.

Öztürk, Recep, and Ahmet Murt. 2020. 'Epidemiology of urological infections: a global burden', *World Journal of Urology*, 38: 2669-79.

Patnaik, S. S., A. S. Laganà, S. G. Vitale, S. Butticè, M. Noventa, S. Gizzo, G. Valenti, A. M. C. Rapisarda, V. L. La Rosa, C. Magno, O. Triolo, and V. Dandolu. 2017. 'Etiology, pathophysiology and biomarkers of interstitial cystitis/painful bladder syndrome', *Arch Gynecol Obstet*, 295: 1341-59.

Peters, Kenneth M., Kim A. Killinger, and Ibrahim A. Ibrahim. 2009. 'Childhood Symptoms and Events in Women With Interstitial Cystitis/Painful Bladder Syndrome', *Urology*, 73: 258-62.

Platón-Corchado, María, Pablo F. Barcelona, Sean Jmaeff, Miguel Marchena, Alberto M. Hernández-Pinto, Catalina Hernández-Sánchez, H. Uri Saragovi, and Enrique J. De La Rosa. 2017. 'p75NTR antagonists attenuate photoreceptor cell loss in murine models of retinitis pigmentosa', *Cell Death and Disease*, 8: e2922.

Rackley, R. R., S. K. Bandyopadhyay, S. Fazeli-Matin, M. S. Shin, and R. Appell. 1999. 'Immunoregulatory potential of urothelium: characterization of NF-kappaB signal transduction', *J Urol*, 162: 1812-6.

Roux, P. 2002. 'Neurotrophin signaling through the p75 neurotrophin receptor', *Progress in Neurobiology*, 67: 203-33.

Rudick, C. N., B. K. Billips, V. I. Pavlov, R. E. Yaggie, A. J. Schaeffer, and D. J. Klumpp. 2010. 'Host-pathogen interactions mediating pain of urinary tract infection', *J Infect Dis*, 201: 1240-9.

Rudick, Charles N., Paul J. Bryce, Laura A. Guichelaar, Ruth E. Berry, and David J. Klumpp. 2008. 'Mast Cell-Derived Histamine Mediates Cystitis Pain', *PLOS ONE*, 3: e2096.

Ryu, Jae Cheon, Katharine Tooke, Susan E. Malley, Anastasia Soulas, Tirzah Weiss, Nisha Ganesh, Nabila Saidi, Stephanie Daugherty, Uri Saragovi, Youko Ikeda, Irina Zabbarova,

Anthony J. Kanai, Mitsuharu Yoshiyama, H. Francis Farhadi, William C. de Groat, Margaret A. Vizzard, and Sung Ok Yoon. 2018. 'Role of proNGF/p75 signaling in bladder dysfunction after spinal cord injury', *The Journal of clinical investigation*, 128: 1772-86.

Sant, Grannum R., Duraisamy Kempuraj, James E. Marchand, and Theoharis C. Theoharides. 2007. 'The Mast Cell in Interstitial Cystitis: Role in Pathophysiology and Pathogenesis', *Urology*, 69: S34-S40.

Schellino, Roberta, Marina Boido, and Alessandro Vercelli. 2019. 'JNK Signaling Pathway Involvement in Spinal Cord Neuron Development and Death', *Cells*, 8: 1576.

Schreiber, S., S. Nikolaus, and J. Hampe. 1998. 'Activation of nuclear factor kappa B inflammatory bowel disease', *Gut*, 42: 477-84.

Schrepf, A., C. S. Bradley, M. O'Donnell, Y. Luo, S. E. Harte, K. Kreder, and S. Lutgendorf. 2015. 'Toll-like receptor 4 and comorbid pain in Interstitial Cystitis/Bladder Pain Syndrome: a multidisciplinary approach to the study of chronic pelvic pain research network study', *Brain Behav Immun*, 49: 66-74.

Shanab, Ahmed Y., Barbara A. Mysona, Suraporn Matragoon, and Azza B. El-Remessy. 2015. 'Silencing p75NTR prevents proNGF-induced endothelial cell death and development of acellular capillaries in rat retina', *Molecular Therapy - Methods & Clinical Development*, 2: 15013.

