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Improvement of bladder voiding dysfunction by $p75^{NTR}$ receptor antagonism in a murine model of Type 2 Diabetes

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Abstract

Bladder dysfunction is a common side effect of diabetes and can impede on quality of life, as well as lead to more threatening complications. Patients exhibit bladder remodelling and overactivity leading to increased urinary frequency and urgency. It has been shown that patients with diabetic voiding dysfunction (DVD) display an imbalance between nerve growth factor (NGF) and its precursor proNGF, to the detriment of NGF, in their urine samples. The receptor $p75^{NTR}$ is also highly expressed in the diabetic bladder. Its activation by proNGF inhibits the micturition reflex and triggers inflammatory pathways, leading to increased tissue degeneration. Urine samples also displayed elevated levels of nitric oxide (NO), implying its role in the DVD mechanism pathway. We have previously demonstrated that using a receptor antagonist molecule THX-B to inhibit the $p75^{NTR}$ pathway has led to positive outcomes regarding bladder dysfunction in mice with type 1 DVD. Similar results were obtained on bladder functions in a model of rodents with spinal cord injuries. Due to the knowledge gaps in current literature, an in-depth understanding of the DVD mechanism is necessary prior to assessing the impact of a potential treatment. As such, the following study was performed into two parts:

1) We first evaluated the role of NO in the mechanism pathway of DVD. NO is an inflammatory signaling molecule, secreted as a result of hyperglycemia. We hypothesized that the synthesis and secretion of NGF and proNGF in bladder cells *in vitro* would be impacted by increased NO in hyperglycemia conditions, contributing to the tissue damage exhibited by the bladder in DVD patients, as the NGF/proNGF ratio decreases. A high glucose environment did in fact increase nitric oxide release which led to a decrease in NGF levels and the NGF-to-proNGF ratio. This secretion was controlled through cGMP signaling increasing, MMP-9 activity in urothelial cells, and decreasing MMP-7 activity in smooth muscle cells.
2) Next, we validated the proNGF/$p75^{NTR}$ interaction as a therapeutic target for the antagonist molecule THX-B in type 2 DVD. We used a novel type 2 DM model, the Tally Ho mouse, and

began by characterizing the changes in the bladder resulting from DM. We found that detrimental changes to voiding patterns and bladder neural function began occurring at 14 weeks of age in the diabetic males, with some similar changes occurring in 14-week insulin resistant females. Following characterization, we evaluated the changes in bladder function, cystometric parameters and contractility analyses. THX-B antagonist had different effect between males and females, mostly improving contractility parameters measured by cystometry and organ bath.

Résumé

Les troubles de la miction (DVD) sont une complication courante du diabète. Les patients diabétiques présentent une inflammation ainsi qu'un épaissement de la paroi vésicale, accompagnée d'une diminution des propriétés contractiles, le tout provoquant une hyperactivité de la vessie. L'urine des patients présentent un déséquilibre entre le « nerve growth factor » (NGF) et son précurseur proNGF, en faveur d'une diminution du rapport NGF/proNGF. Autrement, les vessies de patient diabétiques expriment fortement le récepteur pro-inflammatoire $p75^{NTR}$, dont le ligand préférentiel est le proNGF. Les échantillons d'urine des patients démontrent également une augmentation des niveaux d'oxide nitrique. Nous avons précédemment démontré que l'utilisation de la molécule antagoniste THX-B pour inhiber la voie proNGF/$p75^{NTR}$ a entraîné des résultats positifs concernant le dysfonctionnement de la vessie chez les souris atteintes de DVD de type 1. Des résultats similaires ont été obtenus sur les fonctions vésicales dans un modèle de rongeurs atteints de lésions médullaires. En raison des lacunes dans les connaissances dans la littérature actuelle, une compréhension approfondie du mécanisme du DVD est nécessaire avant d'évaluer l'impact d'un traitement potentiel. Ainsi, l'étude suivante a été réalisée en deux parties :

1) En premier lieu, nous avons évalué le rôle de l'oxide nitrique (NO) dans le mécanisme du DVD. Le NO est une molécule de signalisation inflammatoire, sécrétée à la suite d'une hyperglycémie. Nous avons émis l'hypothèse que la synthèse et la sécrétion du NGF et du proNGF dans les cellules de la vessie *in vitro* seraient affectées par une augmentation du NO dans des conditions d'hyperglycémie, contribuant à la dégénérescence tissulaire présentée par la vessie chez les patients DVD avec une diminution du rapport NGF/proNGF. Un environnement riche en glucose a augmenté la sécrétion de NO, ce qui a entraîné une diminution des taux de NGF et du rapport NGF/proNGF. Ces changements ont été contrôlés par la signalisation cGMP, avec

l’augmentation de l'activité MMP-9 dans les cellules urothéliales et la diminution de l'activité MMP-7 dans les cellules musculaires lisses.

2) Ensuite, nous avons validé l'interaction proNGF/p75^{NTR} comme cible thérapeutique pour la molécule antagoniste THX-B dans le DVD de type 2. Nous avons utilisé un nouveau modèle de diabète de type 2, la souris Tally Ho, et avons commencé par caractériser les changements de la vessie résultant du diabète. Nous avons constaté que des changements préjudiciables aux comportements de miction et à la fonction neurale de la vessie ont commencé à se produire à l'âge de 14 semaines chez les males diabétiques, avec quelques changements similaires chez les femelles résistantes à l'insuline, aussi à 14 semaines. Pour donner suite à la caractérisation, nous avons évalué les changements dans la fonction de la vessie, les paramètres cystométriques et les analyses de contractilité. L'antagoniste THX-B a eu un effet différent entre les mâles et les femelles en raison des différences dans les taux de glycémie. Il y a eu une amélioration principalement dans les paramètres de contractilité mesurés par cystométrie et bain d'organes.

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I would like to start off by expressing my gratitude to my supervisor Dr. Lysanne Campeau. Her constant sincere support and guidance, from the moment I joined her lab has been the most invaluable part of this process. Despite her undoubtedly busy schedule, she has always made herself available for me when I had any questions or concerns, pointing me in the right direction. She has always ensured that I reached my full research potential in my time with her and has constantly encouraged me for my future goals. She has also provided me with the necessary financial support to be successful in my research. I believe that the skills I obtained in my time with Dr. Campeau are those that will remain with me for the rest of my career. I could never thank her enough for being a wonderful mentor to myself and my colleagues.

I am also grateful for all my wonderful colleagues from Dr. Campeau's lab, both past and present. Firstly, I'd like to thank our lab manager Dr. Philippe Cammisotto, without whom I would not have been able to master all the necessary techniques for the completion of this project. Philippe was an integral part of my research process, helping me with data collection, analysis, as well as conception of the project. He often lent me a hand when I needed it, while providing the guidance I needed. I would also like to thank Aalya, Aya, Claudia and Benjamin, who provided me with the utmost kindness and support in my time at the lab. They each played an important role in my success in various ways such as, aiding with data collection, giving feedback on my ideas, and suggesting relevant papers that helped with the progression of the project. I am eternally thankful for the friendships we built and look forward to seeing them all progress in their respective research careers.

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Preface

All author contributions are recorded at the start of their respective chapters.

Chapter 4 presents data in manuscript form, regarding the *in vitro* study on bladder cells (urothelial and smooth muscle) and the potential involvement of nitric oxide in the pathology of overactive bladder syndrome.

Chapter 5 presents data in manuscript form, on the *in vivo* animal study regarding the characterization of bladder changes in a pre-clinical model of type 2 diabetic voiding dysfunction followed by the effect of THX-B treatment on this model.

This work is presented in manuscript-based thesis format.

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Figure 5.9 Organ bath studies using bladder strips from female mice following 4-week treatment with $p75^{NTR}$ antagonist

Abbreviations

α2M: α-2 macroglobulin

ACh: Acetylcholine

AGE: Advanced glycation end products

AKT (or PKB): Protein kinase B

ATP: Adenosine triphosphate

cAMP: Cyclic adenosine monophosphate

cGMP : Cyclic guanosine monophosphate

DM : Diabetes Mellitus

DVD: Diabetic voiding dysfunction

ERK: Extracellular signal-regulated kinases

GLUT4: Glucose transporter 4

GTP: Guanosine triphoshphate

HOMA: Homeostatic Model Assessment of Insulin Resistance

IFN-γ: Interferon gamma

IL: Interleukin

InsR: Insulin receptor

IRS1: Insulin receptor substrate 1

JNK: c-JUN N-terminal kinase

M2, 3: Muscarinic receptor 2, 3

MAPK: Mitogen-activated protein kinase

MEK: MAPK/ERK kinase

MMP 7, 9: Matrix metalloproteinase 7, 9

NA: Noradrenaline

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

NGF: Nerve growth factor

NO: Nitric oxide

NOS: Nitric oxide synthase (endothelial, inducible, neuronal)

OAB: Overactive bladder syndrome

P2X1: purinergic receptor

PI3K: Phosphatidylinositol-3-kinase

PIP2: phosphatidylinositol (4,5)-bisphosphate

PIP3: phosphatidylinositol (3,4,5)-triphosphate

PLCγ: Phospholipase C-γ

proNGF: Proform (precursor) of nerve growth factor

ROS/RNS: Reactive oxygen/nitrogen species

SCI: Spinal cord injury

SD: Sprague Dawley

SGC: soluble guanylyl cyclase

SH2: Src homology region 2

SMC: Smooth muscle cells

SNP: Sodium nitroprusside

STZ: Streptozotocin

TIMP: Tissue inhibitor of metalloproteinases

TH: Tally Ho

T2DM: Type 2 diabetes mellitus

TNF-α: Tumor necrosis factor alpha

TrkA: Tropomyosin kinase receptor A

URO: Urothelial cells

VEGF: Vascular endothelial growth factor

WHO: World Health Organization

Chapter 1: Introduction

1.1 The urinary bladder

1.1.1. Structure and function

The urinary bladder is a vital organ that is composed of several structural, functional, and histologic compartments which all contribute to the filling and voiding cycle of this organ. The bladder is responsible for two main functions. In its relaxed and highly compliant state, it is used as a reservoir that stores the urine, and subsequently voids its contents under conscious control[1]. The structure of the bladder wall can be labelled by 4 layers: the outer serosa, the detrusor smooth muscle layer, the lamina propria (or suburothelium) and the urothelium (or uroepithelium), as shown in **Figure 1A**. The outermost layer can be divided into two components, the serosa and the fibrous coat named the adventitia. The former is responsible for covering the superior and upper lateral walls of the bladder, while the latter covers the rest of the bladder wall, with their ultimate goal being the protection of the underlying tissues[1]. The detrusor muscle, comprised of smooth muscle cells (SMCs) oriented in different directions, is the second outermost layer and forms most of the bladder wall. The diverse alignment of the muscle fibers, in their relaxed state, are what allows the bladder to stretch in the bladder filling phase. The final two layers of the bladder wall, the lamina propria and the urothelium, collectively known as the mucosa, play a role in the sensory function of the bladder[2]. The lamina propria is a layer of loose connective tissue, mostly consisting of interstitial cells, blood vessels, and afferent nerve fibers, while the urothelium, is comprised of several layers of urothelial cells (UROs)[2]. The lamina propria is innervated by both afferent and efferent nerve endings, which can be sensitive to changes in the event of various pathological conditions, such as diabetic voiding dysfunction (DVD)[3]. The top cells of the urothelium are referred to as umbrella cells, whose function is to accommodate the stretching of the bladder. The umbrella cells contain integral membrane proteins named uroplakin proteins. This property of UROs is what allows the formation of tight junctions that prevent urine leakage and allow separation of the underlying bladder wall tissues

from pathogen infiltration[2,4]. The urothelium is also considered the key communicator with underlying afferent innervation for reflex response is the filling and voiding phases[5].

1.1.2. Normal physiology of bladder

The filling and voiding cycle of the bladder occurs multiple times a day. The bladder is a highly innervated organ and normal micturition relies on the complex neural control of the peripheral nervous system ganglia[6]. Under the control of the autonomic nervous system, the contraction of the detrusor muscle in the bladder wall allows for an intravesical pressure increase and simultaneously, the relaxation of the urethral sphincter leads to urination and emptying of the bladder[7]. The urethral sphincter is under the control of the somatic pudendal nerve (S2-4). Voiding is initiated upon parasympathetic nerve activation by signals from the afferent fibers of the bladder wall in the pontine micturition center. Specifically, the signal for the contractions and relaxations occurs by the muscarinic (M3 and M2) stretch receptors, along with the purinergic ($P2X_1$) found within the bladder wall, activated by acetylcholine (ACh) and adenosine triphosphate (ATP) respectively. ACh and ATP are released by the parasympathetic and intramural ganglia from the parasympathetic fibres of the pelvic nerve (S2-4)[8]. Upon activation, cystolic stores release calcium, which in turn will activate myosin light chain phosphorylation, allowing for balance in calcium levels which can dictate contraction and relaxation cycles in the bladder[9] **(Figure 1B)**. Once the bladder has been emptied, the muscarinic receptors become inactivated, the detrusor muscle returns to a relaxed state and the urethral sphincter is constricted, allowing for filling of the bladder. This process is due to the stimulation of the β-3 receptors in the bladder wall and the α-1 adrenoreceptors in the urethral sphincter, via the sympathetic nervous system, which occurs when noradrenaline is released from the postganglionic sympathetic fibers of the hypogastric nerve plexus (T10-L2 spinal cord segments) ((**Figure 1B)**[7,10].

1.2 Diabetes Mellitus

1.2.1. Definition (with emphasis on Type 2)

Diabetes Mellitus (DM) is a common metabolic disorder that involves a deficiency in concentration or activity of insulin, a hormone secreted by the pancreas[11]. The World Health Organization (WHO) reports that the disease affects over 400 million adults worldwide[12]. There exists multiple types of diabetes and patients are classified according to their symptoms. Of the three main types of DM, 90-95% of cases are reported to be of Type 2 nature, which is characterized by insulin resistance followed by a progressive loss of β-cell insulin secretion[11]. There are a number of risk factors for T2DM, such as genetic and environmental influences, age, unhealthy lifestyle, which includes poor eating habits, lack of exercise, smoking, etc. The high levels of adipose tissue in unhealthy patients can secrete several hormones and cytokines such as TNF-α, IL-6, and IL-1β[13]. The current WHO diagnostic criteria for diabetes is a fasting plasma glucose of ≥ 7.0mmol/L or plasma glucose of ≥11.1 mmol/L. Though there is no current cure for DM, various methods of glycemic control and treatment can be used to alleviate the symptoms that impede on quality of life. Using insulin and/or pharmacological therapies can aid in maintaining normal levels of blood glucose as well as prevent worsening of diabetes-related health problems[11]. The first-line pharmacotherapy used for T2DM is metformin (a biguanide), which can reduce glucose levels through reduction of gluconeogenesis in the liver and has insulin-sensitizing properties. Other treatments include insulin secretagogues or sensitizers which regulate insulin levels through pancreas stimulation for insulin secretion or improved insulin sensitivity in peripheral tissues, respectively[11]. That being said, there are still a number of burdensome and costly complications that can be associated with DM, many of which are urologic complications, which will be discussed more in depth later in this chapter.

1.2.2. Periphery organs and insulin resistance (GLUT4, IRS1, PI3K)

Insulin is an anabolic hormone that aids in the body's glucose homeostasis, by stimulating glucose uptake from the blood by surrounding tissues and muscle and suppressing glucose production in the liver[14]. Skeletal muscle has the ability to store glucose as glycogen, oxidizing it to produce energy when needed[15]. Impaired insulin signaling is the process by which insulin resistance develops in insulin-sensitive tissues such as the liver, skeletal muscle, and adipose tissue[16]. The key mediators of insulin signal transduction are insulin receptor (InsR), insulin receptor substrate 1 (IRS1), phosphatidylinositol-3-kinase (PI3K) and insulin-sensitive glucose transporter 4 (GLUT4). Signaling begins when insulin binds to InsR, through insulin-dependent phosphorylation of the tyrosine kinase receptor. This is followed by the association with src homology region 2 (SH2) domain, which subsequently activates PI3K. PI3K will then phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP2) to become phosphatidylinositol (3,4,5)-triphosphate (PIP3). As PIP3 concentrations increase, this recruits phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (or AKT) to the plasma membrane. This will activate PDK1 which in turn phosphorylates AKT. In its active state, p-AKT allows for inactivation of AS160, which normally inhibits translocation of GLUT4. [17,18]. The cascade, as can be seen in **Figure 1C,** leads to the eventual translocation of GLUT4 to the membrane, playing a key role in the uptake of glucose by skeletal muscle and adipose tissue, decreasing the glucose levels in the blood and regulation of glucose homeostasis[15,18]. The glucose channel GLUT-4 plays an important role in transporting glucose into insulin sensitive cells, and when exposed to markers of inflammation, GLUT4 levels decrease, a phenomenon highly correlated with insulin resistance, and even dementia[19,20].

1.3. Diabetic Voiding Dysfunction

1.3.1. Definition, Prevalence, and Incidence

Among the many complications associated with DM, lower urinary tract problems are very common in patients, specifically voiding dysfunction, urinary incontinence, and recurrent urinary tract infections and there remains a large knowledge gap on the onset of the urological symptoms associated with the disease[5,21-23]. Diabetic voiding dysfunction (DVD) is described as an umbrella term, comprising of the group of clinical symptoms including storage problems i.e. overactive bladder disease (OAB) and urge incontinence, voiding issues (poor emptying and overflow incontinence), decreased sensation, and increased capacity[24]. The disease occurs in about 80% of diabetic patients and is more prominent in females[25]. It is typically expressed through a variety of symptoms such as difficulties in bladder storage and emptying, bladder overactivity, impaired bladder contractility, urinary urgency, frequency, nocturia and, as presented in more than 50% of DM patients, urinary incontinence[26,27]. The pathophysiology of DVD is considered to be multifactorial and is associated to dysfunction of bladder smooth muscle and urothelium, as well as the neurogenic control of the bladder, which explains the wide range of symptoms exhibited by patients[24]. Although less detrimental to patients, as compared to other comorbidities of diabetes, the diagnosis does come with costly challenges such as a decreased quality of life and even depression, in the elderly population that suffers from DVD.

1.3.2. Mechanisms of diabetic voiding dysfunction

Urine samples from DVD patients displayed an imbalance between nerve growth factor (NGF) and its precursor proNGF, due to decreased levels of NGF with stable levels of proNGF. Receptor $p75^{NTR}$ is highly expressed in the diabetic bladder and its activation by proNGF inhibits the micturition reflex and triggers inflammatory pathways[28,29]. It is additionally thought that the retrograde axonal transport of NGF to the lumbosacral dorsal root ganglia is altered in DVD and associated to diabetic neuropathy and

voiding dysfunction[26]. As the bladder is highly controlled by neurotransmitters and neuroreceptors (among other neural components), it is without a doubt that the changes in NGF, proNGF, and their respective receptor levels will lead to neural dysfunction. Neural dysfunction is thought to be caused by the altered metabolism of glucose in long-term diabetic patients, which is associated to oxidative stress changes (superoxide-induced free-radical formation and impaired axonal transport) and in turn is believed to cause peripheral nerve degeneration, affecting a number of tissues controlled by the nervous system (i.e. kidneys, eyes, cardiac system)[21,30,31]. DM is also thought to affect the bladder innervation in a way that causes degeneration of NGF, reducing its expression in the spinal dorsal root ganglia[5]. These changes are thought to be associated with the changes exhibited by the detrusor muscle, which will be discussed in the following section[32].

1.3.3. Changes in bladder structure and function

Urodynamic studies by cystometry have shown that DVD can cause several changes to the structure and function of the bladder. The toxic effects leading to neuronal damage due to hyperglycemia are displayed as multifactorial alterations that occur can impact urothelial function, peripheral nerve innervation, reflex mechanisms, and most commonly, detrusor physiology[33]. These changes affect both the innervation and vasculature of the bladder. For the urothelium, this specifically leads to compromised barrier function of the layer by desquamation of the umbrella cells, changes in urothelium mechanosensitivity and cell signaling by afferent nerve endings[8]. Chronic hyperglycemia leads to defects in the barrier and in receptor expression in the urothelium, affecting the release of ATP and subsequently the purinergic receptors, which signal for bladder fullness to the central nervous system[5]. As for the detrusor muscle, the changes occur with regards to their inter- and intra- cellular connection, excitability, and signaling as well as increased muscarinic receptor density and distribution[9]. The temporal DVD theory, as displayed in **Figure 1D**, suggests that the changes occur in a biphasic manner affected by both hyperglycemia and polyuria[34]. The initial phase is described as the overactivity of the detrusor muscle, as a result of

hyperglycemia, where the bladder involuntarily contracts during the filling phase and includes symptoms typical of OAB. The hypercontractile detrusor is the most common symptom reported in DVD patients[21,31]. The later phase of DVD is a result of polyuria and leads to remodelling of the bladder, particularly detrusor underactivity and a reduced afferent nerve threshold and diabetic neuropathy[33]. The weakening of the detrusor muscle leads to incomplete, poor emptying during the voiding phase, consequently resulting in an increased post-void residual volume[8,31]. Studies have shown that the urothelium also undergoes morphological changes, compromising its function of cell signaling with other bladder tissues and its role as a barrier. The changes in urothelial signalling properties, particularly in nerve endings, are part of the contributing factors for altered bladder sensation[31,33]. An alternate theory also suggests that urothelial dysfunction in patients with DVD is the result of cellular senescence in UROs. The DNA is thought to be damaged following prolonged exposure to hyperglycemic conditions and oxidative stress, leading to senescence of the cells[35].

1.3.4. Treatment options

Current treatment forms include both pharmacological and non-pharmacological approaches. Pharmacological treatments are mainly focused on the use of anticholinergic (or antimuscarinic) agents[36]. These pharmacological agents work by reducing the contraction of the detrusor muscle by inhibiting the binding of acetylcholine to the M2 and M3 receptors found mostly on the SMCs of the bladder wall[26]. Other second-line options include antispasmodic agents and β3-adrenoceptor agonists[37] such as mirabegron, which allows for the relaxation of the detrusor muscle through its activation of the β3-receptors.[38] As of more recently, some nerve growth factor inhibitors have been used as investigational therapies for the treatment of DVD/OAB[39-41]. As for non-pharmacological treatments, patients are given tools and strategies to aid in management of urge and urge incontinence using non-invasive strategies. Such tools may include lifestyle changes, fluid intake changes (timing and quantity), bladder retraining to increase bladder capacity and pelvic floor muscle training[37].

