Urinary function and physiology in adult mice are influenced by developmental exposure to a human-relevant mixture of polychlorinated biphenyls

By

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ABSTRACT

Lower urinary tract symptoms (LUTS) are adverse health outcomes estimated to negatively impact two-thirds of adults in the United States. Efforts to ameliorate symptoms or cure the underlying dysfunctions are stymied by an incomplete understanding of LUTS etiologies. Exposure to environmental chemicals can impact the onset, progression, and severity of LUTS; However, work to characterize the underlaying mechanisms of action is complicated because studies often overlook the lower urinary tract for adverse health outcomes, and studies that do focus on endpoints which can contribute to LUTS etiology often consider individual compounds in isolation and do not account for lifetime exposure at different developmental timepoints. The objective of this body of work is to investigate the environmental pollutant polychlorinated biphenyls (PCBs) as a lower urinary tract toxicant, specifically addressing urinary physiology and aging, bladder smooth muscle contractility, and how these can relate to sex-steroid hormone signaling. First, we provided an overview of different mechanisms involved in bladder smooth muscle contractility in healthy physiology and highlighted several ways that exogenous chemical exposures or dysregulation of homeostasis can lead to pathophysiology. Next we characterized the urinary voiding phenotype of mice with developmental exposure (*in utero* and lactational) to a human-relevant mixture of PCBs, and how they respond to a challenge of chemical sensitization, demonstrating PCB effects though aging can persist and newly emerge ranging from 6 to 12 weeks of age. In developmentally exposed mice, we further utilized a muscle contraction assay to probe key mechanisms of both nerve-mediated and myogenic bladder smooth muscle contraction, with a focus on large-conductance voltage- and Ca^{2+}- activated K^{+} (BK) channels. Lastly, we investigated markers of endocrine disruption and demonstrate developmental PCB exposure has sex-, dose-, and tissue-dependent effects on steroid sex

hormone receptor expression at 6 weeks of age. These findings expand upon previous findings that human-relevant PCBs are capable of impacting urinary voiding physiology of mice and expanding the ages mice are assessed, then further delves into both characterizing PCB effects on molecular pathways important for bladder contractile activity and PCB effects on endocrine sensitive endpoints. Taken together, not only does this work contributes to a larger body of knowledge to better understand how external stimuli including exposure to environmental chemicals can contribute to the development of LUTS, but it also identifies key targets of PCB exposure that can contribute to altered bladder function.

TABLE OF CONTENTS

ACKNOWEDGEMENTS **i**

ABSTRACT **iii**

TABLE OF CONTENTS **v**

ABBREVIATIONS **viii**

CHAPTER I: Smooth muscle of the lower urinary tract: BK-RyR coupling in physiology and pathophysiology **1**

1. Abstract **2**
2. Introduction **3**
3. Lower urinary tract physiology **5**
4. Innervation and smooth muscle contraction **7**
5. Pathophysiology **10**
6. BK-RyR coupling in lower urinary tract function **16**
7. Environmental impacts on BK-RyR coupling and future opportunities **21**
8. LUTS treatment **27**
9. Declarations **30**
10. Figures **31**

CHAPTER II: Developmental polychlorinated biphenyl (PCB) exposure impacts on voiding physiology persist into adulthood and influence sensitivity to bladder stimuli in mice **32**

1. Abstract **33**
2. Introduction **35**
3. Materials and methods **39**

4. Results **45**

5. Discussion **50**

6. Declarations **57**

7. Tables **58**

8. Figures **62**

CHAPTER III: Developmental exposure to polychlorinated biphenyls (PCBs) alters contractile strength and sensitivity to contractile stimuli in bladders of young adult mice *ex vivo* **72**

1. Abstract **73**

2. Introduction **75**

3. Materials and methods **79**

4. Results **85**

5. Discussion **96**

6. Declarations **102**

7. Tables **103**

8. Figures **107**

CHAPTER IV: Steroid hormone receptor expression is impacted in a sex-, dose-, and tissue-specific manner in young adult mice with developmental exposure to human-relevant polychlorinated biphenyls **120**

1. Abstract **121**

2. Introduction **123**

3. Materials and methods **128**

4. Results **133**

5. Discussion **136**

6. Declarations **139**

7. Tables **140**

8. Figures **143**

CHAPTER V: Conclusions and future directions **145**

1. Conclusions **146**

2. Future directions **148**

3. Takeaways **153**

WORKS CITED **155**

APPENDIX A **189**

ABBREVIATIONS

ABMA, αβ-methylene ATP, , αβ-methylene adenosine triphosphate

AC, adenylyl cyclase

AhR, aryl hydrocarbon receptor

ATP, adenosine triphosphate

BK , BK_{Ca}, Maxi-K, Slo1, or $K_{Ca1.1}$, big potassium, large-conductance (100-300 pS) voltage- and Ca^{2+}- activated K+

BOO, bladder outlet obstruction

BPA. bisphenol A

BPH, benign prostatic hyperplasia

cDNA, complementary DNA, complementary deoxyribonucleic acid

cGMP, cyclic GMP, cyclic guanosine monophosphate

CMG, cystometry

DO, detrusor overactivity

DU, detrusor underactivity

EC50, effective concentration 50%, half maximal effective concentration

EFS, electrical field stimulation

ER, estrogen receptor

E2, 14β-Estradiol

GnRH, gonadotropin-releasing hormone

Hz, hertz

IP_3R 1,4,5-trisphosphate receptors

LOAL, lowest observable effect level

LUTD, lower urinary tract disfunction

LUTS, lower urinary tract symptoms

MARBLES, Markers of Autism Risk in Babies Learning Early Signs

NIH, National Institute of Health

OAB, overactive bladder

PBDE, polybrominated diphenyl ether

PDE5, phosphodiesterase type 5

PCB, polychlorinated biphenyl

PKA, protein kinase A

PKG, protein kinase G

PMC, pontine micturition center

RNA, ribonucleic acid

RT-qPCR, quantitative PCR, real time quantitative polymerase chain reaction

RyR, ryanodine receptor, ryanodine-sensitive Ca^{2+} release channels

sGC, soluble guanylate cyclase

SR, sarcoplasmic reticulum

TBKC, transient BK channel current

TCDD, dioxin, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin

TRPV1, transient receptor potential cation channel subfamily V member 1

UAB, underactive bladder

VDCC, voltage dependent calcium channel

VSA, void spot assay

β3, beta 3-adrenergic receptors

CHAPTER I: SMOOTH MUSCLE OF THE LOWER URINARY TRACT: BK-RYR COUPLING IN PHYSIOLOGY AND PATHOPHYSIOLOGY

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ABSTRACT

In the lower urinary tract, coordinated function between the bladder and urethra is essential for normal micturition, requiring smooth muscle contraction and relaxation in a tightly regulated cycle. During the bladder filling phase, the bladder remains relaxed while the urethra stays contracted to prevent leakage. During voiding, this coordination reverses, and the bladder contracts to expel urine while the relaxed urethra allows urine flow. These functions are essential for proper micturition and two key molecular regulators of this process are the large-conductance calcium activated potassium (BK) channels and ryanodine receptors (RyRs), both of which modulate smooth muscle excitability and calcium dynamics. This review provides an overview of the roles of BK channels and RyR mediated signaling in regulating smooth muscle activity in the bladder and urethra, with a focus on their contributions to lower urinary tract physiology and pathophysiology. BK channels act as negative feedback modulators, dampening myogenic and nerve-evoked contractions of the detrusor and urethra. RyRs play a role in regulating intracellular calcium signaling that supports both muscle relaxation and contraction. This review highlights alterations in the function of these channels in lower urinary tract dysfunction, and as potential targets for other factors such as environmental exposures to disrupt voiding function. BK channels and RyRs are plausible targets for therapeutic strategies aimed at improving bladder and urethra function in certain patients, particularly those with lower urinary tract symptoms caused by factors such as aging and environmental chemical exposure.

INTRODUCTION

The bladder and urethra work in tandem to properly store and excrete urine in the healthy micturition cycle. When focusing on the lower urinary tract, the healthy micturition cycle can be split into two phases, bladder filling and bladder emptying. During the bladder filling phase, the bladder is relaxed and stretches to comply with increased urine volume (Chakrabarty et al., 2019; Drake et al., 2017), while the urethral sphincter generates sufficient contractile force to maintain continence and prevent urinary leakage (DeLancey et al., 2008; Jung et al., 2012). During the voiding phase, the bladder and urethra coordinate to swap contractile states such that the bladder contracts while the urethral sphincter relaxes to expel urine (Heesakkers and Gerretsen, 2004). The proper function of these two tissues, especially in relation to control of smooth muscle contraction, is vital to regulate micturition.

Dysfunction of the bladder or urethra can give rise to burdensome lower urinary tract symptoms. While there are numerous risk factors such as genetic mutations, environmental insults, comorbid health conditions and aging that can contribute to the onset, progression, and severity of lower urinary tract symptoms, this review will examine insults targeting the smooth muscle layer of the bladder and urethra with a focus on large-conductance (100–300 pS) voltage- and Ca^{2+}-activated K^+ channels (BK, BK_{Ca}, Maxi-K, Slo1, or $K_{Ca1.1}$) and ryanodine-sensitive Ca^{2+} release channels (ryanodine receptors, RyRs) (Yoshida et al., 2001). Together, BK channels and RyRs contribute in part to the regulation of smooth muscle tone of both the bladder and urethra. Although drug therapies for lower urinary tract symptoms do not currently target BK channels and RyRs, this review aims to outline their role in lower urinary tract physiology and pathophysiology to highlight how these ion channels can drive lower urinary tract dysfunction in

preclinical models and be a target for novel therapeutic approaches to improve urinary tract function.

LOWER URINARY TRACT PHYSIOLOGY

Bladder wall histology is primarily segmented into 4 layers: urothelium, lamina propria, smooth muscle, and serosa/adventitia (Bolla et al., 2025). Briefly, starting at the lumen, the urothelium consists of several cell populations including basal, intermediate, and superficial cells. This superficial layer consists of specialized umbrella cells whose surfaces are covered by urothelial plaques composed of uroplakins, which provide barrier function (Wu et al., 2009). The lamina propria separates the urothelium from the detrusor with a layer of extracellular matrix and several cell types including fibroblasts, adipocytes, interstitial cells of Cajal, nerves, and blood vessels (Andersson and McCloskey, 2014). The smooth muscle layer of the bladder, known as the detrusor, consists of myocyte fascicles arranged in spherical bundles in the central section of the detrusor with longitudinal layers on either side (Christ and Hodges, 2006; Kuijpers et al., 2014). As the bladder fills, detrusor tone is maintained through bladder wall stretching and thinning where the myocytes lengthen (Uvelius and Gabella, 1980). Lastly, the serosa and adventitia form connective tissue layers around the bladder and connect it to the peritoneal layer of the abdominal wall (Bolla et al., 2025).

Urethral histology is segmented into the same 4 layers as the bladder, with differences in cellular composition that vary in relation to sex and proximity to the bladder (Henry et al., 2018; Hudson et al., 2001). For example, the urethral urothelium of the prostatic urethra includes additional club and hillock epithelial cells (Henry et al., 2018) and the lamina propria has additional glands in the prostatic urethra and near the female external urethral sphincter (Eggermont et al., 2019; Satoh et al., 2001; Stoddard and Leslie, 2025; Zaviacic et al., 1997). The smooth muscle layer, separated from the lamia propria by a thin fascial layer, is organized into an inner layer of longitudinal smooth muscle and outer layer of circular smooth muscle (Abelson et al., 2018).

Interspersed within the urethra smooth muscle layer are electrically-coupled interstitial cells of Cajal which are involved in the pacemaker activity of urethral contraction during homeostatic conditions (Sergeant et al., 2000). The role of urethral interstitial cells of Cajal was recently expanded to include driving a contractile response to expel pathogens, such as defending against E. coli ascension in a mouse model of urinary tract infection (Ambrogi et al., 2025). The smooth muscle cells of the internal urethral sphincter are primarily responsible for autonomic, involuntary continence while the external sphincter is composed of striated (skeletal) muscle and is responsible for somatic, voluntary control of continence (Greenland et al., 1996; Rother et al., 1996). While the external sphincter plays an important role in voluntary "contingency" continence (such as during coughing, sneezing, or laughter), this review will focus on the smooth muscle and internal urethral sphincter (Ferreira and Duarte Cruz, 2021). Both the internal and external sphincters are necessary for the regulation of micturition, functioning together to regulate voiding in coordination with the bladder (Brading, 1999; Greenland et al., 1996; Koraitim, 2008).

INNERVATION AND SMOOTH MUSCLE CONTRACTION

Blader filling

The average human bladder holds around 500 mL of urine at full capacity, but does not reach full capacity in healthy conditions before a urination event occurs, and healthy individuals hold conscious control over when and where to urinate (Griffiths and Tadic, 2008; Kershen et al., 2002). During the bladder filling phase (Fig. 1), lumbar sympathetic fibers travelling via the hypogastric nerves release norepinephrine, which binds to beta 3-adrenergic receptors (β3) on detrusor smooth muscle cells. This activates adenylate cyclase, increasing cAMP levels and activating protein kinase A, which phosphorylates various downstream targets leading to detrusor relaxation. One downstream effect also includes the activation of transient BK channel currents (TBKCs), which contribute to membrane hyperpolarization and reduced excitability (Michel and Vrydag, 2006; Petkov and Nelson, 2005). In the proximal urethra, sympathetic activity excites smooth muscle via release of norepinephrine, which binds primarily to α1-adrenoceptors, ultimately leading to smooth muscle contraction and closure of the internal sphincter to maintain continence during bladder filling. During this period, intracellular Ca^{2+} waves support sustained smooth muscle contraction. As part of the repolarization phase, BK channels open in response to elevated intracellular Ca^{2+}, promoting membrane hyperpolarization, which limits excessive excitability and contributes to rhythmic control of contractile tone (Drumm et al., 2024; Kunisawa et al., 1985; Kyle et al., 2013; Michel and Vrydag, 2006; Ueda et al., 1984). The role of voltage-dependent calcium channels (VDCCs) in urethral smooth muscle contraction is not fully defined and may vary across species. In murine studies, L-type VDCCs do not appear to be essential for contraction (Drumm et al., 2018). However, studies in pig, rabbit, and human urethra have shown that L-type VDCC mediated Ca^{2+} influx is necessary to trigger transient BK

currents and contractile response to adrenergic and purinergic stimuli (Kyle et al., 2013; Teramoto and Brading, 1996). Species and sex differences in adrenoceptor expression/function have also been reported and are an area of active study (Hsieh et al., 2023). In addition to adrenergic signaling, acetylcholine release from parasympathetic nerve fibers may also contribute to urethral contraction via muscarinic receptors, mainly M_2 and M_3 subtypes, though their exact roles and functional significance remain to be fully elucidated (Mutoh et al., 1997; Nagahama et al., 1998). Somatic efferent activity via the pudendal nerve leads to release of acetylcholine, which binds nicotinic receptors on striated muscle of the external urethral sphincter leading to voluntary contraction important for continence (Damaser et al., 2003; Kerns et al., 2000).

Bladder emptying

During bladder emptying (Fig. 1), efferent parasympathetic fibers from the pelvic nerve release acetylcholine and adenosine triphosphate (ATP), which trigger detrusor smooth muscle contraction via activation of muscarinic acetylcholine receptors and purinergic receptors, respectively (Brading, 2002; Chess-Williams, 2002; Daniel et al., 1983; Giglio and Tobin, 2009; Malysz and Petkov, 2020). Purinergic signaling largely through P2X receptors, causes rapid depolarization and transient contractions, while cholinergic signaling, primarily via M_3 muscarinic receptors, is responsible for the more sustained, tonic contraction necessary for voiding (Heppner et al., 2009). Although M_2 muscarinic receptors are more abundant than M_3 in detrusor, they do not directly mediate contraction, instead they likely contribute indirectly by opposing β-adrenergic mediated relaxation through inhibition of adenylyl cyclase, or by modulating M_3 receptor mediated responses (Chapple et al., 2002; Yamanishi et al., 2001). Membrane depolarization activates downstream calcium signaling pathways. Membrane

depolarization opens L-type VDCCs, which permits Ca^{2+} influx. Additionally, intracellular Ca^{2+} is released from the sarcoplasmic reticulum through IP_3 receptors and RyR, amplifying the contractile response (Brading, 2002; Daniel et al., 1983; Gong et al., 2021; Malysz and Petkov, 2020). Detrusor smooth muscle cells are interconnected by gap junctions, enabling electrical coupling and coordinated contraction, but this coupling is less extensive than in other smooth muscle tissues (Clapham, 1996).

In the urethra parasympathetic nerve terminals release nitric oxide and acetylcholine to relax urethral smooth muscle (Andersson et al., 1992, 1991). Once nitric oxide crosses the smooth muscle membrane, it activates soluble guanylate cyclase, which increases the production of cyclic GMP (cGMP). cGMP then activates protein kinase G (PKG), which inhibits Ca^{2+} release via the IP_3 signaling cascade, ultimately leading to smooth muscle relaxation (Andersson et al., 1992). Purinergic signaling may also be involved in smooth muscle relaxation and contraction, though evidence is limited. ATP administration has been shown to cause both relaxation and contraction depending on urethral tone and ATP concentration (Callahan and Creed, 1981; Sergeant et al., 2019). The purinergic signaling pathway remains unclear but may involve P2Y1, P2X1, or P2X2/3R receptors on the smooth muscle and/or interstitial cells (Bradley et al., 2011; Burnstock, 2014; Salazar et al., 2019).

During bladder emptying, sympathetic efferent activity via the hypogastric nerves to the bladder and urethra is inhibited to allow detrusor contraction and internal urethral sphincter relaxation respectively. In addition, urethral somatic efferent activity via the pudendal nerve is also inhibited to permit relaxation of the external urethral sphincter (Damaser et al., 2003; Kerns et al., 2000).