Skaper, Stephen D. 2017. 'Nerve growth factor: a neuroimmune crosstalk mediator for all seasons', *Immunology*, 151: 1-15.

Skeldal, S., A. M. Sykes, S. Glerup, D. Matusica, N. Palstra, H. Autio, Z. Boskovic, P. Madsen, E. Castrén, A. Nykjaer, and E. J. Coulson. 2012. 'Mapping of the interaction site between sortilin and the p75 neurotrophin receptor reveals a regulatory role for the sortilin intracellular domain in p75 neurotrophin receptor shedding and apoptosis', *J Biol Chem*, 287: 43798-809.

Song, Jeongmin, and Soman N. Abraham. 2008. 'TLR-mediated immune responses in the urinary tract', *Current opinion in microbiology*, 11: 66-73.

Sun, Jie, Xueji Zhang, Mark Broderick, and Harry Fein. 2003. 'Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay', *Sensors*, 3: 276-84.

Sycheva, Marina, Jake Sustarich, Yuxian Zhang, Vaithinathan Selvaraju, Thangiah Geetha, Marla Gearing, and Jeganathan Ramesh Babu. 2019. 'Pro-Nerve Growth Factor Induces Activation of RhoA Kinase and Neuronal Cell Death', *Brain Sciences*, 9: 204.

Szasz, Theodora, Camilla F. Wenceslau, Beth Burgess, Kenia P. Nunes, and R. Clinton Webb. 2016. 'Toll-Like Receptor 4 Activation Contributes to Diabetic Bladder Dysfunction in a Murine Model of Type 1 Diabetes', *Diabetes*, 65: 3754-64.

Takezawa, K., M. Kondo, H. Kiuchi, T. Soda, T. Takao, Y. Miyagawa, A. Tsujimura, N. Nonomura, and S. Shimada. 2014. 'Combination of bladder ultrasonography and novel cystometry method in mice reveals rapid decrease in bladder capacity and compliance in LPS-induced cystitis', *Am J Physiol Renal Physiol*, 307: F234-41.

Tambaro, S., M. A. Casu, A. Mastinu, and P. Lazzari. 2014. 'Evaluation of selective cannabinoid CB(1) and CB(2) receptor agonists in a mouse model of lipopolysaccharide-induced interstitial cystitis', *Eur J Pharmacol*, 729: 67-74.

Tiveron, C., L. Fasulo, S. Capsoni, F. Malerba, S. Marinelli, F. Paoletti, S. Piccinin, R. Scardigli, G. Amato, R. Brandi, P. Capelli, S. D'Aguanno, F. Florenzano, F. La Regina, A. Lecci, A. Manca, G. Meli, L. Pistillo, N. Berretta, R. Nisticò, F. Pavone, and A. Cattaneo. 2013. 'ProNGF\NGF imbalance triggers learning and memory deficits, neurodegeneration and spontaneous epileptic-like discharges in transgenic mice', *Cell Death Differ*, 20: 1017-30.

Ueda, Norichika, Makoto Kondo, Kentaro Takezawa, Hiroshi Kiuchi, Yosuke Sekii, Yusuke Inagaki, Tetsuji Soda, Shinichiro Fukuhara, Kazutoshi Fujita, Motohide Uemura, Ryoichi Imamura, Yasushi Miyagawa, Norio Nonomura, and Shoichi Shimada. 2020. 'Bladder urothelium converts bacterial lipopolysaccharide information into neural signaling via an ATP-mediated pathway to enhance the micturition reflex for rapid defense', *Scientific Reports*, 10: 21167.

United Nations, Department of Economic and Social Affairs, Population Division. 2019. *World Population Ageing 2019: Highlights (ST/ESA/SER.A/430)*.

Vilar, Marçal, and Helena Mira. 2016. 'Regulation of Neurogenesis by Neurotrophins during Adulthood: Expected and Unexpected Roles', *Frontiers in Neuroscience*, 10.

Wang, Tao, Pingping Zuo, and Piu Chan. 2012. 'Oxidative DNA damage triggers ProNGF-Mediated apoptosis in the striatum of 6-OHDA-treated rats', *Molecular Neurodegeneration*, 7: S20-S20.