Figures

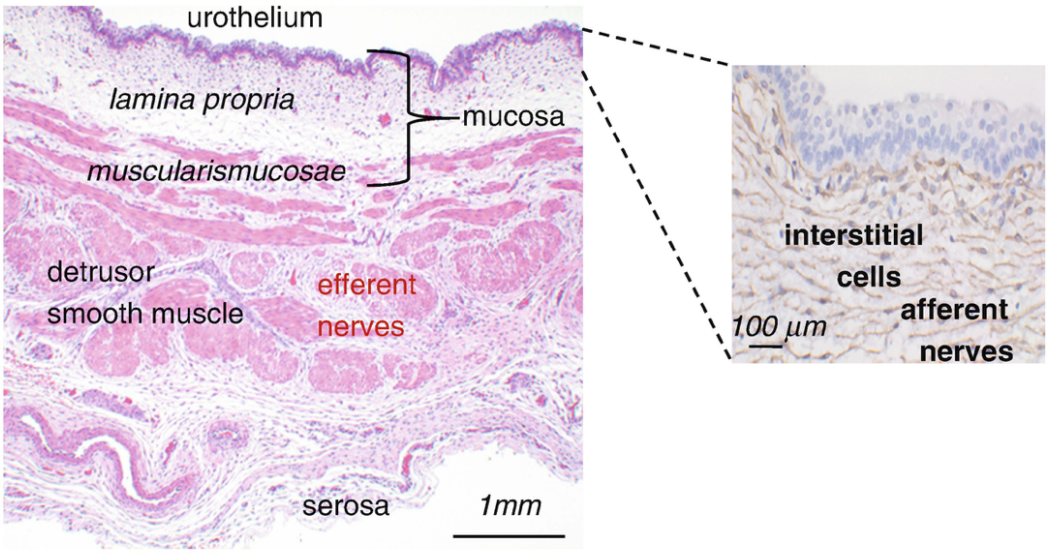

Figure 1.1. Basic overview of the bladder wall composition: The structure of the bladder wall is imperative in its proper functioning. The serosa and the adventitia cover the outermost later and covers the bladder, allowing for protection of the layers below. Smooth muscle cells (SMCs) make up the detrusor muscle, innervated by efferent nerves allow for bladder stretching in the filling phase of micturition. The innermost mucosa layer is composed of the lamina propria and the urothelium. The former is composed of interstitial cells, fibroblasts, and adipocytes, innervated by both afferent and efferent nerves, and works to define bladder compliance and support the urothelium. The urothelium, composed of several layers of urothelial cells (UROs), forms a tight junction barrier, preventing pathogen infiltration and urine leakage.
Figure from: Fry *et al*., 2019[1]

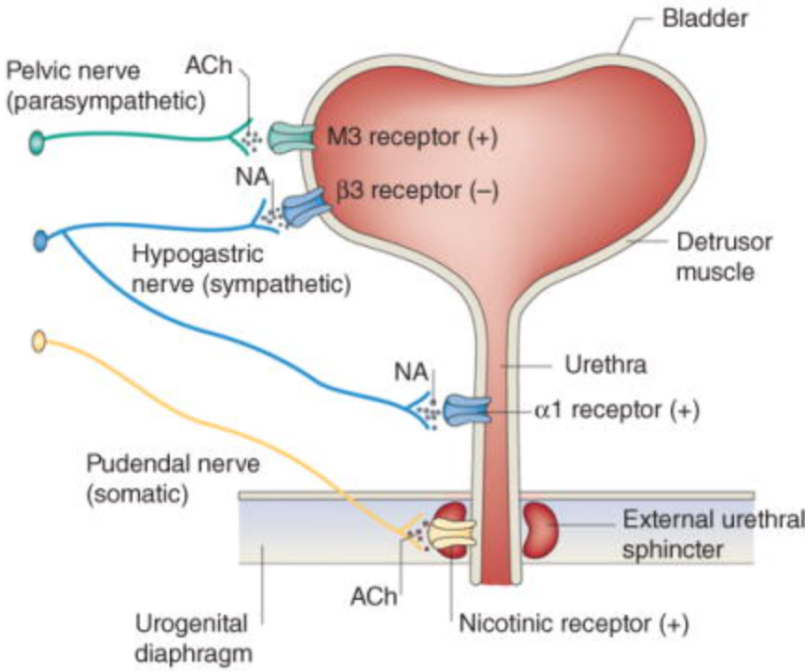

Figure 1.2. Neural signaling in bladder voiding and filling: Acetylcholine (ACh) and Adenosine triphosphate (ATP) are released by the postganglionic axons and intramural ganglia from the parasympathetic fibers in the pelvic nerve (S2-4), stimulating M3 muscarinic receptors found within the detrusor muscle of the bladder wall. This signals for contraction of the detrusor, with increases intravesical pressure, relaxation of the urethral sphincter and subsequent voiding. This process is initiated by signals from afferent nerve fibers in the pontine micturition center. The detrusor relaxes and the urethral sphincter contracts by inactivation of the muscarinic receptors, leading to bladder filling. This is controlled by the sympathetic nervous system, when noradrenaline is released from postganglionic sympathetic fibers of the hypogastric nerve (T10-L2), allowing for stimulation of the β-3 receptors in the bladder wall and the α-1 adrenoreceptors in the urethral sphincter.
Figure by: de Groat and Yoshimura, 2015[6]

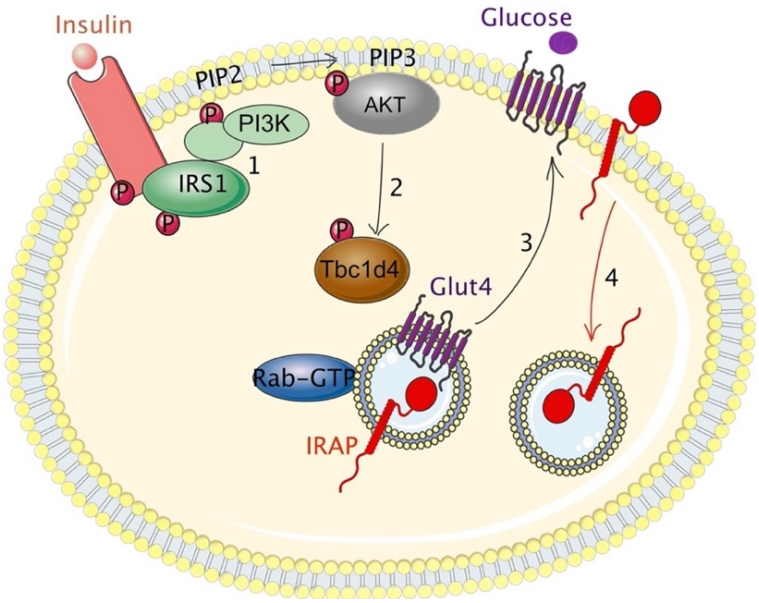

Figure 1.3. Insulin signaling pathway: Insulin is released by the β-cells of the pancreas when high levels of glucose are detected. Signal transduction begins when insulin binds to its receptor (InsR)in the cell membrane. Insulin-dependent phosphorylation occurs on the intracellular domain of the tyrosine kinase receptor. This activated receptor associates with the src homology region 2 (SH2) domain, activating phosphatidylinositol-3-kinase (PI3K). This activated kinase will in turn phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP2) to become phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 recruits AKT to the cell membrane, and once activated, p-AKT will inactivate the AS160 protein which contains a Rab GAP (GTPase-activating protein) domain, allowing for translocation of the glucose translocator GLUT4 to the plasma membrane. GLUT4 facilitates the movement of glucose in and out of the cell, normalizing blood glucose levels.
Figure by: Descamps *et al.*, 2020[42]

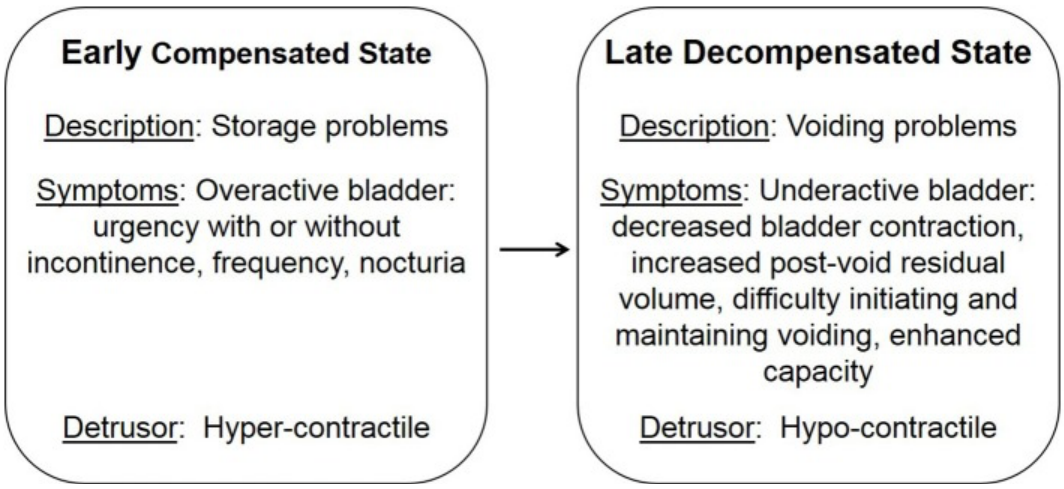

Figure 1.4. DVD mechanism – the temporal theory: Diabetic voiding dysfunction is expressed in a biphasic manner. The early compensated state is a result of elevated glycemia and leads to storage problems reminiscent of overactive bladder disease (OAB). The detrusor muscle is hyper-contractile and leads to symptoms such as urgency with or without incontinence, increased frequency and nocturia. As the disease progresses, there is a transition to the late decompensated state, resulting from polyuria. This leads to underactive bladder symptoms due to a hypo-contractile detrusor muscle. The voiding problems cause decreased bladder contraction, greater post-void residual volumes as well as difficulties initiating and maintaining voiding.
Figure by: Klee *et al*., 2018[35]

Chapter 2: Literature Review

In order to develop new treatments to provide therapeutic options for patients suffering from diabetic voiding dysfunction (DVD), it is imperative to possess a clear understanding of the pathways involved within the disease. The following literature review pinpoints the changes in the levels of nitric oxide secretion as a result of hyperglycemia and provides a comprehensive review of the current literature concerning the function of neurotrophins and of their precursors in diabetic bladder pathophysiology. Gaining a better insight on the disease's intimate molecular mechanisms will help to develop and test new avenues to alleviate the symptoms of the disease.

2.1. Nitric oxide in hyperglycemic conditions

2.1.1. Secretion of NO in normal vs. hyperglycemic conditions

The secretion of nitric oxide is central in a variety of regulatory pathways, with high levels of implication in metabolic, vascular, and cellular systems[43]. It is synthesized as a by-product resulting from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) enzymes, of which there are three isoforms (endothelial, inducible, and neuronal), all of which are expressed in pancreatic β-cells[44,45]. In normo-glycemic conditions, NO is responsible for both physiological and cellular actions such as vasodilation, leading to reduced blood pressure, and preventing platelet aggregation[44]. At low levels, NO keeps inflammation levels balanced, however when concentrations increase, harmful effects may come forth. Specifically, increased NO expression allows for greater production of reactive oxygen and nitrogen species (ROS, RNS), which can negatively affect cellular function[44]. Most pathologies are associated to a disbalance in ROS and RNS levels, resulting in oxidative stress[46]. This is thought to cause endothelial dysfunction, which will be discussed further, later in this chapter.

Under normal conditions, plasma NO is mainly regulated by the activity of eNOS, through Ca^{2+} activation, which regulates blood flow in endothelial cells. This NOS isoform can be found in fibroblasts, skeletal muscle, bone, and various nerve tissue[47]. Vascular tone is dictated by NO levels, constricting and relaxing blood vessels appropriately. This process is critical for proper oxygen and nutrient flow for all cells of the body. The isoform iNOS becomes triggered in different pathological conditions involving inflammation, as it is expressed in immune and glial cells, and will often lead to apoptosis of cells as an immune response[47]. Endothelial dysfunction occurs through inflammatory mediator increase, as well as lipid peroxidation[44]. In fact, most studies have shown a direct correlation between DM and endothelial dysfunction, greatly suggesting a link between the disease and the metabolite. Generally, diabetic patients will display compromised NO synthesis and activity[43].

In hyperglycemic conditions, the secretion of NO, particularly through high iNOS activity, is regulated by insulin through the downstream activation of the Akt pathway[43]. Oxidative stress is enhanced through the production of advanced glycation end products (AGEs), which in turn stimulates pro-oxidative pathways such as the polyol, protein kinase C and hexosamine pathways[44,48]. With regards to specific changes, the literature on NO concentrations in diabetic patients are contradictory. While some studies report increases in NO expression in hyperglycemic conditions due to an increased inflammatory response[49], others report that NO levels decreased in DM patients' serum[43,50]. A resistance to insulin in T2DM patients, associated with hyperglycemia may be responsible for reduced nitric oxide production, as found in urine of diabetic patients[44]. Exposure time and concentration of NO are likely the reason for the conflicting reports, as it can act as both a positive and negative regulator of insulin depending on expression levels. Increased NO and ROS expression has been associated with various macro- and micro-vascular issues affecting the heart, kidneys, eyes, pancreas, among others[46], as displayed in **Figure 2.1**.

2.1.2. Effect of NO secretion in different systems (endothelial, immune, neurological)

As mentioned, nitric oxide can have varying effects on a variety of systems, and this depending on its concentration and location of synthesis and secretion. Endothelial cells are the main site of eNOS expression, which allow for NO synthesis and subsequent secretion into the bloodstream. As described in the previous section, the main physiological effect of NO secreted by endothelial cells is controlling vasodilation of vessels, dictating blood flow, and consequently, oxygen and nutrient delivery to cells[51]. This is specifically done when NO enters smooth muscle cells and binds the ferrous (Fe^{2+}) heme group of soluble guanylyl cyclase (sGC). This initiates the signaling cascade converting guanosine triphosphate (GTP) in the messenger molecule cyclic GMP (cGMP) and subsequently activated cGMP-dependent protein kinases, leading to relaxation of smooth muscle[51].

Different components of immune response regulation are also controlled by NO secreted by the iNOS isoform, making it a key player in the overall immune system. Proper growth and function of immune and inflammatory cell types such as T-lymphocytes, mast cells, antigen-presenting cells, neutrophils, natural killer cells and macrophages, are all partially regulated by NO[52]. Moreover, NO's involvement is present in antigen pattern recognition. Pathogens are recognized by the cells of the innate immune system and activated macrophages will prevent replication of the pathogen through effector molecules such as NO[53].

Though not all components are fully understood, it is well documented that nerve function is also highly correlated with NO, particularly in afferent signaling and innervation of certain organs and muscles[54]. The isoform nNOS, predominant in the central and peripheral neurons, synthesizes NO for cell communication. An example is nerve-mediated relaxation of the gut during digestion, or modulation of various innervated arteries throughout the body[55].

2.1.3. Changes in NO levels in the diabetic bladder

Previous studies report that the detrimental changes in diabetic bladder may be a result of the imbalance in the NGF/proNGF ratio, (to be discussed further in this chapter). Though the role of NO in neuron metabolism is well understood, the direct link between NO, hyperglycemia and neurotrophin levels has yet to be evaluated in the context of voiding dysfunction. As previously mentioned, the iNOS isoform is upregulated in inflammatory conditions, leading to increased amounts of NO being released. This process can be influenced by hyperglycemia-induced oxidative stress and secretion of pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ[56]. In fact, high levels of oxidative and nitrosative stress can result in DNA and protein damage within all cells of the body[44].

Different components of the lower urinary tract and the neural system overlap, and the process is highly regulated with both the detrusor muscle and the urethra subject to contraction and relaxation processes, allowing for normal filling and voiding of the urinary bladder. Noradrenaline (NA) and acetylcholine (ACh), are neurochemical transmitters that play a role in both neural pathways and are key components of the urogenital system[57]. As of more recently, NO has been identified as a key player in these processes as well, specifically acting on muscular and sphincter relaxation[58]. With regards to the urethra, high levels of NO are expressed in the bladder base[59]. NO mediates nerve-stimulated relaxation of the smooth muscle, allowing for micturition when the bladder is full[60]. As the detrusor muscle has a low-sensitivity to NO, it is not as highly involved in relaxation of this component of the bladder wall, although some associations with afferent function of the detrusor have been reported[61]. Complications brought on by diabetes develop over time throughout the duration of the disease, depending on the intensity and length of hyperglycemia exposure and management of glucose levels. It has been reported that the oxidative stress resulting from hyperglycemia has an impact on the pathogenesis of complications occurring in later stages of the disease. Voiding dysfunction is associated to neuron pathology, and studies have shown

a role for changes in NO plasticity with regards to neurodegenerative diseases, as expression of NO and the nNOS isoform have been discovered in various neurons and fibers[60].

2.2. Nerve growth factor (NGF) and its precursor (proNGF)

2.2.1. The balance of NGF and proNGF in healthy bladder physiology

Nerve growth factor (NGF) is a neurotrophin traditionally known for its role in the central and peripheral nervous systems, influencing growth, survival, and differentiation of neurons. It's role of phenotypic and functional maintenance of different systems means the secretion of NGF is localized in various tissues such as salivary glands, smooth muscle cells of blood vessels, immune cells, and most importantly for our purposes, in bladder cells[62,63]. Many aspects of neural function are regulated by neurotrophins and will aid in proper neuronal development with regards to synaptic plasticity, function, and overall neuronal survival[64]. As neurogenic control is a large part of proper bladder function, it is without a doubt that neurotrophins play a key role. The binding of NGF to the receptors TrkA and $p75^{NTR}$ conducts the trophic effect of the neutrotrophin. NGF binds to the tyrosine kinase receptor TrkA with higher affinity, while binding to $p75^{NTR}$ with lower affinity[65,66]. Binding to TrkA allows for activation of the receptor tyrosine kinase through phosphorylation of various residues, at sites promoting different signaling cascades such as phospholipase C-γ (PLCγ), PI3K, MAPK, Ras and Raf/MEK/Erk1[67-69] as displayed in **Figure 2.2**. Nervous system health and overall integrity is dependent on the proper functioning of these signalling cascades. Alternatively, facilitation of apoptosis through activation of the $p75^{NTR}$ receptor, is reliant on proNGF. Upon binding, apoptotic signaling pathways such as JNK signaling cascade, NFκB and ceramide generation are activated[63].

NGF is synthesized through proteolytic cleavage from its precursor proNGF by enzymes matrix metalloproteinase 7 (MMP-7), plasmin and furin. The processing of proNGF is later followed by

digestion of NGF into smaller peptide fragments, mainly dictated by the activity of matrix metalloproteinase 9 (MMP-9)[70]. Under healthy, normo-glycemic conditions, NGF is important in bladder mechanosensitive and neuronal plasticity, and is secreted by both the urothelial cells and the smooth muscle cells of the detrusor[71]. Proper bladder innervation is also reliant on adequate NGF levels as it promotes afferent sensitization in the dorsal root ganglia. Additionally, several mediators important for proper bladder function, such as cAMP, acetylcholine, and noradrenaline play a role in NGF secretion by bladder cells. The fate of bladder tissue health is determined by the collective activity levels of these enzymes, with the ratio between NGF and proNGF being at the core for cell responses.

2.2.2. Changes to NGF/proNGF ratio in the diabetic bladder

The complexity of diabetic voiding dysfunction, as well as the mechanisms regulating the lower urinary tract is evident. Though still not fully understood, the role of NGF, its precursor proNGF and their ratio relative to each other in bladder dysfunction has piqued the interest of researchers. The dysregulation of neurotrophins and their respective receptors have been associated with several neurodegenerative diseases such as Alzheimer's disease, diabetic retinopathies, and Parkinson's disease[72]. Likewise, diabetes has been associated to degeneration of neurons and fibers of the peripheral nervous system, leading to debilitating symptoms, some of which will affect the lower urinary tract[63].

Expression of NGF, various neuromodulators as well as other components of the NGF pathway have been found to be dysregulated in diabetic conditions, particularly in urine and serum[73]. Bladder dysfunction in type 1 diabetic mice showed a significant decrease in the overall NGF to proNGF ratio[74]. A recent study has also shown that an imbalance of proNGF to the detriment of NGF is associated to diabetic retinopathy in animal and human patients[75,76]. This imbalance is believed to be caused by the changes in activity of the enzymes involved in NGF metabolism (such as MMP-7 and MMP-9), due to an increase in oxidative stress brought on by the prolonged exposure to hyperglycemia[75].

Elevated levels of α-macroglobulin (α2M) have also been found in patients with diabetic retinopathies, among other neurodegenerative diseases. In fact, this protein modulator binds both proNGF and NGF. When bound to proNGF, a stable complex is formed, and proteolysis to mature NGF is decreased, increasing the overall levels of proNGF. Moreover, when bound to NGF, the complex is unable to bind and activate its TrkA receptor, decreasing NGF levels. The proNGF-α2M agonist of the $p75^{NTR}$ receptor also demonstrates a more potent effect when bound, leading to increased neuronal death through enhanced TNF-α expression[77]. Increased expression of α2M is reported in vaginal walls of female patients with bladder dysfunction[78]. This suggests that α2M may play role in DVD through decrease in NGF/proNGF ratio. So, although some advances have been made in evaluating the exact role of NGF and proNGF in diabetic bladder pathophysiology, further studies are required.

2.3. $p75^{NTR}$ antagonism by THX-B to re-establish NGF/proNGF balance

2.3.1. The $p75^{NTR}$ receptor signaling and ligands

All neurotrophins, along with their respective precursors, have the ability to bind to the $p75^{NTR}$ receptor. The $p75^{NTR}$ receptor is a part of the TNF-α receptor superfamily and at normal levels, is essential in cell development. The p75 receptor family is categorized as low affinity, as neurotrophins tend to bind to Trk receptors with higher affinity, highlighting the complexity of this signaling axis. Binding of ligands to the p75 receptor lead to a variety of biological effects that are cell- and time- specific. The upregulation of p75 in neurodegenerative states emphasizes its importance in proper biological function[79]. These complex pathways generally lead to cell death signaling and inflammation, however numerous influences play into this[72].

