Correcting Age-Related Overactive Bladder Syndrome Using $p75^{NTR}$ Antagonism

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Abstract

INTRODUCTION: Overactive Bladder Syndrome (OAB) is defined as symptoms of urinary urgency, usually with increased urinary frequency, with or without urgency incontinence. OAB affects approximately 20% of Canadians and increases with age. However, the underlying pathophysiology is unknown and current therapies are mildly effective. Nevertheless, low levels of urinary Nerve Growth Factor (NGF) have been observed in aging patients with OAB and of diabetic rodents, as compared to its precursor, proNGF. NGF ensures nervous system health such that the bladder is properly controlled, while proNGF induces tissue degeneration. Thus, the NGF/proNGF balance determines the extent of tissue health. We previously found that the decreased NGF appeared to originate from overexpression of the Matrix Metalloprotinase-9 (MMP-9) proteolytic enzyme. Further, NGF levels were restored in mice with voiding dysfunction upon inhibition of the proinflammatory receptor, $p75^{NTR}$, by the compound THX-B. This suggests a link between $p75^{NTR}$ and MMP-9. In Chapter 4 we examine the effect of THX-B on the activity of MMP-9 in bladder cells and the consequences on secreted NGF, as well as the functional benefit of THX-B bladder treatment in aging mice with voiding dysfunction in Chapter 5.

METHODS: In Chapter 4, primary culture of urothelial (UROs) and smooth muscle cells (SMCs) are grown from Sprague-Dawley rat bladders. Expression of NGF and MMP-9 are assessed by RT-qPCR, by immunohistochemistry and by immunoblotting. Levels of microRNAs are measured by RT-qPCR. NGF and proNGF secretion are evaluated using ELISA kits and MMP-9 activity by enzymatic assays. In Chapter 5, 18-month, 12-month and 6-month-old C57BL/6J mice received either a $p75^{NTR}$ antagonist, THX-B, or PBS control as weekly intraperitoneal (IP) systemic injections for 4 weeks. Animals were tested at baseline, and after 2 weeks and 4 weeks injection. Voiding parameters as well as patterns and behaviours were assessed in all mice using metabolic

chambers and voiding spot assays, respectively. Cystometry and organ baths were carried out to evaluate bladder contractility.

RESULTS: In Chapter 4, NGF and MMP-9 mRNAs were found to be expressed in both cell types at similar level. Microscopy confirmed the presence of both proteins in the cytoplasm of cells. UROs and SMCs culture supernatant contained significant amounts of NGF and proNGF. On the other hand, MMP-9 protein content was 7 times higher in SMCs than in UROs. However, secretion of active MMP-9 in the medium was 40 times higher in URO medium. Incubation with THX-B (5 μg/mL) for 24 hours abolished the synthesis and secretion of MMP-9 and doubled the concentration of NGF in the medium of UROs. ProNGF secretion levels in the same medium were not affected. On the contrary, THX-B had little effects on SMCs both at the level of NGF and MMP-9. Animal studies in Chapter 5 showed an age-specific response in bladder improvement when treated with THX-B. Specifically, 12-month-old mice treated with THX-B displayed lower total urine volume and voiding frequency as well as lower response to contractile stimuli compared to the 18-month and 6-month-old treated mice as well as the controls.

CONCLUSIONS: UROs *in vitro* secrete most of MMP-9. As well, these cells are the primary target for THX-B, decreasing expression and secretion of MMP-9 and subsequently enhancing secretion of NGF. These results are in accordance with our previous publications on OAB patients and diabetes type 1 in rodents and suggest that THX-B could be a therapeutic tool to improve OAB by targeting primarily the urothelium. Finally, $p75^{NTR}$ antagonism in mice with THX-B shows an age-specific improvement mainly in the 12-month-old mice, which may reflect pathological temporal heterogeneity or a spectrum of severity in OAB.

Resumé

INTRODUCTION: Le syndrome de la vessie hyperactive (VH) se caractérise par des symptômes d'urgence urinaire, avec une fréquence urinaire croissante, avec ou sans incontinence. Le VH touche environ 20% des Canadiens, avec une prévalence accrue dans la population vieillissante. D'autre part, de faibles niveaux de facteur de croissance neuronal (NGF) comparé à son précurseur, le proNGF, ont été observés dans l'urine de patients vieillissants atteints le VH ainsi que chez les rongeurs diabétiques. Le NGF maintient le système nerveux en santé et préserve les fonctions de la vessie, tandis que le proNGF induit la dégénérescence des tissus. Ainsi, l'équilibre NGF/proNGF détermine la bonne santé des tissus. Nous avons précédemment découvert que la diminution du NGF semblait provenir de la surexpression de l'enzyme protéolytique Matrix Metalloprotéinase-9 (MMP-9). En outre, chez des souris présentant un dysfonctionnement de la miction, les niveaux de NGF sont restaurés par l'inhibition du récepteur $p75^{NTR}$ par le composé THX-B. Ceci suggère un lien entre $p75^{NTR}$ et MMP-9. Dans le chapitre 4, nous examinons l'effet du THX-B sur l'activité de la MMP-9 dans les cellules de la vessie et les conséquences sur les niveaux de NGF. Le bénéfice fonctionnel du traitement de la vessie par le THX-B chez les souris vieillissantes présentant un dysfonctionnement de la miction est abordé dans le chapitre 5.

MÉTHODES: Dans le chapitre 4, les cultures primaires de cellules urothéliales (URO) et musculaires lisses (CML) sont obtenues à partir de vessies de rat. L'expression du NGF et de la MMP-9 est évaluée par RT-qPCR, par immunohistochimie et par immunobuvardage. Les niveaux de microARN sont mesurés par RT-qPCR. La sécrétion de NGF et de proNGF est évaluée par ELISA et l'activité de MMP-9 par dosage enzymatique. Dans le chapitre 5, des souris C57BL/6J âgées de 18 mois, 12 mois et 6 mois ont reçu soit un antagoniste de $p75^{NTR}$, THX-B, soit du PBS (contrôle) pendant 4 semaines. Les paramètres de miction ainsi que les modèles et les

comportements ont été évalués à l'aide de chambres métaboliques et par la technique des taches de miction sur papier Whatman, respectivement. Les cystométries et les bains d'organes ont été utilisés pour évaluer la contractilité de la vessie.

RÉSULTATS: Dans le chapitre 4, les ARNm du NGF et de la MMP-9 se sont avérés être exprimés dans les deux types de cellules à un niveau similaire. La microscopie a confirmé la présence des deux protéines dans le cytoplasme des cellules. Le surnageant de culture des UROs et des CMLs contenait des quantités significatives de NGF et de proNGF. D'autre part, le contenu intracellulaire en MMP-9 était 7 fois plus élevé dans les CMLs que dans les UROs. Cependant, l'activité de la MMP-9 était 40 fois plus élevé dans le milieu de culture des UROs. L'incubation avec THX-B (5 μg/mL) pendant 24 heures a aboli la synthèse et la sécrétion de MMP-9 et a doublé la concentration de NGF dans le milieu des cellules urothéliales. Les niveaux de sécrétion de proNGF dans les mêmes milieux n'ont pas été affectés. Au contraire, le THX-B a eu peu d'effets sur les CMLs. Les études du chapitre 5 ont montré une réponse différente selon l'âge des fonctions de la vessie après le traitement avec THX-B. Plus précisément, les souris âgées de 12 mois traitées avec le THX-B présentaient un volume total d'urine et une fréquence de miction diminués ainsi qu'une réponse plus faible aux stimuli contractiles par rapport aux souris traitées âgées de 18 mois et de 6 mois et aux contrôles.

CONCLUSIONS: Les UROs sécrètent la majeure partie de la MMP-9. Ces cellules sont la cible principale de THX-B car elles montrent une expression et sécrétion de MMP-9 réduites et une augmentation de la sécrétion de NGF. Ces résultats suggèrent que le THX-B pourrait devenir un outil thérapeutique en ciblant principalement l'urothélium. Enfin, l'antagonisme p75NTR chez les souris avec THX-B montre une amélioration des fonctions de la vessie, principalement chez les

souris âgées de 12 mois, ce qui pourrait refléter une hétérogénéité temporelle pathologique ou du spectre de gravité dans le VH.

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Preface

This thesis is presented in a manuscript-based format. Chapters 4 and 5 are to be submitted.

Chapter 4 is an in vitro study on cell cultures.

Chapter 5 is an in vivo study in mice.

Contribution of authors

Along with Drs. Lysanne Campeau and Philippe Cammisotto, I contributed to the conception and design of this project.

I contributed to all data extraction in this project with some assistance from Dr. Philippe Cammisotto.

All writing and interpretations in this thesis are entirely my own with minor editorial revisions by Drs. Lysanne Campeau and Philippe Cammisotto.

Dr. Philippe Cammisotto, Aya Hajj, and Stephanie Sirmakesyan assisted in some experimentation in Chapters 4 and 5.

Much of this study is based on previous work and preliminary studies from Dr. Abubakr Mossa.

Dr. Uri Saragovi provided the $p75^{NTR}$ antagonist, THX-B, which was required for this entire study.

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List of abbreviations

α2M: alpha-2-macroglobulin

Ach: Acetylcholine

AD: Alzheimer's disease

ATP: Adenosine triphosphate

BDNF: Brain derived nerve factor

BOO: Bladder outlet obstruction

BPH: Benign prostatic hyperplasia

cAMP: Cyclic adenosine monophosphate

CNS: Central nervous system

DO: Detrusor overactivity

ECD: Extracellular domain

ECM: Extracellular matrix

EFS: Electric field simulation

ENaC: Epithelial sodium channel

ICS: International continence society

JNK: c-Jun N-terminal kinase

KCl: Potassium chloride

KO: Knock out

LUT: Lower urinary tract

M2: Muscarinic type 2

M3: Muscarinic type 3

MAPK: Mitogen-activated protein kinase

miRNA: microRNA

MMP-7: Matrix-metalloproteinase-7

MMP-9: Matrix-metalloproteinase-9

MS: Multiple sclerosis

NE: Norepinephrine

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve growth factor

NO: Nitric oxide

OAB: Overactive bladder syndrome

P38MAPK: P38 mitogen-activated protein kinase

$P75^{NTR}$: p75 neurotrophin receptor

PI3K: Phosphoinositide 3-kinase

PLC- ɣ: phospholipase C-ɣ

PMC: Pontine micturition center

ProNGF: Precursor nerve growth factor

PUO: Partial urethral outflow obstruction

PVR: Post-void residual volume

SCI: Spinal cord injury

SMC: Smooth muscle cell

TIMP: Tissue inhibitor of matrix metalloproteinase

TrkA: Tropomyosin receptor kinase A

URO: Urothelial cell

VSA: Voiding spot assay

Chapter 1: Introduction to bladder physiology and Overactive Bladder Syndrome

1.1 Bladder function and structure

1.1.1 Anatomy of the bladder

The function of the bladder consists of storing and voiding urine[1]. The bladder wall is comprised of several layers, each with different histological, functional, and structural characteristics. The urothelium (made up of transitional epithelium) lines the innermost layer of the bladder and is comprised of several layers of urothelial cells (UROs)[2]. UROs form the tight junctions of the bladder to prevent urine leakage and pathogen infiltration, while accommodating bladder stretching for urine storage[3]. Surrounding the urothelium is loose connective tissue, known as the lamina propria, which functions to support the urothelium and define bladder compliance[4,5]. Cells of lamina propria are mostly fibroblasts, interstitial cells, adipocytes, and more importantly efferent nerve endings. Next, the detrusor muscle, which is comprised of smooth muscle cells (SMCs), surrounds the outer bladder and is responsive to stimuli that lead to bladder contraction for urine release and relaxation for urine storage[2,4]. Finally, the fibrous coat named adventitia and the serosa constitutes the outer layers of the bladder[5]. While each layer provides structure and function to the bladder, the nervous system is crucial for bladder control and regulating urine storage and release.

1.1.2 The role of the nervous system in bladder function

Complex sympathetic and parasympathetic nervous system interactions are important for establishing tight neurogenic control of the bladder and ensuring proper urine storage and release

(Figure 1). When the bladder is full and stretched, muscarinic 3 (M3) stretch receptors on the detrusor muscle activate and become stimulated by acetylcholine (ACh) released by the parasympathetic nervous system[6]. This leads to detrusor contraction, urethral sphincter relaxation and bladder emptying[6]. As the bladder is emptied, stretch is reduced, M3 receptors are inactivated and sympathetic innervation of the bladder induces the release of norepinephrine (NE), activating detrusor muscle β-adrenergic receptors to relax the bladder, constrict the urethral sphincter and mediate filling[6]. The neurological control of the bladder is influenced by several factors, such as growth factors and neurotrophins, including Neurotrophin-3 and Neurotrophin-4, Glial Cell Line Derived Neurotrophic Factor, Brain Derived Neurotrophic Factor (BDNF) and its precursor proBDNF as well as Nerve Growth Factor (NGF) and its precursor proNGF[7,8]. This concept will be discussed further in *Chapter 2: Literature Review*. Moreover, the bladder urothelium exhibits nervous-like properties by transducing chemical and mechanical stimuli, such as urine tonicity and bladder stretching, mainly through release of adenosine triphosphate (ATP) onto purinergic receptors of sensory nerves to relay bladder fullness and pain[9]. Along with the detrusor muscle, the urothelium can also interact with afferent neurons and modulate their sensitization through release of chemical mediators, such as nitric oxide (NO), ATP and prostaglandins, as well as growth factors such as NGF and BDNF[9-11]. Changes in the neurogenic control of the bladder, as well as the interactions between bladder tissue and the neural environment, are often seen in bladder pathologies, such as Overactive Bladder Syndrome (OAB)[12].

1.2 Overview of Overactive Bladder Syndrome

1.2.1 Definition

OAB is defined by the International Continence Society (ICS) as urinary urgency, usually with urinary frequency and nocturia, with or without urgency urinary incontinence[13]. Such symptoms

interfere with daily life, leading to psychological, social, and physical distresses, which reduce the patient's quality of life[13]. OAB affects approximately 18% of Canadians and increases in prevalence with age[13]. In addition, OAB has a higher overall prevalence in women (21.2%) than men (14.8%)[13].

1.2.2 Etiology

OAB can be caused by a variety of conditions, most of which involve detrusor overactivity (DO) leading to symptoms urgency incontinence. Several theories have been used to describe this pathophysiology. The neurogenic theory involves a reduction in the inhibitory impulses that promote storage relative to excitatory impulses that promote voiding[14]. Examples of neurogenic OAB include spinal cord injury (SCI) and multiple sclerosis (MS), among others[15]. Alternatively, the myopathic theory describes the increased sensitivity of the detrusor muscle to excitatory stimuli that leads to contraction[16]. For example, detrusor hypertrophy from chronic retention[17]. This is often seen in aging males with Bladder Outlet Obstruction (BOO) and Benign Prostatic Hyperplasia (BPH); increased voiding pressure results in compensatory detrusor hypertrophy that can lead to symptoms of OAB[18]. OAB is common in the aging population. However, the exact mechanisms underlying age-related OAB is poorly understood but is thought to involve a mix of neurogenic and myogenic causes[14]. While DO is a common component in OAB pathophysiology, changes in the bladder outlet, such as in post-surgical sphincter incompetence as well as dysfunction of the bladder or pelvic floor seen in pregnancy and childbirth, can also lead to OAB[19]. Incontinence in such cases is often associated with stress incontinence caused by increased abdominal pressure or physical exertion[19].

1.2.3 Sex-related differences in OAB pathology

Given that the Canadian prevalence of OAB is higher in women than men across all ages, biological sex may be an important consideration when understanding OAB pathology and clinical presentation[13,20]. A large population-based study conducted by Irwin *et al.* in five countries revealed that women with OAB more commonly exhibit urinary incontinence as a symptom compared to men[21]. Additionally, of those presenting with incontinence, stress urinary incontinence was the most common form among women, whereas urge and mixed (combination of urge and stress) urinary incontinence were more common among men[21]. Further, the underlying cause of OAB and its symptoms may differ between sexes. An important example is DO, whereby bladder contraction occurs spontaneously[22]. Sekido *et al*. reported a higher incidence of DO in male patients with OAB compared to female patients, suggesting that DO may be a more important cause of OAB in men[22]. Moreover, differences in the anatomy of the lower urinary tract (LUT) between males and females may also account for differences in OAB symptoms and pathology. For instance, Yamagushi *et al.* have reported a difference in hypogastric nerve distribution innervating the bladder and ureter between sexes[23]. Specifically, female bladders contained a richer innervation from branches of the hypogastric nerve, suggesting possible sex differences in micturition patterns based on sympathetic nervous system activity and its ability to promote storage[24].

1.2.4 Diagnosis

Given that OAB is a *symptom complex*, diagnosis is primarily based on patient history, reported symptoms, and physical examination[13]. Several definitions provided by the ICS are used as a framework for proper diagnosis. However, symptoms are often patient-specific and may not agree exclusively with ICS definitions. As such, clinical judgement and subjective assessment is often

required for diagnosis[25]. Additionally, various surveys and questionnaires are used to aid in diagnosis and treatment evaluation by measuring urinary symptoms and quality of life. These include, among others, the Overactive Bladder Symptom Score, the International Consultation on Incontinence Questionnaire-Short Form and the Incontinence Impact Questionnaire[26,27]. Several OAB symptoms overlap with other bladder conditions, such as urinary tract infection, benign prostatic enlargement, and bladder cancer[25]. Thus, further assessment of symptoms, such as, pain or hematuria, as well as diagnostic studies, such as, urine analysis and imaging studies may be necessary for accurate diagnosis and treatment evaluation[25]. Finally, the use of biological markers for OAB is an area of interest and may help predict early OAB development and treatment[13].

1.2.5 Treatment

Treatment for OAB is divided into non-pharmacological and pharmacological therapies. Non-pharmacological interventions are used to reduce symptoms of OAB, such as pelvic floor exercises to reduce urgency and urge incontinence[28]. Important lifestyle changes, notably weight management, fluid intake reduction and smoking cessation may help reduce symptoms for some patients[28].

Current pharmacological treatments for OAB are used to alleviate symptoms and include anti-cholinergic and adrenergic agonists[28]. In many cases, OAB symptoms arise from dysregulation of bladder contraction by the detrusor muscle[28]. As such, anti-cholinergic agents are used to inhibit the interaction between ACh and muscarinic receptors on the detrusor muscle in order to reduce the frequency of bladder contraction and voiding[28]. Due to their oral delivery, anticholinergics are associated with several systemic side effects, such as blurred vision, dry mouth, and constipation[28]. Furthermore, the side effects and long-term consequences of anticholinergics are pronounced in

elderly patients who require additional anticholinergic therapies to address comorbidities. Anticholinergic burden refers to the cumulative effect of anticholinergic medication on a patient[29]. In older adults, brain permeability is increased, allowing anticholinergics to enter the central nervous system (CNS) over time, which increases their risk of dementia and cognitive decline[29].

The β_3 agonist, Mirabegron, is also commonly used to treat symptoms of OAB. Mirabegron activates β_3 adrenergic receptors on the detrusor muscle to promote relaxation[28]. Although Mirabegron is preferred over anticholinergics due to reduced side effects and anticholinergic burden, not all patients are responsive[28].

Given the challenges of pharmacological interventions, as well as the absence of curative or reversible therapies, understanding the pathophysiology of OAB is crucial to determine future treatments.