PATHOPHYSIOLOGY

Lower urinary tract symptoms and dysfunction

Lower urinary tract symptoms (LUTS) can be broadly characterized as symptoms related to urine storage, urinary voiding, or following urinary voiding (Abrams et al., 2003). The underpinning lower urinary tract dysfunction that drives these symptoms can be broadly characterized as failure to store urine - including detrusor overactivity, hypersensitivity, stress urinary incontinence, intrinsic sphincter deficiency, or failure to empty the bladder - including neurologic injury, detrusor underactivity, bladder outlet obstruction, urethral obstruction/stricture and striated sphincter dyssynergia (McDonough and Ryan, 2016).

The onset of LUTS can be acquired through congenital anomalies, fetal or early life developmental disorders, injury throughout life, health conditions indirectly influencing lower urinary tract function (i.e. multiple sclerosis, diabetes, metabolic syndrome, obesity), or from aging-related processes including benign prostatic hyperplasia in men (Bharti et al., 2023; Kopač, 2024; Lee and Kuo, 2017; Moussa et al., 2020; Thomas et al., 2021). In a study based in Boston following a prospective cohort in the 2000's, over 5 years moderate to severe LUTS were newly acquired in 11.4% of the individuals (Maserejian et al., 2013). The prevalence of LUTS has been increasing globally since the 1990s, estimated at 45.2% in 2011, and in 2022 the estimated overall incidence of LUTS, and more specifically moderate-to-severe LUTS, is 63.2% and 31.3%, respectively (Huang et al., 2023; Irwin et al., 2011; Tahra et al., 2022).

The cost of LUTS is not solely attributed to medical care, of which the NIH estimates bladder, prostate and other urinary tract diseases cost Americans nearly $11 billion a year, and half this cost is covered through the social welfare program Medicare (National Institutes of Health

(NIH), 2015). LUTS also drives an 8.03% greater overall work productivity loss and a decreased health-related quality of life (Kannan et al., 2009). For hospitalized patients, urinary incontinence is seen as a fate equal to or worse than death (Rubin et al., 2016). This observation underscores the importance of LUTS research, symptom prevention and modulation, as well as treatment.

Over- and underactive bladder

The general clinical presentations of over- and underactive bladder (OAB and UAB) both include urgency and frequency, but UAB also includes weak stream, straining, hesitancy, and sensation of incomplete emptying (Foon and Toozs-Hobson, 2007; Gormley et al., 2015; Hoag and Gani, 2015). The prevalence of OAB in men and women ranges between 11.8% and 35.6% with increased incidence associated with age and body mass index (Eapen and Radomski, 2016; Reynolds et al., 2016; Stewart et al., 2003). OAB is associated with diminished quality of life, quality of sleep, and mental health (Stewart et al., 2003). The prevalence of UAB is predicted to be under-reported as a 2014 study reported 23% of survey respondents reported sensation of incomplete bladder emptying, yet only 11% of survey respondents knew of UAB (Aggarwal and Zimmern, 2016; Valente et al., 2014).

The pathophysiology of OAB and UAB are unclear, but changes to the bladder may include denervation in OAB and UAB, decreased bladder wall tone in OAB, and muscle loss and fibrosis in UAB (Brading, 1997; Drake et al., 2017; Elbadawi et al., 1993; Rosenberg et al., 2016). The occurrence of OAB is often associated with detrusor overactivity with or without urgency incontinence, and UAB is often associated with detrusor underactivity (Chess-Williams, 2002; Osman et al., 2014).

Detrusor over- and underactivity

Detrusor overactivity (DO), as defined by the international continence society, is marked by urodynamic observations of uncontrolled contractions of the detrusor muscle during bladder filling, a consequence of cytometric bladder filling or external stimuli such as bending at the waist, while detrusor underactivity (DU) entails contraction of reduced strength and/or duration resulting in prolonged or incomplete emptying of the bladder (Abrams et al., 2003; Foon and Toozs-Hobson, 2007; van Koeveringe et al., 2011).

DU is observed in approximately 23% of individuals who undergo urodynamic evaluation for non-neurogenic LUTS, yet the overall prevalence of DU in the general population is obfuscated by the requirement for invasive urodynamic evaluation and ambiguity in clinical and literature definitions (Hartigan et al., 2019; Hoag and Gani, 2015; Jeong et al., 2012; Osman et al., 2014). There are no curative treatments for DU, and treatment involves catheterization to aid in bladder emptying (Hartigan et al., 2019). Research on therapeutics for DU may one day include novel drugs, electrical stimulation, and stem cell therapy (Wang et al., 2023).

DO is estimated to occur in approximately 10% of the general population, with increasing prevalence with age with observed occurrence in up to 80% of elderly individuals who undergo urodynamic evaluation for urinary incontinence (Foon and Toozs-Hobson, 2007). DO is associated with LUTS including frequency, nocturia, urgency, and urinary incontinence, and the molecular underpinnings of DO are generally understood to have neurogenic and/or myogenic bases (Foon and Toozs-Hobson, 2007). In patients with DO coincident with benign prostate hyperplasia and several animal models of DO induced through stricture of the urethra causing partial bladder outlet obstruction, studies observe decreased outward BK channel currents at rest and following detrusor contraction, decreased BKα mRNA and protein expression, and changes

to β1 and β4 expression (Aydin et al., 2012; Chang et al., 2010; Hristov et al., 2013; Jiang et al., 2005; Kita et al., 2010; Li et al., 2008; Zheng et al., 2020). Whether the changes to BK channel expression and activity are causal or compensatory in DO remain unclear, however it is likely not a beneficial change considering BK channel's role in diminishing bladder contractility as discussed in a later section.

Although overactivity and underactivity imply the two conditions are opposites, this can be a misnomer because DO and DU can co-occur, with DO occurring during the filling phase and DU occurring during the voiding phase. DO-DU coincidence occurs in 18.8% and 5.5% of the elderly population of men and women, respectively (Jeong et al., 2012). The two dysfunctions seem to occur independently in men, where 46.5% of men with DU also had DO or BOO and 18.8% of elderly men had detrusor activity co-occurring with impaired bladder contractility (Jeong et al., 2021, 2012). In women, DO-DU may be an intermediate step in the process of DO to DU, where 5.5% of elderly women had detrusor overactivity with impaired contractility (Jeong et al., 2021, 2012).

Incontinence

Involuntary urinary leakage accompanied by urgent desire to urinate is defined as urgency incontinence (Abrams et al., 2003). OAB without urgency incontinence is termed "OAB dry," and OAB with urgency incontinence is "OAB wet" which is associated with DO (Corcos and Przydacz, 2018; Foon and Toozs-Hobson, 2007). In women, the prevalence of OAB dry and OAB wet are similar, while in men OAB dry is more prevalent (Reynolds et al., 2016; Stewart et al., 2003).

Stress urinary incontinence (SUI) is defined as involuntary leakage of urine due to abdominal pressure. Two conditions primarily present in patients presenting with SUI are loss of proximal urethra support due to weakened surrounding muscles leading to a lack of structural support for the urethra, and intrinsic sphincter deficiency causing low-pressure urethra closure (Kayigil et al., 1999; Norton and Brubaker, 2006). While these conditions present in both men and women, they are much more common in women due to female-specific traumas to the body, such as vaginal childbirth and hormonal changes in menopause (Brubaker and Saclarides, 1996; Cannon et al., 2003). However, there is growing evidence to suggest that SUI is not only anatomical but may also involve functional deficiencies in the urethral sphincter mechanism (Kayigil et al., 1999; Norton and Brubaker, 2006). In adults with neurogenic bladder disease, urethral internal sphincter dysfunction can occur as an additional effect of spinal cord injury, where the urethra contracts and relaxes in accordance with the bladder instead of oppositely (Myers et al., 2016). Furthermore, denervated rat urethras show significant improvement in muscle contraction following treatment with allogenic muscle-derived progenitor cells (Cannon et al., 2003). This underscores the importance of investigating the roles of BK channels and RyR signaling in models of incontinence.

Urethral obstruction and benign prostatic hyperplasia

The most frequently diagnosed cause of obstruction in males is benign prostatic hyperplasia (BPH), a nonmalignant growth of the prostate that can impede the normal flow of urine from the bladder through the urethra (Ng et al., 2025). BPH is prevalent in older men, with histological evidence observed in approximately 50–60% of men in their 60 s and 80–90% of men over 70 years old (Roehrborn, 2005). Evidence has shown that BK channel signaling may play a role in onset and modulation of BPH. Patients with BPH and detrusor overactivity exhibit

downregulation of BK channel subunits, suggesting that BK channel dysregulation may contribute to detrusor overactivity symptoms seen in men with BPH (Chang et al., 2010; Niwa et al., 2012).

BK-RYR COUPLING IN LOWER URINARY TRACT FUNCTION

BK channels

Large-conductance (100–300 pS) voltage- and Ca^{2+}-activated K^+ channels (BK, BK_{Ca}, Maxi-K, Slo1, or $K_{Ca1.1}$) are potassium ion channels that regulate smooth muscle cell membrane potential through the efflux of potassium ions, and they are the sole K^+ channel member that is gated by both intracellular Ca^{2+} and membrane voltage. BK channels are expressed in diverse tissues and cell types throughout the body including neurons of the central nervous system (Knaus et al., 1996; Misonou et al., 2006; Sausbier et al., 2006), skeletal muscle cells (Dinardo et al., 2012), and epithelial cells (Cornelius et al., 2020). Most importantly for this review, BK channels are expressed in smooth muscle cells of the bladder (Heppner et al., 1997; Herrera et al., 2001, 2000), urethra (Hollywood et al., 2000; Kyle et al., 2013), uterus (Khan et al., 1997; Wakle-Prabagaran et al., 2016), blood vessels (Dopico et al., 2018; Langton et al., 1991), and airways (Cox and Petrou, 1999; Resnik et al., 2006).

The BK channel provides negative feedback for detrusor and urethral contractions, both myogenic and nerve-evoked (Kyle, 2014; Kyle et al., 2013; Meredith et al., 2004). Electrophysical studies of bladder and urethra smooth muscle reveal that while other large and small conductance K^+ channels contribute to depolarization, repolarization and hyperpolarization, the BK channel is the predominant channel involved in maintenance of the resting membrane potential and shaping the current rectification of the repolarization phase (Kyle, 2014; Kyle et al., 2013; Meredith et al., 2004). BK channel opening activity can be categorized as either transient or steady state, with the former activated through Ca^{2+} release from the SR/RyR (Ca^{2+} sparks) and the latter activated through Ca^{2+} influx through VDCCs (Collier et al., 2000; Heppner et al., 1997; Herrera and Nelson, 2002) (Fig. 1). Thus the BK

channel is recognized as a key player in bladder and urethral smooth muscle contraction, and arguably the most important ion channel involved in detrusor contraction (Hayase et al., 2009; Heppner et al., 1997; Kyle, 2014; Lee et al., 2018).

BK channels are located within the myocyte plasma membrane, comprised of four alpha (α) subunits as a homotetramer, and when open allows for K^+ efflux from the myocyte, leading to membrane hyperpolarization and decreased excitability. Changes to the opening probability of the BK channel modulates smooth muscle cell membrane excitability and intracellular Ca^{2+} homeostasis. Increasing intracellular Ca^{2+} concentration and membrane voltage potential both increase the opening probability of the BK channel, but this occurs allosterically with a weak interaction between the two sensors (Gonzalez-Perez and Lingle, 2019; Horrigan and Aldrich, 2002). Changes to BK channel opening probability are achieved by pre- and post-translational changes to the BK channel, such as phosphorylation by PKA which may be triggered indirectly by M_3/IP_3 signaling cascades (Hristov et al., 2014; Parajuli et al., 2015; Parajuli and Petkov, 2013). The BK channel is also modified through auxiliary association with beta regulatory subunits, and the less understood gamma regulatory subunits.

Up to four beta subunits (β1-β4) can associate with each BK channel tetramer, with one β subunit binding to each alpha subunit. Increased β subunit association generally sensitizes BK channel opening kinetics when at elevated Ca^{2+} levels and slows closing kinetics, thus modulating channel gating properties (Brenner et al., 2000b; Gonzalez-Perez and Lingle, 2019; Torres et al., 2014). In the bladder, β1 and β4 are the most abundantly expressed regulatory subunits (Chen and Petkov, 2009; Hristov et al., 2011a; Jiang et al., 1999; Torres et al., 2014). Increased β1 subunit association with the BK channel increases Ca^{2+} sensitivity, decreases voltage sensitivity, prolongs opening bursts, and slows BK channel closing, while β4 exhibits a

biphasic effect on Ca^{2+} sensitivity, reduces voltage sensitivity, and also slows BK channel closing kinetics (Brenner et al., 2000b; Contreras et al., 2012; Gonzalez-Perez and Lingle, 2019; Ha et al., 2004; Li and Yan, 2016; Wang et al., 2006). The presence or absence of β subunits can also impact BK channel sensitivity to pharmacological targeting, with β4 conferring resistance to BK channel blocker, iberiotoxin (Meera et al., 2000).

Ryanodine receptors

Ryanodine-sensitive Ca^{2+} release channels known as ryanodine receptors (RyRs), are homotetrameric, ligand-gated ion channels predominantly expressed in striated and smooth muscle tissues (Chu et al., 1990; Imagawa et al., 1987; Neylon et al., 1995; Otsu et al., 1990) as well as in neurons (Giannini et al., 1995; Hakamata et al., 1992). Within the cell, RyRs are embedded in the membranes of the sarcoplasmic reticulum (SR)/endoplasmic reticula (ER). Cytoplasmic Ca^{2+} binds to RyRs, inducing a "primed" state with a biphasic sensitivity to Ca^{2+} peaking at approximately 10 to 100 μm [Ca^{2+}] (Bezprozvanny et al., 1991). Then ligands (i.e. ATP) bind to and open RyRs to allow Ca^{2+} release from the SR/ER into the cytoplasm (des Georges et al., 2016; Lai et al., 1989; Serysheva, 2004). Ryanodine binds to RyRs at nanomolar concentrations, locking the channel in an open state, whereas at higher concentrations (greater than 100 μm), it inhibits Ca^{2+} release (Meissner, 1986). Each of the three mammalian RyR isoforms (RyR1, RyR2, Ryr3) are detectable in the urinary bladder, and both RyR1 and RyR2 influence the excitation-contraction coupling of the bladder through interactions with the BK channel (Chambers et al., 1999; Fritz et al., 2007; Hotta et al., 2007; Ji et al., 2004).

BK-RyR coupling

As illustrated in Fig. 1, in the bladder, nerve-evoked action potentials trigger release of neurotransmitters (acetylcholine and ATP) which activate muscarinic and purinergic receptors on detrusor smooth muscle. This receptor activation leads to membrane depolarization and Ca^{2+} influx through VDCCs triggering contraction (Herrera et al., 2001; Ohi et al., 2001).

BK channels act as a negative-feedback mechanism to regulate detrusor contraction by promoting membrane hyperpolarization and reducing intracellular calcium levels (Herrera et al., 2001; Ohi et al., 2001). BK channels are activated by increases in intracellular Ca^{2+} and/or membrane depolarization. Once open, they allow for the efflux of K^+, which results in membrane hyperpolarization. This hyperpolarization reduces Ca^{2+} influx through L-type VDCCs, and decreases excitability (Herrera et al., 2001; Ohi et al., 2001). The functional coupling of BK channels with RyRs plays a key role in regulating bladder micromotions, maintaining tone during bladder filling, and modulating sensitivity to nerve evoked contractile stimuli (Hashitani and Brading, 2003; Herrera et al., 2001, 2000; Meredith et al., 2004; Sprossmann et al., 2009; Thorneloe et al., 2005). In the urethra, BK-RyR coupling is similarly involved in maintaining basal tone during bladder filling (Drumm et al., 2018; Feng et al., 2019; Kyle et al., 2013).

RyRs embedded in SR fragments localize near BK channels in detrusor smooth muscle cells (Ohi et al., 2001). While the regulation of BK-RyR colocalization is less studied in myocytes of the lower urinary tract, findings in other tissues support subunit specific interaction, for example β4 subunits promote colocalization of RyR with BK channels in hippocampal neurons of mice (Wang et al., 2016), and pregnancy in sheep increases colocalization of RyR1 and RyR2 with β1 subunits in vascular smooth muscle of uterine arteries (Song et al., 2021). In the bladder, mechanical stretch induces RyR opening and subsequent Ca^{2+} release from the sarcoplasmic

reticulum (Ji et al., 2002). These transient Ca^{2+} events contribute to bladder micromotions that trigger afferent nerve activity, facilitating mechanosensation of bladder filling (Chakrabarty et al., 2019; Drake et al., 2017; Heppner et al., 2016; Tran et al., 2025). The physiological relevance of BK-RyR coupling in maintenance of bladder function is further supported by genetic mouse models. BK channel knockout mice exhibit increased strength and frequency of spontaneous detrusor contractions, and increases in dysfunctional urinary voiding including urine leakage (Herrera et al., 2001, 2000; Jiang et al., 1999; Meredith et al., 2004; Thorneloe et al., 2005). In global RyR1 knockout mice, RyR2 and RyR3 could not compensate for the loss of RyR1 in generating depolarization-induced Ca^{2+} sparks in the detrusor (Fritz et al., 2007). Global RyR2 knockout in mice is embryonic lethal; however, mice with heterozygous RyR2 knockdown are viable and detrusor cells display decreased number of Ca^{2+} spark hot spots when depolarized via voltage clamp (< 20 ms), decreased force development in response to electrical stimulation or direct application of acetylcholine, and decreased BK channel opening frequency, indicating that RyR2 is essential for proper BK channel activation and contractile function (Hotta et al., 2007).

ENVIRONMENTAL IMPACTS ON BK-RYR COUPLING AND FUTURE OPPORTUNITIES

While the etiology of LUTS is complex, there is growing evidence that environmental factors can influence the lower urinary tract and pathophysiology. Below we briefly explore some of these topics and how BK channels and/or RyR signaling pathways could be unexplored areas of considerable interest in improving our understanding of LUTS onset, severity and treatment options.