2.3.2. Effect of $p75^{NTR}$ antagonism

Neurodegenerative conditions, resulting from the imbalance of neurotrophins, are complex and have a very limited treatment profile. The initial treatment approach targeted Trk activity, specifically with the supplement of exogenous neurotrophins[72]. However, since the $p75^{NTR}$ receptor has many ligands, this approach has continuously failed, as the neurotrophins can also activate the p75 receptor, negating the positive effect of neurotrophin upregulation[72,80]. The activation and upregulation of the p75 receptor is associated with neurotoxicity and even neuronal death, in a vast majority of tissues. As such, antagonism of $p75^{NTR}$ has recently become of interest to researchers in various fields as a therapeutic agent in various pathological neurodegenerative disorders in which there is a neurotrophic imbalance involved[72].

A study on diabetic retinopathy by Mysona et al. found that erasing $p75^{NTR}$ expression in patients diminished the changes in proNGF and NGF, as well as the pro-inflammatory cytokines TNF-α, NFκB, and VEGF expression, which were the result of diabetes[67,72,81]. Mossa *et al*., have also demonstrated the positive effect of $p75^{NTR}$ antagonism on bladder dysfunction in a type 1 diabetic murine model. In fact, both functional and morphological benefits were observed following a 4-week treatment with THX-B, a small competitive antagonist molecule of the neurotrophin receptor $p75^{NTR}$ [74]. This was achieved through correction of the NGF/proNGF imbalance. It is important to note that complete deletion of the $p75^{NTR}$ is not entirely beneficial. The receptor is believed to have some role in response to injury, as displayed in mice with bladder dysfunction following a spinal cord injury (SCI). In these SCI rodents, although bladder function improved, believed to be through increased excitatory input to the spinal cord, p75NTR deletion worsened overall voiding efficacy[82].

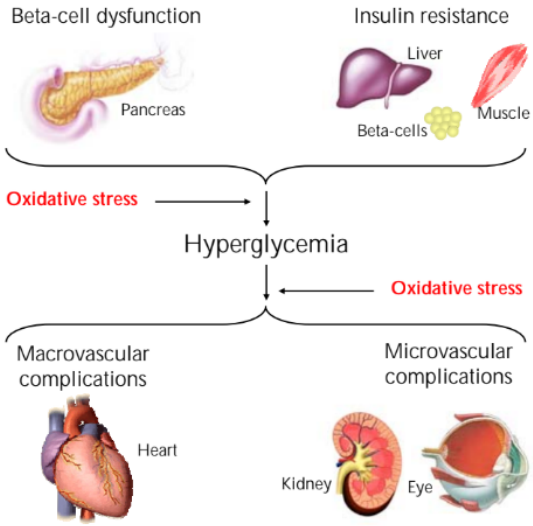

Figure 2.1. Vascular complications resulting from hyperglycemia and oxidative stress.
Figure from: Pitocco et al., 2010[46]

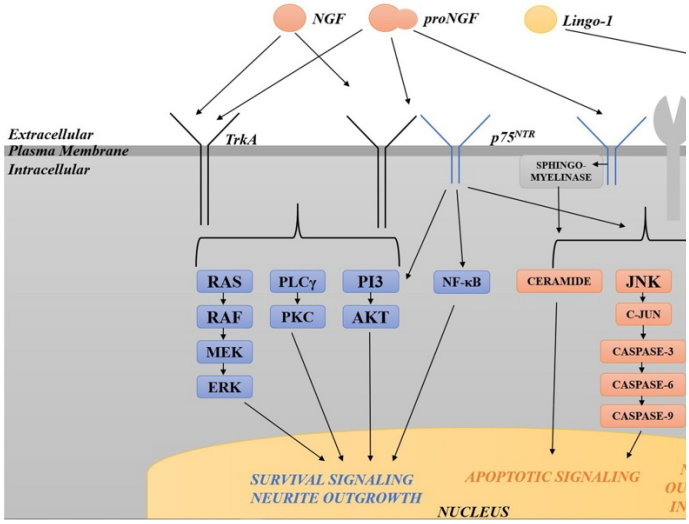

Figure 2.2. Survival and apoptotic pathways activated by NGF-TrkA or proNGF-p75^{NTR} interaction: NGF binding the TrkA receptor activates pro-survival signalling pathways such as Ras/Raf/MEK/ERK, PLCγ/PKC, PI3/AKT. These pathways are responsible for proper growth and maintenance of neurons. NGF can also bind the p75^{NTR} receptor, but to a lesser extent than its precursor proNGF. The p75^{NTR} activation by proNGFleads to inflammatory and pro-apoptotic signalling pathways such as JNK/caspase.
Figure modified from: Fahnestock and Shekari, 2019[83]

Chapter 3: Hypothesis and Objectives

3.1. Rationale

The exact mechanisms underlying DVD are not well understood and there exists a need to fully understand the pathophysiology of the condition. This would bridge the knowledge gap, allowing for the development of better diagnostic tools for DVD, as current methods are limited and not always effective. Additionally, with the important impact diabetes has on the bladder, it is imperative that adequate therapies and treatment options be available for patients, however currently, little-to-no options exist for patients that suffer of these associated complications. Hyperglycaemia and polyuria with local oxidative stress and inflammation[84] are among the many factors contributing to the development of DVD. Oxidative stress is caused by an imbalance between the activity of endogenous reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and antioxidative products[85]. When off-balance, high ROS levels can directly damage lipids, proteins and DNA through oxidation, disturbing cell metabolism and homeostasis, leading to associated pathologies. Nitric oxide (NO) is known to be a signaling molecule that plays a key role in the pathogenesis of inflammation[86] , neural transmission, and oxidative stress. Several inflammatory factors have been associated with NO through the production and/or stimulation of the different isoforms of nitric oxide synthase (NOS). For example, in bone cells, among other cell types, pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) have all been associated with increased NO production through stimulation of the inducible nitric oxide synthase (iNOS) pathway[87]. The upregulation of this pathway will generate high concentrations of NO and thus, ROS and RNS. In high inflammatory responses such as asthma, high levels of NO are also produced[88]. Insulin resistance and beta cell dysfunction, both associated with diabetes, can be induced by high concentrations of ROS. Specifically, these ROS use the same pathway as insulin (i.e., GLUT4 translocation). When under high oxidative stress, the stress-response signalling cascades are activated and the insulin receptor substrate (IRS) will exhibit increased Ser/Thr

phosphorylation, therefore reducing normal metabolic effects and leading to insufficient glucose homeostasis[46]. With regards to the bladder, it has been demonstrated that a cohort of female patients with OAB displayed elevated levels of NO in their urine samples and this was linked to insulin resistance (or HOMA)[70]. Furthermore, the receptor $p75^{NTR}$, which is highly expressed in the urothelium of diabetic bladder and to a lesser extent in smooth muscle cells, is activated preferentially by proneurotrophins (proNGF and proBDNF) and triggers the aforementioned inflammatory pathways that participate in DVD[89]. The overall function of the bladder can be detrimentally affected by the activation of the proNGF/$p75^{NTR}$ axis. This is attributed to the increased expression of the inflammatory cytokine TNF-α in urothelial cells, leading to the activation of RhoA[32]. The inactivation of RhoA, which is involved in the signal transduction pathway of inflammation and apoptosis, has been linked with the inhibition of MAPK/JNK[90]. The MAPK/JNK pathway is an essential signal transduction system that regulates several biological processes such as cell proliferation, development and differentiation, stress, and inflammation[90,91]. Alternatively, the activation of NF-κB mediates the inflammatory process in smooth muscle cells[32]. As such, $p75^{NTR}$ receptor has recently been targeted as a possible therapeutic avenue in diseases involving inflammation and used as a means of delaying disease progression[83,92]. Previous studies have shown that inhibiting the binding of the precursor of the neurotrophin nerve growth factor (proNGF) binding to receptor $p75^{NTR}$ has allowed for reversal of bladder dysfunction in patients with spinal cord injuries[82]. Additionally, $p75^{NTR}$ antagonism can prevent degenerative processes of diabetic retinopathy and microangiopathy[93]. Finally, the inhibition of the $p75^{NTR}$ pathway with a receptor antagonist or an antibody binding proNGF has shown to reverse bladder dysfunction in type 1 diabetic models [74]. However, with type 2 DM being the more prominent type of the disease, accounting for 90-95% of diagnosed cases, the potential therapeutic target needs to be validated in a pre-clinical model of type 2 DM, in order to verify the commonalities in terms of pathophysiology and clinical signs and symptoms.

3.2. Hypothesis

3.2.1 Hypothesis 1

We hypothesize that incubation in a hyperglycemic medium will promote NO secretion in both URO and SMCs in vitro, and subsequently lead to a decreased NGF expression in bladder cells in culture, consistent with what has been shown in urine of OAB patients.

3.2.2 Hypothesis 2

We hypothesize that the activation of the $p75^{NTR}$ receptor by proNGF may cause degenerative and apoptotic changes in the bladder, and that its blockade using THX-B antagonist may reverse these effects. We aim to investigate the proNGF/ $p75^{NTR}$ interaction in a murine model of type 2 DVD and determine how targeting it modulates bladder function.

3.3 Objectives

3.3.1 Objective 1

Our objective is to demonstrate the pathways used by NO, secreted as a result of elevated glycemia, on the levels of NGF and proNGF in bladder cells in culture, as displayed in our clinical observations in patients with OAB.

Experimental Design

Primary cultures of urothelial (URO) and smooth muscle cells (SMC) will be obtained by collagenase digestion of minced healthy rat bladders (two-month-old female Sprague-Dawley rats). The cell extracts will be analyzed by RT-qPCR for relative NGF and MMP-9 mRNA levels. Quantification for NGF, proNGF and cGMP will be determined by ELISA. Immunoblotting will be used to semi-quantify

intracellular MMP-9 content. Enzymatic kits will be used to assess activity of proteases involved in NGF metabolism (i.e. MMP-9, MMP-7, plasmin). The nitric oxide levels in the cell cultures will be measured by the Greiss reaction. And finally, we will use crispr-cas9 for the knockdown of genomic MMP-9 to determine its implication in the pathway involving NO and NGF in hyperglycemic conditions.

3.3.2 Objective 2

As the work by Mossa et al. showed, the blockage of the proNGF/$p75^{NTR}$ interaction in a validated streptozotocin-induced type I diabetic mouse model has prevented the detrimental impact on the bladder, such as remodeling and voiding dysfunction[74]. Our objective is to validate the inhibitory effect of a $p75^{NTR}$ antagonist and its ability to correct the imbalances in NGF and proNGF as well as restoring normal bladder function. This will be done by correlating bladder function from voiding spot assays, cystometric parameters and contractility analysis in relation to proNGF/$p75^{NTR}$ activity in DVD.

Experimental Design

The Tally Ho mice (model for type 2 diabetes) at 10 weeks of age, will be divided into 2 groups of 16 mice each (8 female and 8 male). One group will be treated with weekly intraperitoneal injection of $p75^{NTR}$ small molecule antagonist (THX-B), while the other will receive PBS control, for a period of 4 weeks. SWR/J mice will serve as controls to the Tally Ho mice and will receive the same treatment. Voiding spot assays will be performed weekly to determine the voiding behaviours, specifically by observing the progression of urine patterns on filter papers. This assay will also be used to estimate the voiding frequency and urine volumes. Cystometry and organ baths will be conducted 24 hours following surgical catheter insertion into the mouse bladder. These methods will be used to measure bladder contractile parameters such as basal pressure, capacity and compliance, micturition and residual volumes and spontaneous activity. The $p75^{NTR}$ receptor will be localized in the bladder wall using

immunofluorescence and confocal microscopy. Blood and urine samples will be assessed for quantification of $p75^{NTR}$, NGF, and proNGF, along with other apoptotic markers. This will be done using immunoblotting methods, ELISA kits, and RTqPCR. Markers of inflammation, (NO, TNF-α, CCL2) will be measured with specific ELISA kits.

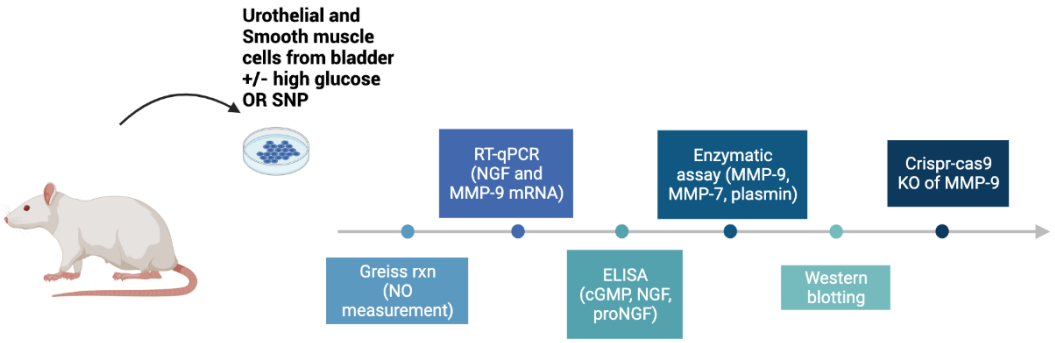

Figure 3.1.: Experimental timeline for *in vitro* study
Figure created with *Biorender*

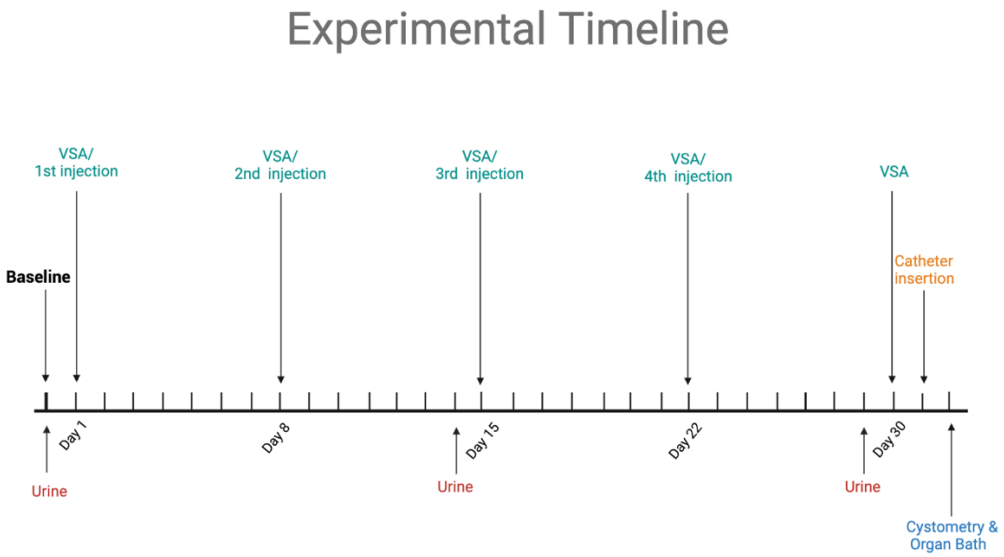

Figure 3.2.: Experimental timeline for *in vivo* treatment study
Figure created with *Biorender*

Chapter 4: Synthesis and secretion of NGF is regulated by Nitric Oxide through cyclic GMP and metalloproteinases in bladder cells

This chapter includes work recently submitted for publishing in *Cellular Signaling*

Here we investigated the *in vitro* effect of an increase in nitric oxide levels by hyperglycemia on the secretion of NGF in bladder cells.

Contribution of authors: The conception of the study was conducted by Dr. Lysanne Campeau, Dr. Philippe Cammisotto, Ms. Aya Hajj, Ms. Aalya Hammouda and myself. The maintenance of all cell cultures was the responsibility of Dr. Cammisotto and myself. The execution of all experiments (including qPCR, ELISA, specific enzymatic assays, western blotting and Crispr-cas9 were performed by myself, along with the help of Ms. Hajj and Ms. Hammouda. The interpretation of the acquired results and statistical analyses of this study, as well as the writing of the manuscript was conducted by myself.

Synthesis and secretion of NGF is regulated by Nitric Oxide through cyclic GMP and metalloproteinases in bladder cells in vitro

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The authors have declared that no conflict of interest exist.

Abstract

Urine samples of female patients with diabetic voiding dysfunction (DVD) are characterized by low levels of nerve growth factor (NGF) and elevated concentrations of nitric oxide (NO). The aim of the present study is to determine how NO regulates the presence of NGF using rat bladder smooth muscle (SMCs) and urothelial (UROs) cells in culture. In UROs, incubation in hyperglycemic conditions increased secretion of NO and concomitantly decreased NGF, except when the NO synthase inhibitor, L-NAME (1 mM) was present. Sodium nitroprusside (SNP) (300μM, 24 hours), a NO generator, decreased NGF levels and decreased cGMP content, a process validated by the cGMP synthase inhibitor ODQ (100 μM). Alternatively, SNP increased mRNA of both NGF and MMP-9. MMP-9 knockout of UROs by Crispr-Cas9 potently decreased the effect of SNP on NGF, implying a dependent role of NO on MMP-9. On the other hand, MMP-7 activity was increased by SNP, which taken together with increase in NGF mRNA, suggests a compensatory mechanism. In SMCs, hyperglycemic conditions had the same effect on extracellular content of NO and NGF than in UROs. SNP also decreased NGF secretion but increased cGMP content. Stable permeable analogs of cGMP 8-(4-Chlorophenylthio)-cGMP (3mM) and N2,2'-O-Dibtyryl-cGMP (1mM) inhibited NGF release. NGF and MMP-9 mRNA expression was unchanged by SNP. Removal of MMP-9 in SMCs by Crispr-Cas9 did not alter the effect of SNP. Finally, SNP decreased MMP-7 activity, diminishing the conversion of proNGF to NGF. These results demonstrate that enhanced NO secretion triggered by high glucose decreases NGF secretion through pathways unique for each cell type that involve cGMP and proteases MMP-7 and MMP-9. These results match our observations from the urine from patients with DVD.

1. Introduction

Normal physiological voiding relies on the complex interaction and activity of both the central and peripheral nervous system[94]. As such, neurological conditions can result in voiding dysfunction[95]. In this respect, neurotrophins are a group of hormones essential to the growth and survival of cells of the nervous systems. In particular, the precursor proNGF is cleaved into mature NGF through specific proteases, mainly furin, plasmin and metalloproteinase-7 (MMP-7)[33]. NGF in turn, can be subjected to proteolysis primarily by the enzyme matrix metalloproteinase-9 (MMP-9)[96]. ProNGF triggers inflammatory processes and apoptosis through its membrane receptor $p75^{NTR}$ while NGF bound to receptor TrkA is essential to the growth, maintenance, and survival of peripheral nerve endings and ganglions[97]. The balance between neurotrophins and their precursors is determined by the activity of proteolytic enzymes, which affects the ratio of NGF/proNGF, subsequently determining the global cellular actions of these neurotrophins and establishing a balance between cell survival versus inflammatory/apoptotic processes. In several other tissues synthesizing NGF and proNGF such as glial cells, NO affects the synthesis and secretion of NGF[98]. Intracellular pathways triggered by NO usually involve cyclic GMP (cGMP) that in turn, involves cGMP regulated protein kinases[98]. So far, no studies have examined this relation between NO and NGF in bladder tissue. This is particularly important in terms of bladder physiology as urothelial and smooth muscle cells of the bladder are major sources of NO, NGF and proNGF[32,99-101]. A considerable amount of research has linked age-related insulin resistance, type 1 and type 2 diabetes mellitus and metabolic syndrome (obesity, lack of exercise, poor dietary habits, etc.) to bladder dysfunction[70,102,103]. Particularly, a recent study on urine samples of an aging population of female patients with OAB reported decreased levels of NGF concomitantly with increased content in nitric oxide (NO), compared to healthy controls[70]. These changes were associated to insulin resistance as rise in HOMA paralleled changes in urine proteomic analysis. Diabetic voiding dysfunction (DVD) affects up to 80% of diabetics and presents with bladder overactivity in early stages and impaired detrusor activity in later stages.[104-106] It leads to debilitating urinary incontinence and recurrent urinary tract infections, with no effective

preventive or curative strategies. As diabetes increases in prevalence with aging, other concurrent factors may come into play (such as prostatic hyperplasia and neurological disorders) making it difficult to determine the exact influence of hyperglycemia in issues of the bladder[26]. As such, in the present study, we linked insulin resistance and nitric oxide to the secretion of NGF and proNGF in primary cultures of rat bladder cells. An emphasis was put on the intracellular pathways involving NO, as well as the activity of the enzymes controlling the synthesis and degradation of NGF and proNGF.

2.Materials and methods

2.1. Cell culture

All animals were housed and handled in accordance with the Canadian Council for Animal Care (CCAC). Protocols received the approval of the Animal Ethics Committee of McGill University (Montreal, Canada). Two-month-old female Sprague-Dawley (SD) rats were sacrificed, and their bladders were used for cell isolation as previously described[107]. Briefly, bladders were placed into cold PBS then opened along the longitudinal axis. To harvest urothelial cells (UROs), the urothelium was carefully scraped and incubated for a duration of 15-20 minutes in DMEM medium containing 100U/mL of collagenase type IV at 37 ºC with gentle shaking. Digested tissue was then washed twice in DMEM containing 10% FBS then cells seeded in T25 flasks with Dubelco's DMEM low glucose/Keratinocyte (50/50) media containing FBS (10%), Glutamax (X1), a mix of hormones (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). Prior to use, cells were starved for 24 hours. In parallel, to harvest smooth muscle cells (SMCs), bladder detrusor muscle was finely 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.

2.2 Cell viability assay (MTT test)

Both UROs and SMCs were seeded on a 96-well plate and after reaching 90% confluency were starved of glucose for 24 and 72 hours respectively. Cells were incubated with SNP (300 µM) for 24 hours, then washed twice with PBS and incubated in culture medium without FBS and with 5µg/mL MTT solution for 30 minutes. Medium was removed, the plate was dried, and each well received 200 µL of DMSO

(Sigma-Aldrich, MO, USA). Readings were done using a microplate spectrophotometer (BioRad, CA, USA) at 535 nm.

2.3 Nitric oxide measurement in medium (Greiss reaction)

Culture medium was used for NO measurement with the Greiss reaction through colorimetry (548 nm) using sulfanilamide and 1-naphtyl-ethylene-diamine-dihydrochloride (NEDD), as previously reported[108].