1.2.6 The Study of OAB

Human studies are useful for the understanding of the nature of OAB, such as symptoms and identification of urinary biomarkers. In parallel, animal models are essential for evaluating the molecular mechanisms leading to the development of OAB and of its pathophysiology as well as assessing potential therapeutic avenues. However, the study of OAB remains difficult to interpret since many subjective and objective patient-reported symptoms seen in a clinical setting cannot be assessed[30]. Therefore, surrogate techniques are used. For example, to assess urinary urgency in animals, voiding frequency and non-micturition contractions are alternative measurements[30]. Considering the difficulty of assessing OAB and modelling complex clinical aspects in an animal, the best tool uses models designed to answer a specific scientific question[30]. Unfortunately, interspecies variabilities complicate animal studies and therefore careful validation of results is

required[30]. Whereas OAB caused by a clear, isolated pathological mechanism is easier to model, idiopathic OAB as well as more complex cases such as age-related OAB that involve several pathological processes are much more challenging to model. Developing novel tools to overcome these challenges is important for answering relevant clinical question using animal models. The current masters project aims to characterize bladder cells *in vitro* to better understand and study OAB *in vivo* in a model of aging mice. The project rational, hypothesis, and objectives are discussed further in *Chapter 3: Rational, Hypothesis, and Objectives.*

1.3 Figures

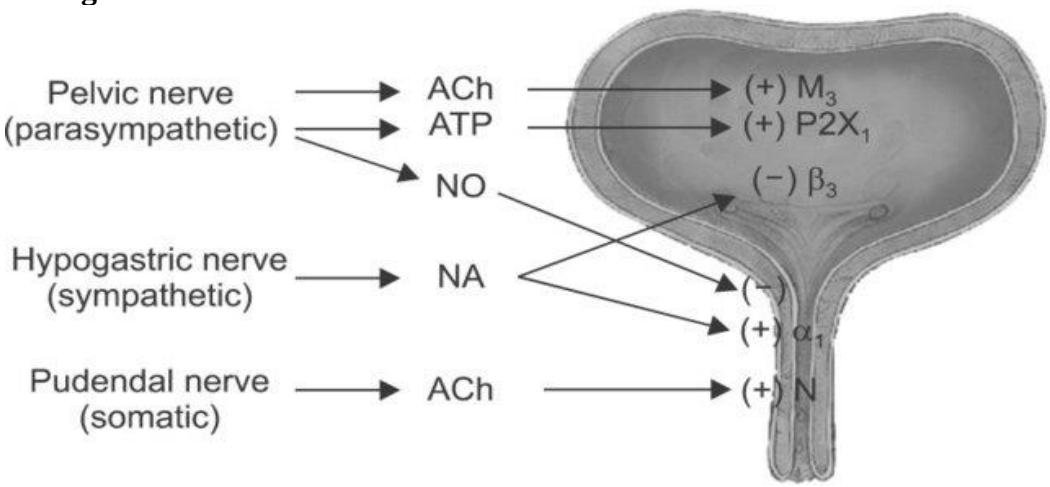

Figure 1. Basic overview of bladder control by the nervous system. Parasympathetic bladder innervation from the Pelvic nerve involves release acetylcholine (ACh) and adenosine triphosphate (ATP) to cause detrusor muscle relaxation through action on M_3 muscarinic and purinergic ($P2X_1$) receptors respectively. The Pelvic nerve also releases nitric oxide (NO) to relax the urethra. Together, detrusor contraction and urethral relaxation allow urine release. Sympathetic bladder innervation from the Hypogastric nerve allows for bladder relaxation and urine storage. This involves release of noradrenaline (NA) or norepinephrine (NE) (not shown) onto the β_3-adrenergic receptor of the detrusor muscle to cause relaxation and α_1 receptor of the urethra to cause

constriction. Somatic innervation is supplied by the Pudendal nerve through release of ACh onto nicotinic (N) receptors on the striated muscle of the external urethral sphincter. Figure from: Yoshimura *et al*., Korean J Urol. 2014 Feb.

Chapter 2: Literature Review

The pathophysiological mechanisms of age-related OAB are poorly understood. Nevertheless, studies on aging and metabolic syndrome related to OAB have been linked to the neurotrophins NGF and proNGF. By exploring cell intrinsic or extrinsic mechanisms as well as the clinical relevance of NGF, proNGF and associated proteins, it appeared that these proteins are affected during OAB. This literature review aims to navigate the current understanding of NGF, proNGF and other factors in the bladder and additional tissues to identify knowledge gaps and provide a foundation for hypothesizing mechanisms that specifically underly age-related OAB.

2.1 Age-related Overactive Bladder Syndrome

OAB is predominant among the elderly population. In fact, Hershorn *et al*., reported a prevalence of OAB symptoms in 24% of patients over 60 years compared to only 12% in those under 60 years of age[20]. OAB continues to be more prevalent in Canadian women than men, where 13.1% of men (mean age 44) and 14.7% of women (mean age 45) experience symptoms and this is also apparent with increasing age[20]. Age-associated voiding dysfunction significantly affects quality of life and presents a large burden on the healthcare system[13]. Unfortunately, the understanding of how aging affects the bladder is not fully established.

2.2 The role of aging on bladder function

Several pathological changes occur in the bladder and nervous system that result in voiding dysfunction and OAB during aging. For example, the pontine micturition center (PMC) that is normally responsible for initiating voiding is dysregulated during aging[31]. In fact, in older women with DO, functional magnetic resonance imaging (fMRI) showed stronger activation of the PMC

pathway at lower bladder volumes, which can explain symptoms of urgency and incontinence that are commonly experienced by OAB patients[31]. In addition to central changes during aging, alterations in the bladder itself also contribute to age-related OAB.

With age, the detrusor muscle of the bladder loses stability such that it becomes overactive and contracts spontaneously[32]. Detrusor instability associated with age can be attributed to gradual denervation or the bladder and tissue degeneration as a result of chronic inflammation and fibrosis linked to wear-and-tear injuries and comorbidities seen with age[33]. In addition to direct changes in the detrusor muscle, alteration in the urothelium, such as, tissue remodelling, indirectly impacts detrusor activity, altering bladder activity, possibly leading to overactivity and reduced bladder compliance[34]. Given that the urothelium has an important role in regulating the release of chemical mediators that affect neuronal and bladder sensitivity as well as overall homeostasis, it is not surprising that a damaged urothelium compromises the proper communication with adjacent structures like the detrusor muscle.

Changes in the bladder's neurogenic control are thought to play an important role in age-related OAB. Bladder tissue health as well as nervous system homeostasis is determined by a variety of factors regulated by bladder tissues, notably neurotrophins[7]. In fact, urine from aging female patients with OAB showed reduced levels of NGF with stable levels of proNGF[44]. Alterations in the NGF/proNGF balance have been associated with other age-related pathologies, such as memory deficit and dementia[44]. Understanding the role of the neurotrophins in the context of aging would be beneficial to further characterize the aging bladder and identify potential therapeutic avenues for age-related OAB.

2.3 NGF and proNGF

NGF is important for cell homeostasis, survival, and nervous system health through binding the extracellular Immunoglobulin C2 set domain of the tropomyosin kinase receptor A (TrkA) of the receptor tyrosine kinase family (Figure 2)[35]. Binding of NGF to TrkA induces receptor dimerization and phosphorylation of tyrosine residues on the cytoplasmic domain, leading to activation of various intracellular signalling cascades, including phospholipase C-γ (PLC-γ), mitogen activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase (PI3K) pathways[35]. In neurons, the NGF/TrkA axis plays an important role in nervous system growth and integrity during embryological development and nervous system health, plasticity as well as overall homeostasis in adulthood[36]. The role of NGF in non-neuronal tissue is not fully understood. However, the expression of TrkA and NGF are not nerve-specific and their neuronal functions have been used as a model to understand organ tissues and specialized cells[37]. The NGF precursor, termed proNGF, confers the opposite functions, facilitating apoptosis and tissue degeneration through binding the p75 neurotrophin receptor ($p75^{NTR}$) (Figure 2)[37]. The binding of proNGF to $p75^{NTR}$ induces mitochondrial dysfunction and cell death through activation of downstream c-Jun N-terminal kinase (JNK), MAPKs, RhoA Kinase, and caspase-3[38]. The proNGF/$p75^{NTR}$ axis may also promote cell proliferation through activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inhibition of JNK in certain cellular contexts that are not fully understood[38].

2.3.1 The role of NGF and proNGF in the normal bladder

In the bladder, the urothelium and detrusor smooth muscle secrete NGF, which promotes afferent sensitization as well as proper bladder innervation by controlling outgrowth of sensory neurons

from dorsal root ganglia and sympathetic ganglia[39]. Interestingly, Schnegelsberg *et al*. have shown that overexpression of NGF in the bladder urothelium increased somatic hypersensitivity and urinary bladder reflex in mice[39]. In addition to ensuring neuronal sensitivity and proper neurogenic bladder control, NGF seems to have similar cell survival mechanisms in non-neuronal tissue. For instance, Teng *et al*. have shown evidence of NGF-mediated urothelial proliferation through TrkA activation[40]. Bladder angiogenesis may also be mediated by urothelial expression of NGF through modulation of the Vascular Endothelial Growth Factor[41]. Studies on proNGF function in the bladder are strongly lacking. This is in part due to the poor specificity of ELISA kits in early studies that did not differentiate between NGF and proNGF immunoreactivity to properly characterize their specific cell-intrinsic mechanisms[42]. Mossa *et al*. have shown that proNGF is important in the proliferation of bladder SMCs through activation of $p75^{NTR}$ and downstream NF-κB[43]. Nonetheless, proNGF's pro-apoptotic and tissue degenerative properties mediated by $p75^{NTR}$ are apparent in neuronal and non-neuronal tissues, such as the bladder urothelium, and should be further studied in the bladder[43,44].

2.4 NGF and proNGF in bladder pathology

Accumulation of proNGF or reduced conversion to NGF have been observed in neurodegenerative disease and other pathological states, such as SCI[45], cancer[46], diabetic retinopathy[47], and voiding dysfunction[48]. With regards to the bladder, *in vitro* changes in the NGF or proNGF expression have been observed in age-related OAB[48], interstitial cystitis[16], BPH[12], diabetic voiding dysfunction[12], neurogenic bladder[12] following SCI[45], among others. Yet, in LUT diseases and bladder dysfunction, the pathophysiology of the NGF/proNGF imbalance warrants further investigation.

Previous studies have identified high levels of NGF and emphasize its use a as a biomarker in OAB[12]. However, due to the prior lack of NGF- and proNGF-specific ELISA kits, such studies are criticized[42]. In fact, newly emerged evidence suggests that NGF and the NGF/proNGF ratio is reduced in the urine of aging female patients with OAB compared to controls[48]. An increase in proNGF relative to NGF shifts the balance between tissue health and degeneration, injuring bladder tissue and the surrounding peripheral nervous system involved in bladder control. Such changes impact bladder modelling and function, resulting in LUT symptoms[49]. Bladder tissues of aged LOU rats with partial urethral outflow obstruction (PUO) showed NGF/proNGF imbalance and increased activation of the proNGF/$p75^{NTR}$ axis compared to young LOU controls[49]. Interestingly, these molecular findings were associated with poor bladder adaptation to PUO during aging confirming pathological consequences of a reduced NGF/proNGF ratio[49].

2.4.1 Factors affecting the NGF/proNGF balance

ProNGF processing

Given that NGF and proNGF have opposing effects on cell survival, the processing of proNGF into NGF has an important influence on relative levels of proNGF and NGF. In the CNS, proNGF secretion is activity-dependent and coordinated with proenzymes[50]. Extracellular processing of proNGF to NGF is regulated by Matrix-Metalloproteinase-7 (MMP-7) and Plasmin[51]. MMP-7 and Plasmin levels are altered in pathological conditions, such as brain injury, thus altering the composition of NGF and proNGF in the extracellular medium[51]. In addition, studies on SMCs in vasculature have shown that proNGF can be processed intracellularly into NGF by Furin to act on TrkA in endosomes or be recycled and secreted to activate TrkA on the cell surface[52]. Therefore,

intracellular and extracellular enzymatic activities are important to gain a better understanding of the ratio of conversion of proNGF to NGF that determinates the fate of tissue health.

NGF metabolism

In addition to proNGF processing, proteolysis of NGF into peptides has an important impact on NGF/proNGF balance. For example, in the urine of aging female patients with OAB, NGF was decreased while levels of proNGF were unchanged, leading to a reduced NGF/proNGF balance[48]. In the same samples, the activity of the proteolytic enzyme responsible for NGF degradation, Matrix Metalloproteinase 9 (MMP-9) was high[48]. Therefore, changes in the amount of NGF digestion have an impact on NGF/proNGF balance in tissues[53].

Regulation of NGF, proNGF and MMP-9

Several proteins influence the function and regulation of NGF, proNGF and MMP-9. Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) prevent the catalytic activity of MMPs, altering the levels of NGF and proNGF through modulating MMP-9 and MMP-7, among other proteases. For example, in hippocampal tissue following kainic acid-induced seizures, proNGF was upregulated due to reduced processing into NGF by MMP-7[51]. This was attributed to an increase in TIMP-1, which blocks enzymatic activity of MMPs, such as MMP-7[51].

In addition to TIMPs, NO has an important role in regulating NGF through MMP-9 modulation. An early study of NO and NGF found that NO reduced glial cell secretion of NGF dose-dependently[54]. In a more recent study, NO was found to regulate the expression of MMP-9 by promoting its conversion from pro-MMP-9 during inflammation[55]. Although the exact mechanism of NO-mediated activation of pro-MMP-9 not clear, it has been suggested that NO disrupts the

zinc-thiolate bond of pro-MMP-9[55]. As such, NO has an effect on NGF regulation through modulation of MMP-9.

Another important example is the soluble protein α_2-Macroglobulin (α_2M). This protein is neurotoxic due to its ability to bind NGF and inhibit proper TrkA activation[52]. It also binds proNGF to prevent processing to NGF, thereby decreasing the NGF/proNGF ratio and increasing the toxic effects of proNGF[56]. Also, the proNGF bound to α_2M has a more potent activation effect on $p75^{NTR}$ than free proNGF, inducing neurotoxic effects more easily[56]. Moreover, MMP-9 degradation is inhibited by α_2M binding, increasing the available MMP-9 for NGF digestion[57].

In addition, regulatory microRNAs (miRNAs) are important in the control of protein synthesis. MiRNAs function by annealing to a target mRNA transcript, preventing protein translation. In fact, dysregulated MMP-9 linked to changes in miR-491-5p have been observed in a variety of different pathologies that are not necessarily related. In cancer studies, miR-491-5p is described as tumor suppressor by negatively controlling MMP-9 translation, which enables migration and invasion of cells[58]. Therefore, changes in miR-491-5p/MMP-9 axis have implications in tumor development. Alternatively, in the urine of aging female patients with OAB, low NGF/proNGF and high levels of MMP-9 were paralleled with a decrease in miR-491-5p[48]. Interestingly, miRNAs involved in the control of proNGF synthesis were unchanged in the same samples, suggesting that the balance of NGF/proNGF ratio occurs at the level of proteases, such as MMP-9[48].

2.5 Inhibition of $p75^{NTR}$ as a therapeutic avenue

Blockade or reduction in the proNGF/$p75^{NTR}$ axis has been explored as a therapeutic avenue in various pathological conditions with neurotrophic imbalance. For example, Shen *et al*. reported

that in mice with frontotemporal lobar degeneration, a neurodegenerative disease, genetic reduction of $p75^{NTR}$ rescued memory defects and suppressed tau pathology, an important hallmark of neurodegeneration[59]. In mice with bladder dysfunction following SCI, systemic blockade of $p75^{NTR}$ using a small molecule, increased excitatory input to the spinal cord and improved bladder function[45]. However, in the same study, genetic deletion of $p75^{NTR}$ reduced voiding efficacy after SCI[45], suggesting that the role of $p75^{NTR}$ and proNGF are nonetheless important in response to injury. Interestingly, in a separate study, $p75^{NTR}$ antagonism was used to restore NGF levels and the NGF/proNGF balance in bladder tissues of mice with diabetic voiding dysfunction, while also improving bladder parameters and voiding behaviour[57]. Thus, antagonism of $p75^{NTR}$ may be of better therapeutic use compared to gene deletion.

2.4 Figures

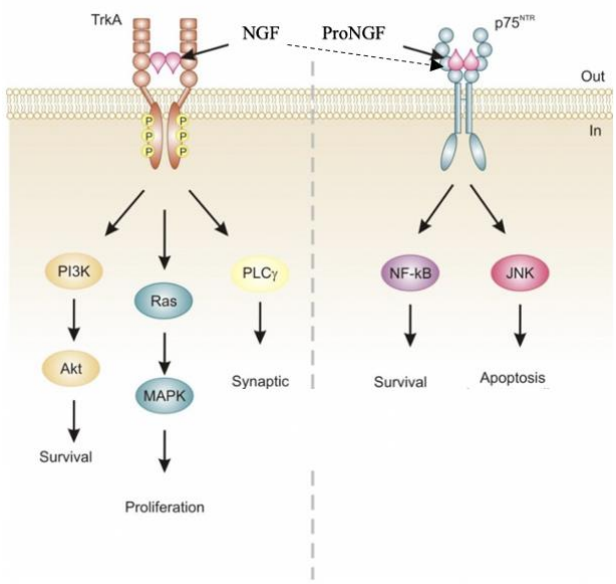

Figure 2. NGF and proNGF signalling pathways. NGF binds and activates TrkA (and $p75^{NTR}$ with low affinity). This leads to cell survival and proliferation in neuronal and non-neuronal tissue as well as synaptic plasticity in neuronal tissue. This is done through PI3K/Akt, Ras/ MAPK and

PLCγ pathways respectively. ProNGF (or NGF) binding to the p75NTR receptor can activate NF-κB for cell survival and JNK for cell apoptosis, both in neuronal and non-neuronal tissue. NGF; Nerve growth factor; PI3K, Phosphatidylinositol-3-kinase; MAPK, Mitogen activated protein kinase; PLCγ, Phospholipase Cγ; NF-κB, Nuclear factor-κB; JNK, c-Jun N-terminal kinase. Figure with slight modifications: Molloy et al., Cancers (Basel). 2011 Feb.

Chapter 3: Rational, Hypothesis, and Objectives

3.1 Rational

Age-related OAB is linked to low NGF/proNGF ratio, which is similarly observed in several age-associated neurodegenerative diseases[4]. In OAB, high urinary MMP-9 leads to low urinary NGF and NGF/proNGF[44]. In bladder tissues of mice with diabetic voiding dysfunction, $p75^{NTR}$ antagonism restored NGF levels and increased NGF/proNGF[57]. However, the link between $p75^{NTR}$ and MMP-9 in bladder tissue and whether $p75^{NTR}$ antagonism is of therapeutic importance in OAB remain to be elucidated.

3.2 Hypothesis and Objectives

3.2.1 General hypothesis

We hypothesized that enhanced activation of $p75^{NTR}$ by increased availability proNGF promotes MMP-9 synthesis and activity which in turn affects NGF levels and impacts bladder function.