Halogenated biphenyls and Diphenyl ethers activate RyRs and disrupt intracellular calcium dynamics

RyR signaling disruption is correlated with a wide range of disease pathologies, particularly in cardiac muscle, skeletal muscle, and the nervous system (Kushnir et al., 2018; Lanner, 2012; Sun et al., 2021). With the increased interest in RyR disruption in the etiology of LUTS, two classes of environmental chemicals of concern are polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). Both PCBs and PBDEs are classes of organo-halogenated compounds that have been widely produced and used for industrial and commercial applications, are resistant to environmental degradation, and bioaccumulate in the food chain (Siddiqi et al., 2003). PCBs have been banned for intentional production in the European Union and the USA, however contemporary PCBs are still produced as unintentional byproducts from several industrial processes (Anh et al., 2021). PBDEs have been banned for intentional production EU but remain partially regulated in the USA. Thus PCBs and PBDEs remain a health concern and alarmingly, the PCBs and PBDEs most detected in human samples are also among the most RyR-active (Chen et al., 2017; Kim et al., 2011; Li et al., 2022; Linares et al., 2015; Mouat et al., 2023; Sethi et al., 2019). PCBs and PBDEs are lipophilic and can passively

cross plasma membranes, once in the cytoplasm they can bind to RyRs causing abnormal calcium release (Chen et al., 2017; Gong et al., 2021; Kim et al., 2011; Wayman et al., 2012).

In neurons, RyR regulation of intracellular calcium dynamics is important for controlling neuronal outgrowth and electrical firing. Both PCBs and PBDEs bind to RyRs and disrupt RyR homeostatic activity (Coburn et al., 2008; Sethi et al., 2021). Yet, while PCBs increase dendritic arborization, increase axonal complexity, and disrupt intracellular calcium dynamics (Feng et al., 2017; Griffin et al., 2024; Lein, 2023; Lesiak et al., 2014; Sethi et al., 2021, 2018; Wayman et al., 2012; Wong et al., 1997), PBDEs delay neuronal polarization and axonal outgrowth (Chen et al., 2017; Kim et al., 2011). This difference in PCB and PBDE effects may be due to PCB effects occurring primarily through RyR, while PBDEs also act on IP_3Rs (Gassmann et al., 2014). Perinatal exposure to PCBs and PBDEs are both associated with neurodevelopmental disorders in rodent models (Kozlova et al., 2022; Li et al., 2021; Sethi et al., 2021) and in longitudinal human studies (Herbstman et al., 2010; Hertz-Picciotto et al., 2018; Lyall et al., 2017; Panesar et al., 2020; Vuong et al., 2018). Neurodevelopmental disorders, including autism spectrum disorder, increase the risk of developing lower urinary tract symptoms, yet the mechanism for how this occurs in children or adults has yet to be determined (Gubbiotti et al., 2024, 2019b, 2019a).

In mouse models, developmental exposure to PCBs results in detectable levels of PCBs in offspring bladder (Keil Stietz et al., 2021a), these offspring also display age-, sex-, and dose-dependent changes to urinary voiding physiology, suburothelial nerve density, and sensitivity to intravesical instillation of capsaicin (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021; Lavery et al., 2023; Ridlon et al., 2025a). In addition, adult female mice given a human relevant mixture of PCBs during gestation and lactation have increased bladder contractility in response to

electrical field stimulation ex vivo (Lavery et al., 2023). These studies highlight the ability of these chemicals to alter the bladder and contractility. Whether this occurs via impacts on bladder or urethra RyR and/or BK channel activity is not currently known but plausible and whether other chemicals like PBDEs could target these pathways to alter voiding function is an ongoing area of study.

Endocrine disruption and BK channel activity

Intentional and unintentional changes to endocrine homeostasis are of interest for their impact on LUTS. Hormone therapy is provided to individuals for a variety of treatments including precocious puberty, peri- and post-menopause, birth control, hypogonadism, hair loss, and prostate enlargement (BPH) (Brabaharan et al., 2022; Jarvis et al., 2015; Oesterling, 1994; Yang et al., 2025). Conditions necessitating hormone treatments themselves can be risk factors for LUTS, but if hormone treatment itself impacts LUTS is an ongoing area of study (Al-Zoubi et al., 2022; Baas and Köhler, 2016; Baruch et al., 2023; Bianchi et al., 2021; Kathrins et al., 2016; Yaish et al., 2025). In mice, treatment with testosterone and estradiol to mimic the hormonal changes of aging men, results in enlarged prostate and an obstruction phenotype (Nicholson et al., 2012). Voiding physiology changes are observed in male mice after undergoing castration or treatment with finasteride (to reduce circulating testosterone levels) or when given exogenous testosterone (Ruetten et al., 2019). Similarly, treatment with testosterone in female mice leads to a more masculine voiding phenotype, and administration of finasteride also impacts voiding even in female mice (Ruetten et al., 2019). Evidence also suggests that BK channels are important for mediating effects of testosterone on the lower urinary tract. Testosterone decreases guinea pig detrusor excitability via increased BK channel open probability and BK channels are necessary for mediating effects of testosterone on detrusor smooth muscle resting potential (Hristov et al.,

2016). In vascular smooth muscle, testosterone causes vasodilation in part through activation of BK channels (Lorigo et al., 2020). Estrogens are also important in lower urinary tract function and disease. Estrogen deficiency during and following menopause can lead to urogenital atrophy increasing the risk of urgency urinary incontinence and urinary tract infection (Fait, 2019; Henn, 2010; Su and Freeman, 2009). A recent study from the RISE FOR HEALTH study, conducted by the Prevention of Lower Urinary Tract Symptoms (PLUS) Research Consortium, demonstrates the transition from pre- to perimenopause increases the risk for urgency urinary incontinence, and overall bladder health is worsened in postmenopausal women when undergoing hormone treatment (Vaughan et al., 2025). While this study included a variety of hormone therapies including vaginal estrogen and systemic menopausal hormone therapy, a meta-review of 17 studies reported vulvovaginal topical estrogen therapy improves LUTS in postmenopausal women (Porcari et al., 2025). Ovariectomy in mice, to reduce estrogen levels, leads to mice which display an overactive voiding phenotype which is reversed by estrogen supplementation (Zhang et al., 2024). BK channels are also linked to the actions of estrogens. 17β-Estradiol decreases human bladder smooth muscle cell excitability in part by activating BK channels (Hristov et al., 2017). Estradiol also directly activates BK channels to reduce guinea pig smooth muscle excitability (Provence et al., 2015). 17β-Estradiol directly binds to BK channels in a β-subunit specific manner which increases the channel opening probability primarily in low Ca^{2+} conditions (Behrens et al., 2000; De Wet et al., 2006; Granados et al., 2019; King et al., 2006; Valverde et al., 1999). Additionally, 17β-Estradiol increases BK channel subunit mRNA and/or protein abundance in vascular smooth muscle of sheep (Hu et al., 2011; Nagar et al., 2005) and in neuronal cell lines of mice and humans (Li and Qiu, 2015a; Nishimura et al., 2008). Using

selective agonist and receptor knockdown strategies, these studies confirmed that ERβ mediates the estrogenic regulation of BK channel expression (Li and Qiu, 2015a; Nishimura et al., 2008).

Environmental chemicals can disrupt the endocrine system broadly by impacting steroidogenesis, mimicking steroid hormones, or altering expression of hormone receptors (Evans, 2022). Disruption of steroidogenesis is observed with a wide variety of xenobiotics including PCBs, PBDEs, TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin), and several families of fungicides (Sanderson, 2006; Whitehead and Rice, 2006). Estrogenic xenobiotics are prevalent environmental contaminants of concern, but the effects of estrogen receptor (ER) activation on BK channel expression/function have not been previously studied in the bladder or urethra of any organism. Environmental estrogen mimicking chemicals including bisphenol A (BPA) and some PCBs can bind to ERs and trigger transcriptional activity (Kuiper et al., 1997). Like estrogens, BPA can bind to BK channels and increase opening probability, but unlike estrogens, the binding site for BPA is on an extracellular site of the α-subunit and sensitivity to BPA is increased by the presence of β1-subunits (Rottgen et al., 2014). BPA has also been shown to increase BK channel expression in rat vascular smooth muscle cells (Costa et al., 2025) and decrease BK channel expression in pancreatic beta cells via ERβ (Martinez-Pinna et al., 2019). Whether BPA or PCBs can similarly alter BK channel expression in the bladder, whether this occurs via Erβ and whether this contributes to voiding dysfunction is currently unknown and is an important area for future research. Further, studies have found that the type of beta subunits present in BK channels can alter the response to steroid hormones (King et al., 2006). Whether this also applies to environmental chemicals is unexplored but potentially impactful as it could add another layer of complexity to understanding how individual responses to environmental chemicals are manifested. Together, these findings underscore the need for further research into how

environmental exposures-through modulation of BK and RyR signaling – may contribute to lower urinary tract dysfunction, and highlight these pathways as promising yet underexplored targets for preventive or therapeutic interventions.

LUTS TREATMENT

Pharmacological interventions for LUTS aimed at controlling detrusor activity are limited to targeting three classes of receptors: muscarinic receptors, the β3 adrenoceptors, or the α1 adrenoceptors (Abreu-Mendes et al., 2020). Non-pharmacologic interventions include pelvic floor exercises and neuromodulation for OAB and stress or mixed incontinence (Yamanishi et al., 2015). In cases of benign prostatic hyperplasia (BPH) associated with LUTS (BPH/LUTS), α1- blockers and β3-agonists, 5α-reductase inhibitors, phosphodiesterase type 5 (PDE5) inhibitors, or a combination drug cocktail may be prescribed (Abreu-Mendes et al., 2020; Laborde and McVary, 2009). Antimuscarinic drugs target the muscarinic acetylcholine receptors and improve OAB symptom severity primarily by reducing detrusor smooth muscle contraction (Athanasopoulos and Giannitsas, 2011). While antimuscarinics drugs are generally well tolerated and improve symptom severity, muscarinic receptors are present in tissues outside of the urinary tract, leading to the drugs having side effects including dry mouth, dry eyes, and constipation (Foon and Toozs-Hobson, 2007; Madhuvrata et al., 2012).

The next generation of LUTS treatments, especially when related to dysfunction of the smooth muscle of the lower urinary tract, may focus on BK channels and RyRs, either alone or in combination. However, it is important to note that BK channels and RyRs are also widely expressed outside of the lower urinary tract, and their individual and coupled functions are important in regulating bladder and urethra contractile states during different phases of the micturition cycle. Therefore, when developing therapeutics aimed at modulating BK channels or RyRs, caution must be exercised to minimize off target effects across lower urinary tract tissue and throughout the body to ensure a quality of care similar to other available therapeutics. For example, NS-8 (CAS RN 186033-14-7) was in a proof-of-concept clinical trials (EudraCT

Number: 2005-001250-24) for overactive bladder treatment (120, 180, or 240 mg twice a day orally for 56 days) in men and women through activation of BK channels and other potassium ion channels; however it was discontinued in phase two due to a lack of efficacy in reducing incontinence episodes (Nardi and Olesen, 2008). Likewise, BMS204352 (CAS RN 187523-35-9, Maxipost) was in clinical trials for ischemic stroke treatment (0.3 mg/kg, i.v.) through activation of BK channels and other ion channels; however it was discontinued in phase three due to a lack of efficiency in acute stroke patients (Jensen, 2002) and intravenous administration (two doses of 1 mg over 20 min) causes migraines in healthy volunteers (Al-Karagholi et al., 2021, 2020). Given that BK channels and RyRs function in diverse cell types throughout the body, and their roles during bladder filling and emptying, increasing BK channel or RyR opening probability without cell or tissue specific targeting is unlikely to provide a universal solution to LUTS (Sancho and Kyle, 2021).

Promising results arose from recent studies targeting BK channels and RyRs separately with greater specificity than previous trials. Recently a potential therapeutic compound, the BK channel opener LDD175, improved erectile dysfunction symptoms and LUTS in rats (Yu et al., 2025). URO-902, a gene therapy recently in clinical trials (NCT04211831), that increases BKα expression targeting smooth muscle cells, reportedly improves symptoms of overactive bladder, urgency urinary incontinence, and erectile dysfunction (Andersson et al., 2021; Enemchukwu et al., 2025; Melman et al., 2006). For treatment of men with Fragile X Syndrome, phase two of clinical trials (NCT06413537) was recently completed using the BK channel opener SPG601 (8 capsules of 100 mg on two separate days). Strategies focused on RyR activity are of interest for treatment of pathologies beyond the lower urinary tract including neurodegenerative diseases such as Alzheimer's Disease and heart failure due to cardiac arrhythmias (Lacampagne et al.,

2017; Liu et al., 2012; Sun et al., 2021); However, like environmental disruptors of RyR activity, chronic activation of RyR results in a loss in Ca^{2+} stores and therefore loss of controlled muscle contraction (Andronache et al., 2007; Bellinger et al., 2008). S 48,168 (ARM 210) was recently in phase 1 clinical trials (NCT04141670) for treatment of skeletal myopathies caused by pathogenic variants in the RYR1 genes leading to Ca^{2+} leak from the SR, with treatment (120 mg or 200 mg daily for 29 days) showing promising results to proceed to further trials (Todd et al., 2024).

Therefore, although BK channels or RyRs pose an exciting opportunity for novel therapeutics due to their central role in smooth muscle contractility, therapies must be developed with specificity and caution to avoid systemic side effects. Nonetheless, as we continue to understand more about the biology of these channels, particularly in relation to risk factors such as environmental contaminants that may disrupt their function, there is significant potential for developing targeted therapies for individuals with these pathways disrupted or with significant environmental exposures which could provide improved personalized and effective treatment options.

DECLARATIONS

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Competing interests

The authors declare no competing interests.

FIGURES

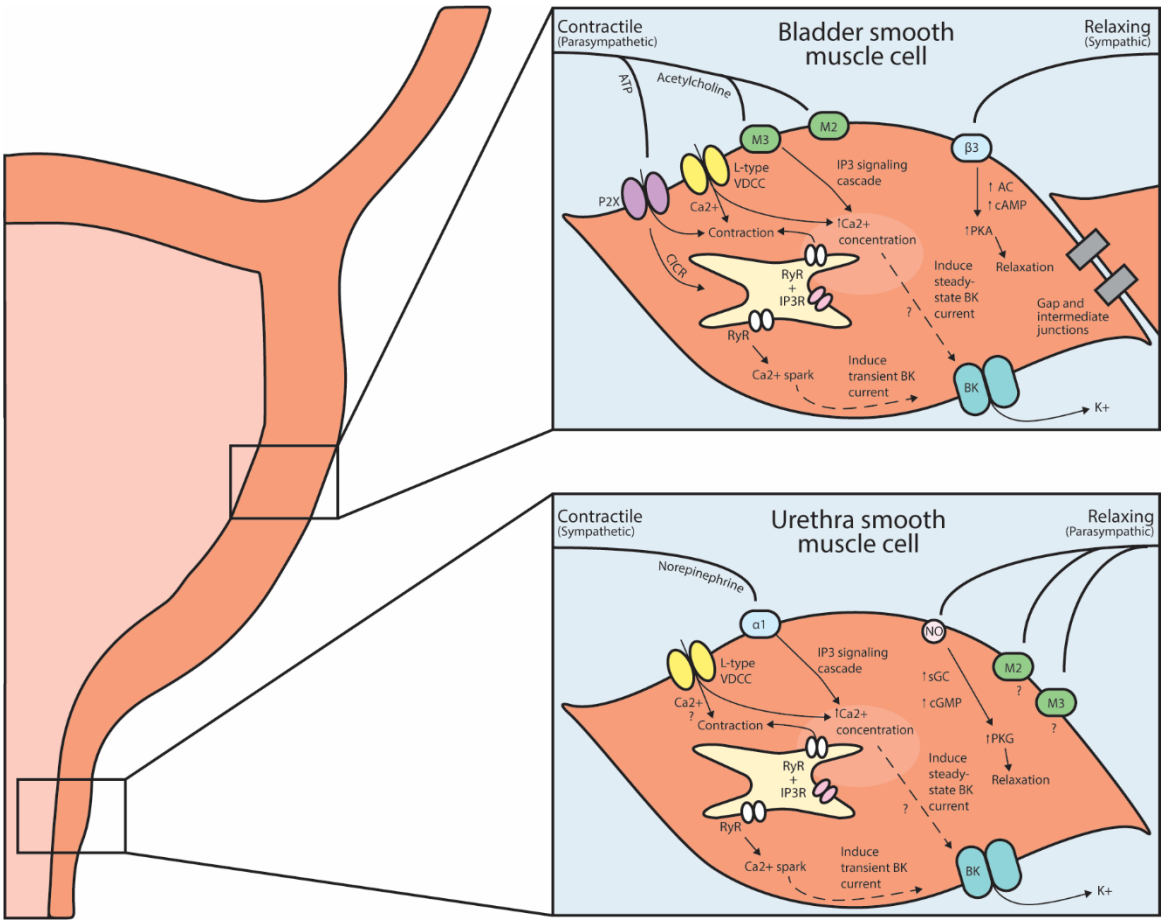

Figure 1. Representation of pathways involved in the contraction and relaxation of bladder and urethra smooth muscle, including relevant neurotransmitters and receptors involved in signaling. ATP: adenosine triphosphate; M_2/M_3: muscarinic receptors; L-type VDCC: L-type voltage dependent calcium channels; P2X: purinergic receptor; RyR: ryanodine receptor; IP_3R: inositol triphosphate receptor; PKA, PKG: protein kinase A, G; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; sGC: soluble guanylate cyclase; cGMP: cyclic guanosine monophosphate; BK: big potassium channel; Ca^{2+}: calcium