2.4 Quantitative PCR

Total RNA was extracted and purified from bladder cells using Trizol (Biomatik Corporation) and chloroform. A Nanodrop spectrophotometer ND-1000 (ThermoFisher Scientific, Wilmington DE) measured the RNA concentration and purity. Synthesis of cDNA relied on a reverse transcriptase kit (OneScript cDNA synthesis kit) according to the manufacture's protocol (ABM, Richmond BC, Canada). 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'), 18S forward primer (5'-GCA ATT ATT CCC CAT GAA CG-3'), 18S reverse primer (5'-GGC CTC ACT AAA CCA TCC AA-3'). Using the Sensifast Probe Low-ROX kit containing SYBR-green, samples were amplified (Applied Bioscience 7500 Fast Real-Time PCR). Conditions of the qPCR were: 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 40 seconds at 57 °C. Samples were prepared in duplicates and melt curve data was analyzed. Assessment of primers was conducted to ensure specificity and 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[109].

2.5 Western Blotting

Bladder cells were homogenized in RIPA buffer containing an anti-protease mix (Roche Diagnostics, Indianapolis, IN, USA). Micro BCA assay kit (Boster, CA, USA) was used to measure protein concentration in lysate. Equal protein amounts varying from 20-40 µg were loaded onto 8% polyacrylamide gels and transferred to PVDF membranes. Proteins were blocked for 1 hour in 5% skim milk in TBST. They were then incubated overnight at 4 ºC with primary antibodies at the following concentrations: anti-MMP-9 (1:2000) and anti-β-actin (1:20000). The following day, secondary antibodies conjugated with HRP were used (anti-mouse or anti-rabbit from Millipore, Billerica, MA, USA) for 1 hour at room temperature, at a concentration of 1:3000 in TBST containing 5% skim milk. Revelation of bands was done using Luminata Crescendo HRP substrate (Millipore, Billerica, MA, USA) and quantified using the ImageJ software (National Institute of Health, Maryland, USA). β -actin was used as a reference "house-keeping" protein for relative quantification.

2.6 Reagents and antibodies

All chemicals and antibodies were purchased as follows: SNP (Sigma-Aldrich, Oakville, ON, Canada), NGF and proNGF ELISA kits (Biosensis, Thebarton, SA, Australia), cGMP Rapid ELISA kit, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), N(ω)-nitro-L-arginine methyl ester (L-NAME) (Cayman, Ann Harbor, MI, USA), MMP-9 and β-actin antibody (Abcam, Cambridge, MA, USA), Fetal bovine serum (FBS) (Wisent, Quebec, Canada), Penicillin/Streptomycin (VWR International, Quebec, Canada), BCA protein assay kit (BosterBio, Pleasantton, CA, USA), collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ, USA), DMEM and MCD153 media (US Biological Life Science, Salem, MA, USA), Glutamax and Keratinocyte medium supplemented with EGF and bovine pituitary extract (Gibco, ThermoFisher Scientific, Waltham, MA, USA), D-glucose, ethanolamine, bovine serum albumin (BSA), analogs 8-(4-Chlorophenylthio)-cGMP, N2,2'-O-Dibutyryl-cGMP,

plasmin activity assay kit (Sigma-Aldrich, Oakville, ON, Canada), adenine (Biomatik, Cambridge, ON, Canada), dihydrocortisone (MP Biochemicals, Solon, OH, USA), Y27632 (Selleckchem, Houston, TX, USA), MMP-9 and MMP-7 enzymatic assay kits (Quickzyme, Leiden, Netherlands).

2.7 Crispr cas-9 knockdown

Sg primers were designed from https://design.synthego.com using the sequence found on the genome of Mus musculus (NM+013599.4). The following sequences were generated and purchased from IDT (Coralville, IOWA, USA): forward 5'-CAC CGC GGC CCG GGT GTA ACC ATA G-3' and reverse 3'-CGC CGG GCC CAC ATT GGT ATC CAA C-5'. Bacteria containing plasmid Crispr-Cas9 were purchased from Addgene (Watertown, MA, USA). After overnight growth on petri dish, single colonies were added to liquid broth containing ampicillin (100 µM) and put at 37°C for 12-18 hours in a humid incubator, with shaking. The following morning, plasmids were extracted using a kit from Sigma-Aldrich (Oakville, Ontario, Canada). Plasmid concentrations were assessed using a Nanodrop system as previously described. Subsequently, plasmids were digested with Bsmb1 (New England Biolabs, MA, USA) and the products were run on a 0.7-1% agarose gel. Digested bands were purified with gel DNA extraction kit (Sigma-Aldrich, Oakville, Ontario, Canada) and plasmid amounts were measured with the Nanodrop system. Sg primers were ligated in the presence of ATP (1mM) and T4PNK enzyme (New England Biolabs, MA, USA) for 30 minutes at 37°C followed by 5 minutes at 95°C. Insertion of ligated primers into digested plasmid was carried out using T4 ligase enzyme, RT for 1 hour. Termination was achieved by incubating at 65°C for 10 minutes. Competent stbl3 cells were transfected by heat shock (42 °C for 45 seconds and cooled down at 4°C) then cultured in SOC media at 30°C for 2 hours. Bacteria were later grown on petri dishes with ampicillin (100 µM), overnight at 37°C. Single colonies were used for characterization of the plasmid, then grown in liquid broth with ampicillin (100 µM). Finally, UROs and SMCs were transfected with plasmids containing the sg primers or not (sham control) for 24 hours,

then selected using puromycin (5 μg/mL). Cells were then allowed to grow until confluence in their respective growth medium.

2.8 Statistical analysis

Results were presented as the mean and standard error of the mean (SEM) for all values. The comparison between groups was done using an independent student t-test and two-way ANOVA (post hoc Tukey HSD test). The statistical significance of the data was presented as a p value where $p<0.05$, $p<0.01$ and $p<0.001$, as described in the legends. The analysis of the statistics was conducted using both Excel and GraphPad softwares.

3. Results

Hyperglycemic media increase nitric oxide (NO) and decrease NGF secretions in bladder cells: After 24-hour incubation in hyperglycemic conditions (glucose 27mM for UROs and 55 mM for SMCs), NO concentrations in media were increased for both cell types (Figure 1A). In the same samples, NGF secretion was decreased, while that of proNGF remained unchanged, leading to a significant decrease in the NGF-to-proNGF ratio as measured by ELISA (Figure 1B and 1C). As UROs are the major source of NO, we tested the effects of the NOS inhibitor L-NAME (1 mM) in hyperglycemic conditions for 24 hours[110]. In the presence of L-NAME, NO secretions were decreased both in normo- and hyperglycemic media, while hyperglycemia-triggered NGF decrease was prevented (Figure 1D). ProNGF levels were stable in the presence of L-NAME and therefore the ratio proNGF/NGF mirrored the change in NGF levels (Figure 1D).

Incubation of UROs and SMCs with SNP decreased expression of NGF with stable levels of proNGF: In order to get rid of other cellular effects potentially linked to hyperglycemia, UROs and SMCs were incubated for 24-hours with the NO generator SNP (300 μM)[111]. MTT test confirmed that SNP has minor effects on cell integrity (results not shown). Using ELISA, NGF secretion in the medium was found to decrease while proNGF levels remained stable, leading to a decrease in to NGF-to-proNGF ratio (Figure 2A), consistent with what was observed with the bladder cells in a high glucose medium. On the other hand, relative NGF expression levels as measured by RT-qPCR were increased by SNP in the UROs while there was no effect observed in SMCs (Figure 2B). The RNA 18S was used as a control for comparison.

Cyclic GMP levels displayed a different pattern between UROs and SMCs incubated with SNP. NO pathway is commonly associated with cGMP synthesis[112]. After incubation with SNP (300 μM, 24

hours), cGMP levels measured by ELISA was found to be decreased in UROs (Figure 3A) and increased in SMCs (Figure 3B). Subsequently, to confirm the link between SNP, cGMP and NGF, UROs were incubated with the cGMP synthetase inhibitor ODQ (100 μM) and SMCs with stable permeable analogs of cGMP, namely cGMP 8-(4-Chlorophenylthio)-cGMP (CPT-cGMP) (1 mM)[113] or N2,2'-O-Dibutyryl-cGMP (DB-cGMP) (3mM)[114], for 24 hours. ODQ and cGMP analogs mimicked the changes observed in NGF and proNGF secretions in their respective cell types (Figure 3C, D). The levels of proNGF remained unchanged in both cell types in the same samples (supplementary data).

The protease MMP-9 is essential to conduct the effect of SNP on NGF in UROs but not in SMCs. MMP-9 is the major protease that degrades NGF into peptides[96]**.** Intracellular and extracellular MMP-9 levels, as well as its mRNA content were measured after incubation with SNP (300 μM) for 24 hours. A significant increase in intracellular MMP-9, extracellular enzymatic activity and MMP-9 mRNA levels were observed in the SNP-treated UROs (Figure 4A). No changes could be seen in SMCs incubated in the same conditions (Figure 4B). To further understand the role of MMP-9, we carried out the deletion of MMP-9 genomic sequence using Crispr-cas9. Knockout was successful as verified by RT-qPCR and immunoblotting, compared to control cells transfected with the empty plasmid (Figure 5A). Transfected bladder cells were then treated with SNP (300 μM) for 24 hours. Deletion of MMP-9 resulted in a significant increase in NGF levels in UROs and SMCs, as compared to the cells transfected with the empty plasmid, confirming that MMP-9 is the main protease degrading NGF (Figure 5B). Upon treating MMP-9 KO cells with SNP, NGF secretion in UROs remained high, suggesting that the SNP-mediated decrease on NGF is partially dependent on MMP-9 (Figure 5B). On the other hand, SNP in MMP-9 KO SMC prevented NGF secretion, suggesting that MMP-9 is not required to mediate SNP-inhibition of NGF secretion in SMCs. ProNGF levels remained unchanged throughout all conditions for both cell types, consistent with previous results obtained. These changes reflected on the NGF-to-proNGF ratios (Figure 5B).

The activity of protease MMP-7 and plasmin were altered in bladder cells treated with SNP. MMP-7 and plasmin are the main proteases responsible for the conversion of proNGF to NGF[115]. In bladder cells treated with SNP (300 μM) for 24 hours, extracellular levels of MMP-7 did not differ in SNP-treated UROs or SMCs. However, an increase in MMP-7 cell content was observed in SNP-treated UROs, while a decrease occurred in SMCs (Figure 6A). Extracellular plasmin activity levels were unchanged by SNP. Conversely, an increase of intracellular plasmin was observed in UROs only for SNP-treated cells. These results suggest that in UROs, a compensatory mechanism is at work to compensate the decrease in NGF while in SMCs, intracellular decrease in MMP-7 activity could mediate the effect of SNP leading to the decrease in NGF secretion.

4. Discussion and Conclusion:

The mechanism of diabetic voiding dysfunction is still poorly understood. Urine and plasma from patients with type 1 or type 2 diabetes is characterized by high levels of NO[44,116,117]. Likewise, animal models of insulin resistance or diabetes also present decreased NGF and increased urinary levels of NO[74,118]. Hyperinsulinemic patients also present significantly higher plasma NO values compared to healthy controls[119]. With the increasing need to understand these clinical observations at the cellular and molecular levels, the present study assesses the role of nitric oxide in the control of the synthesis and secretion of NGF and its precursor proNGF by cells of the bladder in vitro, in a context of hyperglycemia. Short-term hyperglycemia mimics insulin resistance and increases the release of nitric oxide by cells of the bladder. In peripheral tissues, this results in nonspecific tissue damage and various inflammatory and autoimmune diseases[120]. These culture conditions also correspond to early stages of insulin resistance when increases of nitric oxide appear in the first stage of diabetic cystopathy[121]. Concomitantly, NGF secretion was decreased in both bladder cell types, an effect of hyperglycemia already reported in neurons[122]. The specific action of nitric oxide on NGF in our cells was confirmed using L-NAME[110].

To further understand the role of NO, we looked at the pathways commonly associated to this factor. One of the main cellular pathways activated by NO is cyclic GMP-dependent, specifically by activation of soluble guanyl cyclase that synthesize cGMP from GTP[112]. In turn, cGMP-dependent protein kinases induce smooth muscle relaxation and control gene expression in angiogenesis among others[123]. In most tissues, NO increases cGMP levels and decrease NGF secretion[98] as observed here in SMCs. On the contrary, UROs displayed a decrease in cGMP when treated with SNP. The use of cGMP synthetase inhibitor and analogs confirmed these data and sustained the link between NO, cGMP and decrease in NGF secretion. This is the first time, to our knowledge, that a cell type exhibits a decrease rather than increase in cGMP in response to NO. However, previous publications have shown unique differences in signalling pathways in urothelial cells, for example in cyclic AMP synthesis, upon treatment with the β-

3 adrenergic receptor agonist mirabegron or synthesis of TNF-α [32,124]. Differences between the two cell types may involve specific mechanisms controlling synthesis of cGMP by control of cGMP synthase or modulation of phosphodiesterase type 5 enzymatic activity[125,126]. Some examples include nitric oxide regulation of the cardiovascular system and human detrusor smooth muscle tone[125,126].

Matrix metalloproteinases (MMPs) are the main group of enzymes responsible for the degradation of proteins and their expression is under transcriptional and post-transcriptional control[127]. Most of them are synthesized as inactive precursors and require cleavage to be activated [127]. MMP-7 and MMP-9 are important for the balance of NGF/proNGF. MMP-7 is one of the main proteases generating mature NGF from proNGF intracellularly, while MMP-9 is essential in the extracellular proteolysis of NGF. This last point was confirmed in bladder cells by knocking out MMP-9 in vitro, which resulted in the accumulation of NGF in culture medium. The literature highlights the pathophysiological role of these enzymes and makes them a crucial point of study[70,115].

NO and cGMP are positive or negative regulators of MMP-9 expression depending upon the tissue[128,129]. Increased MMP-9 activity has been associated with diabetic retinopathy and diabetic renal dysfunction[130,131]. Nitric oxide also activates precursor pro-MMP-9 directly and indirectly[132]. The increased levels of NO is thought to cause a disruption in the zinc-thiolate bond in pro-MMP-9, which leads to its activation[132]. On the other hand, the NOS isoform iNOS, which is transcriptionally upregulated in prolonged inflammatory processes, produces large amounts of NO, and consequently, leads to an increased generation of oxidative species such as peroxynitrite and superoxide [133,134]. This does not rule out other possible mechanisms involving suppression of TIMP-1, the main inhibitor of MMP-9, a direct action on proMMP-9 leading to activation of its proteolytic activity or increases in MMP-1 and MMP-13 by NO that cleaves proMMP-9 into its active form[135]. In urothelial cells, we observed an increase in the transcription, intracellular content, and extracellular activity of MMP-9. On the other hand, we observed a compensatory mechanism in the same cells, with increases in NGF transcription and increased MMP-7 and plasmin activity, suggesting an increase in proNGF synthesis

and subsequent proteolysis into mature NGF. Yet, extracellular activity of MMP-9 triggered by SNP must be strong enough to significantly decrease NGF content. We speculate that the intracellular increase in the enzyme MMP-7 and plasmin in UROs can be attributed to a potential compensatory mechanism in order to restore balance in the ratio NGF/proNGF[115]. The observed increase in NGF mRNA levels in UROs supports this claim. Regarding smooth muscle cells, MMP-9 is not involved in the control of the of secretion of NGF by NO. Therefore, the decrease in MMP-7 could be the major mechanism leading to decrease in NGF from SMCs. In accordance, we observed that inhibitors of cyclic GMP do decrease the activity of MMP-7 in SMCs and decrease the ratio NGF/proNGF (unpublished observations).

In conclusion, hyperglycemic environment increases nitric oxide release by bladder cells, which in turn decreases NGF secretion and the NGF-to-proNGF ratio. NGF secretion is under control of cGMP, MMP-7, and MMP-9 with specific pathways in UROs and SMCs. These results go along well with recent clinical data displaying lower concentrations of NGF and elevated levels of NO, MMP-7, and MMP-9 in the urine of patients with OAB, suggesting that urine analysis can provide insight into the pathological process of diseases.

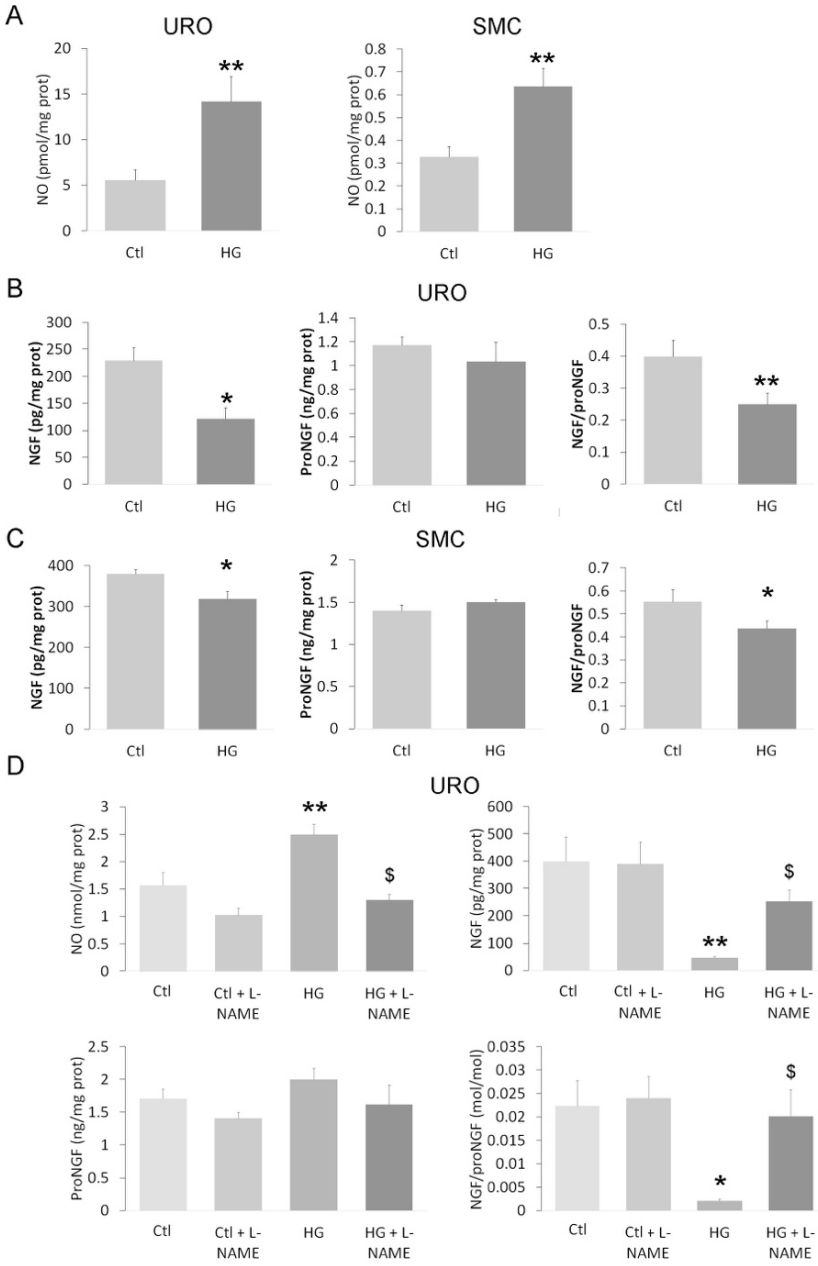

Figure 1. Effects of high glucose on nitric oxide, NGF and proNGF secretion by bladder cells in vitro. (A) Nitric oxide in the medium of urothelial (URO) and smooth muscle (SMC) cells was measured after 24 hours incubation with high glucose (HG) (27 mM for URO and 55 mM for SMC). NGF and proNGF secretion were measured in the same (B) URO and (C) SMC samples. (D) NO, NGF and proNGF were measured after 24 hours in a medium from URO with high glucose with or without L-NAME (1 mM). Student t-test compared to control (n=6), *P<0.05 compared to low glucose, **P<0.01, $P<0.05 compared to high glucose.

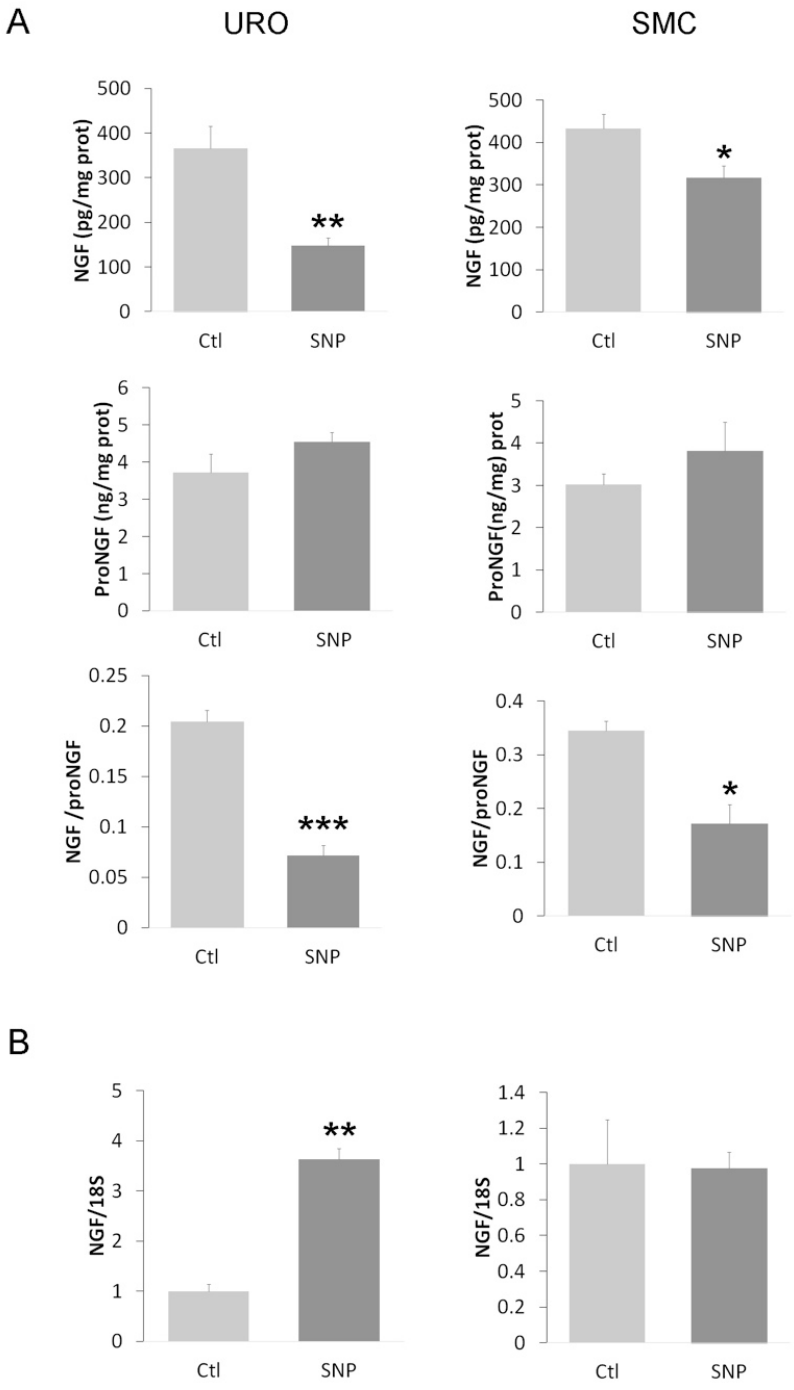

Figure 2. Effect of sodium nitroprusside (SNP) on NGF and proNGF secretion. (A) NGF and proNGF were measured in the medium after 24 hours with SNP (300 µM). (B) Relative expression levels of NGF mRNA were measured by RT-qPCR and reported to RNA 18S. Student t-test compared to control (n=5), *P<0.05, **P<0.01, ***P<0.001.