3.2.2 Hypothesis 1 & Objective 1 with sub-aims (3)

We hypothesized that $p75^{NTR}$ antagonism inhibits MMP-9 activity and restores NGF/proNGF balance in bladder cells. To test this, our objective was first to understand the control of MMP-9 on NGF levels and their link with $p75^{NTR}$ in bladder cells. We aimed to **(1)** Characterize the expression of MMP-9, NGF and proNGF as well as the associated proteins in bladder cell isolates of young (2-month-old) rats. We also aimed to **(2)** measure the *in vitro* effect of $p75^{NTR}$ antagonism on MMP-9 activity, NGF and proNGF levels. Finally, we **(3)** determined the mechanisms by which $p75^{NTR}$ antagonism affects MMP-9 and the NGF/proNGF balance.

Experimental design 1

UROs and SMCs from 2-month-old Sprague-Dawley rat bladders were cultivated to measure the relative expression of NGF and MMP-9 genes by RT-qPCR. Immunoblotting was used to semi-quantify proNGF, NGF, and MMP-9 protein expression. The medium and cellular contents were kept for quantification of NGF, proNGF and α_2M by ELISA and Plasmin, Furin, MMP-7 and MMP-9 activity by enzymatic assays. These studies were repeated in the presence of the small molecule, THX-B, a $p75^{NTR}$ antagonist to understand the effect of $p75^{NTR}$ blockade on bladder cell gene and protein expressions. Immunoblotting was used to semi-quantify phosphorylated MAPKs in the presence and absence of $p75^{NTR}$ antagonism to further understand $p75^{NTR}$ mechanisms.

3.2.3 Hypothesis 2 & Objective 2 with sub-aims (2)

We hypothesize that treating aging male mice with the $p75^{NTR}$ antagonist, THX-B, will correct age-related voiding dysfunction. To test this, our objective is to determine the functional benefit of treating aging male mice with a $p75^{NTR}$ antagonist. We aim to **(1)** assess bladder behaviour as well as voiding parameters, and **(2)** measure the contractile response of the bladder from treated and untreated mice.

Experimental Design 2

We will obtain sixteen 18-month, sixteen 12-month, and nine 6-month-old (age control) male C57BL/6J mice. Voiding parameters (fluid intake, volume of urine, and frequency of urination) will be assessed in all mice using metabolic chambers. Voiding spot assay (VSA) will be used to evaluate voiding behaviours and patterns observed on filter papers for all mice. Half of the mice from each group will receive the $p75^{NTR}$ antagonist, THX-B, and the remaining mice will receive

PBS control (note: for the 6-month-old-mice, five will receive THX-B and four will receive PBS). Administration of PBS or THX-B will be by intraperitoneal (IP) injections once a week for 4 weeks. Voiding parameters and behaviours will be evaluated as previously described. Cystometry and organ baths will be carried out to evaluate bladder contractility. Tissue pieces will be frozen for protein analysis or fixed in paraformaldehyde 4% in PBS for microscopy. Blood and urine samples will be taken for the measurements of NGF, proNGF and associated enzymes.

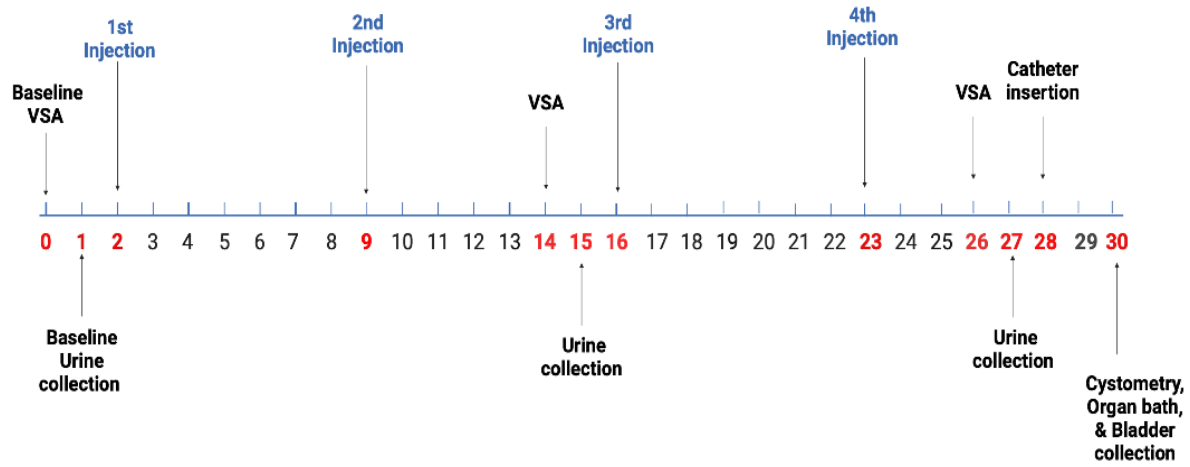

Figure 3. Project timeline. Made with Biorender

Chapter 4: Understanding the control of MMP-9 on NGF levels and their link with the $p75^{NTR}$ receptor in bladder cells

Antagonism of the $p75^{NTR}$ Receptor Decreases MMP-9 Enzymatic Activity and Increases Secretion of NGF by Urothelial Cells in Culture

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Abstract

Urine of aging patients with Overactive Bladder Syndrome (OAB) is characterized by decreased levels of Nerve Growth Factor (NGF) and high activity of the Matrix Metalloproteinase-9 (MMP-9) proteolytic enzyme. In order to understand the origin and relation of these two proteins within the bladder, Sprague-Dawley urothelial cells (UROs) and smooth muscle cells (SMCs) were grown *in vitro*. Expression of NGF and MMP-9 was confirmed by RT-qPCR, immunocytofluorescence and immunoblotting in both cell types. NGF displayed similar levels of synthesis and secretion between both cell types while UROs released most of the MMP-9. Genomic deletion of MMP-9 by Crispr-cas9 potently increased extracellular NGF levels, confirming the essential role of MMP-9 in NGF extracellular survival. On the other hand, THX-B, a $p75^{NTR}$ antagonist that restored normal NGF levels in mice with diabetic voiding dysfunction, increased NGF content in the medium of UROs by decreasing the expression of MMP-9 and increasing the activity of Furin and MMP-7, two enzymes that convert proNGF to NGF. THX-B also decreased the synthesis and release of Alpha-2 Macroglobulin (α2M). Effects of THX-B on SMCs were minor. Finally, THX-B did not affect $p75^{NTR}$ associated intracellular pathways, including cyclic AMP, P38MAPK, Jnk and Erk. This study shows that bladder cells are major sources of NGF and MMP-9 and that MMP-9 controls the amount of NGF released by UROs and SMCs. THX-B could be useful to restore NGF in pathologies where its levels are low.

1. Introduction

Overactive Bladder Syndrome (OAB) is defined by the International Continence Society (ICS) as urinary urgency, usually with urinary frequency and nocturia, with or without urgency urinary incontinence[1]. Such symptoms interfere with daily life, leading to psychological, social, and physical distresses, which reduce the patient's quality of life[1]. OAB is present in approximately 14-18% of Canadians and increases in prevalence with age[1]. The pathophysiological mechanisms of age-related OAB are poorly understood. Nevertheless, studies on aging and metabolic syndrome related to OAB have been linked to the neurotrophins, Nerve Growth Factor (NGF) and its precursor, proNGF.

NGF is expressed by various cell types and has been shown to impart an important survival and homeostatic function on the nervous system through binding the TrkA receptor[2]. On the other hand, proNGF is responsible for apoptosis and degeneration in nervous tissue through activation of the $p75^{NTR}$ receptor[3]. However, recent studies have shown that NGF and proNGF also exhibit similar functions on non-neuronal tissues[3]. An imbalance in the NGF/proNGF ratio and a decrease in NGF have been described in various pathological states such as, among others, spinal cord injury (SCI)[4], cancer[5] and Alzheimer's Disease (AD)[6]. Furthermore, a reduced NGF and NGF/proNGF ratio has shown importance in voiding dysfunction associated with age such as age-related OAB[7,8].

In aging female patients with OAB, urinary NGF levels and the NGF/proNGF ratio were found to be reduced while levels of Matrix Metalloproteinase-9 (MMP-9), the proteolytic enzyme that digests NGF, were increased[8]. In diabetic mice with voiding dysfunction, NGF levels and the NGF/proNGF ratio are also decreased. Systemic treatment (4 weeks) of these mice with a $p75^{NTR}$ antagonist restored NGF levels and the NGF/proNGF balance in bladder tissues while also

improving voiding parameters[9]. So far, the link between bladder $p75^{NTR}$ and NGF, proNGF and MMP-9 is not fully understood.

To better understand the role of NGF and MMP-9 on bladder function, we aimed to characterize the expression patterns of NGF, proNGF and MMP-9 in two major bladder cell types, urothelial cells (UROs) and smooth muscle cells (SMCs). Furthermore, we aimed to understand the control of MMP-9 activity on NGF levels and its link with $p75^{NTR}$ in bladder cells.

2. Materials and methods

Cell culture

Young, 2-month-old Sprague-Dawley rats were handled in accordance with the Canadian Council for Animal Care (CCAC). All protocols were approved by the Animal Ethics Committee of McGill University (Quebec, Canada). Animals received standard Purina chow and had free access to water. Euthanasia by exsanguination under isoflurane anesthesia was performed and bladders were excised and placed in cold sterile PBS (pH 7.4). The urothelium was scraped and placed in DMEM medium containing 100 U/mL of collagenase IV for 15-20 minutes at 37ºC with gentle shaking. UROs were then washed in DMEM 10% fetal bovine serum (FBS) twice, then placed in Dubelco's DMEM low glucose/Keratinocyte (50/50) media containing FBS (10%), Glutamax (X1), hormones mix (insulin 5 µg/mL, dihydrocortisone 0.5 µg/mL, adenine 15 µg/mL, ethanolamine 0.1 mM), Rho Inhibitor Y27632 (10 µM) and 1% penicillin/streptomycin (100 U/mL, 100 µg/mL) in a humidified incubator under 5% CO_2 atmosphere. The medium was replaced every 2-3 days until cell confluency. Cells were starved 24 hours prior to use. On the other hand, bladder detrusor muscle was minced and incubated in DMEM containing 250 U/mL of collagenase IV for 45 minutes, with intense shaking, followed by two washings. SMCs were grown in SK medium

(Wisent, St-Bruno, Canada) and supplemented with FBS (10%), high glucose (27 mM) and penicillin/streptomycin (100 U/mL, 100 µg/mL). The SMCs were starved for a 72-hour period under normoglycemic conditions. For UROs and SMCs, incubation with THX-B (5 µg/mL) was carried out for 24 hours.

Immunocytofluorescence

Cells were grown on sterilized glass coverslips. After confluence was achieved, cells were fixed in paraformaldehyde 4% in PBS for 24 hours then processed for immunocytofluorescence. Cells were permeabilized with a buffer containing Triton X100 0.1%, glycine 300nM, ammonium chloride 75nM, PBS pH 8, for 10 minutes. Wells were then carefully washed with PBS. Buffer with 1% BSA in PBS was added for 30 minutes to block non-specific sites then previously validated primary antibodies were added in the same buffer overnight at 4 C. The next day, cells were washed with PBS 4 times before incubation with secondary antibodies conjugated to DY488 (1:250 in BSA 1%) for 1 hour at room temperature and the dark. After washing with PBS, coverslips were removed from the wells and mounted on individual slides with DAPI. Slides were examined by fluorescence microscopy (Leica).

Quantitative PCR

Total RNA was extracted and purified from UROs and SMCs using Trizol reagent (Biomatik Corporation) and chloroform. RNA concentration and purity were quantified by a Nanodrop spectrophotometer ND-1000 (ThermoFisher Scientific, Wilmington DE). The cDNA synthesis relies on a reverse transcriptase kit (OneScript cDNA synthesis kit) according to the manufacture's protocol (ABM, Richmond BC, Canada). For qPCR, the following primers were purchased from

Integrated DNA Technologies (IDT, Coralville, IOWA, USA): NGF forward primer (5'-CCC GAA TCC TGT AGA GAG TGG-3'), NGF reverse primer (5'GAC AAA GGT GTG AGT CGT GG-3'), MMP-9 forward primer (5'-CCA TGC ACT GGG CTT AGA TCA T-3'), MMP-9 reverse primer (5'-CAG ATA CTG GAT GCC GTC TAT GTC-3'), GAPDH forward primer rat GAPDH (5'-TGC CAC TCA GAA GAC TGT GG-3'), GAPDH reverse primer reverse (5'- TTC AGC TCT GGG ATG ACC TT-3'), 18S forward primer (5'-GCA ATT ATT CCC CAT GAA CG-3'), 18S reverse primer (5'-GGC CTC ACT AAA CCA TCC AA-3'). Samples were amplified (Applied Bioscience 7500 Fast Real-Time PCR) using the Sensifast Probe Low-ROX kit containing SYBR-green. Conditions of the qPCR included 35 cycles of heating and cooling (30s at 95°C for denaturation, 30s at 57°C for annealing and 30s at 72°C for extension) followed by a final extension at 72°C for 10 min. Samples were prepared in duplicates and melt curve data performed each time. Specificity of primers was conducted to ensure efficiency (90-110%). Using purified RNA without reverse transcription, controls were also assessed. Final data analysis was done using the $2^{-\Delta\Delta CT}$method[10].

Immunoblotting

Proteins were extracted in RIPA buffer containing anti-protease cocktail (Roche Diagnostics, Manheim, Germany). Concentration of proteins were measured by Micro BCA assay kit (Boster Biological Technology, CA, USA). Equal amounts of protein (30 μg) were loaded on 8% polyacrylamide gel and electro-transferred to PVDF membranes. After blocking in 5% skimmed (non-fat) milk in 5% TBST for 1 h, overnight incubation with primary antibodies (4ºC) was done at the following concentrations: anti-MMP-9 (1:4000), anti-NGF (1:2000), anti-proNGF (1:2000), anti- α_2-Macroglobulin (1:2000), anti-p-P38 (1:2000), anti-P38 (1:2000), anti-p-ERK (1:2000),

anti-ERK (1:2000), anti-p-JNK (1:2000), anti-JNK(1:2000) anti-β-actin (1:20,000). The next day, secondary HRP-conjugated antibodies (anti-rabbit or anti-mouse antibodies from EMD Millipore Corp., USA) were used at room temperature for 1 hour at 1:3000 concentration in TBST 5% milk. Bands were revealed with Luminata Crescendo horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA) and quantified by ImageJ (Version: 2.0.0-rc-66/1.52n, NIH, USA). B-actin was used as control of protein loading. For p-P38, p-ERK and p-JNK, their respective non-phosphorylated protein levels were used as the control instead of β-actin.

ELISA kits

Cell extracts and supernatants were used to quantify proteins of interest: proNGF and NGF (RapidTM ELISA kit, Biosensis, Australia) and α_2-Macroglobulin (Abcam, Cambridge, MA, USA) as per the manufacturer's protocol.

Enzymatic Activity Assay

MMP-9 and MMP-7 activities were measured using an enzymatic kit from Quickzyme Biosciences (Leiden, The Netherlands). Furin and plasmin activity assay kits were respectively from BioVision (Milpitas, CA, USA) and Millipore-Sigma (St-Louis, MO, USA).

Statistical Analysis

Results were expressed as median and standard error of the mean (SEM). Statistical significance was reported as P value of < 0.05, <0.01 and <0.001.

3. Results

MMP-9 and NGF messenger RNA (mRNA) expression in UROs and SMCs.

Since proNGF and MMP-9 expression levels have not been characterized in bladder tissue, we grew primary cultures of UROs and SMCs and analyzed their cell content. NGF and MMP-9 mRNAs were found to be expressed in both cell types (Figure 1A) with similar expression levels as quantified by RT-qPCR (Figure 1B). Immunoblots revealed lower NGF, proNGF and NGF/proNGF ratio in SMCs compared to UROs, whereas intracellular MMP-9 protein level was significantly higher in SMCs (Figure 1C). Extracellular expression patterns were opposite to the intracellular environment. SMC medium exhibited higher levels of NGF and proNGF compared to URO medium when quantified by ELISA (Figure 1D). Concomitantly, extracellular MMP-9 activity measured by enzymatic assay was 40 times lower in SMCs medium compared to URO (Figure 1D). The relationship between NGF/proNGF and MMP-9 therefore appears to be inverse, similar to our intracellular observations. Immunofluorescence confirmed the expression of MMP-9 and NGF in UROs compared to SMCs (Figure 2A).

MMP-9 proteolytic activity controls NGF levels and regulates the NGF/proNGF balance.

Levels of NGF in part depend on its digestion by proteolytic enzymes[11]. MMP-9 is an important enzyme responsible for NGF degradation in central and peripheral nervous system tissues[12,13]. To determine the importance of MMP-9 in the survival of NGF in bladder cells, genomic deletion was carried out using CRIPSR-Cas9. Successful knockout (KO) of MMP-9 was obtained as revealed by RT-qPCR, immunoblotting (intracellular content) and extracellular proteolytic activity in UROs (Figure 3A) and SMCs (Figure 3B). Extracellular NGF was significantly increased in the MMP-9 KO compared to control while extracellular proNGF levels were unchanged in both SMCs

(Figure 3A) and UROs (Figure 3B) as revealed by ELISA. As a result, the NGF/proNGF balanced significantly increased.

Antagonism of urothelial p75^{NTR} increased extracellular NGF and decreased intracellular MMP-9 levels.

The p75^{NTR} antagonist, THX-B, can restore NGF levels and NGF/proNGF ratio in bladder tissues of diabetic mice[9]. In order to determine how THX-B acts on bladder cells, we incubated UROs and SMCs cells with THX-B (5μg/mL) for 24 hours. RT-qPCR revealed unchanged levels of NGF and MMP-9 mRNA in treated UROs (Figure 4A). Compared to controls, mean extracellular NGF was increased while mean intracellular proNGF decreased with THX-B treatment when measured by ELISA (Figure 4B). As a result, the extracellular NGF/proNGF ratio increased by 82% ($p < 0.001$) (Figure 4B). Mean MMP-9 intracellular content measured by immunoblot and extracellular activity measured by enzymatic assay decreased when treated with THX-B (Figure 4C), suggesting that the increase in NGF could come at least in part from decreased MMP-9 proteolytic activity. Finally, mean α-2 Macroglobulin (α2M), a protein that increases MMP-9 as well as proNGF stability, was decreased intracellularly as revealed by immunoblot and extracellularly when measured by ELISA (figure 4D).

Antagonism of p75^{NTR} in SMCs decreased extracellular NGF protein content.

In THX-B-treated SMCs, gene expression was unchanged for NGF but reduced for MMP-9 when quantified by RTqPCR (Figure 5A). Only extracellular NGF protein content measured by ELISA was reduced by THX-B (Figure 5B), with no significant effect on the SMC extracellular ratio of NGF/proNGF (Figure 5B). The reduction in MMP-9 gene expression was not matched by a similar

decrease in MMP-9 intracellular content measured by immunoblot or extracellular activity measured by enzymatic assay (Figure 5C). Finally, mean α2M release in the medium and quantified by ELISA was increased with THX-B treatment, whereas intracellular α2M showed no significant change on immunoblot (figure 5D).

p75^{NTR} Antagonism decreased activity of extracellular SMC Plasmin and extracellular URO Furin but increased activity of intracellular URO MMP-7 and Furin.