CHAPTER II: DEVELOPMENTAL POLYCHLORINATED BIPHENYL (PCB) EXPOSURE IMPACTS ON VOIDING PHSYIOLOGY PERSIST INTO ADULTHOOD AND INFLUENCE SENSITIVITY TO BLADDER STIMULI IN MICE

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ABSTRACT

Polychlorinated biphenyls (PCBs) are toxicants present in the environment, foodstuff, animal and human tissues. PCBs are linked to numerous adverse health effects; however, impacts of developmental PCB exposure on lower urinary tract function are a comparatively newer area of interest. We have previously found developmental exposure (*in utero* and lactational) to a human-relevant PCB mixture in mice leads to sex- and dose- dependent changes to urinary voiding physiology at 6 weeks of age. This study expands upon previous findings to investigate if developmental PCB-induced urinary voiding phenotypes persist or shift as mice age to 12 weeks of age. Urinary voiding physiology testing through void spot assays, uroflowmetry, and cystometry demonstrated several sex- and dose- dependent effects of PCB exposure at 12 weeks of age. Further, patterns of dysfunction were either maintained, newly acquired, or reversed compared to those from younger adult mice in a previous study. Here, developmental PCB exposure decreased number of small urine spots in adult male and female mice in a dose dependent manner, and female mice had more frequent voiding events assessed by anesthetized cystometry. Mice also had PCB dose-dependent changes to urinary voiding physiology when challenged with intravesical capsaicin infusion to target transient receptor potential cation channel subfamily V member 1 (TRPV1)-mediated pathways. PCBs either blocked or exacerbated capsaicin induced responses depending on the endpoint examined, suggesting this pathway may play a role in PCB-dependent changes in voiding. PCBs also had subtle impacts on prostate wet weight, with high PCB doses reducing tissue mass compared to low PCB doses, while none differed from vehicle. This study demonstrates developmental exposure to PCBs continues to impact lower urinary tract function in adulthood to at least 12 weeks of age both during homeostatic conditions and upon challenge of capsaicin. Better understanding of how

early life stressors like PCBs contribute to aging-associated voiding dysfunction are imperative as these findings may help mitigate risk or improve treatment strategies for patients suffering from lower urinary tract symptoms.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous, synthetic, persistent organic pollutants that have been produced in the United States for over a century, and research continues to elucidate potential adverse health outcomes from PCB exposure. Despite the 1979 Toxic Substances Control Act banning intentional PCB production in the United States, studies estimate that in 2020 the United States has retained 1000 metric tons of pure PCBs and 13,755 metric tons of PCB materials in electrical transformers alone (Jahnke and Hornbuckle, 2019; Melymuk et al., 2022). In addition to legacy sources of PCBs produced before the bans, PCBs are unintentionally produced as by-products of manufacturing processes that use chlorine, salts, and hydrocarbons or chlorinated hydrocarbons at high temperatures such as pigment production (Grossman, 2013; Hu and Hornbuckle, 2010). Although the US Environmental Protection agency and US Food and Drug Administration set safety limits to mitigate PCB exposure, legacy and contemporary PCB sources continue to bioaccumulate and biomagnify in animal foodstuff resulting in chronic PCB exposure for humans (Guo et al., 2014; Koh et al., 2015; Melymuk et al., 2022) and the US-based production of contemporary PCBs in 2019 could have surpassed peak production of legacy PCBs in the 1970 s (Megson et al., 2024). In the environment, PCBs are ubiquitous and can be transported throughout air, water, and soil (Anh et al., 2021; Grimm et al., 2015a; Melymuk et al., 2022). In biota, PCBs accumulate in adipose and other fatty tissues with an elimination half-life on the order of months to years (Quinn and Wania, 2012; Ritter et al., 2011). Thus, the adverse outcomes from legacy and contemporary PCB sources continue to be relevant for environmental and human health.

PCBs can transfer to the next generation through developmental exposure via transplacental transfer in utero and through lactational exposure via breast milk (Ando et al., 1985; Björvang et

al., 2021; Eguchi et al., 2018; Jeong et al., 2018; Lancz et al., 2015; Needham et al., 2011). Developmental PCB exposures are hypothesized risk factors for adverse health effects later in life including neurodevelopmental disorders such as autism spectrum disorder (Balalian et al., 2024; Panesar et al., 2020). Several types of neurological dysfunctions and disorders, including autism spectrum disorder, are comorbid with the occurrence of lower urinary tract dysfunction (LUTD) (Gubbiotti et al., 2024, 2019b, 2019a; Moussa et al., 2020). However, a role for PCBs in LUTD etiology alone or in the context of neurological disorders is not well understood. Studying the etiology of LUTD has been historically challenging because the heterogeneity of symptom onset, progression, and severity in patients likely arises from a combination of risk factors which are not fully elucidated (Peterson and Vezina, 2022). This is further complicated by the temporal consideration where challenges to the lower urinary tract in adolescence may predispose or exacerbate the effects of aging and incidence of LUTD later in life (de Wall et al., 2021; Nishii, 2021; Peterson and Vezina, 2022; Suskind, 2017). While pharmacological interventions exist for palliative treatment, the incomplete understanding of LUTD etiology remains a barrier for targeted, more effective treatments (Abreu-Mendes et al., 2020; MacNevin et al., 2022).

In our investigation of risk factors for LUTD, we have identified the bladder as a novel target for PCB effects in rodent models (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021). Using a human-relevant PCB mixture (termed the MARBLES PCB mixture) (Hertz-Picciotto et al., 2018), several developmental PCB exposure effects on mouse offspring have been observed. These include increased suburothelial nerve density (Keil Stietz et al., 2021a), increased bladder $CD45^{+}$ immune cells in ~ 4-week-old offspring (Kennedy et al., 2021); and altered voiding physiology with a phenotype reminiscent of overactive bladder in ~ 6-week-old offspring (Kennedy et al., 2022). These PCB effects were observed in the absence of signs of overt

toxicity, with no changes to body mass, bladder mass, bladder muscle thickness, or urothelium composition (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021). Whether the voiding alterations persist, return to control levels or new phenotypes arise as mice age, is completely unknown and the first goal of this study.

Together this work aims to better define the long-term outcomes of developmental PCB exposure on lower urinary tract function and begin to identify underlying mechanisms. This study fits into our greater understanding of PCB effects on lower urinary tract function by considering the temporal shifts associated with voiding as mice age and how PCBs could impact these processes. We found PCBs induced several sex- and dose- dependent effects on urinary voiding in 12 week old mice, and the overall phenotype is incongruent with previously described effects in younger mice at 6 weeks of age, signifying that PCB effects on urinary voiding are dynamic over time. Additionally, this is the first study to examine whether transient receptor potential cation channel subfamily V member 1 (TRPV1)-mediated pathways are involved in PCB effects on urinary voiding function. TRPV1 is involved in physiological and pathological processes throughout the body, especially in bladder mechanosensation (Storozhuk et al., 2019), making TRPV1-pathway activity of interest as a potential mechanism for PCB action in the bladder. By infusing the bladder with capsaicin, a TRPV1 agonist, during anesthetized cystometry, we reveal several capsaicin responses in voiding which are modified by PCB exposure. Since prostate morphology can impact voiding function (Lee and Kuo, 2017; Lee et al., 2015; Ruetten et al., 2019) and we previously observed PCB effects on prostate tissue mass at 6 weeks of age (Spiegelhoff et al., 2023), these tissues were collected to determine sex-specific effects of PCBs in male mice at this 12 week timepoint. PCBs had subtle impacts on prostate wet weight, with high PCB doses reducing tissue mass compared to low PCB doses, while none differed from vehicle. Together

these results suggest that developmental PCB exposure can influence urinary voiding physiology at least through 12 weeks of age, and TRPV1 dependent pathways may be disrupted. Improving the basic understanding of how early life stressors such as PCBs can contribute to aging-associated urinary voiding dysfunction are imperative as these findings may help mitigate risk or improve treatment strategies for LUTD.

MATERIALS AND METHODS

Animals

All procedures involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Madison Animal Care and Use Committee. Wild type C57BL/6J mice (#000664, Jackson Labs, Bar Harbor, ME), were used in this study. All mice were housed in clear plastic cages containing corn cob bedding and maintained on a 12 h light and dark cycle at 22 ± 2°C. Feed (Diet 2019 (breeder) and 2020x, Teklad, Indianapolis, IN) and water were available ad libitum.

Developmental PCB exposures

The MARBLES PCB mixture was chosen since it is derived from the MARBLES (Markers of Autism Risk in Babies Learning Early Signs) study and represents a contemporary relevant mixture of PCBs found in pregnant women (Hertz-Picciotto et al., 2018) which was characterized for endocrine activity (Sethi et al., 2019) and has been used in prior mouse studies on neurological and urological function (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021; Matelski et al., 2020; Panesar et al., 2020; Sethi et al., 2021; Spiegelhoff et al., 2023). This mix contains each of the following PCBs at the specified percentage: 28 (48.2 %), 11 (24.3 %), 118 (4.9 %), 101 (4.5 %), 52 (4.5 %), 153 (3.1 %), 180 (2.8 %), 149 (2.1 %) 138 (1.7 %), 84 (1.5 %), 135 (1.3 %), and 95 (1.2 %). PCBs were synthesized and authenticated by the Synthesis Core of the University of Iowa Superfund Research Program previously described (Li et al., 2018; Sethi et al., 2019), and mice were dosed as described previously (Kennedy et al., 2022). Briefly, female nulliparous adult mice were dosed orally daily with the MARBLES PCB mixture mixed in organic peanut oil and peanut butter (Spectrum Organic Products, LLC, Melville, NY, Trader

Joe's, Monrovia, CA) or vehicle control (peanut oil and peanut butter only) starting two weeks prior to mating and continuing through gestation and lactation until pups were weaned at postnatal day (P) 21. The 0.1, 1, or 6 mg/kg body weight/day doses and dosing paradigm were chosen because it results in exposed offspring bearing detectable levels of PCBs in bladder and other organs at levels relevant to human health and lead to varied phenotypes in offspring (Kania-Korwel et al., 2017; Keil Stietz et al., 2021a; Sethi et al., 2021; Yang et al., 2009). After weaning, male and female offspring were group housed with same sex and dose littermates. In an effort to reduce and refine animal numbers, mice were generated as part of a larger study (Kennedy et al., 2022), being allowed to age to 12 weeks while some littermates were collected at earlier timepoints. A total of 8–13 mice per group derived from 3 to 7 litters per group were used in this study.

Void spot assay (VSA)

VSA was conducted using best practices as described previously (Kennedy et al., 2022; Wegner et al., 2018). Briefly, mice (84.4 ± 0.7 days old, n = 8–13 mice derived from 3 to 7 litters per group) were acclimated to the testing room for at least 1 h prior to starting. Mice were singly placed into an empty plastic cage with 3 MM chromatography paper (057163E, Fisher Scientific) lining the bottom and had access to food but not water during the 4-hour testing period. Filter papers were allowed to dry and then imaged using a UVP ChemStudio PLUS (Analytik Jena, Jena, Germany) under UV light with 7 s exposure with VisionWorks (Analytik Jena) software. VSA image analysis was performed using the Void Whizzard freely available analysis software designed for Image J using default parameters by an individual blinded to treatment conditions (Wegner et al., 2018).

Uroflowmetry

Uroflowmetry was conducted as described previously using Void Sorcerer open access design and software (Kennedy et al., 2022; Wang et al., 2019). Briefly, uroflowmetry was performed in the same room as VSA testing, and mice (86.1 ± 1.1 days old, n = 8–13 mice derived from 3 to 7 litters per group) were acclimated to the room for at least 1 h prior to testing. Mice were placed into uroflowmetry chambers with access to water but not food during the 4-hour testing period. Uroflowmetry data was analyzed by an individual blinded to treatment conditions. Only urine events that fell without inference from the grid floor bars or without co-occurrence of a fecal event were used in analysis. This resulted in a final n = 7–12 mice derived from 3 to 6 litters per group. To determine a stream rating, a scale from 1 to 3 was used where 1 was a drop pattern void and 3 was a strong stream void. Urine mass was converted to urine volume by dividing the change in urine mass by 1.0046 g/ml. Flow rate was then calculated as change in volume over change in time for urine events.

Cystometry

Anesthetized cystometry was performed essentially as described previously (Kennedy et al., 2022). Briefly, mice (87.5 ± 1.3 days old, n = 8–13 mice derived from 3 to 7 litters per group) were anesthetized with a subcutaneous injection of urethane dissolved in saline (AC32554-0500, Fisher, Waltham, MA) at a dosage of 1.43 g urethane/kg mouse from fresh stock 86 mg/ml. Approximately 30 min following urethane administration, the abdomen was opened and a purse string suture (6–0 Silk, 501180809, Fisher) was placed in the dome of the bladder. PE-50 tubing (NC9140178, Fisher) was used as a catheter and placed into the dome of the bladder using a 25 G 1.5-inch needle. The needle was removed and a purse string suture was tied to secure the catheter and prevent fluid from retrograde leaking from the bladder dome. The abdominal wall

and skin were closed with a suture, and the mouse was placed on a heating pad for ~ 60 min. Mice were then connected to an in-line pressure transducer and infusion pump. Saline was infused at a rate of 0.8 ml/hr, and intravesical pressure was recorded using an MLT844 physiological pressure transducer (ADInstruments, Colorado Springs, CO) connected to an FE221 Bridge Amp (ADInstruments) with a Power lab 2/26 (PL2602) data acquisition system. Cystometrograms were recorded and analyzed using LabChart software (ADInstruments). Recordings were conducted for ~ 1 h to achieve a steady pattern. Immediately following saline infusion, mice were then infused with a fresh stock solution of capsaicin (CAS 404–86-4, Millipore Sigma, Burlington, MA) dissolved in saline (30 μM). While transitioning between the two solutions, the in-line tubing was disconnected from the catheter and was flushed with the 30 μM capsaicin solution to ensure capsaicin solution immediately entered the bladder upon reconnection of the tubing to the catheter. The cystometrogram was recorded for the first 5 consecutive voids during capsaicin infusion. 5 consecutive voids were selected from both saline and capsaicin infusions, analyzed, and averaged per animal by an individual blinded to treatment conditions. Parameters measured from the cystometrograms include void duration, intervoid interval, normalized threshold pressure, normalized maximum void pressure, non-voiding contractions (minimum 5 mmHg pressure change), and compliance (infused volume/change in pressure). If intravesical pressure did not build over the hour and was not recovered with a subsequent repositioning of the catheter, then the mouse was excluded from analysis. Additionally, if there was not a clear threshold pressure, that parameter was not analyzed, resulting in a final n = 5–11 mice derived from 3 to 7 litters per group. If disconnecting and switching to capsaicin resulted in removal of the catheter or failure to elicit voids, then that

animal was excluded from the paired capsaicin comparisons, resulting in a final n = 4–10 mice derived from 3 to 6 litters per group.

The order of testing was void spot assay (VSA), uroflowmetry, and then anesthetized cystometry with first a saline infusion immediately followed by infusion of capsaicin in saline. Testing was carried out at the same time of day; however, cytometry can only accommodate 4 mice per day, so mice were randomly counterbalanced to start in the AM or PM. After cystometry, mice were euthanized with CO_2 and necropsy was immediately performed to collect prostate lobes, seminal vesicle, and testes for wet weight masses from male mice.

Tissue collection

Following CO_2 euthanasia, testes were removed, and seminal vesicle and prostate lobes were micro-dissected. Tissue wet weights were measured immediately after necropsy from n = 4–11 mice derived from 3 to 6 litters per group.

Statistics

Statistics were run using GraphPad Prism software version 10 or R. Data were assessed using mixed effects models to account for litter effects. In Prism this is termed a nested one-way ANOVA (littermates are entered into subcolumns within a treatment group), the same analysis can be conducted in R using the lme function from the nlme package. R was used for paired or repeated measures data since this structure could not be accounted for in nested Prism analyses. Nested one-way ANOVAs were followed by Tukey's multiple comparisons tests. The lme function in R followed by the emmeans package for pairwise comparisons with Tukey adjustment was used to determine PCB effects within urine spot size distribution in male and female mice (Fig. 1B-C). Pairwise analysis of saline and capsaicin infusion during anesthetized

cystometry were limited to mice with endpoints collected in both tests. Each parameter analyzed the effect of 30 μM capsaicin infusion compared to saline infusion within a group using lme function in R (Fig. 4). Descriptive statistics of frequent spotting patterns (Table 1) were assessed with Fisher's exact tests. Statistical tests run on each data set and n values of animal and litter are stated within the figure legends, p values ≤ 0.05 were considered significant and are indicated by asterisks in the figures and reported in the results. Trends were noted only in the text for data in which $p \leq 0.09$. The test statistical details are provided in Supplemental Table 1. Data were assessed for normality using D'Agostino and Pearson test, Anderson-Darling test, Shapiro-Wilk test, Kolmogorov-Smirnov test or QQ plots. If a log or square root transformation improved normality the test was also performed on transformed data and is also included in Supplemental Table 1. For nonparametric paired data (Fig. 4) for which transformation did not restore normality, ranked values were used in the lmer function and are also included in Supplemental Table 1.

RESULTS

PCBs decrease the number of voided small diameter urine spots

Voiding phenotypes are dynamic over the course of aging in mice (Keil et al., 2016). Therefore, it is important to assess voiding function throughout life in response to early life environmental hits. Here we focus on testing voiding function in male and female mice developmentally exposed (*in utero* and lactational) to PCBs and then aged to 12 weeks of age. To examine voiding physiology in a non-invasive manner, we first conducted void spot assay (VSA). Changes to distribution of spotting frequency were limited to small spot sizes 0–0.1 cm2 (Fig. 1A-C, Supplemental Table 1). In male mice, there was a significant decrease in small urine spot sizes in the 1 mg/kg/d PCB group compared to vehicle control and 6 mg/kg/d PCB dose groups (Fig. 1B, $p = 0.02$, $p = 0.03$ respectively). In female mice, there was a significant decrease in small urine spot sizes in the 1 and 6 mg/kg/d PCB dose groups compared to the vehicle control group (Fig. 1C, $p = 0.05$, $p = 0.003$, respectively; the 0.1 mg/kg/d PCB group versus vehicle control group was trending $p = 0.08$). There were no significant differences in the total spot number, total urine area, or the percentage of urine area located in the center or corners of the cage in male or female mice (Fig. 1D-K). A normal effect of aging is for a subset of mice to acquire a ‘frequent’ spotter phenotype (Keil et al., 2016). We assessed this parameter by determining the number of mice that produced a total number of urine spots greater than 50, 100, 200, or 300 (Table 1). No significant changes in proportion of mice with each spot frequency was observed.