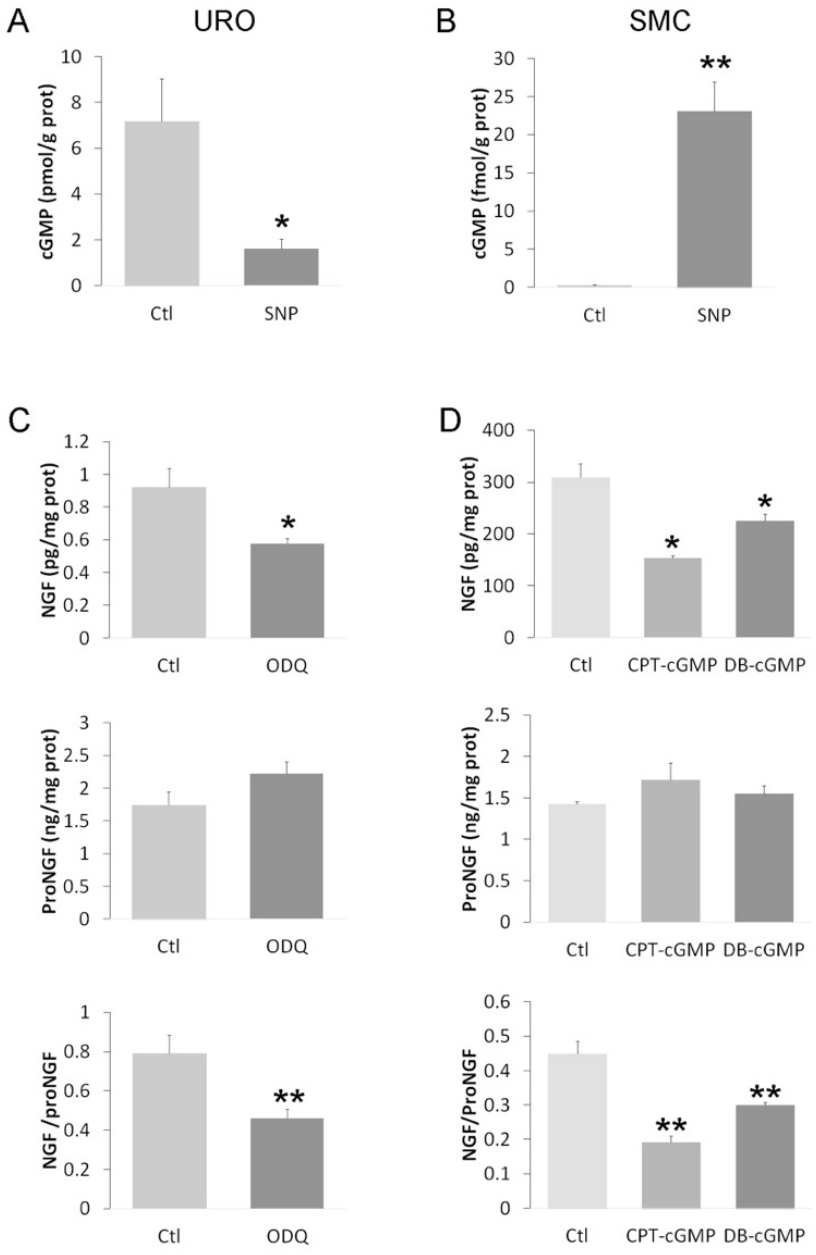

Figure 3. Cyclic GMP involvement in the secretion of NGF and proNGF. Cyclic GMP was measured in urothelial (A) and smooth muscle (B) cell extracts after incubation with SNP (300 µM) for 24 hours. (C) In URO cell medium, NGF and proNGF were evaluated in the presence of the cGMP inhibitor ODQ (100 µM). (D) In SMC medium, NGF and proNGF were measured in cell medium after incubation (24 hours) with the stable analogs 8-(4-Chlorophenylthio)-cGMP (CPT-cGMP) (1 mM) or N2,2'-O-Dibutyryl-cGMP (DB-cGMP) (3mM). Student t-test compared to control (n=4-5), *P<0.05, **P<0.01.

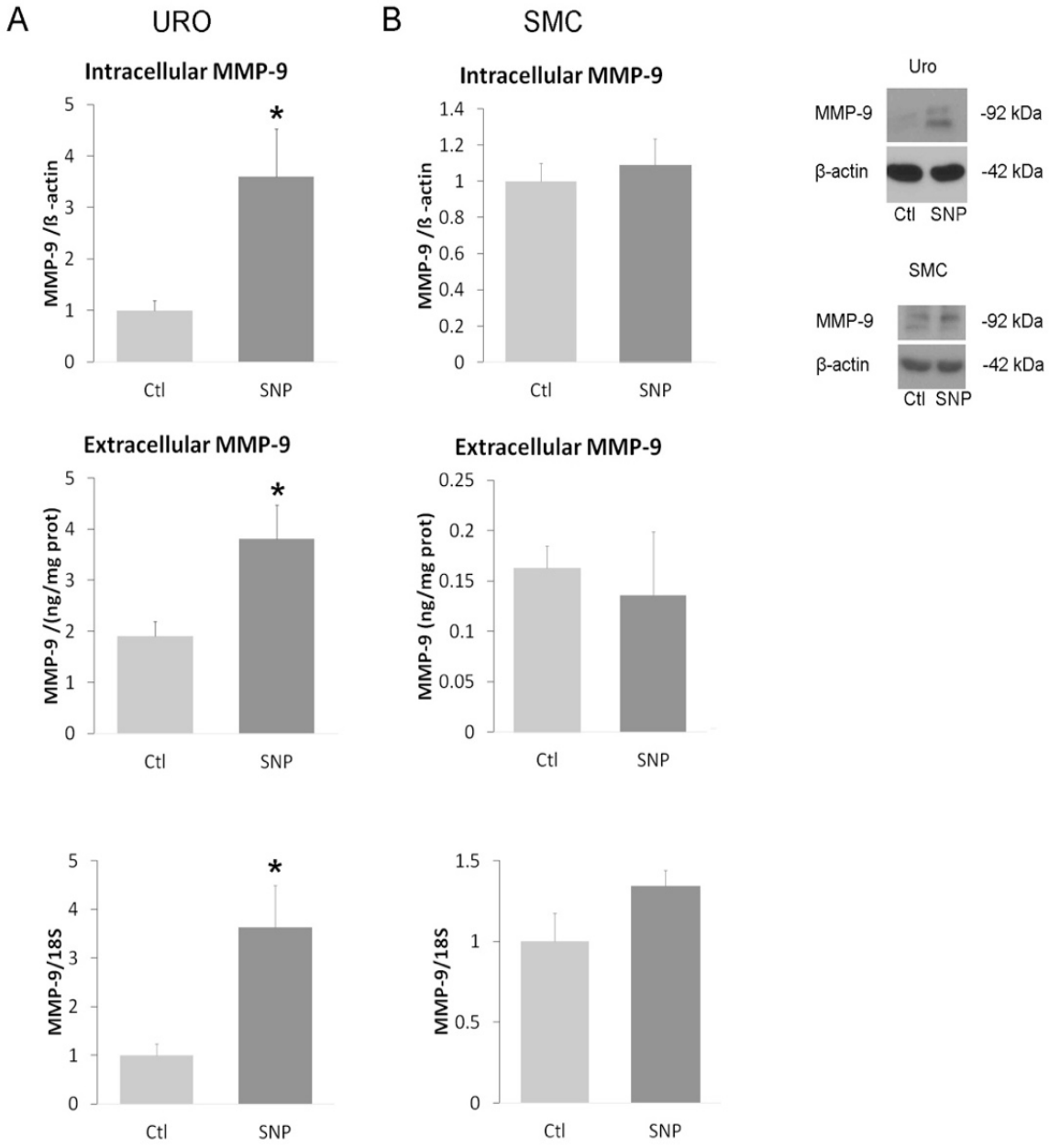

Figure 4. Effect of SNP on metalloproteinase-9 (MMP-9) in vitro. Cells were incubated for 24 hours with SNP (300 μM). In (A) urothelial (URO) and (B) smooth muscle (SMC) cells intracellular MMP-9 protein content, extracellular MMP-9 enzymatic activity and relative expression levels of MMP-9 mRNA were measured (top to bottom). Student t-test compared to control (n=5), *P<0.05.

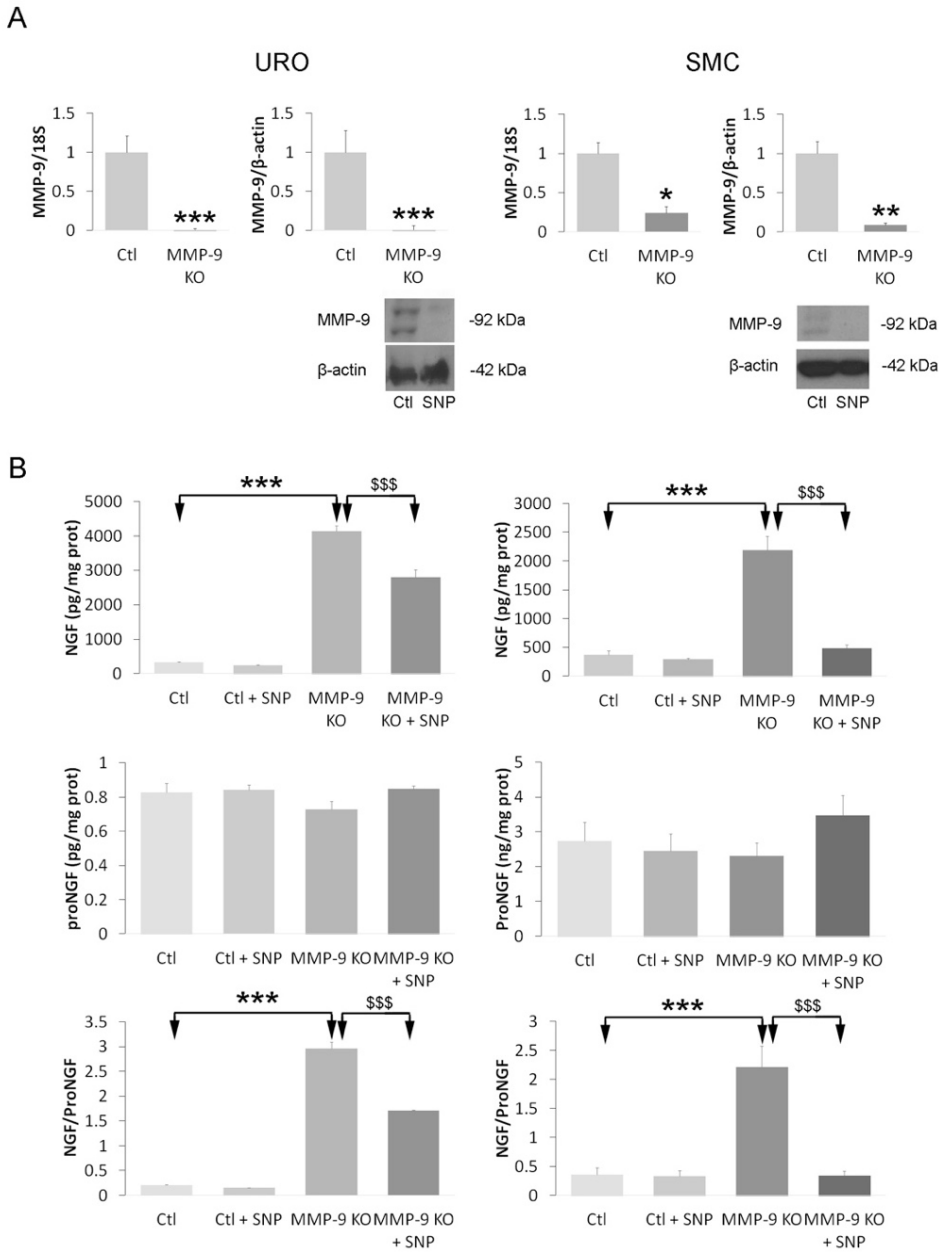

Figure 5. Deletion of MMP-9 by Crispr-cas9 in the effect of SNP on cells. (A) Cells were transfected with an empty plasmid (Ctl) or Crispr-cas9 (MMP-9 KO). MMP-9 expression and synthesis were measured. (B) Levels of extracellular NGF and proNGF were measured after 24 hours with SNP (300 µM) in cells transfected with empty plasmid (Ctl) or with MMP-9 Crispr-cas9 (MMP-9 KO). (A) Student t-test immunoblotting (n=5), *P<0.05, **P<0.01, ***P<0.001. (B) ANOVA two-ways (n=6). ***P<0.001 compared to ctl, $$$P<0.001 compared to MMP-9 KO without SNP.

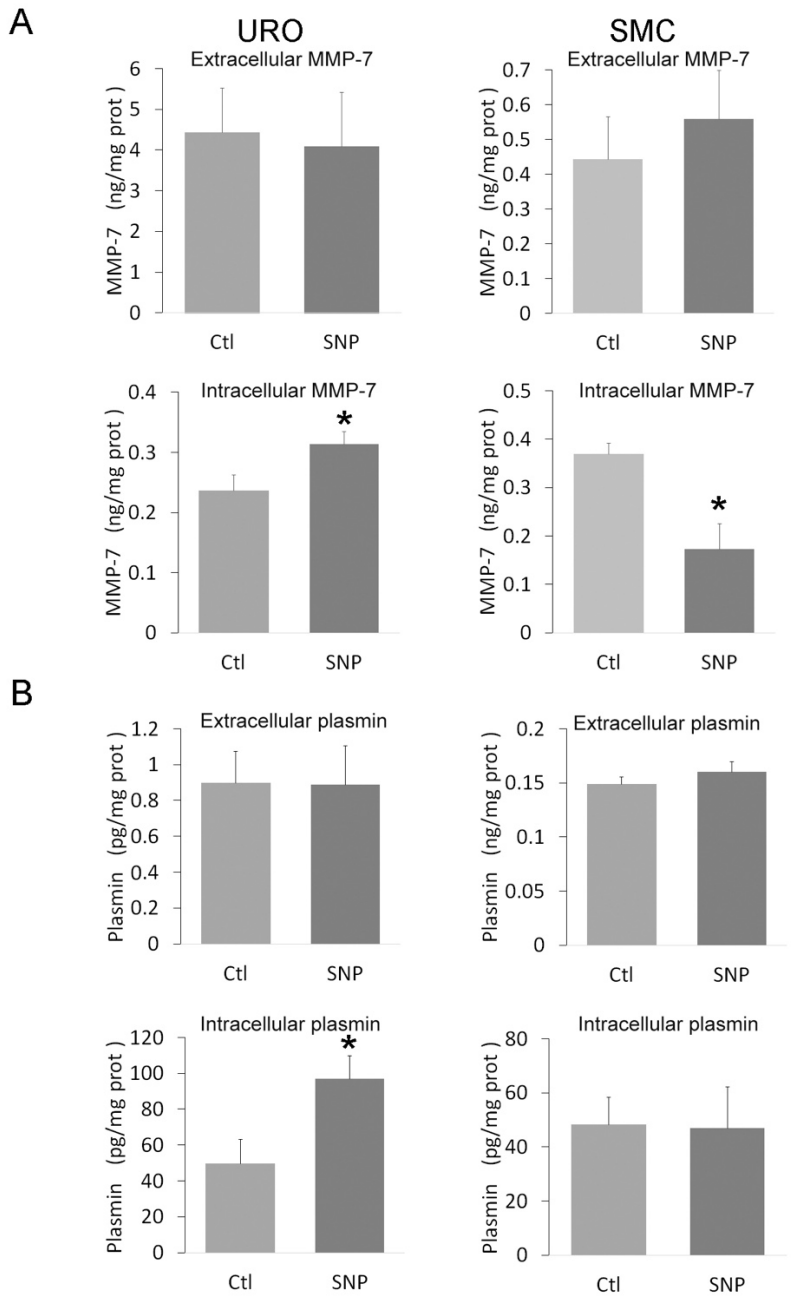

Figure 6. Measurements of Matrix Metalloproteinase 7 (MMP-7) and plasmin enzymatic activity after SNP incubation. Culture medium and cell protein contents were taken after 24 hours incubation with SNP (300 µM). Extracellular and intracellular activity of MMP-7 (A) and plasmin (B) were measured in both cell types. Student t-test compared to controls (n=6), *P<0.05.

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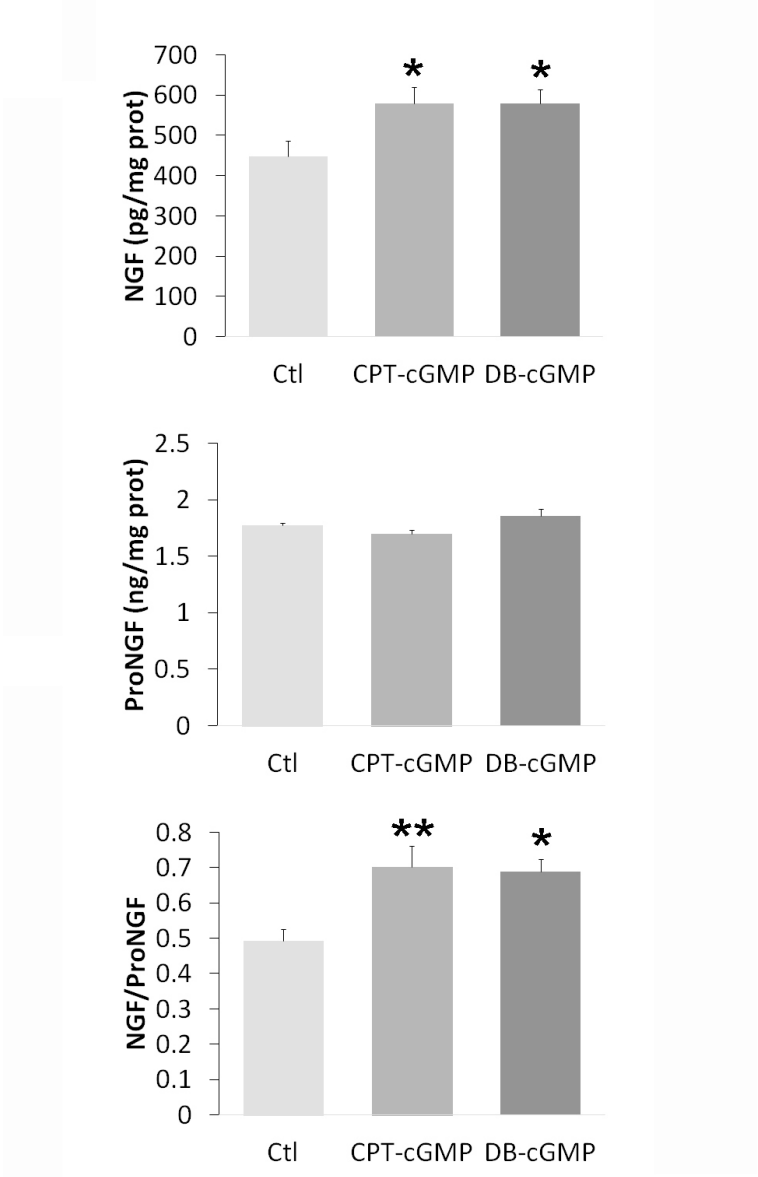
Supplementary Figures



Supplementary Figure 1: In URO medium, NGF and proNGF were measured in cell medium after incubation (24 hours) with the stable analogs 8-(4-Chlorophenylthio)-cGMP (CPT-cGMP) (1 mM) or N2,2'-O-Dibutyryl-cGMP (DB-cGMP) (3mM). Student t-test compared to control (n=4-5), *P<0.05, **P<0.01.

Bridging Manuscript

The overall objective of this body of work is to have a greater understanding of diabetic voiding dysfunction (DVD), particularly in patients exhibiting type 2 diabetes mellitus (T2DM). Complex, multifactorial diseases such as DVD require an appreciation of the different layers to develop potential therapies, through different avenues such as novel biomarkers. It is known that DVD results not only from elevated glycemia, but polyuria with local oxidative stress and inflammation as well, among other factors. Nitric oxide, a key regulator in the signalling pathways of inflammation has been thought to also play a role in the development of the disease.