MMP-9 controls the survival of extracellular NGF, yet we observed decrease in mean extracellular NGF in SMCs without changes in intracellular or extracellular MMP-9 with THX-B. Similarly, intracellular proNGF was decreased in treated UROs. Enzymes converting proNGF to NGF are Furin intracellularly and Plasmin and MMP-7 extracellularly. Intracellularly, enzymatic assay revealed that THX-B-treated UROs showed no change in mean Plasmin activity (Figure 6A), but an increased mean MMP-7 activity (Figure 6B). There was also an increased activity of intracellular Furin by 390% ($p < 0.001$) (Figure 6C), suggesting an increase in the proteolysis of proNGF to NGF. Treated SMCs showed an increased activity of intracellular MMP-7 (Figure 6B). Extracellularly however, there was reduced Furin activity in URO media (Figure 6C) and a decreased Plasmin activity (Figure 6A) in SMC media with THX-B treatment, which could account for the decrease in extracellular NGF. These findings also demonstrate that UROs and SMCs present different pathways in the synthesis and secretion of NGF.

P75^{NTR} antagonism did not alter the MAPK pathway activity.

To further understand the relationship between p75^{NTR} antagonism and the changes in NGF and MMP-9 that were observed in treated UROs or SMCs, we studied the effect of THX-B on the

pathways linked to $p75^{NTR}$ using immunoblot. Neither treated UROs nor SMCs showed changes in the levels of cyclic AMP (cAMP) or activated p38MAPK, JNK, or ERK (Figure 7) by THX-B compared to controls.

4. Discussion and conclusions

Evidence of the pathological role of proNGF/$p75^{NTR}$ axis activity has been demonstrated in many disease states such as neurodegeneration and voiding dysfunction associated with age and metabolic syndrome[4,9,14]. NGF levels in urine and bladder tissue are altered in voiding dysfunction in female mice and female patients with OAB[8,9] Specifically, there is a decrease in the NGF/proNGF ratio in these conditions that lead to an abnormal balance of cell survival through the NGF/TrkA axis versus tissue degeneration through the proNGF/$p75^{NTR}$ axis[15,16]. As well, overexpression of MMP-9 has been observed in these conditions and in other pathological states, such as cancer and ocular disease[17,18]. This study successfully characterized the genetic and protein expression patterns of NGF, proNGF and MMP-9 in normal UROs and SMCs of young (2-month-old) female rat bladders. We found that NGF, proNGF and MMP-9 have distinct protein expression patterns depending on the intracellular and extracellular environment as well as cell type. Future studies characterizing bladder cells in female mice and rats may be important for identifying sex and species-related differences that contribute to our understanding of bladder behaviour observed in preliminary animal and human studies.

The secretion of proNGF and NGF is activity-dependent such that secretion changes based on cell requirements[19]. ProNGF and NGF mainly exhibit their effects through binding extracellular domains of $p75^{NTR}$ and TrkA, respectively[12]. Both cell types secrete NGF and proNGF while MMP-9 appeared to be produced mainly by UROs. These patterns might explain migratory,

regenerative, and proliferative capacities of the bladder urothelium and detrusor smooth muscle. For example, proNGF has been shown to have adaptive proliferative and migratory effects on SMCs through activating $p75^{NTR}$ and downstream nuclear transcription factor, NF-κB[15,16]. In the bladder, SMCs have a highly regenerative and proliferative nature compared to UROs, which are normally mitotically quiescent, likely explaining our results[20]. Moreover, Ryu *et al*. have demonstrated that TrkA and $p75^{NTR}$ expression is greater in detrusor smooth muscle tissue compared to the urothelium, explaining why we observed high levels of their respective ligands, NGF and proNGF, in SMC media[4].

NGF/proNGF extracellular ratio may be particularly important not only in regulating SMCs themselves, but also in regulating surrounding nervous tissue[7,15]. Detrusor contraction and relaxation is under tight neurogenic control, requiring relatively greater pro-survival and homeostatic signals from NGF than apoptotic and degenerative signals from proNGF on surrounding nerves[15]. Importantly, NGF has an important role in sensitizing afferents, which allows for better transduction of mechanical and chemical stimuli such as urine contents and bladder fullness[21].

Extracellular MMP-9 mediates cell migration, tissue invasion and adhesion by degrading the extracellular matrix (ECM)[22]. Additionally, MMP-9 digests NGF, reducing its concentration in the extracellular space[13]. In URO media, we observed high extracellular MMP-9, which might explain the lower extracellular NGF compared to SMCs. This was confirmed by Cripsr-Cas9 mediated KO of MMP-9 that resulted in an increased NGF without changes in proNGF. High extracellular URO MMP-9 also suggests UROs have an increased MMP-9-mediated cell migration, invasion and adhesion capacity compared to SMCs (which may rely mainly on proNGF for migration instead, as previously described). This concept is apparent in cancer pathogenesis.

For example, upregulated MMP-9 is involved in URO migration and invasion in urothelial carcinoma of the urinary tract[17]. Additionally, Sutherland *et al*. have shown that in an acellular tissue matrix studying bladder ontogenesis and wound healing processes, the urothelium extends and migrates early, while aiding the development of the detrusor smooth muscle later on[23]. UROs might therefore require the ability to migrate quickly when crucial formation and repair of the watertight barrier is needed[23].

The opposite protein expression profile was observed in the intracellular contents; URO showed higher intracellular NGF, proNGF and overall higher NGF/proNGF ratio, with lower MMP-9 compared to SMCs. We confirm the findings of several prior studies in the literature that describe high NGF immunoreactivity in the urothelium[7]. De Boer *et al*., have shown that although UROs proliferate slowly in normal conditions, they shift to a highly mitotic state during injury[24]. Therefore, one explanation for our observations is that UROs may have large intracellular reserve of NGF and proNGF since they exhibit high regenerative and proliferative capacities only during injury. Intracellular storage of these proteins may be required to ensure ready secretion and response to injury, especially given that the urothelium is the first layer in contact with noxious urinary components. Alternatively, there is evidence that intracellular NGF interacts with recycled TrkA in endosomes in axons for retrograde signalling[25]. In bladder cells, signalling endosomes can be particularly important in relaying specific information towards the basolateral versus apical membranes for specialized cell functions[26]. Whether or not NGF is involved in signalling endosomes in UROs and SMCs remains to be studied.

As opposed to the extracellular environment, UROs had a higher expression of intracellular MMP-9 compared to SMCs. Intracellular MMP-9 has been shown to interfere with mitochondria in neurons and retinal cells to induce apoptosis through activation of caspase-3[27]. In SMCs, the

high extracellular NGF/proNGF ratio favoring cell survival and homeostasis that we also observed may therefore be important for balancing out potential apoptotic effects of intracellular MMP-9.

MMP-9 controls NGF levels and NGF/proNGF balance which we confirmed using MMP-9 KOs. Diabetic mice with voiding dysfunction treated with the $p75^{NTR}$ antagonist, THX-B, showed restored NGF and NGF/proNGF in bladder tissues and improved voiding parameters such as contractility, frequency, and bladder capacity[9]. Additionally, aging female patients with OAB show increased urinary MMP-9 with reduced NGF and NGF/proNGF balance[8]. The role of THX-B on NGF and NGF/proNGF balance was confirmed in this study through inhibition of MMP-9 in UROs and SMCs. More precisely, $p75^{NTR}$ antagonism targeted Furin, α2M and MMP-9 in the bladder urothelium. The increase in extracellular NGF from UROs can be explained by reduced extracellular MMP-9 activity and therefore reduced NGF proteolysis. Since Furin is responsible for intracellular conversion of proNGF to NGF, the increase in intracellular Furin activity in UROs could also explain the observed reduction in intracellular proNGF. We also observed a decrease in extracellular Furin activity with THX-B treatment of UROs. While the function of extracellular Furin is not well characterized, it has been suggested that the protease can be processed and shed into the extracellular space and found in the urine[29]. In fact, urinary Furin stimulates Epithelial Sodium Channels (ENaCs) of the kidney, increasing sodium and water retention and its levels are increased in patients and animal models with congestive heart failure and urinary retention[29]. Thus, THX-B may show an added benefit by decreasing extracellular Furin from UROs.

In addition to a decrease in extracellular MMP-9, $p75^{NTR}$ antagonism by THX-B also reduced intracellular MMP-9. While the role of intracellular MMP-9 in bladder tissue is not known, intracellular MMP-9 has been shown to have pro-apoptotic functions in some cells. For example, MMP-9 induced activation of caspase-3 in endothelial cells in hyperglycemic states to

promote apoptosis[30]. As well, intracellular MMP-9 has been shown to promote cytokine release and neuroinflammation[31]. Bladder inflammation is known to negatively affect bladder function and can lead to changes such as detrusor overactivity[32]. In addition, chronic inflammation has been suggested to be crucial in the pathogenesis of OAB. In fact, anti-inflammatory medications have been shown to reduce certain bladder symptoms[33]. Given that intracellular MMP-9 has important pro-inflammatory functions, a reduction in intracellular urothelial MMP-9 as seen with $p75^{NTR}$ antagonism by THX-B may be of therapeutic potential and merits further investigation.

In the plasma, α2M has been found to bind MMP-9 and protects it from protease degradation, prolonging it functions, notably NGF digestion[34]. Furthermore, previous studies in mouse retinal ganglion cells have shown that α2M also complexes with proNGF to increase its stability and prevent proteolysis by Furin, while also increasing the potency of proNGF as a $p75^{NTR}$ agonist[12]. The same study found that $p75^{NTR}$ antagonism with THX-B reduced α2M-induced neurotoxicity[12]. Here, $p75^{NTR}$ antagonism reduced intracellular and extracellular α2M levels as well as intracellular MMP-9 and its extracellular activity in UROs, suggesting that $p75^{NTR}$ modulates MMP-9 activity through altering urothelial α2M levels, which in turn may participate in the increased extracellular NGF/proNGF ratio. In addition, given that α2M protects proNGF from Furin's proteolytic activity, the reduction in α2M by THX-B can also explain why intracellular proNGF is reduced and secreted NGF is increased in treated UROs.

$P75^{NTR}$ antagonism had minimal effects on SMCs. In treated SMCs, there were no changes in intracellular or extracellular MMP-9 nor intracellular α2M. Surprisingly however, extracellular α2M activity did increase without altered MMP-9. Thus, it is possible that changes in extracellular α2M, although significant, were not enough to effect MMP-9 activity. This suggests that $p75^{NTR}$ differentially regulates α2M depending on the cell type. Nonetheless, the increase in extracellular

α2M may in part explain the decreased extracellular NGF levels as α2M protects proNGF from conversion to NGF. Further, the reduced extracellular NGF levels can also be partly explained by the decrease in SMC extracellular Plasmin activity, which converts proNGF to NGF. In vascular SMCs, Plasmin has been shown to promote SMC proliferation[35]. Detrusor muscle hyperplasia alters bladder physiology and is a common pathological finding in voiding dysfunction[15]. Therefore, our results suggest an added benefit of THX-B treatment through reducing extracellular SMC Plasmin and possibly the bladder's synthetic phenotype in voiding dysfunction.

Finally, we were interested in better understanding pathways downstream of $p75^{NTR}$ activation, specifically the MAPKs, and their function under THX-B treatment. $p75^{NTR}$ antagonism with THX-B did not alter the levels of activated (phosphorylated) p38MAPK, ERK or JNK proteins involved in $p75^{NTR}$ intracellular pathways, suggesting that any changes induced by treatment involve other pathways.

In conclusion, $p75^{NTR}$ antagonism mainly targets the bladder urothelium through inhibiting the expression of MMP-9, α2M, and Furin and allowing an increase in NGF and the NGF/proNGF ratio. Given that aging patients with OAB exhibit low urinary NGF and NGF/proNGF ratio with increased MMP-9, our results suggest that $p75^{NTR}$ blockage has therapeutic potential. Further studies evaluating the *in vivo* functional and behavioural effects of $p75^{NTR}$ antagonism on the aging bladder will be important in assessing it's benefits in age-related voiding dysfunction.

5. Figures

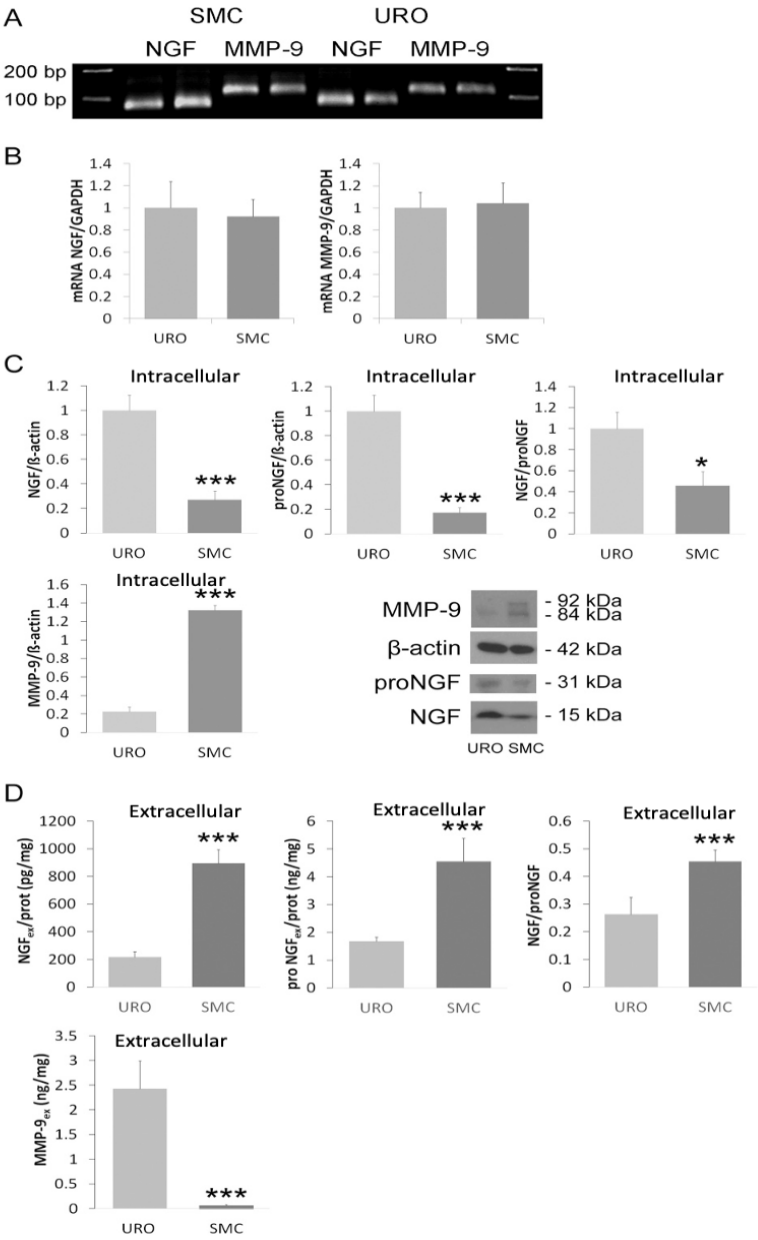

Figure 1. **UROs and SMCs are sources of NGF and MMP-9.** (A) NGF and MMP-9 mRNAs were expressed in both cell types. (B) Quantification by RTqPCR revealed similar levels of expression between URO and SMC. (C) Immunoblotting was used to semi-quantify intracellular NGF, proNGF and MMP-9 content. (D) Extracellular concentrations of NGF and proNGF were assessed by ELISA and enzymatic activity of MMP-9 by enzymatic assay. (n=6), student t-test (*P<0.05, ***P<0.001).

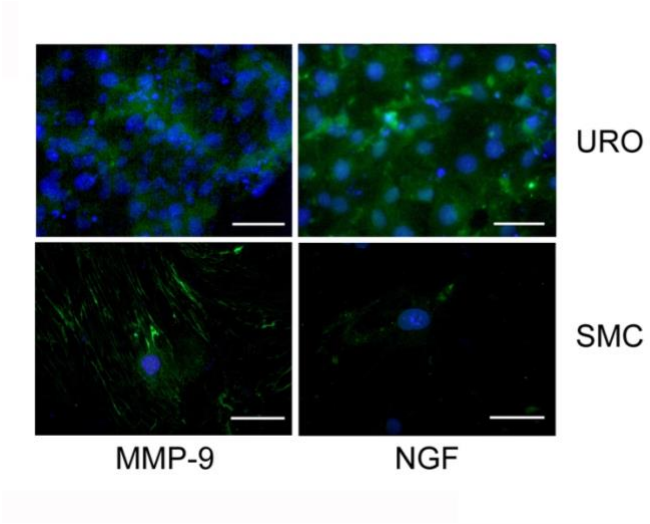

Figure 2. Immunocytofluorescence detecting MMP-9 and NGF expression in URO and SMC. Both cells revealed NGF and MMP-9 protein expression (bars= 10 µm).

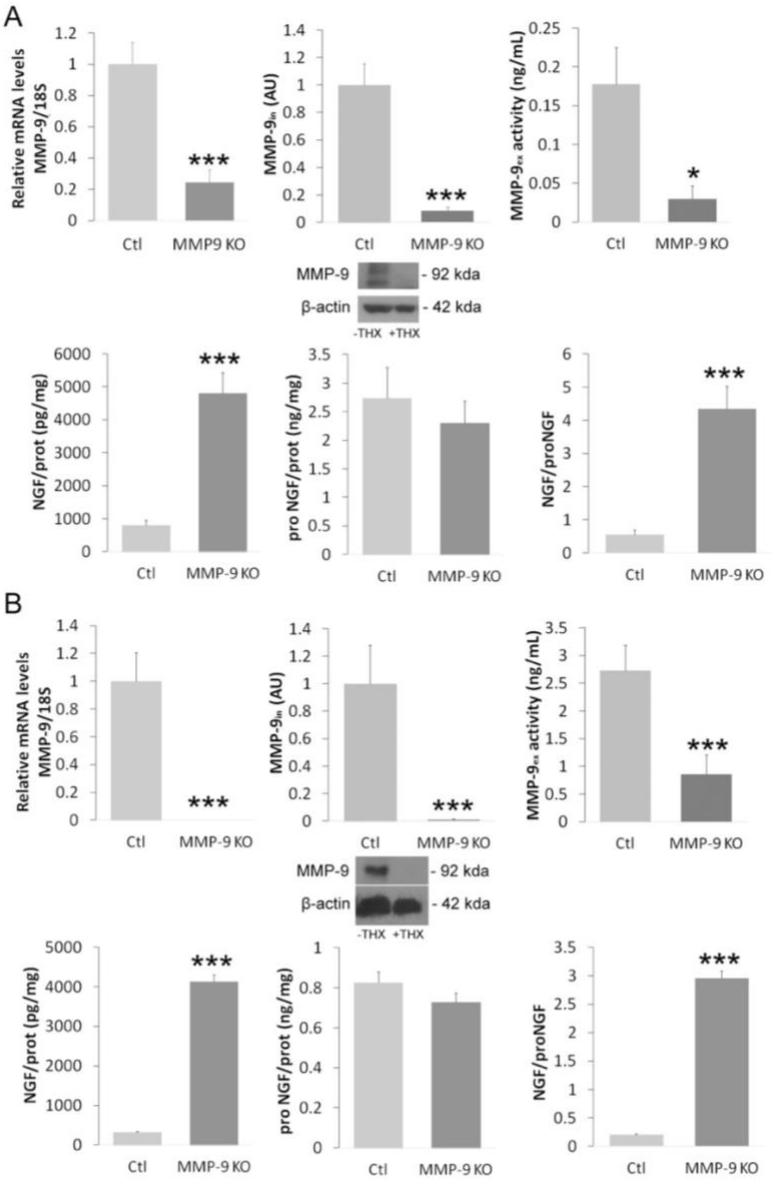

Figure 3. Crispr-cas9 on NGF and proNGF secretion in (A) SMCs and (B) UROs. Transfection was carried out using an empty plasmid (Ctl) or a plasmid containing sg primers targeting specific sequences of the MMP-9 gene. Efficiency of the MMP-9 knockout was confirmed by measuring MMP9/18S RNA ratio by RT-qPCR, intracellular MMP-9 relative content by immunoblotting and extracellular MMP-9 activity by enzymatic assay. In parallel, NGF and proNGF were assessed in extracellular medium using ELISA. (n=6), student t-test (*P<0.05, ***P<0.001).