Since VSA is unable to discriminate between discrete void events, we also conducted uroflowmetry on 12 week old male and female offspring following developmental PCB exposure. Uroflowmetry parameters of stream rating, void mass, void duration and flow rate were unchanged in male and female mice (Fig. 2, Supplemental Table 1). However, there was a

trend for reduced male stream rating driven by 0.1 mg/kg/d PCB group compared to the vehicle control group (Fig. 2A, $p = 0.09$).

PCBs decrease the time between urinary void events during anesthetized cystometry in female mice

In order to examine voiding physiology in response to bladder filling in a controlled environment, we conducted anesthetized cystometry (CMG) on 12 week old male and female offspring following developmental PCB exposure (Fig. 3, Supplemental Table 1). No changes were observed for either sex regarding void duration, normalized peak pressure, non-voiding contractions, normalized threshold pressure, or compliance (Fig. 3A-D and Fig. 3G-L). In male mice, there were no PCB effects on intervoid interval (Fig. 3E). However, in female mice, intervoid interval was significantly decreased in the 1 mg/kg/d PCB and 6 mg/kg/d PCB groups compared to vehicle control (Fig. 3F, $p = 0.04$, $p = 0.01$ respectively). Therefore, developmental PCB exposure increases the frequency of urinary voiding events of female mice undergoing anesthetized cystometry at 12 weeks of age.

PCBs alter TRPV1-mediated voiding physiology

Since we observed PCB induced changes to urine spot sizes in male and female mice and intervoid intervals in female mice, we sought to test whether PCBs influence bladder sensitivity to filling. To test this, we examined the bladder's response upon challenge with intravesical instillation of capsaicin. Capsaicin is a specific agonist of TRPV1 (transient receptor potential vanilloid 1) receptors which are important for mechanosensation. Fig. 4 presents the results of comparing CMG parameters before (saline) versus after capsaicin (30 μM capsaicin in saline) infusion in the same animals. There were several effects of PCBs on capsaicin responses in a

sex- and dose- dependent manner. There were three parameters, void duration, intervoid interval and non-voiding contractions, which displayed capsaicin effects in only one of the sexes. In male mice, void duration was significantly increased by capsaicin in the vehicle control group (Fig. 4A, $p = 0.004$, Supplemental Table 1) but not in any PCB groups (though a trend was noted for an effect in the 1 and 6 mg/kg/d PCB groups, $p = 0.08$), nor was this parameter altered in female mice (Fig. 4B). Likewise, nonvoiding contractions were significantly decreased by capsaicin in the vehicle control group (Fig. 4K, $p = 0.01$) but not in any PCB groups (though a trend was noted for an effect in the 1 mg/kg/d PCB group, $p = 0.07$), nor was this parameter altered in female mice (though a trend was noted for an effect in the 0.1 mg/kg/d PCB group, $p = 0.07$, and the 1 mg/kg/d PCB group, $p = 0.09$) (Fig. 4L). Conversely, male mice showed no changes in intervoid interval (Fig. 4C), while in female mice intervoid interval was decreased by capsaicin only in the 0.1 mg/kg/d PCB group (Fig. 4D, $p = 0.05$). Several parameters had effects of capsaicin in both males and females that varied by PCB dose. In male mice, normalized threshold voiding pressure was decreased by capsaicin in the vehicle control group (Fig. 4E, $p = 0.007$), but not in any of the PCB groups. In female mice, this parameter was also decreased by capsaicin in the vehicle control group, 0.1 mg/kg/d, 6 mg/kg/d PCB groups (Fig. 4F, $p = 0.004$, $p = 0.03$, $p = 0.01$ respectively) but not the 1 mg/kg/d PCB group. In male mice, maximum voiding pressure was increased by capsaicin only in the 1 mg/kg/d and 6 mg/kg/d PCB groups (Fig. 4I, $p = 0.05$, $p = 0.007$) (though a trend was noted for an effect in the vehicle control group, $p = 0.07$), while in female mice capsaicin decreased maximum voiding pressure only in the 6 mg/kg/d PCB group (Fig. 4J, $p = 0.03$). In male mice capsaicin also significantly increased compliance in the 1 mg/kg/d PCB group (Fig. 4G, $p = 0.02$) (though a trend was noted for an effect in the vehicle control group and 6 mg/kg/d PCB group both $p = 0.08$), while in female mice capsaicin increased

compliance in the vehicle control group and 6 mg/kg/d PCB groups only (Fig. 4H, $p = 0.05$, $p = 0.003$ respectively). These results indicate that capsaicin infusion alone induces a measurable effect on bladder sensitization, and PCBs are capable of influencing this response in a sex- and dose- dependent manner, both blocking capsaicin responses seen in vehicle control animals and enhancing responses not otherwise seen in vehicle control animals. Table 2 summarizes all significant changes in voiding parameters.

PCBs decrease ventral prostate mass

Since we previously observed in younger (6 weeks of age) male mice that developmental PCB exposure led to a decrease in ventral prostate mass in the 1 mg/kg/d PCB dose group compared to the 0.1 mg/kg/d and 6 mg/kg/d PCB dose groups only (Spiegelhoff et al., 2023), we wanted to confirm whether changes in prostate persist to 12 weeks of age or could be responsible for any of the male voiding phenotypes observed. In this study, at 12 weeks of age, body mass of male and female mice was unchanged across all dose groups (Fig. 5A-B). Anterior prostate mass and dorsal prostate mass were unchanged both as raw wet weights and when normalized to body mass (Fig. 5C-D, G-H). Ventral prostate mass of both the 1 mg/kg/d and 6 mg/kg/d PCB groups were significantly decreased compared to the 0.1 mg/kg/d PCB group male mice both when comparing raw wet weight (Fig. 5E, $p = 0.01$, $p = 0.03$ respectively) and when normalized to body mass(Fig. 5F, $p = 0.01$, $p = 0.03$ respectively). Aside from changes in prostate mass, changes in wet weight to testes and seminal vesicle can also be influenced by hormone disruption. There were no significant differences in testes wet weight alone or normalized to body mass (Fig. 5I-J), a trend was noted for testes mass alone and normalized to body mass driven by the 0.1 mg/kg/d PCB group versus 1 mg/kg/d PCB group (Fig. 5I, $p = 0.08$, Fig. 5J, $p = 0.06$). Seminal vesicle mass was not significantly altered (Fig. 5K), but a trend was noted for a

decrease in the 6 mg/kg/d PCB group compared to the 0.1 mg/kg/d PCB group when normalized to body mass (Fig. 5L, $p = 0.06$). Together, these data indicate that the higher doses of PCBs tend to have reduced wet weight of steroid hormone sensitive tissue compared to the low dose of PCBs. While PCB induced changes to ventral prostate tissue mass was reserved to between PCB groups and not the vehicle control, it suggests that different doses of PCBs may trigger different effects on steroid hormone dependent endpoints.

DISCUSSION

This study investigated persistence of adult urinary voiding dysfunction resulting from developmental exposure to MARBLES PCBs. Previous work demonstrated developmental exposure to MARBLES PCBs results in sex- and dose- dependent voiding dysfunction, generally characterized as more frequent, drop-like voids ((Spiegelhoff et al., 2023). Here we investigated whether detectable changes in voiding physiology persisted to 12 weeks of age, 9 weeks removed from the last possible lactational PCB exposure (summarized in Table 2). We demonstrate developmental PCB exposure is sufficient to decrease small urine spot sizes and intervoid interval in a sex- and dose-dependent manner. Strikingly, we provide evidence that upon subsequent challenge with capsaicin (TRPV1 agonist/desensitizer) PCB exposed mice do not respond similarly to vehicle control, suggesting subtle PCB induced changes in voiding physiology − which were not necessarily apparent under saline cystometry conditions − were revealed when hit with a second challenge. This is important as changes to voiding through normal aging or response to additional stressors later in life could be influenced by developmental PCB exposures.

Urinary voiding physiology is dynamic and can be influenced by genotype, age, sex, and xenobiotic exposures (Bjorling et al., 2015; Keil et al., 2016; Ruetten et al., 2019). This study illustrates the persistence of voiding dysfunction into adulthood following developmental MARBLES PCB exposure, highlighting the dynamic nature of urinary voiding abnormalities over time. While the PCB exposure paradigm is the same between the current study and a previous study of younger mice (Kennedy et al., 2022), the 6 week difference in age coincides with sexual maturity. Age is expected to shift baseline metrics of voiding phenotypes in both sexes (Bjorling et al., 2015; Keil et al., 2016). Therefore, this may also impact the maximum

influence of PCBs as animals age. Two major differences in VSA outcomes were observed when comparing the effects of developmental PCB exposure between the previous 6 weeks of age (Kennedy et al., 2022) and current study (12 weeks of age) – urine spot count and size. At 6 weeks of age, PCBs increased frequent spotting in 0.1 mg/kg/d and 6 mg/kg/d PCB male mice and in 0.1 mg/kg/d PCB female mice, and PCBs increased number of small urine spots (0–0.1 cm^2) in 0.1 mg/kg/d and 6 mg/kg/d PCB male mice and all doses in female mice (Kennedy et al., 2022). In this study, no PCB effects on frequent spotting were observed. The number of small spots was not increased, rather fewer small urine spots were observed in the 1 mg/kg/d PCB male mice and the 1 and 6 mg/kg/d PCB group female mice when compared to vehicle controls. Since the quantity of urine spots in untreated wild type mice increases with age (Bjorling et al., 2015; Keil et al., 2016), these observed changes suggest two possibilities. 1) PCBs may have a protective effect on age associated increases in small urine spots, or 2) because PCBs increase small urine spots earlier in life, the difference with aging is masked in PCB exposed mice. The drop like stream of 0.1 mg/kg/d PCB male mice at 6 weeks of age undergoing uroflow (Kennedy et al., 2022) was not as severe at 12 weeks of age, though the same trend persisted. Anesthetized cystometry also demonstrated a shift in PCB effects over time. In male mice at 6 weeks of age, PCBs increased max void pressure in 0.1 mg/kg/d and 1 mg/kg/d PCB groups versus vehicle control (Kennedy et al., 2022). This was no longer observed at 12 weeks of age. In female mice at 6 weeks of age PCBs decreased intervoid interval at 0.1 mg/kg/d and 6 mg/kg/d PCB doses compared to the vehicle control group (Kennedy et al., 2022). Here, at 12 weeks of age, decreased intervoid interval in female mice was retained in the 6 mg/kg/d PCB group, no longer observed in the 0.1 mg/kg/d PCB group, and newly observed in the 1 mg/kg/d PCB group. These CMG results suggest developmental PCB exposure produces more severe effects in female mice

versus male mice at 12 weeks of age. Overall, results of the three voiding assays clearly underline that PCBs continue to impact voiding at 12 weeks of age, generally resulting in fewer small urine spots in both sexes, and more frequent voiding in female mice (summarized in Table 2). Furthermore, by comparing PCB effects on voiding here at 12 weeks of age to previously reported mice at 6 weeks of age, this study demonstrates PCB effects on voiding can converge, diverge, or persist over the course of normal aging.

PCB effects on TRPV1-mediated voiding activity to our knowledge has never been studied. TRPV1 pathway signaling is involved in physiological and pathological processes throughout the body, especially bladder mechanosensation (Storozhuk et al., 2019). TRPV6, another calcium channel in the TRPV family, is implicated in PCB effects on skeletal development in zebrafish and rats (Ju et al., 2012; Ronis et al., 2020) and cytotoxicity in the osteoblast cell line MC3T3-E1 (An et al., 2012). Intravesical infusion of capsaicin is useful for studying TRPV1-mediated urinary voiding physiology; Capsaicin specifically binds to and activates TRPV1, sensitizing C-type afferent nerves which are generally silent in homeostatic conditions, causing a period of increased bladder sensitivity and subsequent refractory period of desensitized tachyphylaxis (Avelino et al., 2002; Fischer et al., 2020; Mohapatra and Nau, 2005; Sanz-Salvador et al., 2012; Smutzer and Devassy, 2016; Tian et al., 2019; Yoshimura and Chancellor, 2003). Interestingly, developmental PCB exposure is sufficient to alter responses to capsaicin which are sex- and dose-dependent (summarized in Table 2). In male mice PCB exposure at all doses prevented any capsaicin induced change that was seen in vehicle control mice on void duration, threshold pressure, and non-voiding contractions. Newly acquired sensitivity to capsaicin (not seen in vehicle control) was observed in 1 mg/kg/d and 6 mg/kg/d PCB male mice with capsaicin induced increases in max void pressure, and in 1 mg/kg/d PCB male mice with capsaicin induced

increases in compliance. In female mice, the 1 mg/kg/d PCB exposure prevented any capsaicin induced change seen in vehicle control on threshold pressure and compliance. The 0.1 mg/kg/d PCB exposure also prevented capsaicin induced increases in compliance. Newly acquired sensitivity to capsaicin (not seen in vehicle control), was observed in the 0.1 mg/kg/d and 6 mg/kg/d PCB female mice with capsaicin induced decreases in intervoid interval and max void pressure respectively. Looking across all parameters during capsaicin challenge, a capsaicin effect was never observed in male 0.1 mg/kg/d PCB groups or female 1 mg/kg/d PCB groups (Table 2) suggesting these doses produce a capsaicin resistant/insensitive phenotype. Together these data demonstrate that PCBs are capable of inducing sex- and dose-dependent changes to sensitivity to capsaicin, enhancing or eliminating the sensitivity, suggesting that capsaicin sensitive pathways may be important targets mediating developmental PCB exposure on voiding function. With the ability of capsaicin to both sensitize and desensitize bladder TRPV1 receptors (Avelino et al., 2002; Fischer et al., 2020; Mohapatra and Nau, 2005; Sanz-Salvador et al., 2012; Smutzer and Devassy, 2016; Tian et al., 2019; Yoshimura and Chancellor, 2003), it is not surprising that PCB effects could prime the bladder to either be more sensitive or less sensitive to capsaicin. Since treatment with capsaicin or other agonists of this pathway are used in humans to desensitize C-fiber afferents thus reducing overactive bladder symptoms, whether PCBs can influence response to this treatment strategy is a future area of study (Andersson et al., 2022; Charrua et al., 2009; Liu et al., 2014). Overall, TRPV1-mediated signaling is directly and/or indirectly altered under this PCB dosing paradigm, which could underlie perturbed voiding physiology in 12 week old mice. Assessing this pathway further by quantifying expression, using TRPV1 knockout mice and determining whether TRPV1 in urothelium or sensory nerves are important in mediating PCB effects are areas of future study.

Hormone induced changes to prostate size are implicated in voiding dysfunction in mice, typically with increased prostate mass attributed to obstruction, large bladder volumes, and small voided urine volumes (De Falco and Laforgia, 2021; Nicholson et al., 2018; Turco and Vezina, 2023). Given the absence of increased prostate mass (Fig. 5), prostate enlargement and bladder outlet obstruction are unlikely contributing to altered voiding in male mice seen here. Instead, we found males had decreased ventral prostate mass in the 1 mg/kg/d and 6 mg/kg/d PCB groups compared to the 0.1 mg/kg/d PCB group but not compared to vehicle control. The difference among PCB groups in prostate size was more extensive at this age compared to younger mice, which only showed a decrease in ventral prostate mass in the 1 mg/kg/d PCB group compared to the two other PCB groups (Spiegelhoff et al., 2023). While we did not examine prostate histology in this study, in the previous study of mice at 6 weeks of age there were no observed developmental PCB effects on prostate smooth muscle thickness, caspase 3 positive cells, Ki67 positive cells, bladder mass or bladder thickness (Kennedy et al., 2022; Spiegelhoff et al., 2023). While we cannot rule out these changes in the current study it is unlikely that these would arise in mice at 12 weeks of age when they were not present at 6 weeks of age (Kennedy et al., 2022; Spiegelhoff et al., 2023). Collecting prostate in PCB exposed mice at a younger timepoint could help to determine whether PCB induced reductions in prostate size occur early in development and are then maintained throughout adulthood or whether any histological changes are evident. Although the MARBLES PCB mixture itself has not yet been tested for pro- or anti-estrogenic or androgenic activity, individual congeners in the MARBLES PCB mix can mimic sex steroid hormones and disrupt hormone receptor dependent signaling (Bonefeld-Jørgensen et al., 2001, p. 2005; Plíšková et al., 2005; Sechman et al., 2016; Tam et al., 2022; Wang et al., 2021). Further, anogenital distance (an endocrine responsive endpoint) in postnatal day 7 male mice was found

to be decreased following developmental exposure to MARBLES PCB at the 0.1, 1 and 6 mg/kg PCB groups vs vehicle control (Matelski et al., 2020). Since the prostate, seminal vesicle, and testes are sensitive to changes in circulating hormones and endocrine disruption by exogenous chemicals, it is plausible that PCBs decrease steroid hormone levels leading to reduction in tissue size (Bianco et al., 2002; De Falco and Laforgia, 2021; Nicholson et al., 2018; Ruetten et al., 2019; Turco and Vezina, 2023). Endocrine disruption can both decrease seminal vesicle and prostate lobe masses, and drive changes in voiding (Bianco et al., 2002; Ruetten et al., 2019). Androgens and estrogens, especially 5α-dihydrotestosterone and estradiol-17β, are both implicated in prostatic proliferation and maintenance (Nicholson et al., 2012; Nicholson and Ricke, 2011; Wynder et al., 2015), and exogenous endocrine disruptors including PCBs may interfere with these pathways (Bianco et al., 2002; De Falco and Laforgia, 2021; Nicholson et al., 2018; Turco and Vezina, 2023). Finasteride, a type II 5α-reductase inhibitor, inhibits the transformation of testosterone to the more potent 5α-dihydrotestosterone, and finasteride treatment results in a 25 % reduction in total prostate volume in men with benign prostatic hyperplasia (Kaplan et al., 2008); PCBs have been shown to decrease 5α-reductase RNA and protein expression in rat testes (Thangavelu et al., 2018; Yamamoto et al., 2005), and reduce 5α-reductase activity in LNCaP cells (Endo et al., 2003). Likewise, activation of estrogen receptor beta (ER-β) inhibits prostate proliferation (Nicholson and Ricke, 2011; Wynder et al., 2015). PCBs and their hydroxylated metabolites can bind ER-β and induce estrogen receptor-mediated transcriptional activity (Bonefeld-Jørgensen et al., 2001; Kuiper et al., 1998; Tam et al., 2022). These are two examples of how changes to endocrine signaling could result in decreased prostatic tissue mass, and the potential for PCBs to interact with these mechanisms either early in development or in adulthood to alter lower urinary tract function is an ongoing area of study.