The previous chapter highlights the role for hyperglycemia and nitric oxide synthesis and secretion and allows for an enhanced knowledge on the mechanism for diabetic voiding dysfunction. The findings displayed in *Chapter 4: Synthesis and secretion of NGF is regulated by Nitric Oxide through cyclic GMP and metalloproteinases in bladder cells in vitro* specifically link high glucose levels with increased nitric oxide release, leading to a decrease in the NGF/proNGF ratio in bladder cells *in vitro*. These changes are linked to changes in matrix-metalloproteinase enzymatic activity, another factor commonly associated with DVD. Evaluating therapeutic effects in a pre-clinical model is the next step in drug development, once a more in-depth understanding of a disease is achieved. As such, *Chapter 5: Bladder characteristics in a murine model with Type 2 Diabetic Voiding Dysfunction following a 4-week treatment with p75^{NTR} antagonist THX-B* aims to validate the inhibitory effect of THX-B, a p75^{NTR} receptor antagonist molecule, verifying its ability to stabilize imbalanced NGF and proNGF levels, allowing for normal bladder function. The upcoming chapter begins with the characterization of bladder changes in a novel T2DM murine model. To our knowledge, the Tally Ho mice have not been assessed in this regard. This model will be appropriate for the second part of the chapter, in which the proNGF/p75^{NTR} interaction will be evaluated as a potential therapeutic target. Upon treatment of mice, we will correlate bladder function from voiding spot assays, cystometric parameters and contractility analysis in relation to proNGF/p75^{NTR} activity in type 2 DVD.

Chapter 5: Bladder characteristics in a murine model with Type 2 Diabetic Voiding Dysfunction following a 4-week treatment with THX-B

This chapter includes work recently submitted to the Journal of Diabetes Research

Here we investigated the effect of diabetic voiding dysfunction on a novel murine model for type 2 diabetes, as well as validate $p75^{NTR}$ antagonism as a potential therapeutic avenue.

Contribution of authors: The conception of the study was conducted by Dr. Lysanne Campeau, Dr. Philippe Cammisotto, Ms. Aya Hajj, Ms. Aalya Hammouda and myself. I, with the help of Ms. Hajj and Ms. Hammouda completed all in vivo experiments, including acquiring physiological data from the mice, conducting voiding spot assays, cystometry measurements, and organ baths. The interpretation and statistical analysis of the acquired results of this study was conducted by myself, with the guidance of Dr. Philippe Cammisotto. The creation of the manuscript was done entirely by myself.

Bladder characteristics in a murine model with Type 2 Diabetic Voiding Dysfunction following a 4-week treatment with $p75^{NTR}$ antagonist THX-B

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The authors have declared that no conflict of interest exist.

Abstract

Around 80% of diabetic patients suffer from bladder voiding dysfunction, yet therapeutic solutions are scarce. To provide an in-depth understanding of the pathology, we characterized the Tally Ho mouse, a novel, polygenic model for type 2 diabetes, to determine how the disease affects the structure and function of the bladder. Diabetic Tally Ho and genetic control SWR/J mice, aged 10 or 14 weeks, received weekly systemic injections of $p75^{NTR}$ antagonist molecule (THX-B) for 4 weeks. Body weight, glycemic index, and changes in voiding patterns (via voiding spot assay) were recorded at baseline and prior to each weekly injection. Bladder contractility parameters were measured with conscious cystometry and organ bath. We observed that voiding parameters (total volume, voiding frequency and volume per micturition) significantly increased in male Tally Ho mice starting at 14 weeks of age, implying that the diabetes-induced bladder changes occur within this time frame. Organ bath studies revealed a decreased stimulus response to KCl, to electrical field stimulation (EFS) and to high concentrations of carbachol (30μM and 100μM). Treatment of 14-week-old mice with THX-B for a 4-week period yielded neither changes in body weight, glycemic index, or bladder weight, nor in bladder contractile properties, as compared to PBS-treated controls. Improvements were seen when treatment began at 10 weeks of age. Cystometric parameters in males included decreases in intermicturition pressure, basal pressure, and spontaneous activity, while intercontraction interval, bladder capacity, bladder compliance and micturition volume increased. In females, decreases were found in threshold pressure, spontaneous activity, intercontraction interval, micturition volume, residual volume, and bladder capacity. Contractile responses to KCl and carbachol decreased in both sexes, however a difference in electrical field stimulation was only observed in females. This study establishes the Tally Ho mice as a new model to study the bladder in the context of insulin resistance and diabetes. Antagonism of $p75^{NTR}$ by THX-B improved contractile parameters and stimulus response, leading the way for a potential therapeutic treatment in early stages of DVD.

1. Introduction

Diabetic voiding dysfunction (DVD) is among the many complications associated with diabetes and occurs in about 80% of patients[136]. The compilation of symptoms categorized as DVD include urinary incontinence, increased urinary frequency and urgency, impaired bladder contractility, as well as bladder overactivity[26,27,136]. The current literature regarding the changes in bladder structure and function in patients with DVD is limited. The underlying mechanisms of the disease are still poorly understood and there are little-to-no treatment options for patients. It has been shown that there is an imbalance between nerve growth factor (NGF) and its precursor proNGF in the urine of patients with diabetes, which is thought to be the cause of the tissue and neural degeneration of the bladder in DVD[70]. Recent evidence has also shown implication of these neurotrophins in other diabetic complications such as retinopathies and angiopathies[74]. The diabetic oxidative stress conditions are thought to be the cause of lower mature NGF expression, as a result of low proteolysis of proNGF or high degradation of NGF[76,137-139]. A recent study by Mossa *et al*. evaluated the effectiveness of $p75^{NTR}$ receptor antagonism by small molecule THX-B in a type 1 diabetic murine model of DVD[74]. With the target validation of the $p75^{NTR}$/proNGF axis in type 1 DVD, there remains a need to also corroborate the role of the antagonist molecule THX-B in type 2 DVD. Type 2 diabetes mellitus (T2DM) remains the most common of all diabetes subtypes, accounting for over 90% of cases[11]. With the prevalence of T2DM increasing each year, as well as the need to validate this therapeutic avenue in type 2 DVD, the model chosen for this study was crucial. In the development of T2DM, there are various pathologic pathways, and a multitude of genes are said to be involved[140,141]. The Tally Ho (TH) mouse is a novel, polygenic mouse model for type 2 diabetes, hyperinsulinemia and obesity. Although both sexes show signs of moderate obesity, the type 2 DM phenotype is limited to males, though the females do develop insulin resistance. These mice have normal leptin and leptin receptor expression, which allows for their condition to be less extreme than the traditionally used streptozotocin (*STZ*) mice[142,143]. The impact of insulin resistance and diabetes has not

yet been evaluated in the context of bladder pathophysiology in the TH mice. As such, it remains a topic that requires further examination prior to evaluating potential treatment options. A better understanding of the molecular pathogenesis, along with metabolic and bladder characteristics in TH mice will allow for more insight on DVD, in turn bridging the knowledge gap in the literature and allowing for further pharmacological studies. Evaluating $p75^{NTR}$ antagonism *in vivo* offers the possibility for translational research and can be clinically valuable moving forward. In the present study, we aim to characterize the changes occurring in the bladder of the type 2 diabetic mouse model, the TH. We also seek to validate the proNGF/$p75^{NTR}$ interaction as a therapeutic target in the pre-clinical model, as well as evaluate the potential therapeutic benefits in bladder functionality, cystometric parameters and contractility stimulus responses following treatment.

2. Materials and Methods

2.1 Animal housing and treatment

All experiments received the ethical approval from the Lady Davis Institute Ethics Board (No. 7859), Montreal, Canada. Housing and handling conformed with the Canadian Council for Animal Care (CCAC). Male and female Tally Ho (aged 10, 14, and 18 weeks) and SWR/J (aged 14 weeks) mice were kept with free access to food (standard Purina chow, Teklad Global, WI, USA) and water, on a 12h light/dark cycle. Fasting glucose serum (FSG) was verified at 8 weeks of age with glucometer (Contour next EZ, Bayer, ON, Canada) to confirm diabetic and insulin resistant conditions. FSG over 14 mmol/L was considered diabetic and insulin resistant respectively. For bladder characterization, three age groups (10, 14 and 18 week) of both male and female Tally Ho mice were used. For treatment, each strain of mice was divided into 4 groups: diabetic male control with PBS treatment, diabetic male with small molecule p75NTR antagonist (THX-B)-treatment, insulin resistant female control with PBS treatment, insulin resistant female small molecule $p75^{NTR}$ antagonist (THX-B)-treatment. The small molecule THX-B ((1,3-diisopropyl-1-[2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-acetyl]-urea); MW 540 Da) is a synthetic chemical with an estimated systemic circulation half-life in vivo of 6–8 h. It is a pure antagonist that inhibits proNGF from binding the receptor $p75^{NTR}$ and its ligand-independent signaling. Dose given was 50 μg/mouse under 100 μL in sterile PBS, as per previous studies. Mice were given i.p. injections of THX-B or the vehicle (PBS) weekly (for 4 weeks) starting at 10 and 14 of age, without blinding. Experimental time points (to carry out cystometry, contractility, protein extraction and histology) were baseline (10, 14, or 18 weeks of age), week 2 after treatment (following two injection cycles) and week 4 after treatment (following four injection cycles). Body weight and FSG were measured weekly for all groups.

2.2 Voiding Spot Assays

Voiding Spot Assays (VSA) were conducted by fitting cages with chromatography paper (grade 3mm CHR, Whatman, GE Healthcare, UK). Mice were placed in the cages with access to food, without water, for 4 hours. Voiding patterns were assessed by imaging urine spots on the chromatography paper using ultraviolet light (SynGene Bioimaging system, USA) through the GeneSnap software (Version 7.02, USA) and analysed 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,)[144]

2.3 Bladder catheter insertion

One week following the last injection, mice were injected with subcutaneous slow-release buprenorphine (1.0 mg/kg) and anaesthetised under 2.5% isoflurane. The bladder was exposed by a lower abdominal midline incision. The dome was punctured and a polyethylene PE10, 0.28/0.61 mm catheter (Stoelting Wood Dale, IL, USA) was inserted. The catheter was fixed using a suture and passed subcutaneously out through a small incision made at the base on the neck and sealed thermally.

2.4 Conscious cystometry

At 24 hours after catheter insertion (as previously described)[145], conscious cystometry was performed in metabolic cages. The end of the catheter exposed at the base of the beck was unsealed and connected to a pressure transducer (Grass Technologies, USA) and a saline solution (154 mmol/L NaCl) was injected into the bladder by an infusion pump at the rate of 1.5 mL/hour (for a 1-hour duration). The parameters recorded were basal pressure, maximal pressure, threshold pressure, intermicturition pressure and inter-contraction interval. The micturition volume of saline and urine voided was measured in a container connected to a force displacement transducer. Using these measured parameters, spontaneous activity,

bladder capacity and bladder compliance were calculated [146]. Data was analyzed using LabScribe2 Data Recording and Analysis Software (iWorx, Dover NH, USA). Mice were euthanized by exsanguination under 3% isoflurane.

2.5 Organ bath studies

Following conscious cystometry measurements, mouse bladders were removed under anaesthesia, once they were euthanized by exsanguination. Bladders were emptied, weighed, and placed in ice-cold Krebs-Ringer solution. The bladder dome and base were removed and intact longitudinal strips of urothelium (2 x 5 mmm) were pinned in the wells of a Tissue Bath System – 720MO (DMT-USA, Ann Arbor, MI, USA) filled with 6 mL of Krebs-Ringer solution (pH 7.4, 37°C, under 95% O_2/5% CO_2 bubbling). Tension of strips was kept at 0.5g for 1 hour, and Krebs solution in wells was replaced every 15 minutes. Strips were then stimulated twice with KCl (60 mM), electrical field stimulation (EFS at 1, 2, 4, 8, 16, 32 Hz) using a Grass Technologies S88 Stimulator (West Warwick, RI, USA) and carbachol (3nM to 100 μM final). Wells were washed between each stimulation and the tissue strips to rest for 10 minutes. Obtained values were normalized with respective strip weight and analyzed using LabChart 7 (ADInstruments, CO, USA)

2.6 Statistical Analysis

The data was presented as the mean and standard error of the mean (SEM) for all results. Comparison between groups was done with an independent student t-test and two-way ANOVA (post hoc Tukey HSD test). Statistical significance was presented as a p value with $p<0.05$, $p<0.01$ and $p<0.001$, as described in the legends. The analysis of the statistics was conducted using both Excel and GraphPad softwares.

3. Results

Voiding Spot Assay (VSA) and Organ Bath (OB) studies revealed significant changes in voiding patterns and stimulus response in male and female Tally Ho mice starting at 14 weeks of age. Male and female 10-, 14-, 18-week-old Tally Ho mice were placed on filter paper for a 4-hour VSA, and spots were subsequently revealed by UV light. Void spot area and frequency were assessed and revealed a significant increase in total urine volume in 14- and 18-week-old male mice (Figure 1A), a significant increase in voiding frequency in 14- and 18-week-old male mice (Figure 1B) and a significant increase in average volume per micturition in 14-week-old male mice only (Figure 1C). OB studies showed that the KCl (60 mM) stimulus response significantly decreased in male and female Tally Ho mice at 14- and 18- weeks of age (Figure 2A). Electrical field stimulation significantly decreased at all frequencies (1, 2, 4, 8, 16, 32 Hz) in males only, starting at 14 weeks (Figure 2B). Carbachol stimulus response decreased at 30μM and 100μM in 14-week-old males only (Figure 2C, D, E).

Treatment with p75^{NTR} antagonist for 4 weeks showed improvement in voiding patterns in male Tally Ho mice only when starting injections at 10 weeks of age. Weekly 4-hour VSAs were conducted on male and female SWR (injections starting at 14 weeks) and Tally Ho (2 groups: injections starting at 14 and 10 weeks). Male SWR and 14 week-Tally Hos showed no changes in any voiding patterns observed. When starting injections at 10 weeks, Tally Ho males showed an increase in total urine volume (Figure 3A), a decrease in voiding frequency (Figure 3B) and an overall increase in volume per micturition (Figure 3C). Changes were evident on last measurement, one week after final injection. All 3 female groups showed no changes in voiding patterns (Figures 4A, B, C).

No differences were observed in body weight, glycemia or bladder weight throughout 4-week treatment with THX-B. We recorded weekly body weight and glycemia changes in all 3 groups. Body

weight did not significantly change in male (Figure 5A) or female (Figure 5D) groups of SWR, Tally Ho (14 week) or Tally Ho (10 week) when treated with THX-B antagonist for 4 weeks, compared to PBS-control groups. Fasting serum glucose measured weekly showed no changes in male (Figure 5B) and female (Figure 5E) THX-B treatment groups, compared to PBS treatment groups. Bladders were retrieved, emptied, and weighed following mouse sacrifice. No changes were observed in weight bladders of male and female THX-B treated SWR, Tally Ho (14-week) and Tally Ho (10-week) as compared to PBS-control groups (Figure 5C, F).

Most bladder parameters measured by conscious cystometry showed changes in male and female Tally Ho mice when 4-week treatment began at 10 weeks of age. When compared to PBS-controls, treatment with $p75^{NTR}$ antagonist THX-B led to significant decrease in intermicturition pressure, spontaneous activity, basal pressure (Figures 6C, D, G) in male Tally Ho when treatment started at 10 weeks. Significant increases were observed in bladder compliance (Figure 6E), intercontraction interval (Figure 6F), micturition volume (Figure 6H) and bladder capacity (Figure 6J) in the same group. Maximal pressure (Figure 6A), threshold pressure (Figure 6B), residual volume (Figure 6I) did not improve following 4-week THX-B treatment in male Tally Hos. In female mice, decreases were observed in threshold pressure (Figure 7B), spontaneous activity (Figure 7D), intercontraction interval (7F), micturition volume (7H), residual volume (7I), and bladder capacity (7J) in Tally Hos, when treatment began at 10 weeks of age. No differences were observed between groups for maximal pressure (Figure 7A), intermicturition pressure (Figure 7C), bladder compliance (Figure 7E), and basal pressure (Figure 7G).

Changes in all parameters measured by organ bath were observed in female Tally Ho mice, while males showed changes in KCl and carbachol response only, when treatment started at 10 weeks. Stimulus by KCl (30mM), EFS (1, 2, 4, 8, 16, and 32 Hz) and Carbachol (3nM to 100 μM) showed no changes in male SWR (Figure 8A) and Tally Hos when treatment began at 14 weeks of age (Figure 8B). When 4-week treatment began at 10 weeks of age, male Tally Hos showed decrease in KCl response, as well as carbachol response at 30μM and 100μM (Figure 8C). In females, no changes were observed in SWR (Figure 9A) or Tally Ho mice with treatment starting at 14 weeks (Figure 9B) for KCl, EFS and Carbachol stimulus. When treatment began at 10 weeks, we observed a decrease in response for KCl, EFS (at 16 Hz and 32 Hz) and carbachol (10μM, 30μM, 100μM) Tally Ho females as compared to PBS controls (Figure 9C).

4. Discussion and Conclusions

The key in early stages of pharmacological advancements in disease therapy is the selection of an appropriate model of the disease. The Tally Ho (TH) mouse is a recently discovered model for type 2 diabetes mellitus (T2DM). This polygenic model mimics human T2DM as multiple genes are involved, and different pathways lead to development of the pathology[142]. The expression of hyperglycemia is sex-specific in TH mice, where males become diabetic, and females remain insulin resistant. The timing of changes in metabolic characteristics is debated, however, more recent studies show that complete diabetic status is seen at 14 weeks, with impaired glucose tolerance starting as early as 8 weeks[142]. The traditionally used *STZ* mouse model for diabetes (Type 1) studies are not considered an ideal translational model, due to the cytotoxicity of streptozotocin[147]. Alternatively, the Tally Ho mouse model is already well viewed as a model for study, due to its similarities with humans in genetic characteristics and mode of inheritance and development of type 2 diabetes, but translational work still needs to be explored. It is well documented in the literature that prolonged exposure to diabetic and insulin resistant conditions result in changes in the bladder, leading to complications such as diabetic voiding dysfunction (DVD)[148,149]. The Tally Ho mouse is a potential model for studies on DVD, however to our knowledge, there have been no studies conducted on diabetes-associated bladder alterations and complications. Voiding patterns were evaluated in both male and female TH at different ages. Overall urine volume and voiding frequency drastically increased at 14 weeks of age. These changes led to an overall increase in volume per micturition. These results are consistent with the observation that bladder capacity and volume, as well as frequency from increased muscarinic-mediated bladder contractions as seen in diabetic rodents[149,150]. A study by Xiao *et al*. also revealed a decrease in bladder compliance, along with decreased intercontraction intervals of voiding, which also supports our observed results[151]. Bladder overdistention, resulting from bladder retention and polyuria may also explain the changes in bladder morphology[22]. Contractility parameters evaluated by cystometry (results not shown) were inconclusive,

potentially due to bladder injury occurring during catheter insertion surgery and/or inexperience. Organ bath studies evaluate non-specific stimulations through KCl and electrical field stimulation (EFS) and muscarinic receptor specific stimulations by carbachol. Diabetic voiding dysfunction can cause multifactorial alterations in bladder function, specifically pertaining to urothelial and detrusor physiology, as well as nerve innervation and reflex mechanisms[33]. As such, the decreased receptor specific and non-specific stimulus responses starting at 14 weeks of age in males, implies reduced contractillty in the bladder. The reduced responsiveness is consistent with the later, decompensated stage of diabetic voiding dysfunction, where there is remodelling of the bladder, leading to a reduced afferent nerve threshold. Urothelial signaling properties are altered, and this compromises cell signaling within the bladder wall, leading to changes in bladder sensation through nerve ending modifications[8,31,33].

In DVD pathologic conditions, it is well established that the NGF/proNGF ratio decreases, leading to debilitating symptoms within the bladder, caused by degeneration of neurons and fibers of the peripheral nervous system[63]. The correction of the dysregulation of neurotrophins has been evaluated through $p75^{NTR}$ antagonism in type 1 DVD and showed promising results[74]. The 4-week treatment of the T2DM TH mouse model with $p75^{NTR}$ antagonist THX-B did not alter body weight or glycemia, regardless of the age at which treatment began. This is consistent with results obtained in type 1 DVD mice[74]. Diabetes-induced bladder alterations as well as nerve-response dysregulation is believed to result from the prolonged exposure to hyperglycemic conditions[152]. The lack of improvements observed in Tally Ho mice when injections started at 14 weeks of age may be due to the irreversible changes in bladder morphology from longer hyperglycemia exposure. These conditions may warrant a higher dose of THX-B treatment, though this point is left to be examined further. With regards to voiding patterns, changes were observed with increased total urine volume, decreased voiding frequency, and increased volume per micturition only after the last of 4 injections was administered, in male Tally Hos only (when

injections started at 10 weeks). In DVD conditions, the detrusor muscle is hyper-contractile, particularly in early stages of the disease[21]. Treatment with THX-B may have improved neurogenic control of the detrusor smooth muscle, leading to improved emptying efficiency. There is also less spontaneous activity, which is potentially why a decreased voiding frequency is observed. THX-B had no effect on female Tally Ho voiding parameters. This may be due to the differences in neurotrophin dysregulation in insulin resistant conditions, as compared to type 2 diabetic conditions. Although both conditions show a decrease in the NGF/proNGF ratio, it is perhaps the baseline differences in neurogenic control within the bladder wall along with time-dependent symptom development that yields different results.

Bladder contractility parameters as measured by cystometry in males and females when treatment began at 10 weeks of age. In male Tally Hos, basal and intermicturition pressure, along with spontaneous activity decreased with THX-B treatment, as compared to PBS controls. Spontaneous activity increase, associated with increased bladder contractions and overactive bladder phenotype has been well highlighted in rats with spinal cord injury, emphasizing the role of altered detrusor properties and an increased myogenic response[153]. Antagonizing $p75^{NTR}$ receptor may restore NGF/proNGF balance, and subsequently correct hypercontractility of the detrusor muscle, as observed in the initial stages of type 2 DVD[21]. Intercontraction intervals and micturition volumes increased, which was consistent with VSA results, where voiding volume and volume per micturition increased, while frequency decreased following treatment. These results show improved bladder neural control through increased urinary retention and better emptying efficiency. Bladder compliance and capacity both significantly increased. We were surprised by these results, as we expected a decrease in both parameters, since $p75^{NTR}$ antagonism has corrected bladder hypertrophy and thus decreased compliance and capacity in type 1 DVD female mice[74]. We believe these changes were due to the improvement in neurogenic control of the bladder as NGF/proNGF ratios were corrected, improving detrusor overactivity, and thus mice were able

to retain their urine for a longer period. Correction detrusor overactivity through botulinum toxin has previously been linked to increase in bladder compliance and capacity, which corroborates this hypothesis[154]. In female THs, threshold pressure decreased, again correcting the hypercontractility of the detrusor muscle in the bladder wall. Spontaneous activity decreased, as it did in males, again displaying improved neurogenic control, with less spontaneous contractions from the bladder wall. Residual volume and bladder capacity both decreased as well. THX-B was able to improve voiding efficiency with a more complete emptying of the bladder, as well as correct the bladder hypertrophy that has been previously linked to increased glycemia[74,155]. Intercontraction intervals decreased in females, though when taken collectively with other cystometric parameters, this came to a surprise, as we expected an increase. A potential cause could be, with the decrease in bladder capacity, the females will need to empty their bladders more often. It will be necessary to further examine the total water consumption, as well as changes kidney function in the female insulin resistant Tally Hos to gain further insight on this point. When starting injections at 10 weeks, both males and females displayed decreased KCl stimulus response, while only females had decreased EFS response. These stimulations are both non-receptor specific, and so treatment normalized the overall contractile response to stimulus. Carbachol stimulations, specific to muscarinic receptors decreased starting at higher concentrations, implying there may be a certain threshold before the treatment has effect on the contractile parameters. The muscarinic receptors modulate bladder contractions in the voiding phase[8,156]. As their contractility response decreases with $p75^{NTR}$ antagonist treatment, it shows correction in hyperresponsiveness, and overactivity exhibited in the early, compensated stage of DVD.