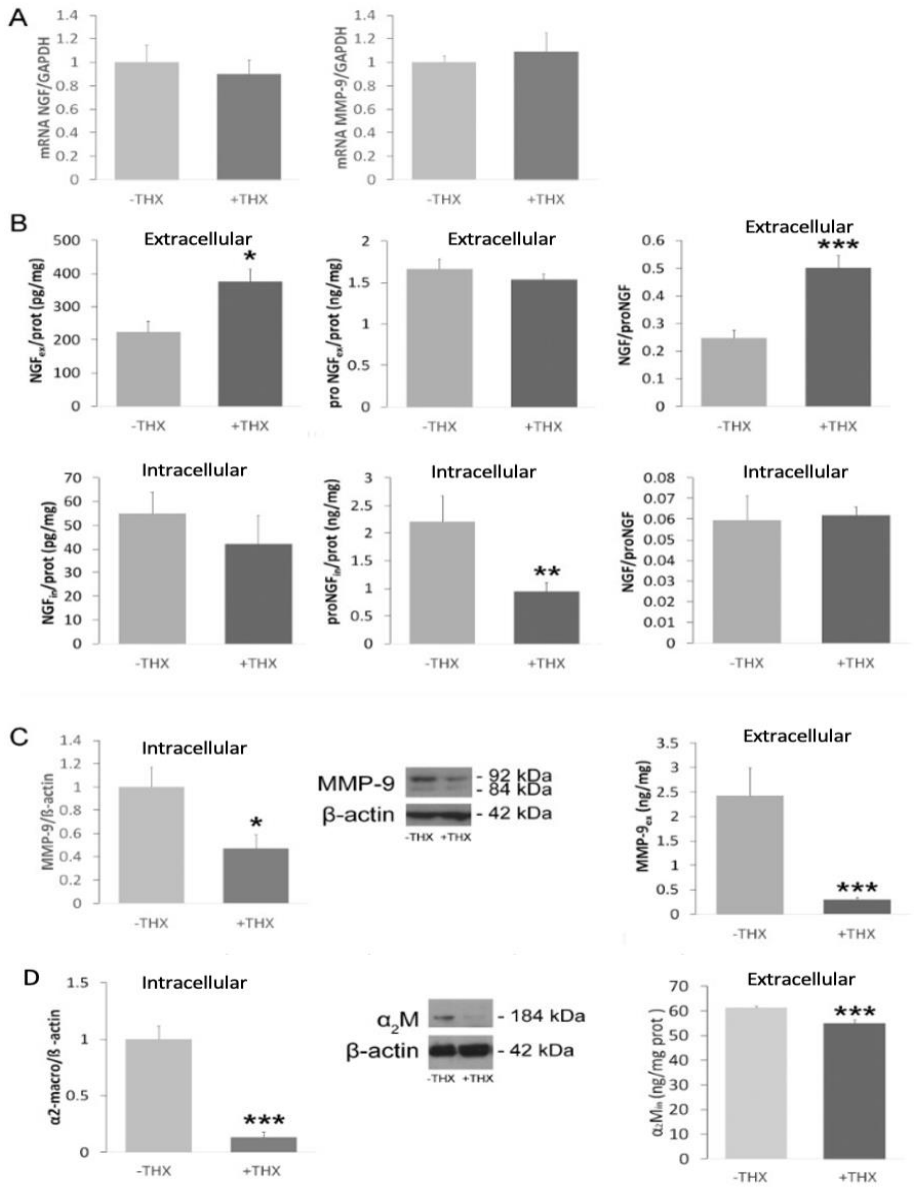

Figure 4. Effect of THX-B on UROs. (A) Incubation of cells with THX-B (5 µg/mL) for 24 hours did not affect NGF or MMP-9 mRNAs as quantified by RTqPCR. (B) Extracellular and intracellular NGF and proNGF were measured by ELISA kits, revealing increased extracellular NGF and NGF/proNGF, and reduced intracellular proNGF. (C) MMP-9 cell content as assessed by immunoblot was decreased as well as extracellular MMP-9 activity measured by enzymatic assay. (D) Levels of intracellular α2M measured by immunoblot as well as extracellular α2M measured by ELISA were both decreased. (n=6), student t-test (*P<0.05, **P<0.01, ***P<0.001).

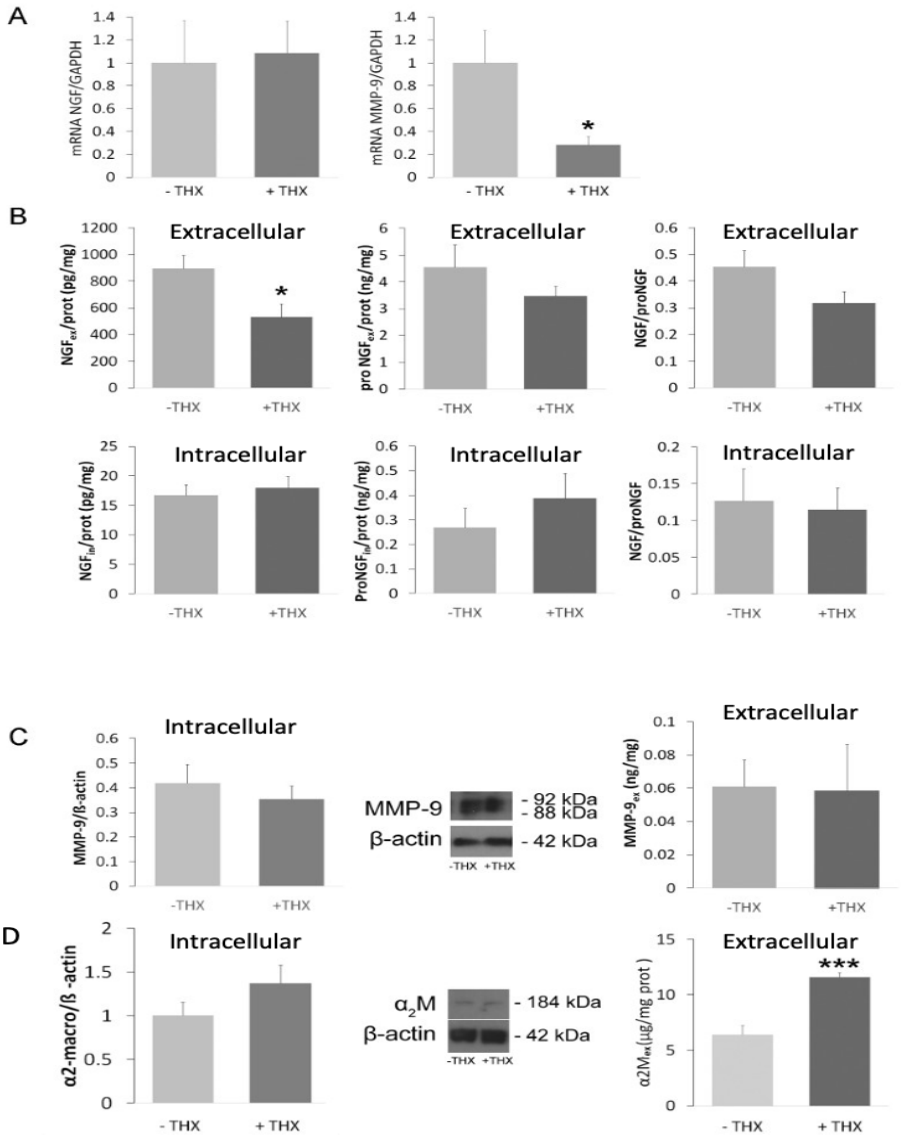

Figure 5. Effect of THX-B on SMCs. (A) Incubation of cells with THX-B (5 µg/mL) for 24 hours did not affect expression of NGF but decreased MMP-9 mRNA revealed by RTqPCR. (B) Extracellular and intracellular NGF and proNGF were measured by ELISA kits, revealing a decrease in extracellular NGF. (C) MMP-9 cell content as assessed by immunoblot was unchanged as well as extracellular MMP-9 activity measured by enzymatic assay. (D) Intracellular α2M content measured by immunoblot was not affected whereas and extracellular content measured by ELISA was increased with THX-B. (n=6), student t-test (*P<0.05).

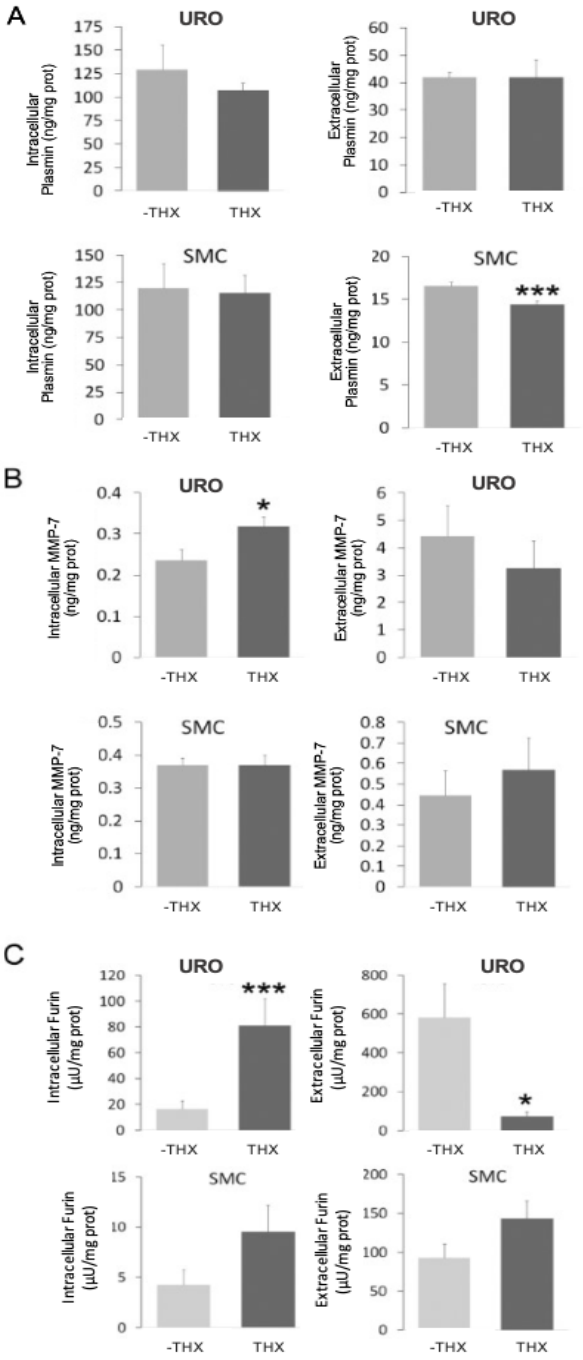

Figure 6. Plasmin (A), MMP-7 (B) and Furin (C) activity measured in UROS and SMCs, both intracellularly and extracellularly by enzymatic assay. (n=6), student t-test (*P<0.05, **P<0.01, ***P<0.001).

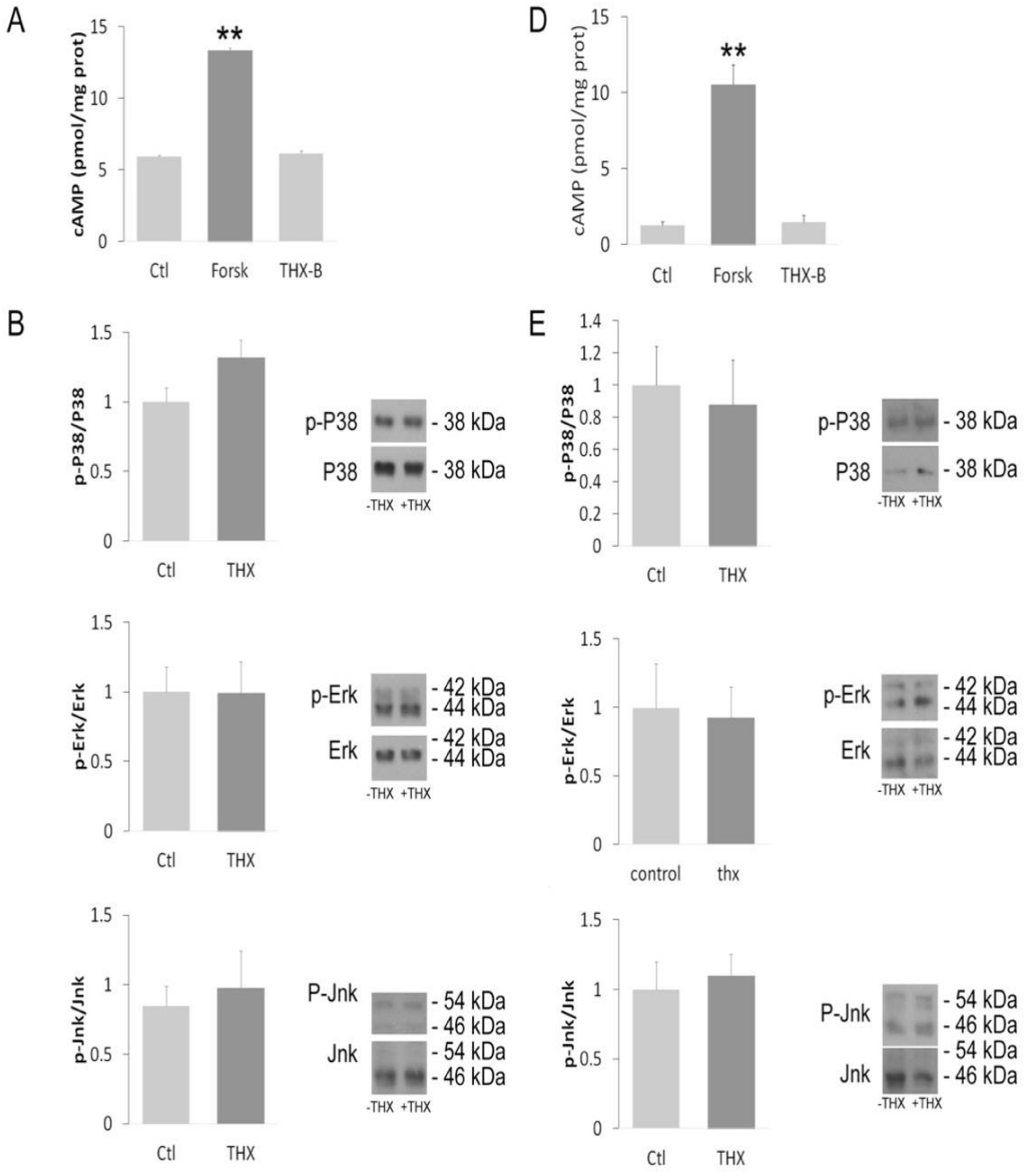

Figure 7. Measurements of intracellular pathway activation by THX-B. In (A) UROs and (D) SMCs, increase in cyclic AMP content in the presence of THX-B (5 µg/mL) for 24 hours was compared to forskolin (10 µM). (B) UROs and (E) SMCs were also assessed for *p*-P38MAPK, *p*-Erk and *p*-Jnk and their respective non-phosphorylated forms using immunoblot (n=6), student t-test (**P<0.01).

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Bridging Manuscripts

The previous chapter focused on characterizing two major cells of the bladder, UROs and SMCs in terms of NGF, proNGF and MMP-9 expression derived from young female rats. Given that aging patients with OAB have shown urinary NGF/proNGF imbalance with high urinary MMP-9, this study was essential in order to understand the normal (young) bladder environment with regards to protein function[57]. Further, we used an antagonist of the proNGF receptor, $p75^{NTR}$, as a tool to understand the control of NGF, proNGF and MMP-9, which is important in understanding future therapeutic solutions to age-related OAB. We identified that $p75^{NTR}$ antagonism primarily targets UROs as it increased extracellular NGF and NGF/proNGF through inhibition of MMP-9. This is therefore clinically valuable since an imbalance in NGF/proNGF has been observed in several studies examining age-related pathologies, including age-related OAB.

Studies have shown that aging mice displayed similar changes in bladder function to aging humans, such as altered contractility and urine storage[58]. Understanding the use of $p75^{NTR}$ antagonism *in vivo* in mice will bridge the gap between our *in vitro* findings and whole system pharmacodynamics that are clinically relevant and a step towards necessary translational research. Therefore, the purpose of the following chapter is to illustrate functional improvements in the bladder of aging male mice treated with a $p75^{NTR}$ antagonist.

Chapter 5: Determining the functional benefit of treating aging mice bladders with a $p75^{NTR}$ antagonist

The p75 neurotrophin receptor antagonist THX-B improves voiding behavior and reduces bladder contractility in aging mice

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The authors have declared that no conflict of interest exist.

Abstract

Introduction: Overactive Bladder Syndrome (OAB) is characterized by low urinary levels of the Nerve Growth Factor (NGF) in aging female patients, which was linked to high activity of the proteolytic enzyme Matrix Metalloproteinase-9 (MMP-9). NGF provides important homeostatic functions and promotes tissue health, particularly for cells of the nervous system. We previously found that THX-B, an antagonist of the pro-inflammatory p75 neurotrophin receptor ($p75^{NTR}$), increased NGF levels by decreasing MMP-9 activity in urothelial cell (URO) culture. Here, we aimed to assess *in vivo* the effect of THX-B on bladder behaviour and parameters.

Methods: Male C57BL/6J mice of 6-, 12- and 18-months were injected intraperitoneal with either PBS (control) or THX-B (50 μg) once weekly for four weeks. Mice were separated based on age and expression of urinary $p75^{NTR}$ extracellular domain (ECD). Voiding behaviors and patterns, notably, total urine volume, volume per micturition, and frequency of urination were assessed using Voiding Spot Assay (VSA). Conscious cystometry was conducted to measure bladder contractile parameters. Bladders were collected for organ bath to evaluate contractility using electrical field stimulation (EFS), potassium chloride (KCl) and carbachol as contractile stimuli.

Results: Compared to age-matched controls, voiding behavior and bladder contractility was improved only in the 12-month-old mice treated with THX-B. Specifically, total urine volume and voiding frequency were low following four weeks of treatment. Residual volume and bladder contractility were lower in the same mice compared to age-matched controls.

Conclusion: Our results illustrate an age-specific effect of THX-B on age-related bladder dysfunction. Better bladder behavior and activity were observed only in 12-month-old treated mice. These results are in accordance with our previous studies and suggest that $p75^{NTR}$ might be a pharmacological target for the treatment of voiding dysfunction.

1. Introduction

The pathophysiologic process of aging is complex and poorly understood. Nonetheless, various age-related pathologies have been linked with distinct neurodegenerative changes, such as Overactive Bladder Syndrome (OAB). OAB is defined by the International Continence Society (ICS) as urinary urgency, usually with urinary frequency and nocturia, with or without urgency urinary incontinence[1]. Approximately 14-18% of Canadians present with OAB, and this prevalence increases with age[1]. More specifically there is a higher overall incidence in women (21.2%) compared to men (14.8%)[1]. Given that the pathophysiological mechanisms of age-related OAB are poorly understood, curative strategies are non-existent and current pharmacologic agents are of limited benefit. However, models of age and metabolic disease have recently been linked to changes in the levels of Nerve Growth Factor (NGF) and its precursor, proNGF.