Taken together, this study demonstrates that urinary voiding dysfunction following developmental PCB exposure persists to at least 12 weeks of age and suggests a possible role for TRPV1 dependent mechanisms in mediating PCB effects on voiding. While changes in prostate mass were only seen between PCB groups and not vehicle control, this also highlights that low and high doses of PCBs may have divergent mechanisms in relation to endocrine disruption. This work highlights the importance of ongoing investigations into PCBs' and other xenobiotics' roles in the etiology of lower urinary tract symptoms throughout life, especially in elucidating mechanisms which may lend to identifying susceptible populations disproportionately affected by these toxicant exposures or improving therapeutic treatments. The MARBLES PCB mix was used here since it represents the top PCBs found in a contemporary cohort of pregnant women, thus these PCBs are present in these women and their offspring have been exposed (Hertz-Picciotto et al., 2018; Sethi et al., 2019). Whether PCBs impact the voiding phenotypes of individuals directly exposed through the environment, or if PCBs can predispose to LUTD from developmental exposure (in utero and lactational), remains to be determined. This study provides an area of opportunity; future work may assess voiding function in humans with known PCB exposure, alone or in combination with epidemiology studies focused on measuring PCB concentrations in human tissues. Further, examining PCB concentrations in patients with well documented lower urinary tract dysfunction could provide useful in determining risk factors for LUTD, and may provide insight for developing possible future treatment options for patients.

DECLARATIONS

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

TABLES

	Male				Female			
mg/kg/d PCB	**0**	**0.1**	**1**	**6**	**0**	**0.1**	**1**	**6**
Frequent spotters (50+)	80%	100%	100%	100%	38.5%	46%	20%	37.5%
Non-frequent spotter	20%	0%	0%	0%	61.5%	54%	80%	62.5%
Frequent spotters (100+)	80%	87.5%	81.8%	90%	23%	15.4%	20%	0%
Non-frequent spotter	20%	12.5%	18.2%	10%	77%	84.6%	80%	100%
Frequent spotters (200+)	60%	62.5%	36.4%	50%	15.4%	7.7%	0%	0%
Non-frequent spotter	40%	37.5%	63.6%	50%	84.6%	92.3%	100%	100%
Frequent spotters (300+)	50%	50%	27.3%	50%	7.7%	0%	0%	0%
Non-frequent spotter	50%	50%	72.7%	50%	92.3%	100%	100%	100%

Table 1. Urine spotting frequency unchanged by developmental PCB exposure in either sex. Developmental PCB exposure did not alter the distribution of a frequent spotter phenotype. Mice were developmentally exposed to PCBs via the dam and VSA testing conducted on adult male and female mice. Total number of urine spots were analyzed at each cutoff value and the number of mice in each category was assessed using Fischer's exact test, no significant differences from vehicle control were found. Results are from n = 8–11 male mice per group, n = 8–13 female mice per group derived from 3 to 7 litters per group.

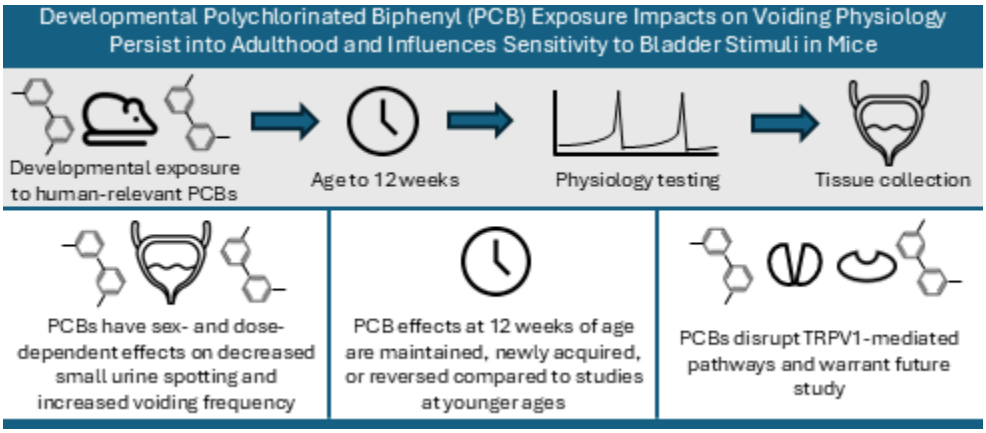
Figure	Panel	Values	Post hoc p values
1	B	Full model fixed effects of relevant comparisons: 0mg v 0.1mg t(17)=-0.7, P=0.5; **0mg v 1mg t(17)=-3.2, P=0.005**; 0mg v 6mg t(17)=-0.2, P=0.8; All other spot sizes were significantly different than smallest spots, **t(245)=-10.7 to -7, p=0.0000**	***0-0.1cm spots: 0 vs 1 P=0.02; 1 vs 6 P=0.03***
1	C	Full model fixed effects of relevant comparisons: **0mg v 0.1mg t(18)=-2.6, P=0.02**; **0mg v 1mg t(18)=-2.8, P=0.01**; **0mg v 6mg t(18)=-4.2, P=0.005**. All other spot sizes were significantly different than smallest spots, **t(245)=-8.4 to -8, p=0.0000**	***0-0.1cm spots:*** *0 vs 0.1 P=0.08****; 0 vs 1 P=0.05; 0 vs 6 P=0.003***
1	D	F(3,17) = 0.6, P = 0.6	
1	E	F(3, 17) = 0.9, P = 0.5	
1	F	F(3, 17) = 1.7, P = 0.2	
1	G	F(3, 17) = 1.3, P = 0.3	
1	H	F(3, 18) = 0.5, P = 0.7 log transform F(3,18)=0.3, P=0.8	
1	I	F(3, 18) = 1.1, P = 0.4	
1	J	F(3, 18) = 1.4, P = 0.3 sqrt transform F(3,18)=0.9, P=0.5	
1	K	F(3, 18) = 0.09, P = 1	
2	A	F(3, 33) = 2.2, P = 0.1	*0 vs 0.1 P=0.09*
2	B	F(3, 33) = 1.7, P = 0.2	
2	C	F(3, 17) = 1.8, P = 0.2 log transform F(3,17)=2.5, P=0.1	
2	D	F(3, 17) = 1.0, P = 0.4	
2	E	F(3, 16) = 1.0, P = 0.4	
2	F	F(3, 37) = 0.3, P = 0.8 log transform F(3,37)=0.1, P=1	
2	G	F(3, 37) = 0.4, P = 0.8	
2	H	F(3, 37) = 0.2, P = 0.9	
3	A	F(3, 30) = 0.8, P = 0.5	
3	B	F(3, 18) = 0.5, P = 0.7	
3	C	F(3, 31) = 1.2, P = 0.3	
3	D	F(3, 18) = 2.1, P = 0.1	
3	E	F(3, 15) = 1.1, P = 0.4	
3	F	***F(3, 36) = 4.2, P = 0.01***	***0 vs 1 P=0.04; 0 vs 6 P=0.01***
3	G	F(3, 15) = 0.7, P = 0.6 sqrt transform F(3,15)=0.7, P=0.6	
3	H	F(3, 18) = 0.04, P = 1	
3	I	F(3, 15) = 1.1, P = 0.4	
3	J	F(3, 18) = 0.7, P = 0.5	

3	K	F(3, 15) = 0.8, P = 0.5	
3	L	F(3, 18) = 1.1, P = 0.4 log transform F(3,18)=0.5, P=0.7	
4	A	***0mg t(6)=-4.5, P=0.004***; 0.1mg t(3)=-0.4, P=0.7; *1mg t(7)=-2.0, P=0.08; 6mg t(9)=-2.0, P=0.08*	
4	B	0mg t(6)=0.6, P=0.6; 0.1mg t(5)=0.07, P=0.9; 1mg t(5)=0.3, P=0.8; 6mg t(7)=-0.7, P=0.5	
4	C	0mg t(6)=1.4, P=0.2; 0.1mg t(3)=-2.0, P=0.1; 1mg t(7)=-0.7, P=0.5; 6mg t(9)=-0.1, P=0.9 : 0.1mg nonparametric t(3)=-1.2, P=0.3	
4	D	0mg t(8)=1.6, P=0.2; **0.1mg *t(6)=2.4, P=0.05***; 1mg t(5)=-0.4, P=0.7; 6mg t(7)=1.4; P=0.2	
4	E	***0mg t(6)=4.1, P=0.007***; 0.1mg t(3)=1.1, P=0.4; 1mg t(7)=0.7, P=0.5; 6mg t(9)=0.8, P=0.4	
4	F	***0mg t(6)=4.6, P=0.004***; ***0.1mg t(5)=2.9, P=0.03***; 1mg t(5)=0.5, P=0.6; **6mg *t(7)=3.4, P=0.01:*** log transform 1mg t(5)=0.5, P=0.6	
4	G	*0mg t(6)=-2.1, P=0.08*; 0.1mg t(3)=-0.9, P=0.4; **1mg *t(7)=-2.9, P=0.02;*** *6mg t(9)=-2.0, P=0.08*	
4	H	***0mg t(6)=-2.5, P=0.05***; 0.1mg t(5)=0.6, P=0.5; 1mg t(5)=-1.4, P=0.2; **6mg t(*7)=-4.5, P=0.003:*** log transform 0.1mg t(5)=-0.6, P=0.6	
4	I	*0mg t(6)=-2.2, P=0.07*; 0.1mg t(3)=-2.0, P=0.1; **1mg *t(7)-2.4, P=0.05; 6mg t(9)=-3.4, P=0.007***	
4	J	0mg t(8)=0.2, P=0.8; 0.1mg t(6)=0.05, P=1; 1mg t(5)=0.6, P=0.6; **6mg *t(7)=2.7, P=0.03***	
4	K	***0mg t(6)=2.6, P=0.04***; 0.1mg t(3)=-1.7, P=0.2; 1mg *t(7)=-2.1, P=0.07*; 6mg t(9)=1.2, P=0.2: nonparametric ***0mg t(6)=3.5, P=0.01***, 0.1mg t(3)=-1.7, P=0.2, 6mg t(11.9)=-0.2, P=0.9	
4	L	0mg t(8)=-0.7, P=0.5; 0.1mg *t(5)=-2.3, P=0.07*; 1mg t(5)=-1.3, P=0.2; 6mg t(7)=0.4, P=0.7 nonparametric *1mg t(7)=-2, P=0.09*	
5	A	F(3, 17)=0.7, P=0.6	
5	B	F(3, 18) = 2.3, P = 0.1	
5	C	F(3,15)=0.5, P=0.7	
5	D	F(3,15)=0.5, P=0.7	
5	E	***F(3, 15)=5.4, P=0.01***	***0.1 vs 1 P=0.01; 0.1 vs 6 P=0.03***
5	F	***F(3, 15)=5.1, P=0.01***	***0.1 vs 1 P=0.01; 0.1 vs 6 P=0.03***
5	G	F(3, 14)=0.07, P=1	
5	H	F(3, 14)=0.06, P=1	
5	I	F(3,31)=2.3, P=0.1	*0.1 vs 1 P=0.08*

5	J	*F(3, 31)=2.5, P=0.08*	*0.1 vs 1 P=0.06*
5	K	F(3, 14)=2.2, P=0.1	
5	L	*F(3, 14)=2.7, P=0.08*	*0.1 vs 6 P=0.06*

Table S1. Test statistics of mixed effects models which account for litter effects. Bold and italic indicate p values of <0.05, trends are indicated by italics only at $p < 0.09$. If tranformation helped normality, that is noted and the corresponding test statistics included.

FIGURES



Graphic 1. Graphical abstract of the main takeaways for chapter 2.

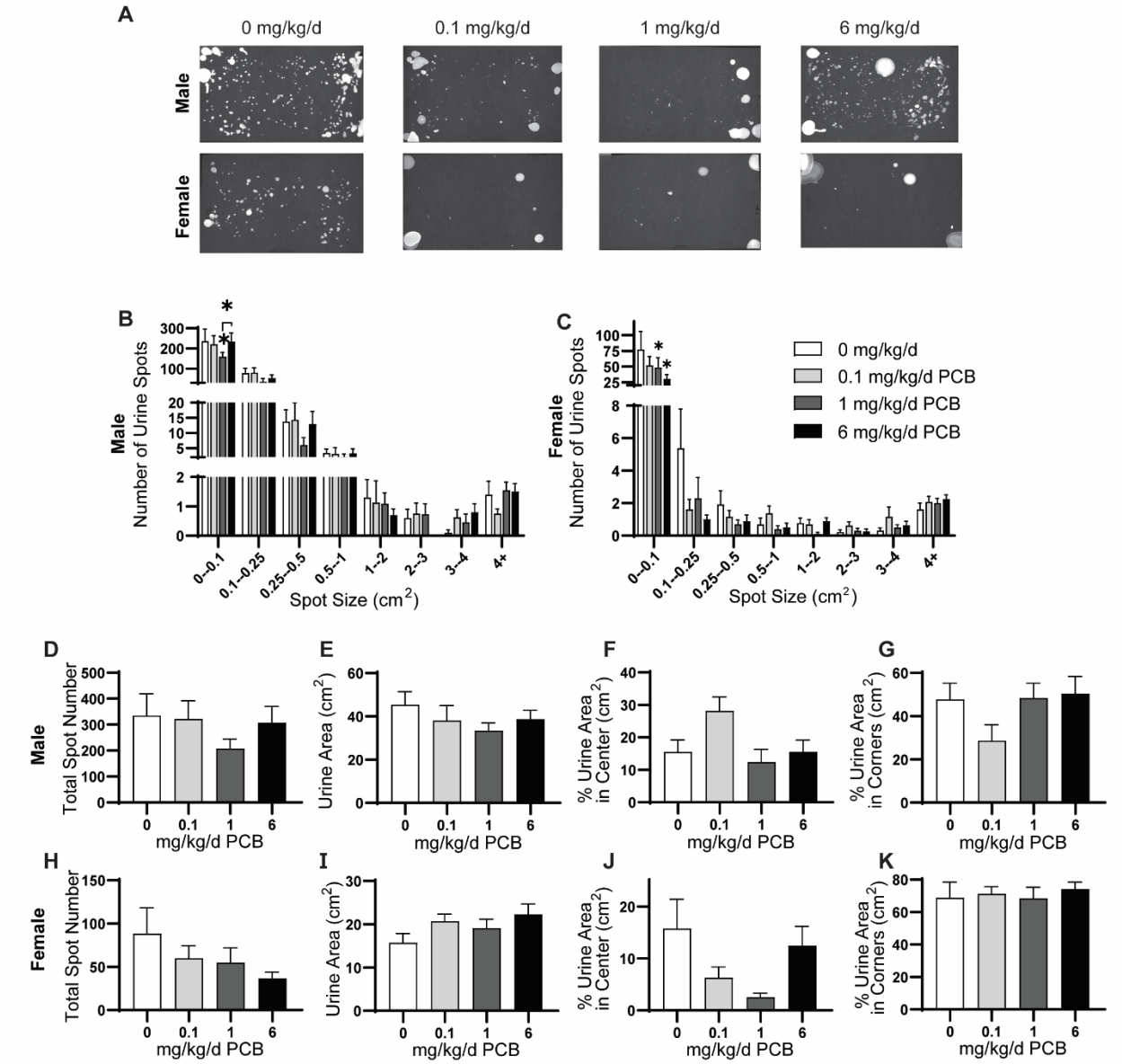


Figure 1. Developmental PCB exposure decreased the number of small 0–0.1 cm^2 spots in male and female offspring while undergoing VSA. Mice were developmentally exposed to PCBs via the dam and VSA testing conducted on adult male and female mice. (A) Representative images of VSA for both male and female PCB dosage groups, paper length 16.5 x 27 cm. Parameters examined following the 4-hour VSA include for male and female include; (B,C) urine spot size distribution, (D,H) total urine spot number, (E,I) total urine area, (F,J) percent of the urine area in

the center of the paper and (G,K) percent of the urine area in the corners of the paper. Results are mean ± SEM n = 8–11 males derived from 4 to 6 litters per treatment group, n = 8–13 females derived from 3 to 7 litters per treatment group. * indicates significant difference from vehicle control. Bar and * indicate significant differences between dosage groups. $p \leq 0.05$ were considered significant as determined by mixed effects models to account for litter: (B,C) Mixed effects model (R code: m = lme(Value ~ Treatment * Spot, random = ~ 1| Litter/Animal, data = data) followed by pairwise comparisons with Tukey adjustment (R code: c = emmeans(m, ~ Treatment | Spot); effects = pairs(c), (D-K) Nested one-way ANOVA to account for litter followed by Tukey's multiple comparisons test.