The improvements we observed in voiding patterns and contractility parameters were sex-specific, owing not only to biological differences, but also differences in glycemia levels and duration. So, although our result show promise, particularly in correcting hypercontractility in early stages of diabetic voiding

dysfunction, it remains difficult to pinpoint systemic effects of our treatment, due to the complexity of the disease that is DVD.

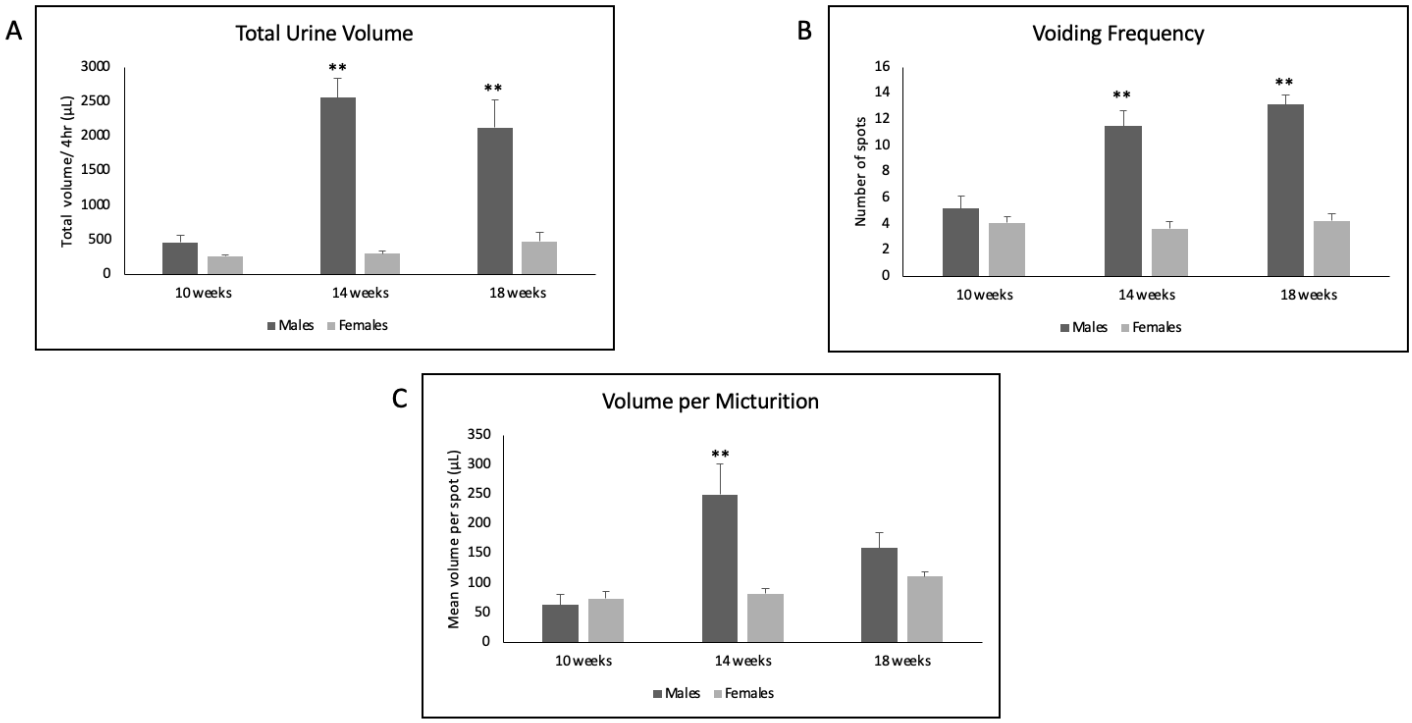

Figure 1: Voiding spot assay (VSA) parameters measured over 4-hour period (N=5-11): Comparison of (A) average total urine volume, (B) voiding frequency, and (C) volume per micturition between male and female 10-, 14- and 18- week age groups of Tally Ho mice. ANOVA one-way, **P<0.01, compared to 10-week mice.

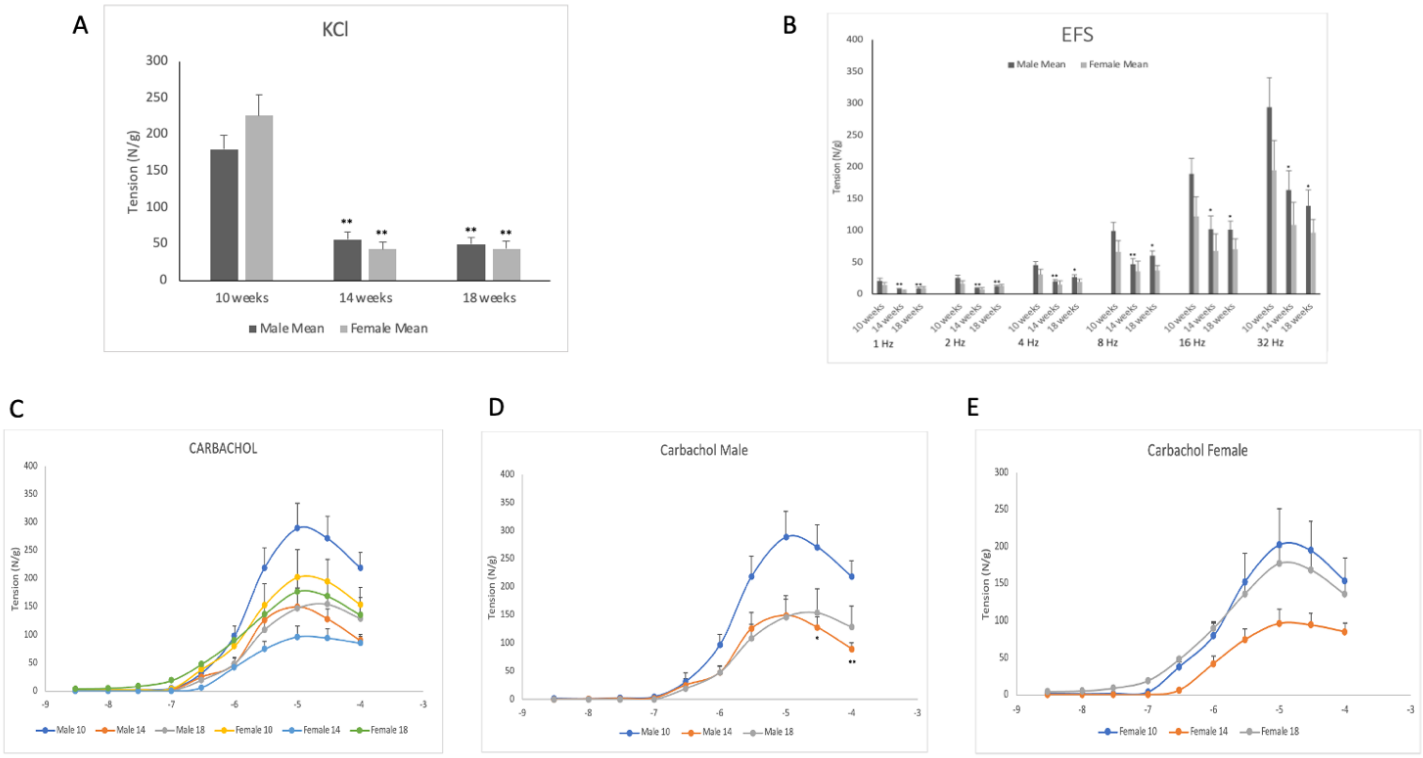

Figure 2. Organ Bath Studies: Tally Ho mouse bladder strips (N=5-8) A) incubated with KCl (30 mM) B) electrically stimulated at various frequencies C, D, E) incubated with Carbachol at various concentrations. Bars represent mean ± SEM. ANOVA one-way. *p<0.05, **P<0.01, compared to 10-week mice.

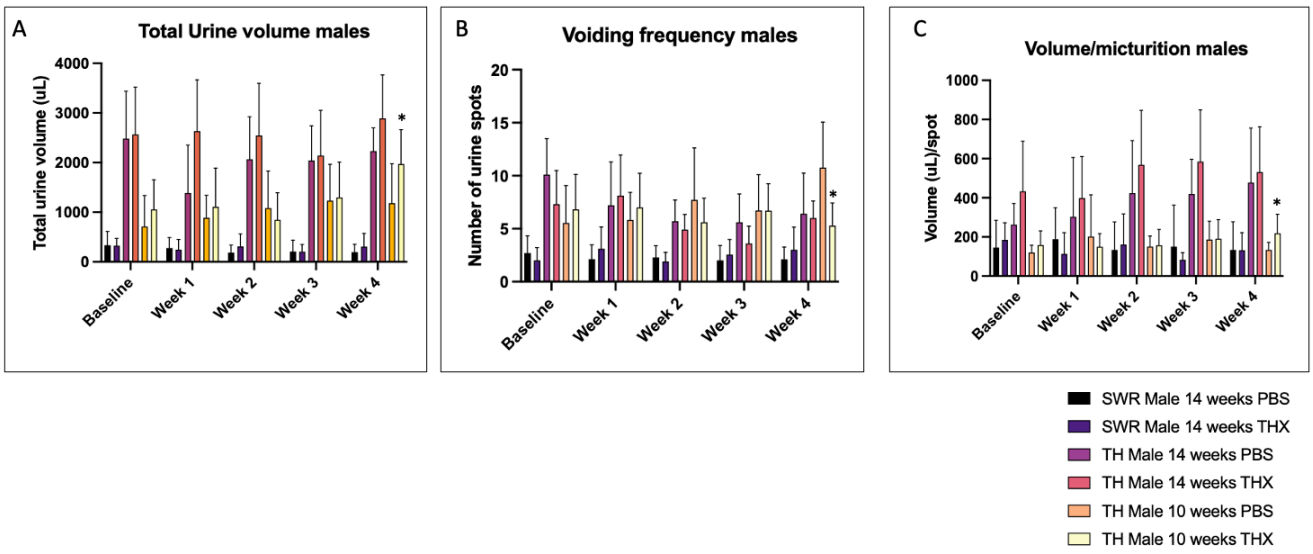

Figure 3. Voiding spot assay (VSA) over 4-hour period (N=9-11): Comparison of (A) average total urine volume, (B) voiding frequency, and (C) volume per micturition between male SWR (14 wk), Tally Ho (14 wk) and Tally Ho (10 wk) after 4-week THX-B treatment. Student t-test, *p<0.05, compared to PBS control.

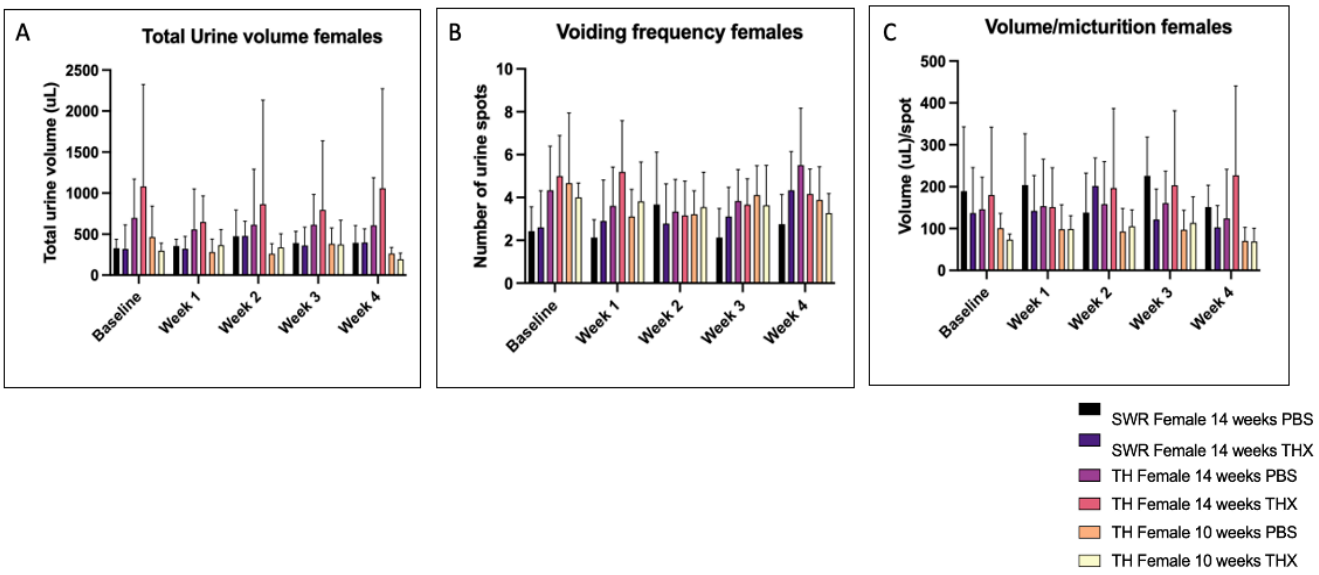

Figure 4. Voiding spot assay (VSA) over 4-hour period (N=6-11): Comparison of (A) average total urine volume, (B) voiding frequency, and (C) volume per micturition in female SWR (14 wk), Tally Ho (14 wk) and Tally Ho (10 wk) after 4-week THX-B treatment. Student t-test, *p<0.05, compared to PBS control.

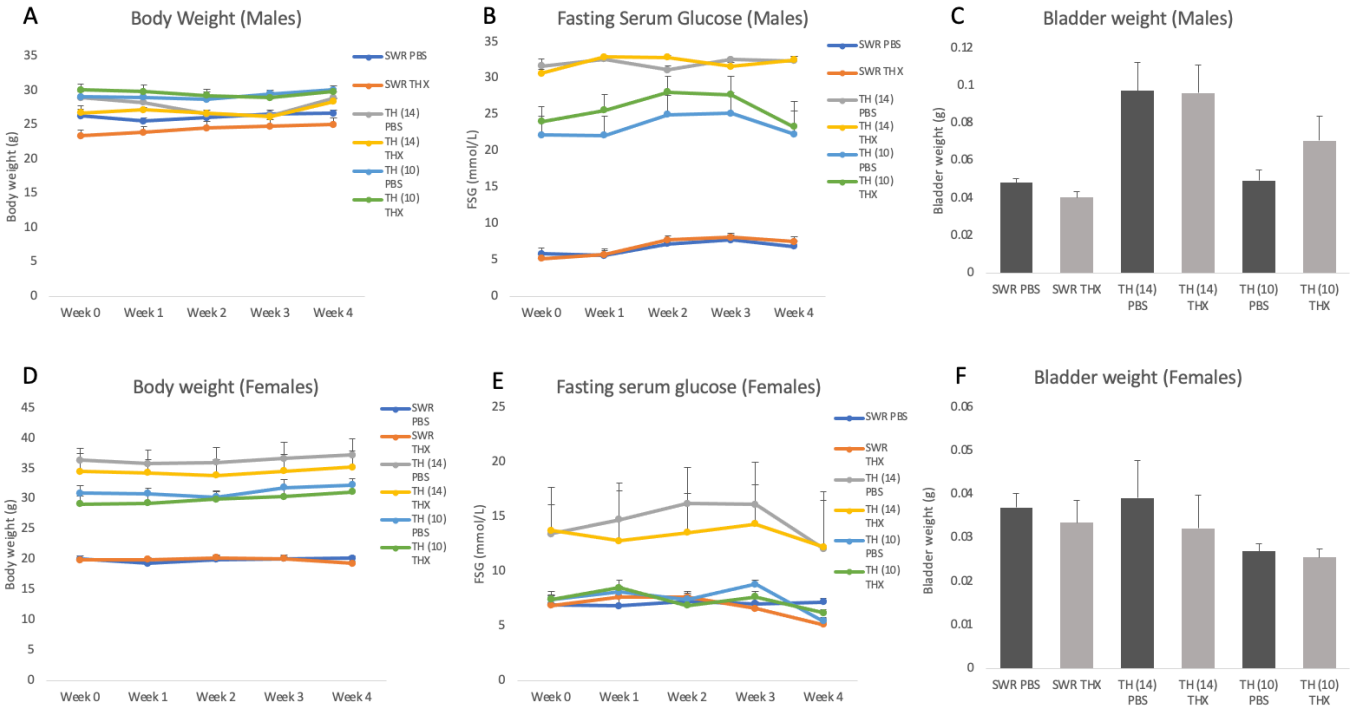

Figure 5: Male SWR (14 wk), Tally Ho (14 wk) and Tally Ho (10 wk) mouse A) body weight, B) fasting serum glucose and C) bladder weight changes over course of 4-week treatment with THX-B (N=9-11). Female SWR (14 wk), Tally Ho (14 wk) and Tally Ho (10 wk) mouse D) body weight, E) fasting serum glucose and F) bladder weight changes over course of 4-week treatment with THX-B (N=5-10).

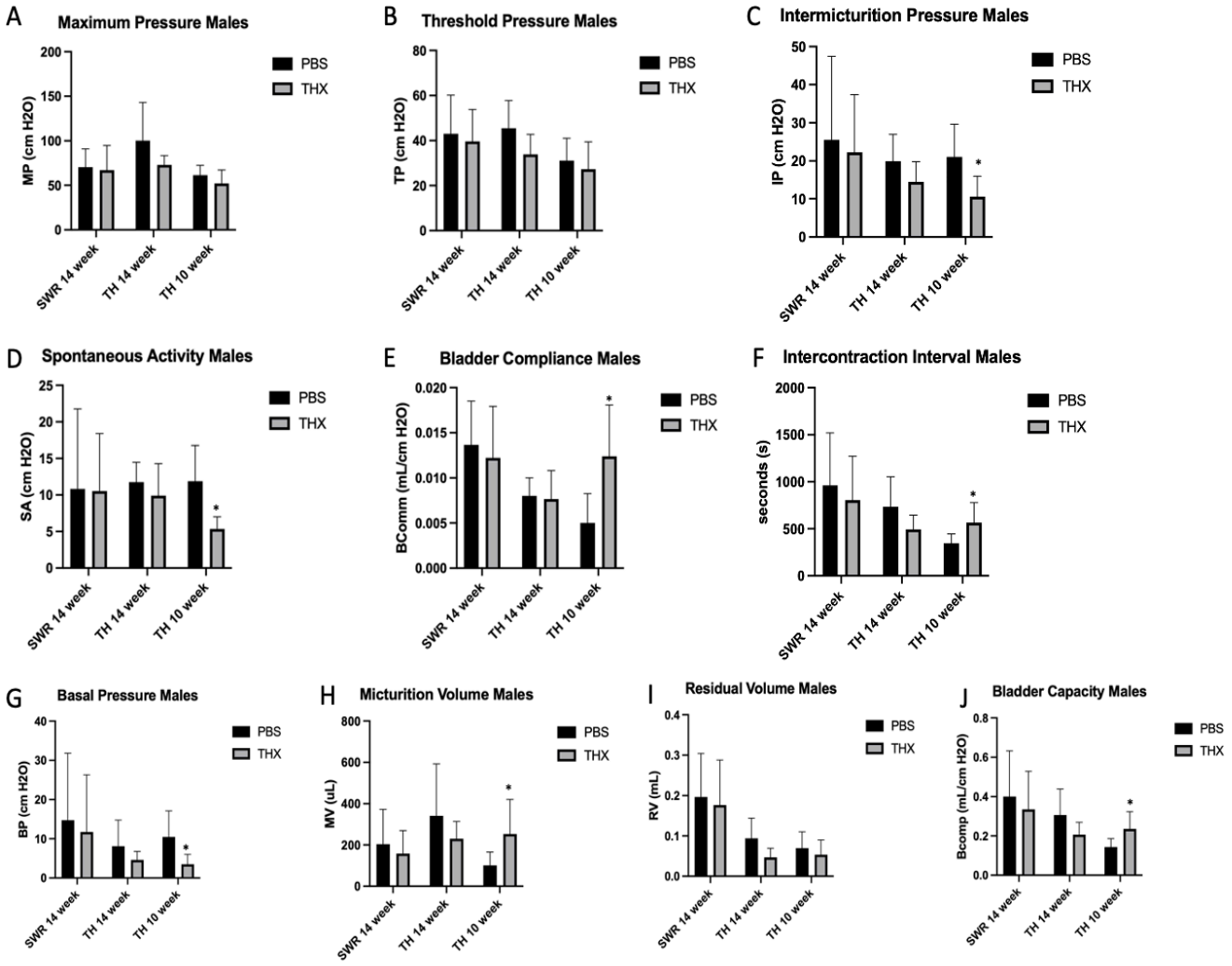

Figure 6: Conscious cystometry for male SWR (14 wk), Tally Ho (14 wk), Tally Ho (10 wk) with 4-week THX-B treatment (N=6-9). Recordings represent A) Maximum pressure, B) Threshold pressure, C) Intermicturition pressure, D) Spontaneous Activity, E) Bladder compliance, F) Intercontraction interval, G) Basal pressure, H) Micturition volume, I) Residual volume, J) Bladder capacity. Bars represent mean ± SEM. Student t-test. *p<0.05, **P<0.01 compared to PBS control.