Warren, J. W., V. Brown, S. Jacobs, L. Horne, P. Langenberg, and P. Greenberg. 2008. 'Urinary tract infection and inflammation at onset of interstitial cystitis/painful bladder syndrome', *Urology*, 71: 1085-90.

Weng, T. I., H. Y. Wu, P. Y. Lin, and S. H. Liu. 2009. 'Uropathogenic Escherichia coli-induced inflammation alters mouse urinary bladder contraction via an interleukin-6-activated inducible nitric oxide synthase-related pathway', *Infect Immun*, 77: 3312-9.

White, Rachel S., Betsir G. Zemen, Zulqarnain Khan, Jenna R. Montgomery, Gerald M. Herrera, and Andrea L. Meredith. 2015. 'Evaluation of mouse urinary bladder smooth muscle for diurnal differences in contractile properties', *Frontiers in Pharmacology*, 5.

Winder, Michael, Gunnar Tobin, Daša Zupančič, and Rok Romih. 2014. 'Signalling Molecules in the Urothelium', *BioMed Research International*, 2014: 297295.

Wislet, Sabine, Geoffrey Vandervelden, and Bernard Rogister. 2018. 'From Neural Crest Development to Cancer and Vice Versa: How p75NTR and (Pro)neurotrophins Could Act on Cell Migration and Invasion?', *Frontiers in Molecular Neuroscience*, 11.

Wyndaele, Jean Jacques J., Claus Riedl, Rajesh Taneja, Sándor Lovász, Tomohiro Ueda, and Mauro Cervigni. 2019. 'GAG replenishment therapy for bladder pain syndrome/interstitial cystitis', *Neurourology and Urodynamics*, 38: 535-44.

Xiao, F., H. Wang, X. Fu, Y. Li, and Z. Wu. 2012. 'TRAF6 promotes myogenic differentiation via the TAK1/p38 mitogen-activated protein kinase and Akt pathways', *PLOS ONE*, 7: e34081.

Yang, Wenbin, Timothy J. Searl, Ryan Yaggie, Anthony J. Schaeffer, and David J. Klumpp. 2018. 'A MAPP Network study: overexpression of tumor necrosis factor-α in mouse urothelium mimics interstitial cystitis', *American journal of physiology. Renal physiology*, 315: F36-F44.

Yoshimura, Naoki, Tomohiko Oguchi, Hitoshi Yokoyama, Yasuhito Funahashi, Satoru Yoshikawa, Yoshio Sugino, Naoki Kawamorita, Mahendra P. Kashyap, Michael B. Chancellor, Pradeep Tyagi, and Teruyuki Ogawa. 2014. 'Bladder afferent hyperexcitability in bladder pain syndrome/interstitial cystitis', *International journal of urology : official journal of the Japanese Urological Association*, 21 Suppl 1: 18-25.

Yu, L., V. P. O'Brien, J. Livny, D. Dorsey, N. Bandyopadhyay, M. Colonna, M. G. Caparon, E. D. Roberson, S. J. Hultgren, and T. J. Hannan. 2019. 'Mucosal infection rewires TNFα signaling dynamics to skew susceptibility to recurrence', *Elife*, 8.

Zabbarova, Irina V., Youko Ikeda, Evan J. Carder, Peter Wipf, Amanda S. Wolf-Johnston, Lori A. Birder, Naoki Yoshimura, Samuel E. Getchell, Khalifa Almansoori, Pradeep Tyagi, Christopher H. Fry, Marcus J. Drake, and Anthony J. Kanai. 2018. 'Targeting p75 neurotrophin receptors ameliorates spinal cord injury-induced detrusor sphincter dyssynergia in mice', *Neurourology and Urodynamics*, 37: 2452-61.

Zhao, Jiang, Liang Wang, Xingyou Dong, Xiaoyan Hu, Long Zhou, Qina Liu, Bo Song, Qingjian Wu, and Longkun Li. 2016. 'The c-Jun N-terminal kinase (JNK) pathway is activated in human interstitial cystitis (IC) and rat protamine sulfate induced cystitis', *Scientific Reports*, 6: 19670.