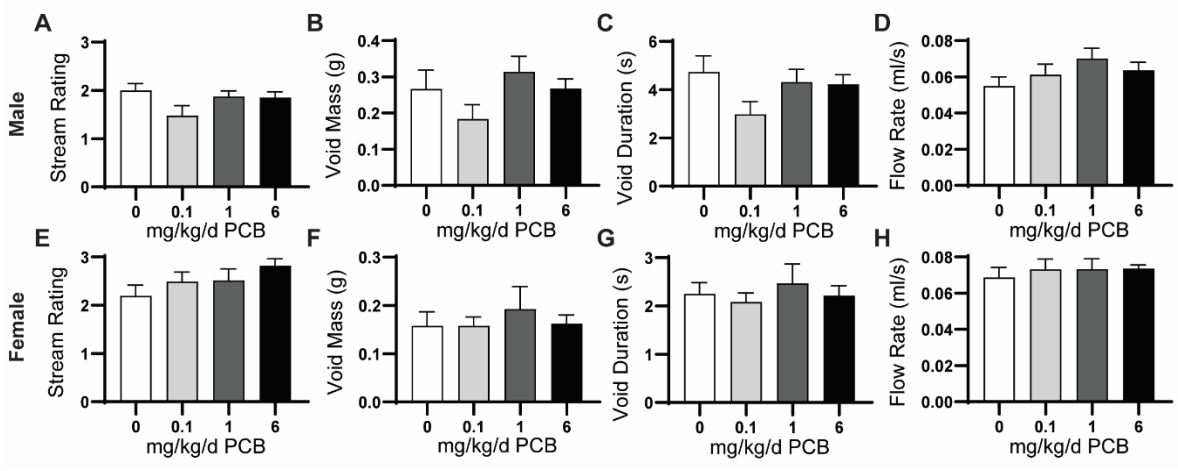


Figure 2. Developmental PCB exposure did not alter urine flow rating, mass, duration, or flow rate while undergoing uroflowmetry. Mice were exposed to PCBs via the dam and uroflowmetry testing conducted on young adult male and female mice. Examined parameters include (A,E) urine stream rating, (B,F) void mass (g), (C,G) void duration (s), and (D,H) flow rate (mL/s). Results are mean ± SEM n = 8–10 males derived from 4 to 6 litters per treatment group, n = 7–12 females derived from 3 to 6 litters per treatment group. No significant differences, $p \leq 0.05$ as determined by nested one-way ANOVA to account for litter followed by Tukey's multiple comparisons test.

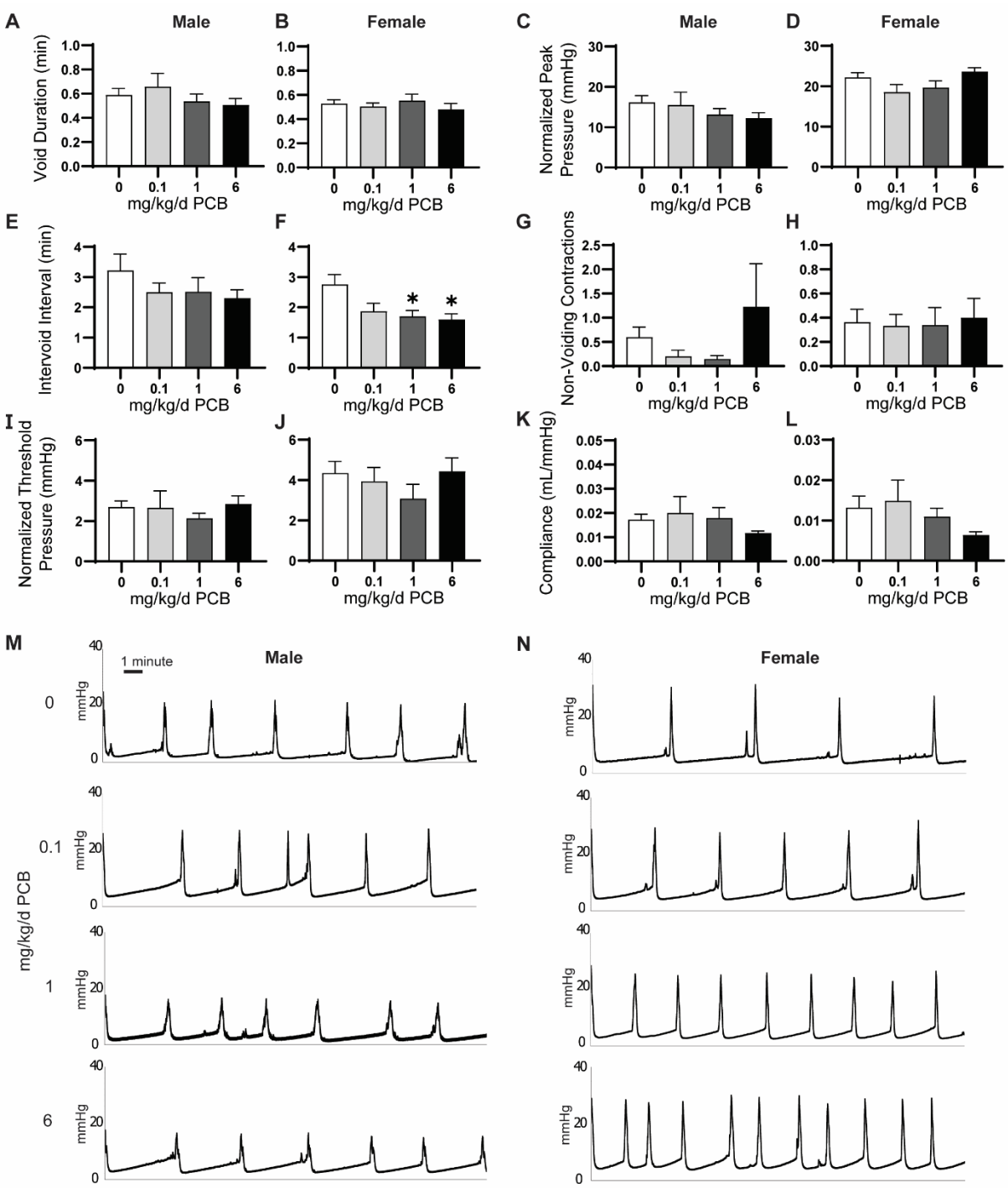


Figure 3. Developmental PCB exposure decreases intervoid interval in female offspring. Mice were exposed to PCBs via the dam and anesthetized cystometry testing was conducted with saline infusion on young adult male and female mice. Parameters examine include (A, B) void duration (min), (C, D) normalized peak pressure (mmHg), (E, F) intervoid interval (min), (G, H)

non-voiding contractions, (I, J) normalized threshold pressure (mmHg), and (K, L) compliance (ml/mmHg). (M, N) Representative cystometrograms. Results are mean ± SEM n = 5–11 males derived from 4 to 6 litters per treatment group, n = 8–11 females derived from 3 to 7 litters per treatment group. * indicates significant difference from vehicle control $p \leq 0.05$ as determined by nested one-way ANOVA to account for litter followed by Tukey's multiple comparisons test.

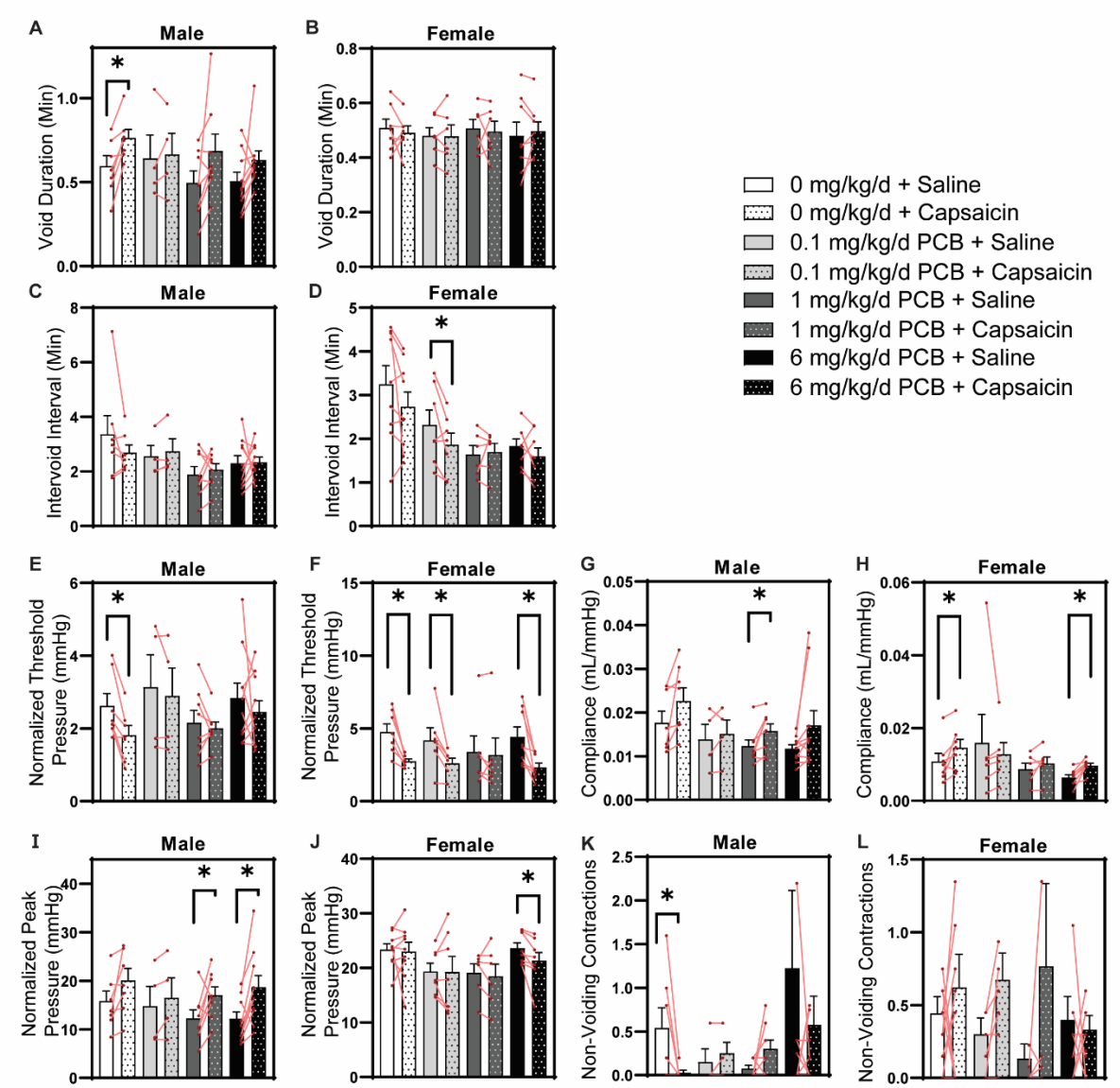


Figure 4. Developmental PCB exposure had sex- and dose- dependent effects on CMG parameters when comparing saline to capsaicin infusion. Mice were exposed to PCBs via the dam and anesthetized cystometry testing was conducted with saline infusion followed by capsaicin infusion on young adult male and female mice. The average values for each mouse are shown as red points, and a red line connects data points collected from the same individual mouse. Parameters examine include (A, B) void duration, (C, D) intervoid interval, (E, F) normalized threshold pressure, (G, H) compliance, (I, J) normalized peak pressure, and (K, L)

non-voiding contractions. Results are mean ± SEM n = 4–10 males derived from 3 to 6 litters per treatment group, n = 6–9 females derived from 3 to 6 litters per treatment group. * indicates significant difference from saline of that group $p \leq 0.05$ as determined by mixed effects model to account for litter using R lme function (R code: m = lme(Value ~ Treatment, random = ~ 1| Litter/Animal, data = data). For nonparametric data the mixed effects model using R lmer function (R code: m = lmer(ranked_values ~ Treatment + (1 | Animal) + (1 | Litter), data = data) on ranked values was used (C 0.1 mg; K 0 mg, 0.1 mg, 6 mg; L 1 mg). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

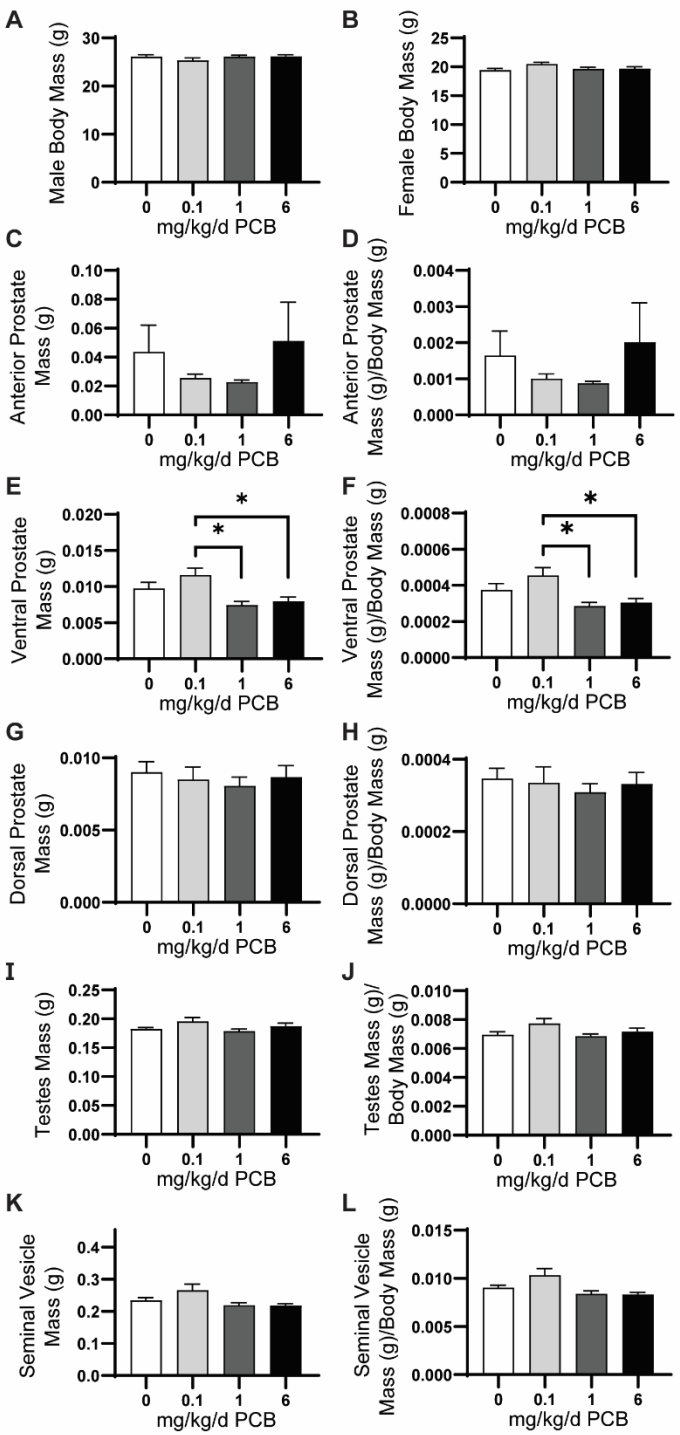


Figure 5. Developmental PCB exposure decreased the mass of ventral prostate in 1 mg/kg/d and 6 mg/kg/d compared to 0.1 mg/kg/d PCB dose group in male offspring. Mice were exposed to PCBs via the dam and weighed prior to euthanasia. Prostate lobes, testes, and seminal vesicle were collected immediately following euthanasia and weighed. Results are mean ± SEM n = 8–

11 males derived from 4 to 6 litters per treatment group (body mass), 4–11 males derived from 3 to 6 litters per treatment group (prostate/SV), n = 8–13 females derived from 3 to 7 litters per treatment group. Bar and * indicate significant differences $p < 0.05$ as determined by nested one way ANOVA to account for litter followed by Tukey's multiple comparisons tests.