NGF is expressed by various cell types and binds the TrkA receptor to carry out important survival and homeostatic functions within the nervous system[2]. Conversely, proNGF imparts apoptotic and degenerative effects in nervous tissue through binding the pro-inflammatory p75 neurotrophin receptor ($p75^{NTR}$)[3]. Importantly, recent studies have also shown that NGF and proNGF exhibit their functions on non-neuronal tissues[3]. A decrease in NGF and the NGF/proNGF ratio has been linked with aging and neurodegenerative processes, such as, Alzheimer's Disease (AD)[4], spinal cord injury (SCI)[5], diabetic retinopathy[6], and voiding dysfunction[7].

We recently found that in the urine of aging female patients with OAB, NGF urinary levels were decreased with stable proNGF such that the NGF/proNGF ratio was reduced[1]. These results were linked to a high level of the proteolytic enzyme Matrix Metalloprotinase-9 (MMP-9)[1]. Given that the bladder is under tight neurogenic control, changes in the central and peripheral nervous

system activities as well as bladder physiology may be important in voiding dysfunction, warranting investigation in the control of NGF and proNGF expression.

We also previously found that the small compound THX-B, which is a $p75^{NTR}$ antagonist, restored the NGF and the NGF/proNGF balance in bladder tissues of mice with diabetic voiding dysfunction[8]. Furthermore, the antagonist was found to inhibit MMP-9 activity and increases NGF and NGF/proNGF in the media of urothelial cells (UROs) derived from young, 2-month-old, rat bladders. The current study therefore aims to determine the functional benefit of treating aging mice with bladder dysfunction with THX-B by assessing bladder behaviour, voiding parameters, and bladder contractility.

2. Materials and methods

Animal Housing and Treatment

All experiments were approved by the McGill University Animal Ethics Committee (Montreal, QC, Canada). Housing and handling followed the standards of the Canadian Council for Animal Care (CCAC). Nine 6-month (age control), sixteen 12-month, and sixteen 18-month-old C57BL/6J mice we housed in a 12-hour light/dark cycle with access to food (standard Purina chow, Teklad Global, WI, USA) and water. Mice were injected with THX-B (50 μg in 125 μL PBS) or PBS control intraperitoneally (IP) once a week for 4 weeks. THX-B is a small synthetic compound (((1,3-diisopropyl-1-[2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-acetyl]-urea), molecular mass 540 Daltons) with a systemic circulation half-life of 8 hours[8]. THX-B blocks proNGF binding to $p75^{NTR}$ receptor and inhibits ligand independent signalling and can therefore be considered a pure $p75^{NTR}$ antagonist[8]. Mice ages 12-months and 18-months were divided into control PBS and treatment with THX-B based on urinary levels of $p75^{NTR}$ extracellular domain

(ECD) in correlation to their bladder behaviours measured by voiding spot assay (VSA). 6-month-old mice were randomly divided into control and treatment groups rather than division based on bladder behaviour correlations with urinary $p75^{NTR}$ ECD levels. This is because the 6-month-old mice were composed of a smaller sample size to which correlations may not be accurate. Total body weight and fasting serum glucose (FSG) was measured before each injection to monitor for side effects. FSG was measured with a glucometer (Contour next EZ, Bayer, ON, Canada) from the tail following 4-5 hours of fasting.

ELISA measurements for urinary $p75^{NTR}$ Extracellular Domain (ECD)

Urine presence of $p75^{NTR}$ ECD was measured using ELISA (RapidTM ELISA kit, Biosensis, Australia) as per manufacturer's protocol and results were expressed as protein to creatinine (Cr) ratio.

Voiding Spot Assay (VSA)

Each mouse was placed for 4 hours in a cage fitted with a chromatography paper, grade 3 mm CHR (Whatman, GE Healthcare, UK) covered by a fine metallic mesh (2-3 mm pore size) with food, but without water. Urine patterns were visualized with ultraviolet light using SynGene Bioimaging System (USA) connected to GenSsnap software Version 7.02, USA). Urine patterns on each filter paper were analyzed by ImageJ software (version: 2.0.0-rc- 66/1.52n, NIH, USA) using a calibration curve and Void Whizzard free software plugin for FIJI (distribution of ImageJ, Version: 2.0.0-rc-66/1.52n, NIH, USA) to measure sum of urine spot sizes reflecting total urine volume (μL), number of spots reflecting voiding frequency, and mean size of urine per spot

reflecting volume (µL) per micturition. VSA was done on each mouse once before treatment (baseline), and later at 2 and 4 weeks of treatment.

Bladder Catheterization

Mice were anesthetized under isoflurane (2.5%) and oxygen (2%). Buprenorphine slow release (1 mg kg^{-1}) was injected subcutaneously for analgesic purposes. A lower abdominal incision was made to expose the bladder. The dome of the bladder was punctured for the insertion of a polyethylene PE50, .58/.99 mm catheter (Stoelting, Wood Dale, IL, USA) catheter. The catheter was secured with 6.0 Perma-Hand silk braided (Johnson and Johnson, ON, Canada) and tunneled subcutaneously from the bladder to the back of the neck. A small incision at the back of the neck was made to expose the end of the catheter, which was sealed thermally to prevent leakage. Incisions at the neck and abdomen were sutured using 3.0 coated vicryl (Johnson and Johnson, ON, Canada).

Conscious Cystomety and Animal Euthenasia

Conscious cystometry was performed 48 hours following bladder catheter insertion. The exposed end of the catheter was unsealed and attached to a pressure inducer (Grass Technologies, USA) and an infusion pump instilling saline 0.8% into the bladder at 1.5 ml/h for 1 hour. Voided saline and urine were collected in a container connected to a force displacement transducer to measure voided volume (mL). Intravesical pressure during micturition as well as the volume voided were measured simultaneously. The following parameters were recorded: maximal pressure (highest pressure during a micturition cycle), threshold pressure (pressure immediately preceding micturition), basal pressure (lowest pressure between two consecutive voids), intermicturition pressure (mean calculated pressure between two consecutive voids), micturition volume (volume

voided per micturition), and inter-contraction interval (time between two consecutive voids). The aforementioned recordings were used to calculate spontaneous activity (intermicturition pressure – basal pressure), bladder capacity (instillation rate inter-contraction interval), and bladder compliance (bladder capacity/ [threshold pressure – basal pressure])[8]. The data was analyzed using Labscribe2 Data Recording and Analysis Software (iWorx, Dover, NH, USA). Mice were euthanized the same day by exsanguination under isoflurane (3%) and cervical dislocation, as recommended by the McGill University Animal Ethics Committee. The bladder was then excised and collected in cold PBS pH 7.4 on ice to be used immediately for organ bath studies.

Contractility Studies (Organ Bath)

Immediately following euthanasia and excision and emptying of the bladder, bladder weight was measured. For each bladder, two bladder strip rings were excised below the bladder dome and placed in wells of a 4-channel Tissue Bath System – 720MO (DMT-USA Inc., Ann Arbor, MI, USA). Each well contained 6 mL of Krebs–Ringer solution: NaCl (119 mM), KCl (4.6 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1.2 mM), NaH_2PO_4 (1.2 mM), $NaHCO_3$ (15 mM), glucose (5 mM), pH 7.4 at 37 °C. The tissue bath wells connected to a tank delivering 95% O_2/ 5% CO_2 mix. Strips were kept at a tension of 0.5 g for 1 h and medium changes every 15 minutes. Then, strips were stimulated with 60 mmol/L KCl (120 uL of 3 mol/L KCl solution to 6 ml Krebs-Ringer in each well). Strips were washed with 6 mL Krebs-Ringer 4 times every 2 minutes without adjusting the tension to 0.5 g until the end of the final wash. This KCl stimulation and wash procedure was repeated a second time to confirm consistency. Electrical field stimulation (EFS; 1, 2, 4, 8, 16, 32 Hz) was performed using Grass Technologies S88 Stimulator (West Warwick, RI, USA). Washing was done and strip tension adjusted back to 0.5 g. Finally, strips were stimulated with increasing concentration of

carbachol (3nmol/L to 100mmol/L). All values were normalised to their respective strip weights for the purpose of reflecting the effect of bladder wall thickness increase and weight on contractility. LabChart 7 (ADInstruments, CO, USA) was used for recording and data analysis.

Statistical Analysis

All values are expressed as mean ± SEM. Two-way ANOVA analysis was carried out to account for both the treatment (PBS control *vs*. THX-B) and age (6-month *vs*. 12-month *vs*. 18-month-olds) effects on our findings. Post-hoc Tukey was conducted to compare age and Šidák to compare treatment. Student t-test was performed to look at the effect of treatment alone within each age group. Statistical significance was expressed as P value of < 0.05, <0.01 and <0.001. Graphpad Prism Software was used to compute two-way ANOVA.

3. Results

3.1. Baseline urine p75^{NTR} extracellular domain (ECD) expression levels correlated with bladder behaviour: Prior to treatment interventions, urinary p75^{NTR} extracellular domain (ECD) level was measured using ELISA in each age group. The results from the 12-month and 18-month-old mice were correlated with baseline bladder behaviours measured by VSA, namely total urine volume (µL), voiding frequency (number of spots) and volume (µL) per micturition (volume/spot). In the 12-month-old mice, levels less than 400 pg of p75^{NTR} ECD per mg of creatinine (Cr) and correlated positively with total urine volume (µL) and voiding frequency, but negatively with volume (µL) per micturition (Figure 1 A-C). The same was observed in the 18-month-old mice greater than 179 pg of p75^{NTR} ECD per mg of Cr (Figure 2 A-C). Correlations were weak when levels were greater than 400 pg in the 12-month-old mice (Figure 1 D-F) and less than 179 pg of

p75^{NTR} ECD per mg of Cr in the 18-month-old mice (Figure 2 D-F). These correlations were not done with the 6-month-old mice given a small sample size.

3.2. Baseline urine p75^{NTR} ECD expression levels were highest among 12-month-old mice compared to 6-month-old mice: The ELISA results of urinary p75^{NTR} ECD levels were also used to compare expression between age groups. However, given that the ELISA plates were not large enough to accommodate urine from all age groups at once, each plate contained urine from the 6-month-old mice (control for age) with either urine from each 12-month-old mice or 18-month-told mice. The 12-month-old mice group was found to have 170 times higher urinary p75^{NTR} ECD concentration compared to the 6-month-old mice ($p < 0.001$) (Figure 3 A). The 18-month-old mice showed no significant difference in urinary p75 ECD expression compared to the 6-month-old mice (Figure 3 B).

3.3. Only the 12-month-old mice treated with a p75^{NTR} antagonist showed better voiding behaviour compared to age-matched controls: Voiding behaviour was measured by VSA as total urine volume (µL), voiding frequency (number of spots) and volume (µL) per micturition (volume/spot) at baseline, 2 weeks and 4 weeks. Age was a significant source of variation at all time points for total urine volume (µL) (Figure 4 A-C), at 2 weeks for volume (µL) per micturition (Figure 5 B) and at 4 weeks for voiding frequency (Figure 6 C). Age and treatment interacted significantly at 4 weeks for voiding frequency (Figure 6 C). Only the 12-month-old mice treated with THX-B showed better voiding behaviour compared to their respective PBS group. Specifically, total urine volume (µL) (Figure 4 C) and voiding frequency (Figure 6 C) were both lower following 4-weeks of treatment in 12-month-old mice treated with THX-B compared to the

age-matched PBS control group. No important differences in volume (μL) per micturition was observed in any treatment group compared to age-matched controls (Figure 5).

3.4. 12-month-old mice had lower residual volume when treated with a p75^{NTR} antagonist compared to age-matched controls: Cystometry (described in Table 1) measures bladder parameters for each age and treatment groups. Interaction between age and treatment was a significant source of variability. The 12-month-old mice treated with THX-B showed 65% lower mean residual volume compared to the age-matched PBS controls ($p < 0.05$). All other parameters were not statistically different between THX-B and PBS treated 12-month-old mice. Neither the 6-month nor the 18-month-old THX-B treated mice showed differences in bladder parameters compared to respective controls.

3.5. 12-month-old mice bladders had lower response to contractile stimuli following 4-weeks of treatment with a p75^{NTR} antagonist: Following bladder parameters and voiding behaviours, contractile response of bladders from mice treated with THX-B were compared to the untreated ones within each age group. After 4 weeks of treatment, bladder strips derived from 12-month-old THX-B-treated mice showed lower contractile response to KCl (60 mmol/L) (Figure 7), EFS at the highest frequency (32 Hz) (Figure 8 B), and at high concentrations of carbachol (30nM and 100nM) (Figure 9 B) compared to the age-matched PBS controls. Bladders from 18-month and 6-month-old THX-B-treated mice did not show any important changes to contractile stimuli compared to respective controls (Figures 7, 8 A&C, 9 A&C).

3.6. The p75^{NTR} antagonist had no important effect on bladder weight: Bladder weights where measured following euthanasia (post 4 weeks of treatment). Bladders treated with THX-B for 4 weeks showed no significant difference in mean weight compared to their respective age-matched PBS controls (Figure 10). However, age and treatment were each found to be significant sources of variation between bladder weights within each treatment arm, but not the interaction between the two. Within the THX-B arm, 6-month-old weighed less than the 18-month-old bladders (Figure 10).

4. Discussion

Bladder behaviour and parameters

We have demonstrated that systemic injection of a p75^{NTR} antagonist provided an age-specific therapeutic effect on bladder function of mice with voiding dysfunction. Only the 12-month-old treated mice benefitted from THX-B compared to controls with regards to bladder behaviour measured by VSA. Total urine volume (µL) and frequency of micturition was lower in the 12-month-old treated mice at 4 weeks of treatment. This is clinically valuable as voiding frequency is a surrogate measure for urinary urgency incontinence, an important symptom of OAB that observed in patients[9]. Volume per micturition, was also measured and may reflect bladder capacity[10]. This was unchanged following treatment in all groups, indicating no effect of THX-B on bladder capacity. Nonetheless, volume per micturition is an important measurement as it accounts for potential differences in water consumption before conducting VSA as opposed to total urine volume, which partially depends on kidney function[10].

Given the systemic delivery of THX-B and that total urine volume depends on kidney function, changes in urine volume may reflect an effect of p75^{NTR} antagonism on the kidney. While

changes in urine production during aging is not well described, previous studies have found alterations in NGF in diseased kidneys, such as glomerulonephritis and end-stage kidney disease[11]. Whether $p75^{NTR}$ antagonism alters kidney function during aging and effects total urine volume and volume per micturition is unknown and studies on this would provide better insight into the uses of $p75^{NTR}$ antagonism.

After 4 weeks of treatment, cystometry was carried out to measure bladder parameters. Residual volume was 65% lower in the 12-month-old THX-B treated mice compared to age-matched controls. Increased post-void residual volume (PVR) indicates poor bladder emptying, which contributes to urinary symptoms[12]. Our results are clinically meaningful as Milleman *et al*. previously found that 19% of females with OAB had an increase in PVR[13]. Further, the authors found that age was an important risk factor for increased PVR in OAB patients[13-15]. Interestingly, PVR remains a difficult problem to solve in patients despite current therapies[14]. As such, $p75^{NTR}$ shows potential translational benefit in treating OAB in the future.

We did not find any differences in bladder capacity with THX-B treatment measured by cystometry as expected given that this parameter can be inferred from the volume per micturition measured by VSA, which was also unaffected by treatment[10]. There was also no observed difference in bladder weights between treated bladders and the respective controls, suggesting no important difference in bladder hypertrophy. Since bladder capacity is affected by hypertrophy, our results are consistent since neither weight nor capacity measured by cystometry or inferred by VSA were different between control and treated bladders[8].

Further, while VSA indicated changes in voiding frequency and total urine volume in the treated 12-month-old mice, there were no changes in cystometry to account for these changes in bladder behaviour. The use of cystometry requires an invasive surgery for bladder catheterization.

Therefore, a possible explanation for the lack of cystometry results may be due to bladder injury, inflammation and compensation during surgery that interfered with the quality of the results[16]. Moreover, anesthesia administered during surgery may influence urethral tone, altering the results of cystometry performed afterwards[17].

Bladder contractility

Consistent with the VSA results, *in vitro* bladder contractility also showed to be lower only in the 12-month-old bladder strips treated with THX-B for 4 weeks compared to control. In aging males, hypercontractility of the detrusor muscle leads to symptoms of OAB. This can be due to higher voiding pressures caused by conditions such as Benign Prostatic Hyperplasia (BPH) and Bladder Outlet Obstruction (BOO)[18]. Additionally, OAB can be accompanied with detrusor overactivity (DO), whereby the detrusor muscle is unstable and contracts involuntarily[19]. We observed lower contractile responses to KCl, EFS at the highest frequency (32 Hz), as well as carbachol at the highest concentrations (30 μM and 100μM) compared to the 12-month-old mice controls. Given that carbachol is a muscarinic receptor agonist, our results suggest possible changes in the parasympathetic and acetylcholine pathway that have led to reduced smooth muscle tone in the presence of $p75^{NTR}$ antagonism[20]. Further studies looking at the expression of acetylcholine and the muscarinic type 2 (M2) and 3 (M3) receptors after THX-B treatment are required to test this hypothesis. Current therapies for OAB and DO are antimuscarinic and target the parasympathetic-bladder axis through inhibition of M2 and M3 receptors, but have limited long-term benefit[21]. Whether THX-B has long-term benefit through additional mechanisms of action remain to be elucidated. In addition, reduced bladder contractility in response to non-specific stimuli, specifically EFS and KCl, may suggest changes in the intrinsic contractile

characteristic of the detrusor muscle or the neural control of the bladder under the influence of $p75^{NTR}$ antagonism[22].

Age-Specific effects of $p75^{NTR}$ antagonism

Several factors can explain the age-specific effects of $p75^{NTR}$ antagonism in which the majority of the functional and behavioural benefits were observed in the 12-month-old mice. Firstly, this suggests temporal heterogeneity in the pathophysiology of age-related OAB. Bladder changes and remodelling events that occur during aging may be heterogenous and encompass many other factors including from NGF and proNGF. Specifically, the role of NGF and proNGF may be less important in determining alterations in bladder function in the 6-month and 18-month-old mice compared to the 12-month-old mice. Moreover, studies have shown that with advanced age chronic bladder ischemia is observed[23]. Greater ischemia associated with age may result in either irreversible changes or reversible changes, but requiring greater intervention, in the bladder and thus, reduced therapeutic effects of $p75^{NTR}$ antagonism in the 18-month-old mice.

Further, 12-month-old mice showed a significantly higher concentration of the ECD of $p75^{NTR}$ in urine compared to 6-months prior to intervention. Immunoreactivity of urinary $p75^{NTR}$ ECD may arise from kidney cells as well as cleaved $p75^{NTR}$ resulting from ligand binding, such as proNGF. A larger $p75^{NTR}$ ECD content in the 12-month-old urine at baseline may indicate that the proNGF/$p75^{NTR}$ axis is more largely responsible for bladder dysfunction relative to the 6-month-olds. This may explain why $p75^{NTR}$ antagonism has a therapeutic effect on the 12-month-old treated bladders but not the 6-month-old bladders. Further, the 18-month-old mice did not show any difference in urinary $p75^{NTR}$ ECD compared to the 6-month-old mice. The urine from all age groups were not combined on one ELISA plate because quantification of $p75^{NTR}$ ECD was initially used for the purpose of correlating protein levels with VSA results within each age group to divide

mice into treatment groups. At the time, the purpose of the experiment was not to find a general comparison of urinary $p75^{NTR}$ ECD levels across all ages. We did not expect to observe an age-specific effect of THX-B on bladder function and thus, the need to compare $p75^{NTR}$ ECD levels between all three age-groups. In the future, it would be valuable to compare urine from all age groups on one ELISA plate to properly conclude a relationship between age and urinary $p75^{NTR}$ ECD expression.