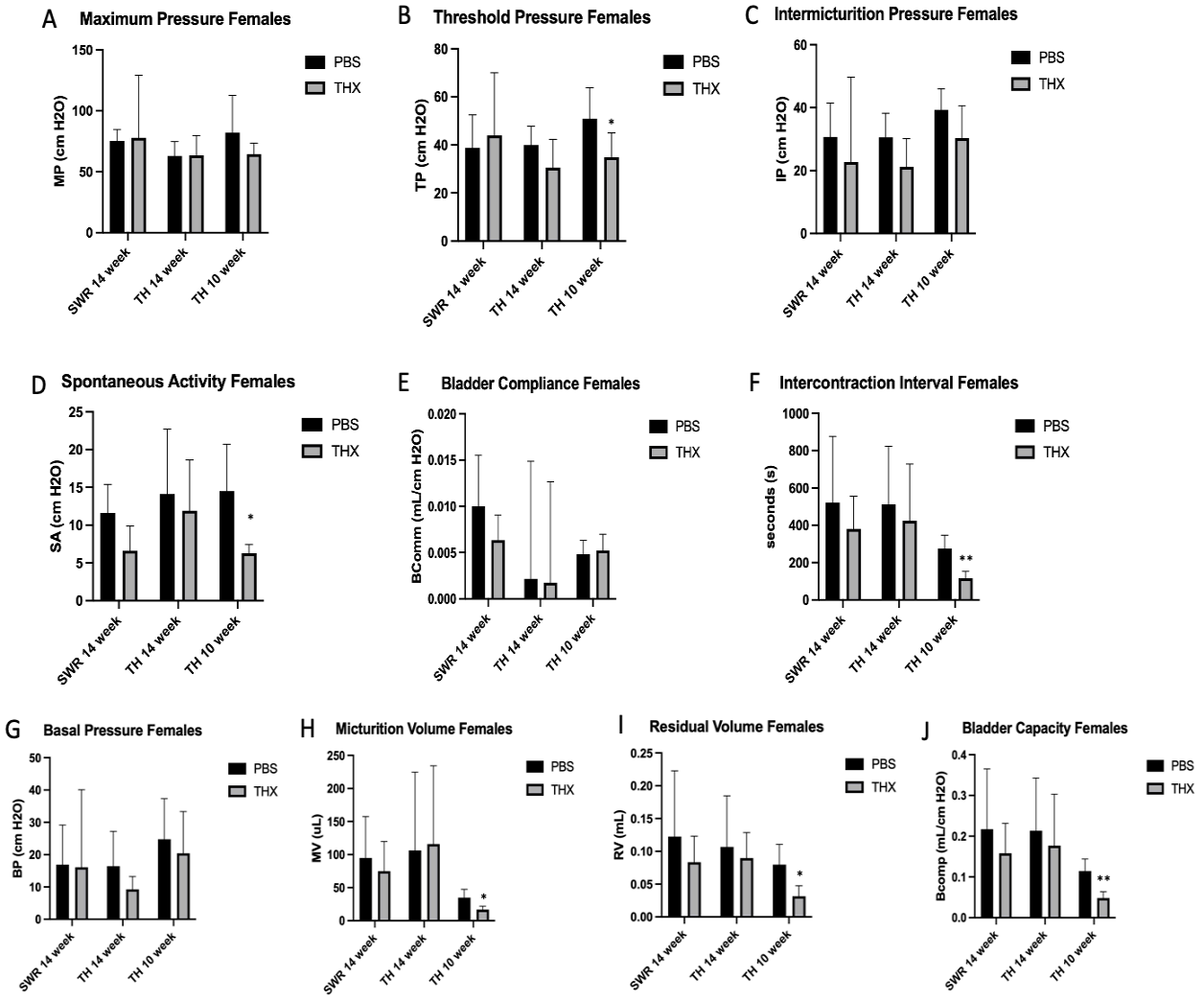

Figure 7: Conscious cystometry for female SWR (14 wk), Tally Ho (14 wk), Tally Ho (10 wk) with 4-week THX-B treatment (N=6). Recordings represent A) Maximum pressure, B) Threshold pressure, C) Intermicturition pressure, D) Spontaneous Activity, E) Bladder compliance, F) Intercontraction interval, G) Basal pressure, H) Micturition volume, I) Residual volume, J) Bladder capacity. Bars represent mean ± SEM. Student t-test. *p<0.05, **P<0.01 compared to PBS control.

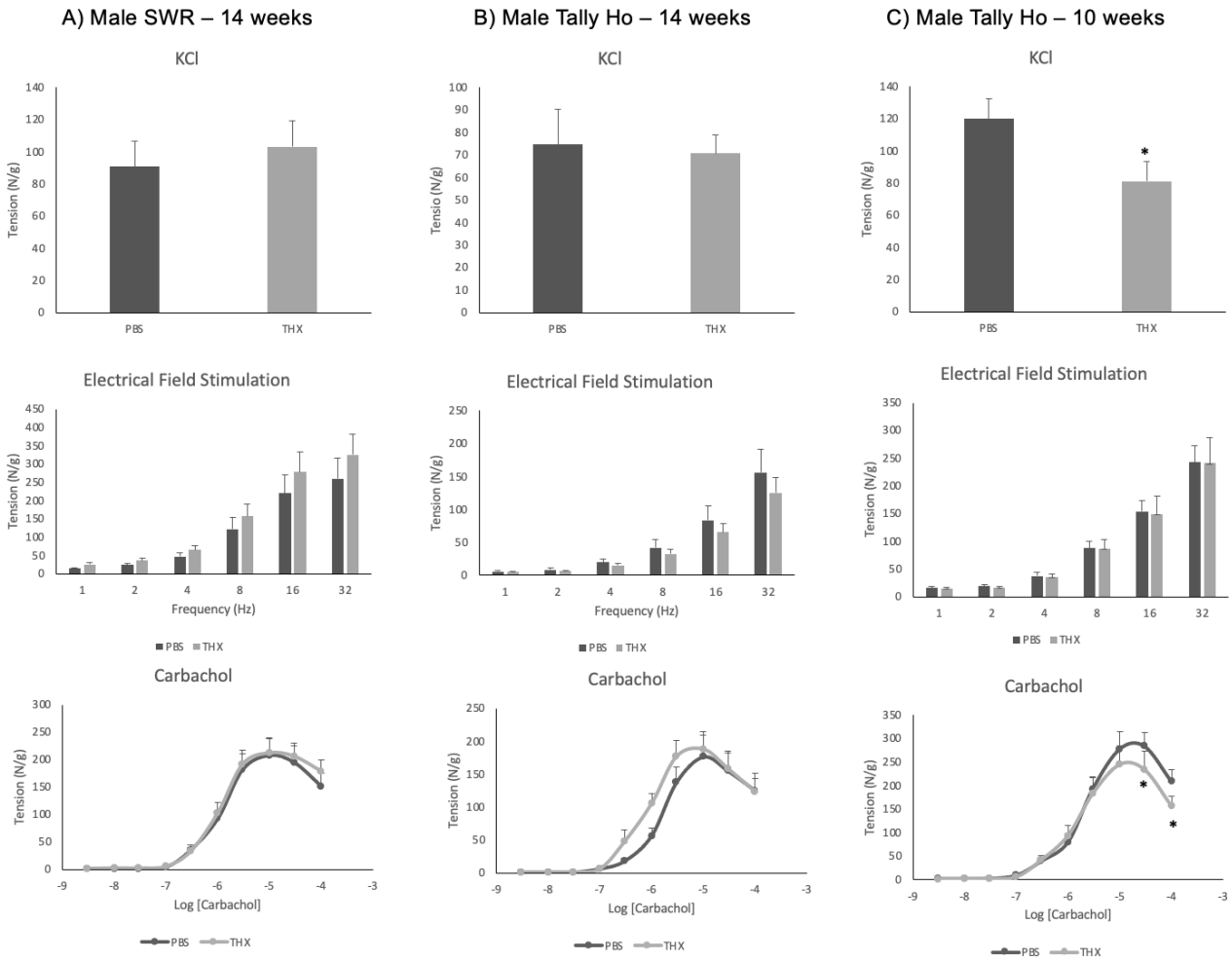

Figure 8: Organ Bath studies (males) (N=8-16): 4-week THX-B treated A) Male SWR (14 wk); B) Male Tally Ho (14 wk); C) Male Tally Ho (10 wk) bladder strips incubated with KCl (30 mM), electrically stimulated at various frequencies, and incubated with carbachol at various concentrations. Bars represent mean ± SEM. Student t-test. *p<0.05, compared to PBS control.

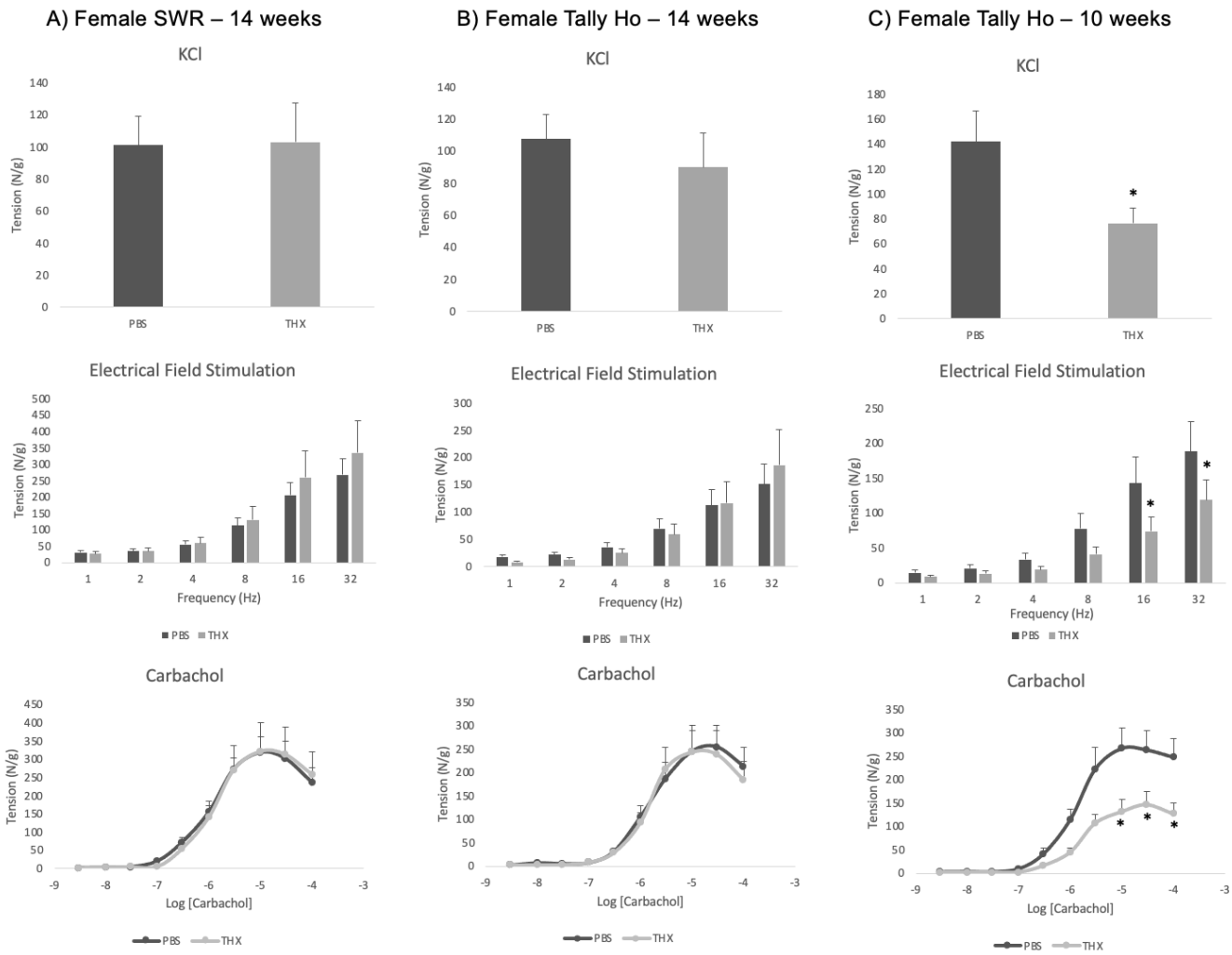

Figure 9: Organ Bath studies (females) (N=8-13): 4-week THX-B treated A) Female SWR (14 wk); B) Female Tally Ho (14 wk); C) Female Tally Ho (10 wk) bladder strips incubated with KCl (30 mM), electrically stimulated at various frequencies, and incubated with carbachol at various concentrations. Bars represent mean ± SEM. Student t-test. *p<0.05, compared to PBS control.

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Chapter 6: Comprehensive Discussion

Bladder pathologies have been linked to imbalances in neurotrophins, specifically changes in nerve growth factor (NGF) and its precursor proNGF, to the detriment of NGF. Despite recent advances in the literature, the concept is still poorly understood. Patients with diabetic voiding dysfunction (DVD) have been found to express these changes in their urine samples, leading to speculation that NGF and proNGF are greatly implicated in the development of DVD. However, studies on specific intra- and extra-cellular mechanisms leading to DVD are lacking and are essential to obtain progress in treatments. To our knowledge, this is the first body of work that addresses the role of nitric oxide in the pathway of diabetic voiding dysfunction. It is also the first time $p75^{NTR}$ antagonism has been validated as a potential therapeutic target in treating type 2 diabetic voiding dysfunction.

Our objectives were split into an in vitro and in vivo study. In bladder cells in culture, we met our in vitro objective, which was to demonstrate the link between nitric oxide, hyperglycemia and decreased NGF secretion, all while demonstrating the pathways involved. Our results provide a molecular and cellular explanation for our previously published clinical data on lower urinary tract pathophysiology and pharmacology. By establishing that increases in nitric oxide levels lead to a downregulation of NGF and upregulation of matrix metalloproteinase- 9 (MMP-9) in urothelial cells, we were able to mirror our observations in this study on aging women with overactive bladder disease and hyperglycemia[70]. These results are also consistent with unpublished findings of our team, stating that urothelial cells appear to be the main source of MMP-9 secretion, with values close to 40 times higher as compared to smooth muscle cells.

Our in vivo objectives were also met, which included the characterization of the bladder changes in the T2DM mouse model, the Tally Ho. Moreover, we were able to use our latest findings on the Tally Ho

mouse to select an appropriate timeframe to begin treating, while validating the proNGF/$p75^{NTR}$ interaction as a therapeutic target using THX-B and evaluating the changes occurring in bladder function and contractility parameters. Our results on male diabetic mice were consistent with results obtained using THX-B in a type 1 diabetic mouse model. However, some changes in contractility parameters differed in female insulin resistant mice, potentially due to the differences in glycemia levels and duration of diabetes. A separate (unpublished) study by our team also evaluated the potential of $p75^{NTR}$ antagonism to address age-related overactive bladder syndrome (OAB), which is caused by similar underlying conditions such as an imbalance in the NGF/proNGF ratio. Urine and blood samples were collected and stored from all mice in order to evaluate the changes in the NGF-to-proNGF ratio. This particular objective of the study will be investigated in future studies.

6.1. Study Limitations

A limitation of this work, particularly for the in vivo portion of the study is the inability required for the female Tally Ho mice to develop type 2 diabetes. This sex dimorphism for diabetes is speculated to be the result of a decrease in the activity of hepatic estrogen sulfotransferase, leading to in an increased density of insulin receptor in the hepatocyte membrane[142,157,158]. Although insulin resistance does lead to morphological changes in the bladder leading to symptoms of the lower urinary tract, the sex-specific development of type 2 diabetes did not allow us to study exact differences between treated male and female Tally Ho mice at the same age. Biological discrepancies must be addressed and although the Tally Ho male mimics many characteristics of human non-insulin dependent type 2 DM, a female T2DM model would be useful in studying these differences.

It is standard practice to use a murine model to obtain cell cultures for testing, as they are quite similar to human models, are easy to manipulate, and are cost-effective. However, using human cell cultures

could improve external validity of the study, removing the limitation of inter-species variability. Furthermore, although rat urothelial and smooth muscle cells were treated with adequate levels of glucose and sodium nitroprusside to mimic the diabetic voiding dysfunction condition, the accuracy in representation is lacking. Diabetes is a multigenic disease with many complex, contributing factors that could lead to fluctuations in nitric oxide levels, as well as responses in neurotrophin levels. This is another reason why human cell cultures may be more representative and ease the translational aspect of this research.

6.2. Future directions of research

This body of work further enhances our knowledge of type 2 diabetic voiding dysfunction, paving the way for future translational research. A missing link between our in vitro and in vivo studies is to identify the changes in nitric oxide levels in before and after treatment with $p75^{NTR}$ antagonist molecule. This next step can be taken in an in vitro or in vivo perspective. An unpublished study by our team has found that THX-B affects urothelial cells in vitro, particularly through changes in MMP-9 expression. Combined with the knowledge that nitric oxide is a regulator of the precursor pro-MMP-9, it would be valuable to study the changes in NO secretion upon incubation with THX-B. Measuring NO levels in mouse bladder and urine samples throughout (and after) the 4-week treatment period would also allow us to monitor these changes in vivo.

Additionally, as the proNGF/$p75^{NTR}$ axis is highly linked with inflammation and cell death signalling, a more in depth understanding of the pathways downstream of $p75^{NTR}$ activation and blockade would be useful in further understanding the effects of the treatment. Our results show promise; however, we limited our findings to changes in voiding behaviour and contractility parameters and responses. Throughout the 4 weeks of treatment, we collected urine samples and kept bladder tissue following

sacrifice. Evaluating expression of proteins such as nitric oxide, TNF-α and the phosphorylated form of JNK, in these samples, would allow us to monitor the changes THX-B can have on the tissue degeneration pathways controlled by $p75^{NTR}$ activation. Moreover, inspecting bladder tissues by microscopic examination could give better insight on neurotrophin and receptor localization, once again allowing us to evaluate the changes in bladder morphology upon treatment with THX-B.

6.3. Barriers to translation

The transition from novel research into clinical practice is essential in drug research and development. Using a mouse model in the pre-clinical stages is common practice in research, however there are always barriers to translating the obtained results to be relevant in humans. Aside from the aforementioned limitations of the study regarding inter-species variability leading to reduced external validity, we must address other difficulties.

The Tally Ho mouse model for type 2 diabetes is still relatively new and although its polygenic genotype helps account for the multifactorial origin of diabetes, it is still not yet fully understood with regards to bladder function and pathophysiology. A portion of our study gave good insight on the timeline of changes in the bladder, but further studies are required. Using a murine model with a relatively short lifespan, as compared to humans, is also difficult. As the natural history of diabetes, symptom progression and subsequent urological complications, can extend through decades, diabetic voiding dysfunction exhibits in a time-dependent manner which adds a layer of complexity [24].

Symptoms of DVD have been shown to detrimentally impact quality of life of patients. Using questionnaires and bladder diary measurements is generally how diagnosis and disease progression are measured, which is clearly an aspect that cannot be evaluated in an animal model[24]. So, although we

observed improve outcomes with regards to bladder function and voiding behaviour, we must keep this in mind moving forward in a clinical direction.

Finally, in functional urology, selectivity of drug targets is a barrier to consider. With the wide expression of NGF and proNGF within different human cell types, it can be difficult to target $p75^{NTR}$ receptors in the bladder only. This would allow for adverse effects that would need to be considered, weighing out pros and cons, prior to translation. A previous study on $p75^{NTR}$ antagonism for diabetic retinopathy evaluated different delivery methods to optimize pharmacological benefits and localization of the drug. They found that using subconjunctival delivery rather than the traditional intravitreal drug injection helped achieve their delivery goals, which was specifically to avoid systemic exposure[159]. Our study used intraperitoneal delivery of THX-B, however, evaluating the changes using an intravesical approach may be beneficial and help address this issue when moving to a clinical setting.

Chapter 7: Conclusions

7.1. Summary of findings

Both the in vitro and in vivo portions of this study helped us gain valuable insight on type 2 diabetic voiding dysfunction, both with regards to the mechanism in which it manifests, as well as a potential therapeutic avenue that can be explored on a translational level. Chapter 4 highlights the role of nitric oxide, resulting from hyperglycemia as a contributing factor in NGF/proNGF imbalance in diabetic voiding dysfunction. The signaling pathways involved were further explored, associating NO with cGMP through increased MMP-9 activity in urothelial cells and decreased MMP-7 activity in smooth muscle cells. In Chapter 5, when evaluating type 2 diabetic voiding dysfunction in an in vivo context, we first were able to characterize the Tally Ho mouse, highlighting the significant changes occurring in voiding patterns and contractility stimulus response starting at 14 weeks of age (mainly in male mice). We then took these results and used them to evaluate $p75^{NTR}$ antagonism starting at the onset of urinary symptoms (14 weeks) or with a more preventative approach, starting treatment at 10 weeks. We found that THX-B was not an effective treatment when starting at 14-weeks, however its effects, which were sex-specific, showed improvements in bladder contractility parameters and responses when starting treatment at 10 weeks. The effects were mostly seen in correcting hyper-contractility of the detrusor muscle, a symptom expressed in early stages of DVD. This leads us to believe that, pending further studies, THX-B could be a potential therapeutic tool to treat early DVD with a more preventative approach.

7.2. Contribution to literature

The gaps in literature regarding diabetic voiding dysfunction makes it a crucial topic of study. There is a high prevalence in diabetic patients and various health complications have been associated with this condition. Not only is there a reduction in quality of life, but the deterioration of bladder function can

cause life-threatening infections, as well as renal impairment. Although there are some treatment options to alleviate symptoms, there are very few treatment options that have been evaluated through clinical trials that are specific to the disease. Current knowledge indicates that the proNGF/p75^{NTR} interaction has displayed degenerative and apoptotic changes, leading to tissue damage. Blocking the interaction has been able to correct the imbalance in the NGF/proNGF ratio and correct resulting symptoms in diabetic retinopathy, glaucoma, and other forms of nerve damage, including bladder dysfunction resulting from spinal cord injuries[75,82]. Results from a study conducted on a Type 1 DVD model treated with THX-B antagonist to block the proNGF/p75^{NTR} interaction were promising and showed improvement in bladder dysfunction and remodelling[74]. The proNGF/p75^{NTR} axis has been evaluated as a potential therapeutic target in different pathologies, however, has never been evaluated specifically in the context of diabetic voiding dysfunction in patients with Type 2 DM. Combining the findings of this work with current knowledge of the disease can potentially provide translational treatment for DVD, using the proNGF/p75^{NTR} interaction as a new drug target with more extensive clinical applications following.

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