5. Conclusions

The current study demonstrates that $p75^{NTR}$ antagonism has age-specific benefits on bladder behaviour and function in mice and can be used as a tool to further understand changes in bladder physiology with age. Given its effects, there is translational potential in using $p75^{NTR}$ antagonism to improve outcomes of patients with age-associated voiding dysfunction. However, further studies are required to elucidate its mechanism of action on the bladder and surrounding nervous system tissue, especially in different ages.

6. Figures and tables

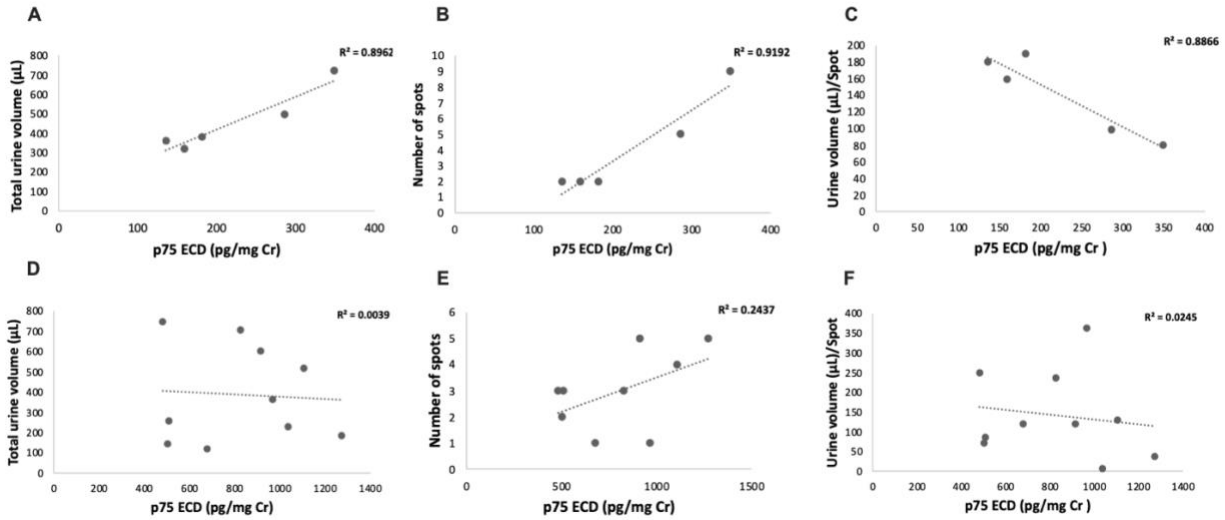

Figure 1. Correlation between urinary p75^{NTR} ECD levels and VSA measures in 12-month-old mice. Correlation between P75^{NTR} ECD levels measured by ELISA and total urine volume (µL) (**A, D**), number of spots reflecting voiding frequency (**B, E**) and urine volume (µL) per spot reflecting volume per micturition (**C, F**) in 12-month-old mice. **A-C** describe levels of p75^{NTR} ECD less than 400 pg per mg of Cr and **D-F** describe levels of p75^{NTR} ECD above 400 per mg of Cr.

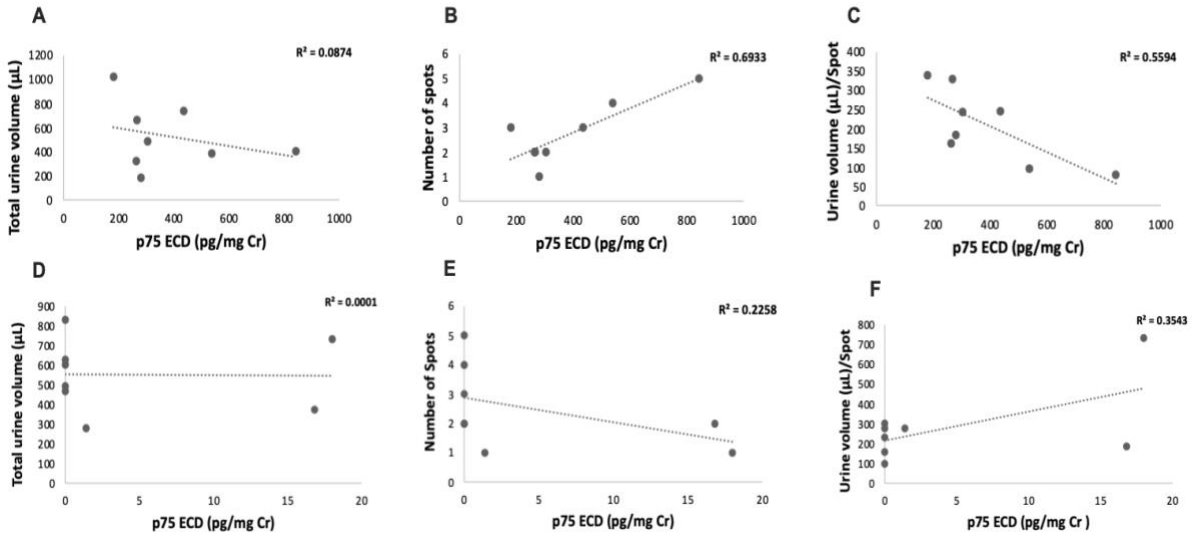

Figure 2. Correlation between urinary p75^{NTR} ECD levels and VSA measures in 18-month-old mice. Correlation between urinary p75^{NTR} ECD measured by ELISA and total urine volume (µL) (**A, D**), number of spots reflecting voiding frequency (**B, E**) and urine volume (µL) per spot reflecting volume per micturition (**C, F**) in 18-month-old mice. **A-C** describe levels of p75^{NTR} ECD above 179 pg per mg of Cr. **D-F** describe levels of p75^{NTR} ECD below 179 pg per mg of Cr.

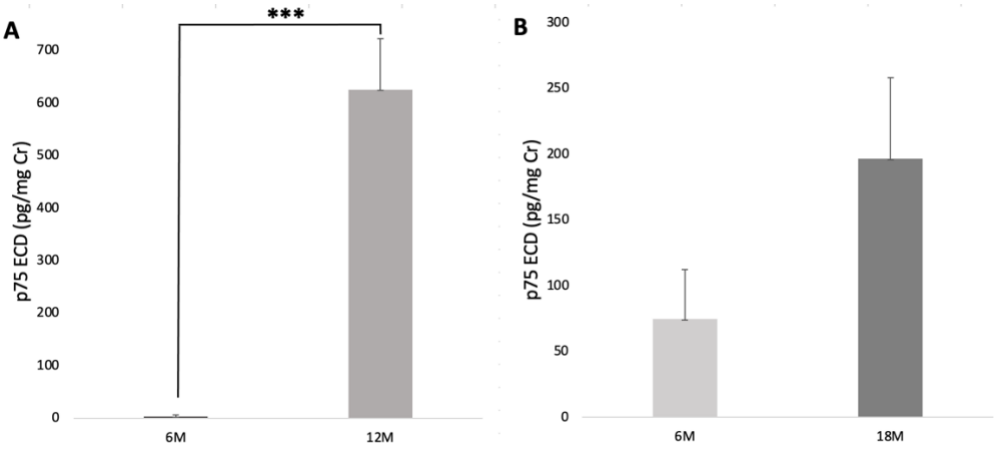

Figure 3. Urinary p75^{NTR} ECD. Mean levels of urinary p75^{NTR} ECD from 6-month and 12-month-old mice (**A**) and 6-month and 18-month-old mice (**B**). (n=5-16), student t-test (***P<0.001).

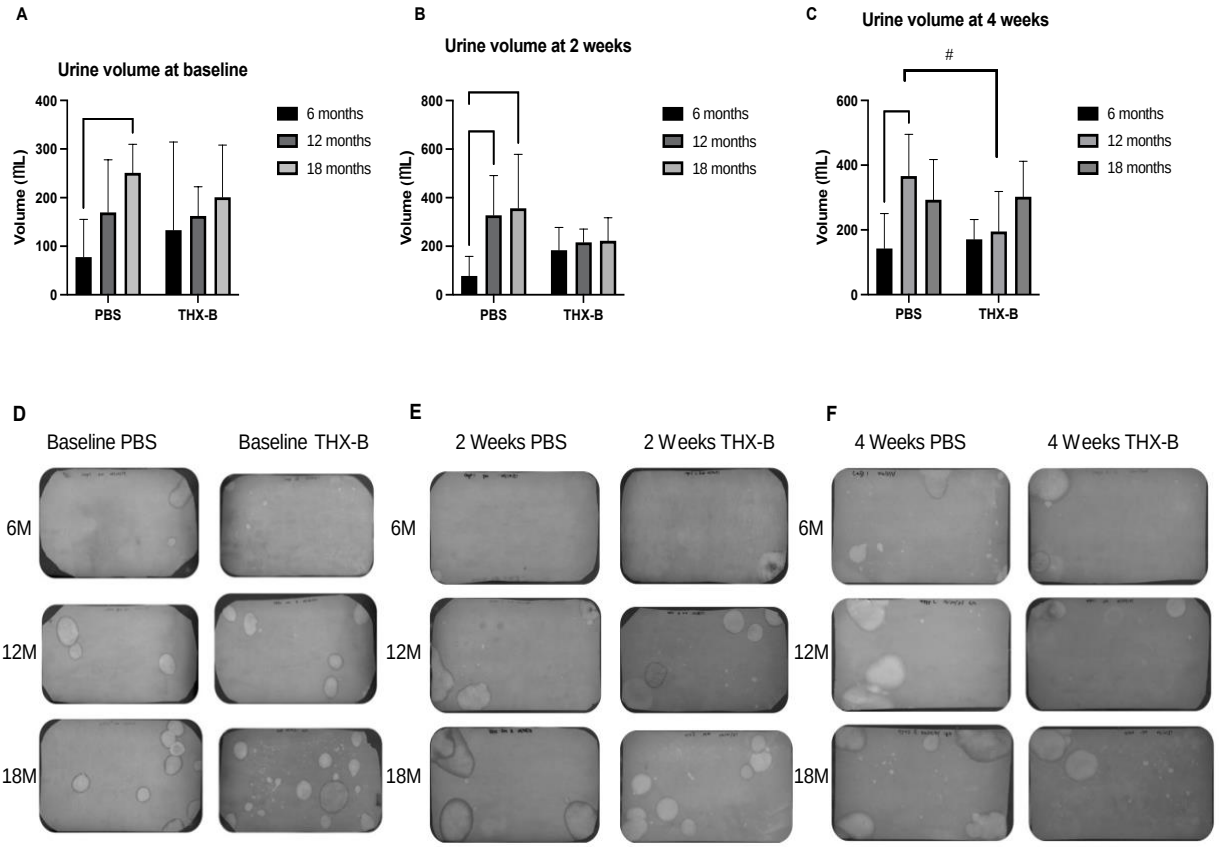

Figure 4. VSA measuring total urine volume (µL) over a 4-hour period at baseline (before treatment) (**A**), at 2-weeks (**B**), and 4 weeks (**C**) of treatment. n=4-8 mice in each group at each time point. All comparisons were done at a particular time point using two-way ANOVA with post-hoc Tukey to compare age (*P <0.05) and Šidák to compare treatment (#P <0.05). (**D-F**) Representative images of the VSA paper for the two treatments and three age groups at baseline (before treatment) (**D**), 2 weeks (**E**), and 4 weeks of treatment (**F**).

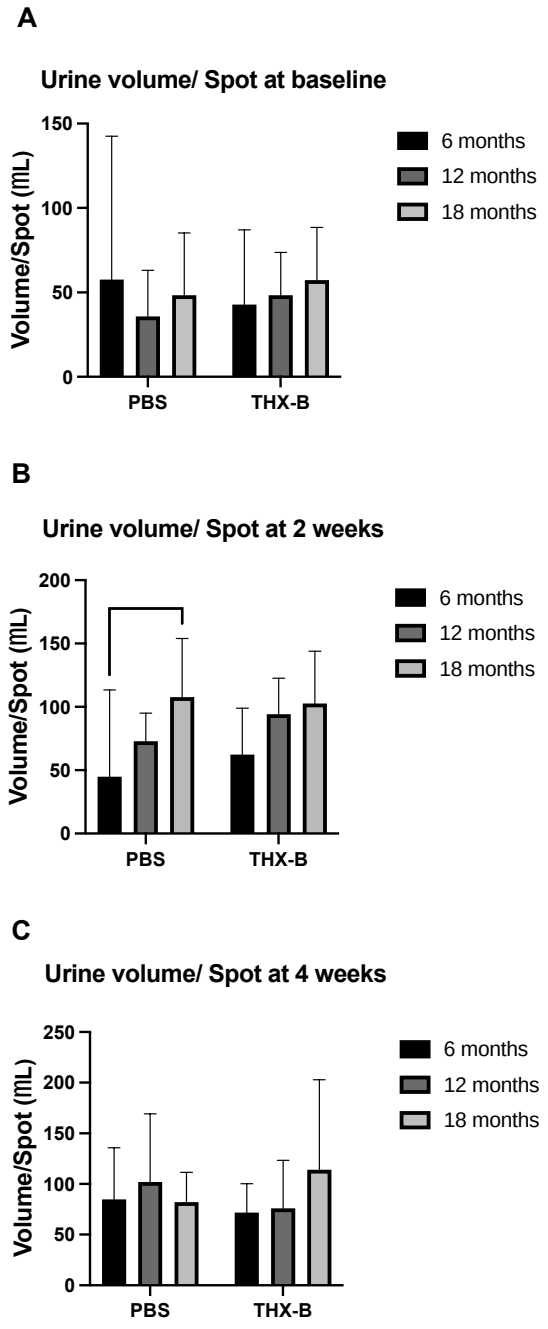

Figure 5. VSA measuring urine volume per spot, reflecting urine volume (µL) per micturition over a 4-hour period at baseline (before treatment) **(A)**, at 2-weeks **(B)**, and 4 weeks **(C)** of treatment. n=4-8 mice in each group at each time point. All comparisons were done at a particular time point using two-way ANOVA with post-hoc Tukey to compare age (*P <0.05) and Šidák to compare treatment (#P <0.05).

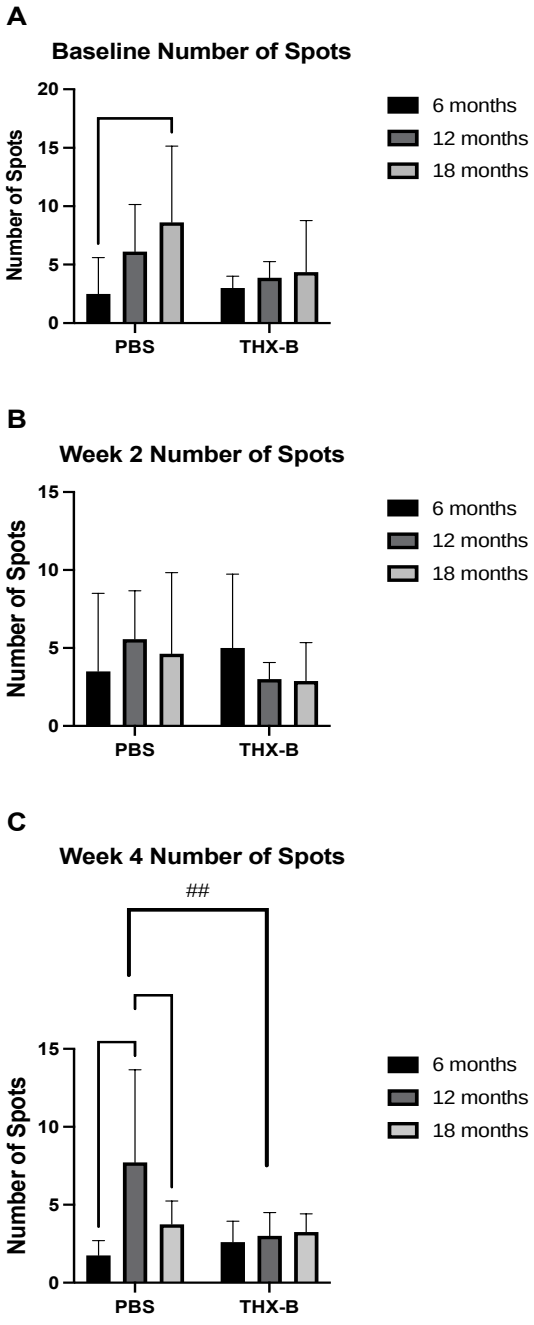

Figure 6. VSA measuring number of spots, reflecting urine frequency over a 4-hour period at baseline (before treatment) (**A**), at 2-weeks (**B**), and 4 weeks (**C**) of treatment. n=4-8 mice in each group at each time point. All comparisons were done at a particular time point using two-way ANOVA with post-hoc Tukey to compare age (*P <0.05) and Šidák to compare treatment (#P <0.05).

Parameter	Age	PBS	THX-B
Maximal Pressure (cm H_2O)	6 Months	75.75 ± 22.54	90.52 ± 17.97
	12 Months	95.27 ± 8.88	89.05 ± 13.43
	18 Months	99.54 ± 5.51	104.52 ± 12.45
Threshold Pressure (cm H_2O)	6 Months	35.30 ± 3.94	35.83 ± 5.99
	12 Months	50.46 ± 4.16	45.07 ± 8.95
	18 Months	44.51 ± 6.11	47.31 ± 6.96
Basal Pressure (cm H_2O)	6 Months	15.11 ± 6.19	17.92 ± 7.23
	12 Months	27.51 ± 3.68	24.78 ± 7.07
	18 Months	23.22 ± 5.60	25.32 ± 8.33
Intermicturition Pressure (cm H_2O)	6 Months	37.04 ± 8.01	26.72 ± 6.88
	12 Months	40.49 ± 2.58	37.01 ± 7.76
	18 Months	33.54 ± 4.89	37.12 ± 7.78
Spontaneous activity (cm H_2O)	6 Months	21.92 ± 1.83	8.80 ± 1.07
	12 Months	12.98 ± 2.38	12.23 ± 4.76
	18 Months	10.32 ± 0.90	11.81 ± 2.70
Intercontraction interval (s)	6 Months	376.23 ± 155.57	671.04 ± 141.64
	12 Months	473.88 ± 147.00	297.66 ± 79.83
	18 Months	457.62 ± 105.72	348.26 ± 71.35
Bladder Capacity (mL)	6 Months	0.16 ± 0.06	0.28 ± 0.06
	12 Months	0.19 ± 0.06	0.12 ± 0.03
	18 Months	0.19 ± 0.04	0.15 ± 0.03
Micturition Volume (mL)	6 Months	0.07 ± 0.03	0.10 ± 0.03
	12 Months	0.04 ± 0.01	0.05 ± 0.007
	18 Months	0.07± 0.01	0.07 ± 0.01
Residual Volume (mL) *	6 Months	0.09 ± 0.04	0.18 ± 0.03
	12 Months	**0.20 ± 0.05 ***	**0.07 ± 0.03**
	18 Months	0.12 ± 0.04	0.07 ± 0.02
Bladder Compliance (mL cm H_2O^{-1})	6 Months	0.008 ± 0.004	0.02 ± 0.004
	12 Months	0.009 ± 0.002	0.007 ± 0.002
	18 Months	0.010 ±0.003	0.007 ± 0.001

Table 1. Summary of conscious cystometry for PBS control and THX-B treated mice

Values are expressed as mean ± SEM. Results of two-way ANOVA are summarized next to the parameter to represent effect of age and treatment interaction for that parameter (*P<0.05).

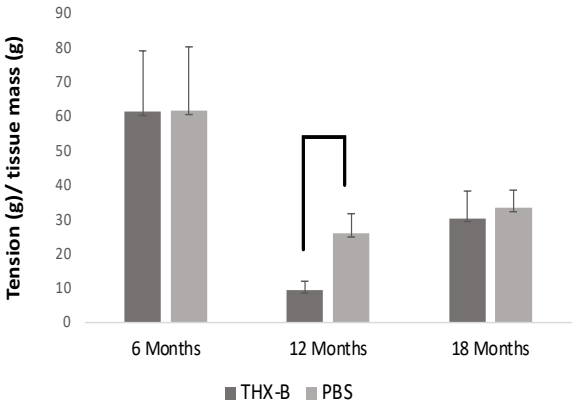

Figure 7. Bladder strip mean response to potassium chloride (KCl) (60 mmol/L). Strips derived from 6-month, 12-month, and 18-month-old mice treated with THX-B or PBS control. (n=10-15), student t-test (*P<0.05).

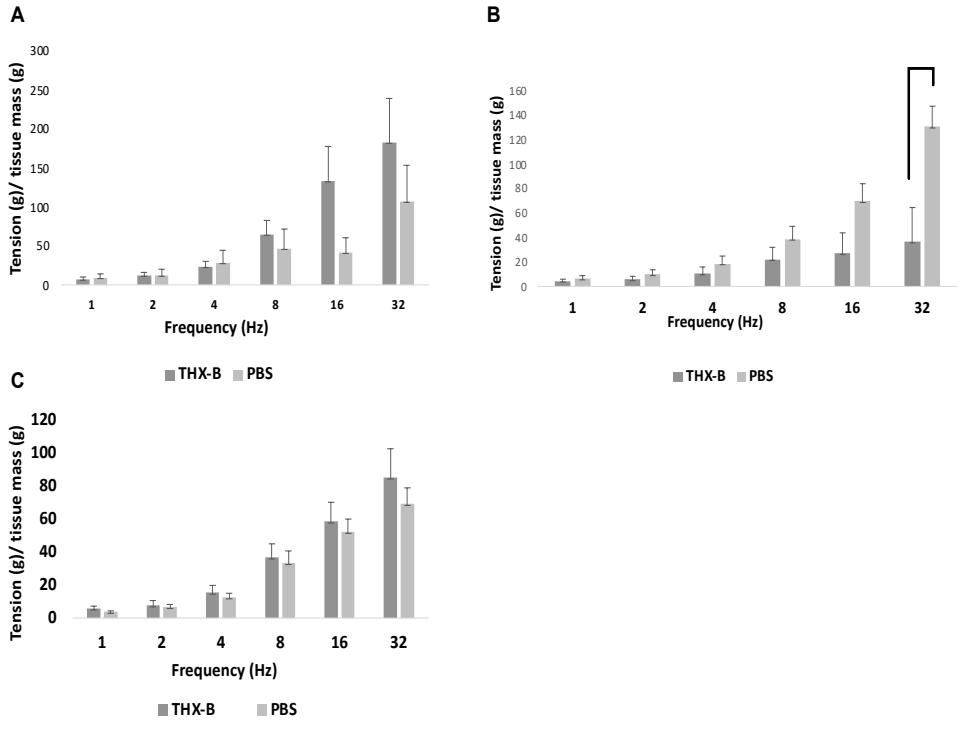

Figure 8. Bladder strip mean response to EFS at increasing frequencies from 1Hz-32Hz. Strips derived from 6-month (**A**), 12-month (**B**), and 18-month (**C**) old mice treated with THX-B or PBS control. (n=10-15), student t-test (*P<0.05).

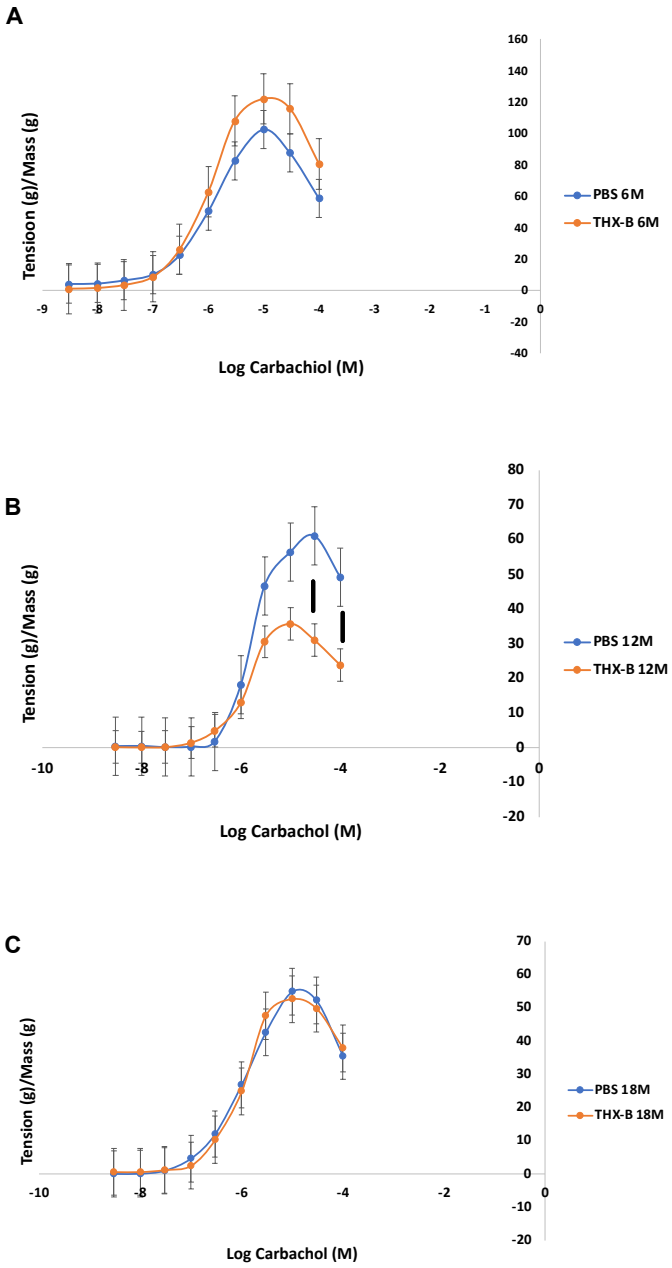

Figure 9. Bladder strip dose-response to carbachol at increasing concentrations (3nM-100mM). Strips derived from 6-month **(A)**, 12-month **(B),** and 18-month **(C)** old mice treated with THX-B or PBS control. (n=10-15), student t-test (*P<0.05).

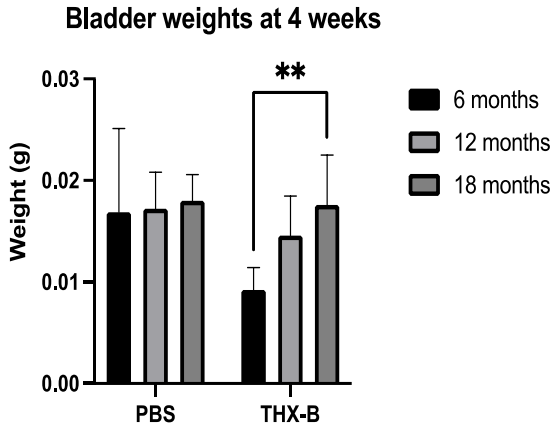

Figure 10. Difference in mean bladder weight between 4-weeks of treatment with PBS control and THX-B. n=4-8 mice in each group at each time point. All comparisons were done at a particular time point using two-way ANOVA with post-hoc Tukey to compare age (*P <0.05) and Šidák to compare treatment (#P <0.05).

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Chapter 6: Overall Discussion

6.1 Linking *in vitro* to *in vivo* findings

To our knowledge, this master's project is the first to successfully understand the mechanism of action of the $p75^{NTR}$ receptor in UROs and SMCs of the bladder, specifically in relation to MMP-9, NGF, proNGF and the NGF/proNGF ratio while also evaluating the therapeutic potential of the $p75^{NTR}$ antagonist, THX-B, *in vitro* in cell culture and *in vivo* in aging mice. By looking at intracellular and extracellular protein expression in UROs and SMCs after incubation with THX-B, we found that $p75^{NTR}$ antagonism essentially effects UROs. Specifically, antagonism increased NGF and NGF/proNGF in this environment through modulation in α_2M, Furin, and MMP-9 expression. α_2M is important in allowing MMP-9 to escape degradation as well as increasing the strength between proNGF and $p75^{NTR}$ to potentiate its pro-apoptotic and degenerative effects[56]. Our data are in accordance with results obtained by our team in a separate study which found that UROs synthesize 6 times more $p75^{NTR}$ than SMCs, as revealed by immunoblotting and ELISA kits (Mossa *et al.*, under review).

Studies identifying NGF/proNGF imbalance in age-associated diseases have largely been limited to neuronal tissue, leaving the aging bladder a poorly defined concept[46]. Nonetheless, our team previously found that in aging female patients with OAB, urinary NGF and NGF/proNGF were decreased and paralleled with an increase in MMP-9, suggesting increased NGF digestion[48]. This is consistent with our current study's findings in URO media that show an increase in NGF and the NGF/proNGF ratio in parallel to a decrease in MMP-9 after treatment with THX-B. We subsequently found using Crispr-Cas9 that MMP-9 is essential in the proteolysis of NGF, making the protease an important target for future therapies. Thus, there is important translational and

therapeutic potential in using a $p75^{NTR}$ antagonist as an MMP-9 modulator to address age-related OAB, encouraging us to study this in animal models.

In mice models of age-related voiding dysfunction, 12-month-old mice were mainly seen to benefit from $p75^{NTR}$ antagonism compared to 6-month and 18-month-old mice. This age-specific effect suggests temporal heterogeneity in voiding dysfunction. Possible differences in pharmacokinetics, for example, due to altered kidney, cardiac or gastrointestinal functions, based on age may require different drug doses[60]. It is also possible that older mice require higher doses of THX-B to allow a tighter control of MMP-9 expression.

Further, differences in pharmacodynamics may also account for the age-specific observations, suggesting that a different mechanism of voiding dysfunction in 6-month and 18-month-old mice and the need for a new target[60]. One example of this which was previously discussed in Chapter 5 is the significantly lower urinary $p75^{NTR}$ extracellular domain (ECD) found in 12-month-old-mice compared to 6-month-old mice. Thus, levels of the main drug-target are different in various ages. Further studies should also compare urinary $p75^{NTR}$ ECD in the 18-month and 12-month-old mice as well to better understand the correlation between age, OAB, and $p75^{NTR}$ expression.

While the bladders of THX-B treated 6-month and 18-month-old mice did not show significantly lower bladder contractility compared to their controls, they did show higher contractility at various frequencies for EFS and concentrations of carbachol, yet insignificant. Despite statistical insignificance, the increase in contractility in the 6-month and 18-month-old treated bladders might suggest an opposite effect of THX-B on these aged bladders. For example, an increase in NGF content driven by THX-B may have led to over-sensitization of afferents controlling bladder contractility[39]. In fact, Schneglesberg *et al.*, have found that mice with

urothelial NGF overexpression had neuronal hyperinnervation and increased non-voiding contractile amplitude measured cystometry as well as increased referred somatic hypersensitivity[39]. Although administration of the THX-B was adjusted by weight, further dose-response analysis is required to better understand the therapeutic effect of THX-B in different ages.

To confirm that the effects observed in THX-B-treated mice are led by changes in MMP-9, NGF, and NGF/proNGF, we collected urine before and throughout treatment as well as bladders and kidneys after euthanasia (4 weeks of treatment). ELISA of urinary NGF and proNGF, as well as immunoblotting of MMP-9 and analysis other proteins previously analyzed throughout the *in vitro* study, namely, α_2M, Furin, Plasmin, MMP-7, and MAPKs will be assessed. Further, immunohistochemistry identifying such proteins will be carried out on the preserved bladder tissue. This analysis will be important as it will allow better interpretation of our *in vivo* results. Similar analysis in sampled kidneys will be important in understanding changes in kidney function that may explain the observed changes in urine volume and content in the 12-month-old treated mice.

6.2 Study limitations

A potential limitation of the current project is the use of young, 2-month-old female rat bladder cell samples to study normal cell characteristics and protein expression profiles instead of male mice samples for the *in vitro* study (Chapter 4). Rat bladders were used due to easier manipulation. However, the *in vivo* study (Chapter 5), male mice rather than female rats were used as models for age-related voiding dysfunction. As well, the youngest group of 6-month-old mice was used as the control for age, rather than a 2-month-old group similar to the *in vitro* study used to understand normal cell profiles unaffected by age. Given the differences in species, age of

control and biological sex between the models used in each study, there may be pathological and biological discrepancies that are unaccounted for within the conclusion generated from the *in vitro* study that was used to interpret results of the *in vivo* study. Nonetheless, mice were chosen over rats for the *in vivo* study as we had previous success using $p75^{NTR}$ antagonist in mice[61]. As well studies observing bladder function changes in aging mice showed similar trends to the human aging bladder, such as increase in spontaneous activity during storage phase and altered sensitivity to chemical stimuli controlling bladder contractility[62]. In the future, mice similar to those used in the *in vivo* experiment should be studied *in vitro* to better understand and interpret our results[63]. Furthermore, *in vivo* study length and number of doses may be a potential limitation. Ideally, treatment with THX-B may require multiple doses per week for more than 4 weeks given its relatively short half-life (8 hours)[61]. Differences in blood flow and liver metabolism between age groups may signify that a more frequent and longer dosing schedule is required to observe beneficial effects of THX-B in the 6-month and 18-month-old mice[64].

6.3 Barriers to translation research and future directions

While our findings show a positive outcome in aging mice and a newly built foundation for cellular mechanisms that may explain this outcome, important barriers to translational research must be addressed. Firstly, while animal models are necessary to evaluate the initial therapeutic use of $p75^{NTR}$ antagonism, important differences between animal and human physiology, pathology, and pharmacology must be acknowledged[65]. For example, OAB is diagnosed mainly through evaluation of patient experience and symptoms using questionnaires[26,27]. Therefore, in mice models of age-related voiding dysfunction, surrogate measures using cytometric parameters and Voiding Spot Assay (VSA) are often used to evaluate treatment benefit[30]. Another crucial

component of OAB is quality of life, which includes, among others, mental, physical, and social, aspects of one's life[13]. Evidently, these factors are not evaluated in animal models, even if a given therapeutic agent appears to provide better functional and physiological outcomes[30]. Therefore, animal models should mainly be used to reflect a specific hypothesis, while keeping in mind that other facets of the disease are unaccounted for but can be studied separately and progressively as scientific questions become more clear[30]. In our case, we were primarily interested in studying the functional and voiding behaviours in mice treated with a $p75^{NTR}$ antagonist based on clinical data that showed changes in urinary NGF and NGF/proNGF in aging females with OAB[48].

Secondly, drug delivery remains an important challenge in translational research and even in late-stage clinical trials[66]. While this study uses a $p75^{NTR}$ antagonist, it was mainly used as a tool to understand the mechanisms of NGF control and preliminary potential for altering age-related voiding dysfunction in an animal model. Yet, given that we identified potential therapeutic benefit only in a select age group of mice, options for better drug delivery may be worth exploring for other ages. Alternatively, now that we have identified the use of MMP-9 as a target in bladder cells to increase NGF and NGF/proNGF, other drugs can be studied to optimize MMP-9 inhibition and the therapeutic benefits that may follow. For example, miRNA mimics that inhibit MMP-9 expression may be of therapeutic benefit[67]. Synthetic miRNAs have previously shown potential in correcting neurogenic muscle control by targeting gene expression[67]. In fact, we previously identified low levels of urinary miR-491-5p, a miRNA that inhibits MMP-9 translation, in the same aging female patients with high urinary MMP-9 and low NGF and NGF/proNGF ratio[68]. However, miRNA mimics are limited in their long-term use due to instability[69]. Nonetheless, whether treating bladder cells with miRNA mimics that have a similar function to miR-491-5p

increases NGF and NGF/proNGF more effectively than a $p75^{NTR}$ antagonist and shows functional benefit in mice remains to be elucidated and would be an important study for the future.

Chapter 7: Final conclusions

7.1 Meeting objectives

The purpose of this project was to contribute to important translational research aimed at improving treatment of age-related OAB. We set out to study the therapeutic potential of $p75^{NTR}$ antagonism in improving bladder function in age-related OAB.

To do this, we met our first objective, which was to understand the control of MMP-9 on NGF and the link with $p75^{NTR}$ in bladder cells. We identified that $p75^{NTR}$ influences NGF and NGF/proNGF mainly through modulation of urothelial MMP-9. In fact, $p75^{NTR}$ antagonism increased NGF and NGF/proNGF in these cells while decreasing MMP-9, confirming our first hypothesis. Our study is therefore important in identifying the novel use of MMP-9 as a therapeutic target for age-related OAB treatment, looking beyond its conventional function of extracellular matrix degradation. Other mechanisms may also account for the increase in NGF and NGF/proNGF that was mediated by $p75^{NTR}$ antagonism. For example, α2M was decreased as well, which may indicate increased degradation of MMP-9 as well as reduced proNGF-$p75^{NTR}$ axis activity. Further, intracellular Furin was increased with treatment, reflecting a possible increase in proNGF to NGF conversion.

These findings encouraged us to evaluate a second objective, which was to determine the bladder-related functional benefit of treating aging mice with a $p75^{NTR}$ antagonist. Of three different age groups, only the treated 12-month-old mice showed better bladder behaviour, voiding patterns, and reduced contractility compared to age-matched controls. Since the effect was age-specific, our second hypothesis was only partly confirmed. Despite this, our results are still valuable in identifying the uses of $p75^{NTR}$ antagonism in age-related voiding dysfunction. Several

reasons may explain the age-specific effect of $p75^{NTR}$ antagonism on bladder function, such as the concentration of $p75^{NTR}$ in bladder tissue responsible for voiding dysfunction at different ages, as well as a difference in drug pharmacodynamics at different ages. Nonetheless, these results contributed to further understanding of $p75^{NTR}$ in the bladder by combining the *in vitro* results.

7.2 Implications of findings

This project is important for addressing future, non-invasive solutions aimed towards reducing age-related OAB symptoms, such as urgency, that interfere with daily life and reduce the quality of life. Current drugs for OAB are anticholinergic and adrenergic agonists, which are non-curative and are often limited by their side-effects[28]. Our study has explored MMP-9 beyond its conventional functions, such as extracellular matrix degradation. We studied the potential of MMP-9 as a therapeutic target for age-related OAB treatment through modulation of NGF and NGF/proNGF which are important in nervous system and bladder physiology. As well, this project has provided clarity to the protein expression profiles of UROs and SMCs of the bladder, an otherwise poorly studied area.

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