CHAPTER III: DEVELOPMENTAL EXPOSURE TO POLYCHLORINATED BIPHENYLS (PCBS) ALTERS CONTRACTLE STRENGTH AND SENSITIVITY TO CONTRACTILE STIMULI IN BLADDERS OF YOUNG ADULT MICE EX VIVO

ABSTRACT

Lower urinary tract symptoms (LUTS) pose a significant health burden and greatly reduce quality of life. Changes to bladder contractile strength, bladder innervation, and nerve evocation of bladder contraction are all ways that altered bladder function can lead to LUTS. The etiology of altered bladder function is not completely known, and one possible contributing factor is exposure to environmental contaminants. Polychlorinated biphenyls (PCBs) are persistent environmental toxicants widely reported in animal and human tissues. We have previously reported female mice exposed to a human-relevant mixture of PCBs during pregnancy have altered bladder contractility, and offspring mice exposed developmentally have altered urinary voiding physiology as adults. Here, we aim to assess if mice with developmental exposure to PCBs have altered bladder contractile strength, bladder innervation, and changes to genes important for mediating contractility - such as cholinergic and purinergic receptors and big potassium (BK) channel members as well as pathways related to calcium signaling and nerve growth factors. An *ex vivo* bladder tissue bath assay was conducted to measure the bladder contractile force induced by stimuli including electrical field stimulation (EFS), cholinergic agonist carbachol, and purinergic agonist adenosine triphosphate (ATP) stimulation. We also assess the response to EFS when treated with cholinergic inhibitor atropine, purinergic inhibitor αβ-methylene ATP, BK channel opener NS11021, BK channel closers paxilline and iberiotoxin, and ryanodine receptor modulator ryanodine. In the absence of external contractile stimuli, spontaneous contractile activity was also measured. Immunohistochemistry was performed on bladders to quantify suburothelial nerve density, and quantitative PCR was performed to quantify RNA abundance of target genes. PCBs did not change bladder innervation but did have sex- and dose-specific effects on bladder contractility and gene expression in the bladder related to

calcium signaling pathways, neurotropic factors, neurotransmitter receptors, and BK channels. Decreases to nerve-evoked contractile strength was observed in both male and female mice at 1 mg/kg/d PCB, but in male mice PCB effects are associated with changes to BK channel expression and distribution, while in female mice PCB effects are associated with changes at the level of the neurotransmitter receptors, increased efficacy of BK channel opening drugs, and changes to BK channel distribution. Spontaneous bladder contractions, which are associated with micromotions during bladder filling, are also changed by PCB exposure with increased spontaneous contractile activity when treated with BK channel inhibitor, paxilline, in male mice at 1 mg/kg/d and masculinization of the spontaneous contractile activity in female mice. Together, these results indicate developmental exposure to human-relevant PCBs in male and female mice is sufficient to change bladder contractility, both nerve-evoked and spontaneous. Overall, this work highlights the potential for environmental chemicals to contribute to LUTS, and PCBs can change murine bladder contractility with a critical role of timing of exposure in influencing voiding function.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are persistent organic pollutants which have been banned for intentional commercial production; yet, legacy sources remain in historical materials and the environment, as well as continue to be produced as unintentional biproducts of material production processes such as dye synthesis (Anh et al., 2021; Grossman, 2013; Guo et al., 2014; Hannah et al., 2022; Hu and Hornbuckle, 2010). Structurally, PCBs are relatively stable as a bridged, aromatic biphenyl with 1-10 electron withdrawing chlorine groups, making 209 possible configurations of PCB congeners. Coplanar PCBs are considered dioxin-like PCBs, as they induce aryl hydrocarbon receptor (AhR) signaling which is the binding site for the canonical ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), hence the moniker "dioxin-like." Conversely, PCBs with 1-4 chlorines in the *ortho*-position assume a non-coplanar conformation which inhibits binding to and activation of AhR, thus the non-coplanar PCBs are referred to as non-dioxin-like. Contrary to dioxin-like PCBs, non-dioxin-like PCBs bind to a variety of targets, including the ryanodine receptors (RyR) (Wong et al., 1997). PCB effects in the central nervous system have been linked to RyR activity, but PCB effects on other organs are less well understood. PCB exposure can impact systems and processes associated with lower urinary tract symptoms and dysfunction, yet the molecular mechanisms responsible for altering lower urinary tract function has not been fully elucidated and is a goal of this work

Bladder contractility is an integral component of healthy bladder emptying, and bladder contraction pathophysiology can contribute to lower urinary tract symptoms (LUTS) including under- and overactive bladder, urge incontinence, and incomplete bladder emptying (Ridlon et al., 2025b). Elucidating the etiology of LUTS is an ongoing goal of urological research, and progress towards identifying the molecular mechanisms that drive LUTS serves to inform

prevention, intervention, and treatment strategies. Insults to the lower urinary tract system itself cause immediate or delayed LUTS, and indirect insults to the LUT including via nervous, immune, or endocrine systems can also adversely affect lower urinary tract function. Thus, in an effort to uncover molecular mechanisms that contribute to the onset, progression, and severity of LUTS, we have focused on understanding how perinatal insults from environmental chemicals can alter lower urinary tract function and bladder contractility into adulthood. We have previously found that developmental exposure to a mixture of PCBs is capable of altering voiding function in young adult mice (Kennedy et al., 2022); however the mechanisms driving these phenotypes are unknown. This study bridges this research gap by assessing the murine bladder contractile strength and sensitivity in an *ex vivo* preparation allowing us to focus on nerve-evoked pathways of bladder contraction as well as spontaneous contractions.

The initiation of nerve-evoked contraction in the bladder begins with the propagation of an action potential in the parasympathetic nerves which innervate the smooth muscle of the bladder. At the nerve terminus, the neurotransmitters acetylcholine and ATP bind to cholinergic and purinergic receptors, respectively. The binding events of these neurotransmitter receptors initiate the opening of voltage dependent calcium channels and drives sparks of cytoplasmic calcium ions and subsequent smooth muscle contraction. Purinergic receptors in the membrane of the urinary bladder smooth muscle quickly potentiates contraction through calcium ion influx in response to ATP (Heppner et al., 2009). This calcium influx and action potential is sustained through acetylcholine stimulation of cholinergic receptors in the membrane of the urinary bladder smooth muscle (Heppner et al., 2009).

Regulation of the urinary bladder smooth muscle membrane voltage charge and intracellular calcium ion concentration are both vital for mediating bladder relaxation and contraction. The

sarcoplasmic reticulum (SR) is a membrane structure in muscle cells which acts as an intracellular storage site for Ca^{2+}. Cytoplasmic Ca^{2+} ions enter the SR through sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, and Ca^{2+} are released through inositol 1,4,5-trisphosphate receptors (IP3Rs) and ryanodine receptors (RyRs). Ryanodine receptors along with IP3s release a sarcoplasmic intracellular store of calcium ions during nerve-evoked contractile events as calcium-induced calcium release (CICR). Bladder relaxation is also tightly regulated and key to terminating voiding contractions. Large-conductance (100-300 pS) voltage- and Ca^{2+}- activated K+ channels (BK, BK_{Ca}, MaxiK, *Slo1*, $K_{Ca}1.1$) are potassium ion channels that modulate the polarization of UBSM through the efflux of potassium ions, and it is the sole K^+ channel member that is gated by both Ca^{2+} and K^+. The BK channel provides negative feedback for bladder smooth muscle contractions, both spontaneous and nerve-evoked (Meredith 2004), through maintenance of the resting membrane potential and shaping the current rectification of the repolarization phase (Hayase et al., 2009; Heppner et al., 1997), thus the field recognizes the BK channel as arguably the most important ion channel involved in UBSM contraction.

Here we for the first time examine the effects of developmental PCB exposure on bladder contractility, assessing functional changes as well as changes in RNA abundance of the key receptors mediating bladder contractions, both spontaneous and nerve-evoked. Using an *ex vivo* bladder contractility assay, we can test the contractile strength of the mouse bladder in response to different stimuli and challenge different targets along the bladder contraction pathway (Kullmann et al., 2014). Through this work, not only are the baseline contractile properties of the mouse bladder further elucidated, but also the impacts of developmental PCB exposure are

explored and further opens the door for continued exploration into how dose and sex specific effects drive bladder associated changes upon exposure to PCBs.

METHODS

Animal generation

Mice with developmental (*in utero* and lactational) exposure to human-relevant PCBs were generated for this study. The PCB mixture, termed MARBLES PCB after the cohort of pregnant woman the mixture is derived from (MARLBES, (Markers of Autism Risk in Babies Learning Early Signs) (Hertz-Picciotto et al., 2018), and this MARBLES PCB mixture been used in prior mouse studies on neurological and urological function (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021; Matelski et al., 2020; Panesar et al., 2020; Ridlon et al., 2025a; Sethi et al., 2021; Spiegelhoff et al., 2023; H. Wang et al., 2026a; K. Wang et al., 2026). This mixture contains each of the following PCBs at the specified percentage: 28 (48.2%), 11 (24.3%), 118 (4.9%), 101 (4.5%), 52 (4.5%), 153 (3.1%), 180 (2.8%), 149 (2.1%) 138 (1.7%), 84 (1.5%), 135 (1.3%), and 95 (1.2%). PCBs were synthesized and authenticated by the Synthesis Core of the University of Iowa Superfund Research Program and through Accustandard previously described (Li et al., 2018; Sethi et al., 2019). Briefly, female nulliparous adult mice were orally dosed daily with peanut butter (Spectrum Organic Products, LLC, Melville, NY, Trader Joe's, Monrovia, CA) spiked with vegetable oil alone for the vehicle control group, or vegetable oil with MARBLES PCBs dissolved. Dosing started two weeks prior to mating and continued through gestation and lactation until pups were weaned at postnatal day (P) 21. The 0.1, 1, or 6 mg/kg body weight/day doses and dosing paradigm were chosen because it results in exposed offspring bearing detectable levels of PCBs in bladder and other organs at levels relevant to human health and lead to varied phenotypes in offspring (Kania-Korwel et al., 2017; Keil Stietz et al., 2021a; Sethi et al., 2021; H. Wang et al., 2026a; Yang et al., 2009). After weaning, male and female offspring

were group housed with same sex and dose littermates, aged to ~6 weeks of age without additionally exposure to PCBs.

Immunohistochemistry nerve quantification

Immunohistochemistry was performed as previously described (Abler et al., 2011; Keil Stietz et al., 2021a; Turco et al., 2019; Wegner et al., 2018). Briefly, following euthanasia, bladders from mice at 6 weeks of age (42.9 ± 2.4 days) were removed (n= 4-5 per group, representing at least 3 litters per group) and fixed in 4% paraformaldehyde (Fischer, Madison, WI) overnight, and dehydrated into 100% methanol for long-term storage. Samples were rehydrated into phosphate buffered saline (Fischer), embedded in bacto agar (VWR, Radnor, PA) to maintain bladder orientation. Then the block was dehydrated into 100% ethanol, washed in xylenes (Fischer), embedded into paraffin, and sectioned at 5 μm thickness. Multiple tissues from each exposure group were embedded in the same block to reduce variability in staining. using antibodies listed in Table 1. Images were captured using an Eclipse Ci compound microscope (Nikon Instruments Inc., Melville, NY) with a Photometric DS RI2 camera interfaced to NIS elements imaging software (Nikon Instruments Inc). Within Image J, the entire stroma or muscle was outlined and saved in the region of interest manager tool within Image J for analysis. For the suburothelium, a region 10 μm thick was drawn along the entire epithelial border labeled by E-cadherin and saved for analysis. The nerve fiber channel alone was then opened, a threshold applied and the area of positive pixels within the saved regions was measured using the analysis-measure feature of Image J. Total nerve area was calculated from at least two images of each bladder section, then averages of each bladder across multiple sections (2-5) were used for a total of 4-5 bladders per treatment group and sex.

RNA isolation and qPCR

Following euthanasia, bladders from mice at 6 weeks of age (43.1 ± 1.8 days) were removed (n= 4-5 per group, representing at least 3 litters per group). RNA was isolated using the Cytiva RNAspin Mini RNA Isolation Kit with on-column DNase I digestion according to manufacturer recommendations. RNA concentrations were quantified using a Qubit 4 fluorometer (Thermo Fischer Scientific). cDNA was synthesized from 300 ng of total RNA using the GoScript Reverse Transcription Reaction Kits (Promega). Singleplex quantitative real-time PCR was performed using SSoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad; cat. #1855201). Real-time qPCR primers were designed using primer3 (Kõressaar et al., 2018; Koressaar and Remm, 2007), and sequences are listed in Table 2. Target gene specificity was confirmed using the National Center for Biotechnology Information Primer Basic Local Alignment Search Tool (NCBI Primer-BLAST). PPIA was used as the housekeeping gene, and analyses were conducted with the $2^{-\Delta\Delta Ct}$ method.

Bladder bath

Bladder bath assays were conducted as described previously (Keil et al., 2015; Lavery et al., 2023; Turco et al., 2021). Briefly, mice were euthanized via CO_2 and the bladder removed and bifurcated longitudinally. Thread (5-0 silk, Fine Science Tools, Foster City, CA) is secured on the top and bottom of each bladder half and connected a force pressure transducer (FT-03, Grass Instruments, Quincy, MA). Tissues were submerged in a thermostatic 37°C water jacketed tissue chamber filled with Krebs solution (133 mM NaCl, 16 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 1.4 mM NaH_2PO_4, 2.25 mM $CaCl_2$ $2H_2O$, 7.8 mM d-glucose, pH 7.2 with glacial acetic acid) and aerated with 95% O_2-5% CO_2. Bladder halves were maintained at a baseline of 1 gram of tension for 60 minutes before experimentation began, with Krebs solution changed every 15

minutes. For electrical field stimulation (EFS), bladders were stimulated with 10 V, duration of 0.5 ms, at frequencies of 1 Hz, 2 Hz, 5 Hz, 10 Hz, 20 Hz, and 50 Hz (Grass Instruments, West Warwick, RI). Bladders were stimulated at each frequency for 5 seconds, with 3 minutes recovery between each stimulation. Once completed, bladders recovered for 30 minutes. During all recovery periods, Krebs was changed every 15 minutes and the tension was readjusted to 1 gram as needed. Increasing concentrations (0-10 μM final bath concentration, added from 1000× fresh stocks) of the cholinergic agonist carbachol (AAL0667403, Thermo Fisher Scientific) were then added to baths, with each addition occurring as the response plateaued to the previous addition to elicit a stepwise response. After final application of carbachol, bladders were washed with Krebs solution 3 times and tension readjusted to 1 gram as necessary. Bladders recovered for 30 minutes. ATP (A1852-1VL, Sigma) was then applied to baths (1000 μM final bath concentration, added from 1000× fresh stocks). After final addition of ATP, bladders were washed with Krebs solution 3 times, and tension readjusted to 1 gram as necessary. Bladders recovered for 30 minutes. Following this period, 60 mM KCl was added to the baths to elicit a maximum response.

In a separate cohort of mice, bladders were prepared for bladder baths as described above. After performing EFS with a 30 minute recovery period, atropine (A05505G, Thermo Fisher Scientific) or α,β-methylene ATP (ABMA, 5054190001, Thermo Fisher Scientific) were then added to baths (Atropine: 1 μM final bath concentration, added from 1000× fresh stocks; ABMA: 10 μM final bath concentration, added from 1000× fresh stocks). After a 10 minutes delay to allow for full tissue saturation and receptor binding, then EFS was performed. After the final EFS, bladders were washed with Krebs solution 3 times and tension readjusted to 1 gram as necessary. Bladders recovered for 30 minutes. Following this period, 60 mM KCl was added to

the baths to elicit a maximum response. Bladders were washed with Krebs solution 3 times and tension readjusted to 1 gram as necessary. After bladders recovered for 30 minutes, NS11021 (478810, Thermo Fisher Scientific), paxilline (AAJ61341LB0, Thermo Fisher Scientific), or iberiotoxin (I5904, Sigma-Aldrich) were then added to baths (NS11021: 3 μM final bath concentration, added from 1000× fresh stocks; Paxilline: 10 μM final bath concentration, added from 1000× fresh stocks; Iberiotoxin: 100 nM final bath concentration, added from 1000× frozen stocks). After a 10 minutes delay to allow for full tissue saturation and receptor binding, then EFS was performed.

In a separate cohort of mice, bladders were prepared for bladder baths as described above. After performing EFS with a 30 minute recovery period, ryanodine (sc-201523A, Santa Cruz Biotechnology) was added to baths (10 μM final bath concentration, added from 1000× fresh stocks). After a 10 minutes delay to allow for full tissue saturation and receptor binding, then EFS was performed. Bladders were washed with Krebs solution 3 times and tension readjusted to 1 gram as necessary. After bladders recovered for 30 minutes, both ryanodine and paxilline were added to baths (Ryanodine: 10 μM final bath concentration, added from 1000× fresh stocks; Paxilline: 10 μM final bath concentration, added from 1000× fresh stocks). After a 10 minutes delay to allow for full tissue saturation and receptor binding, then EFS was performed. Bladders were washed with Krebs solution 3 times and tension readjusted to 1 gram as necessary. Following this period, 60 mM KCl was added to the baths to elicit a maximum response.

Bladder contractility was recorded and analyzed by an individual blinded to treatment conditions using Labchart software (ADInstruments). Baseline values were subtracted from each response, and each response normalized to baseline subtracted KCl response and expressed as percent.

Bladder halves were averaged, if a bladder half failed to respond to KCl or electrical field stimulation that half was excluded from analysis.

Statistics

Statistics were run with GraphPad Prism software version 10. Data was analyzed in a structure format appropriate for the data type. For RNA abundance and ATP contractile response: Data were analyzed for normality using Shapiro-Wilk and Kolmogorov-Smirnov normality tests, and variance using Brown-Forsythe and Bartlett's tests. Data that did not pass normality were assessed via Kruskal-Wallis test followed by Dunn's multiple comparisons tests. Data that passed normality and variance tests were assessed via one-way ANOVA followed by Tukey's multiple comparisons test. Data that passed normality and did not pass variance tests were assessed by Brown-Forsythe ANOVA and post-hoc multiple comparisons test. RNA abundance data was analyzed a single time for outliers using the identify outliers function using the ROUT method at Q=1%. For carbachol dose response: Dose responses were assessed via two-way repeated measure ANOVA with Tukey's multiple comparisons tests. The EC50 best fit values from bladder bath assays were generated from nonlinear fit analysis for log agonist vs. response curves within Prism. For repeated measure of response to Hz stimuli: 2-way Anova and Dunnett's multiple comparisons test. For % change in response to Hz stimuli: Mixed effect analysis 2-way Anova and Dunnett's multiple comparisons test. Results with $P \leq 0.05$ were considered statistically significant. Where $0.05 < P \leq 0.09$ the P value is reported as a numerical value and considered trending. N values for each treatment group and parameter, along with the statistical test run, are indicated in the figure legends.

RESULTS

Developmental exposure to PCB significantly alters bladder contractility in response to electrical field stimulation and cholinergic agonist carbachol

Electrical field stimulation (1-50 Hz) induces frequency-dependent, neurogenic contractions of the bladder, mimicking excitation during micturition and excitatory neurotransmitter release from efferent nerve terminals, this effect can be blocked through application of the neurotoxin tetrodotoxin (Ambrogi et al., 2025). Here, baseline KCl response is unchanged by PCB exposure (Fig. S1). Contractility of bladder strips was assessed in tissue bath assays with responses to stimuli normalized to KCl induced contraction (Fig. 1) to mimic nerve evoked smooth muscle contraction, electrical field stimulation (EFS) at 1, 2, 5, 10, 20, and 50 Hz was applied to bladders. Developmental exposure to 1 mg/kg/d PCB decreased the contractile strength in male mice at 10 Hz and 20 Hz, and in female mice at 20 Hz when compared to the sex-matched vehicle control group at the respective Hz stimulation (Fig. 1A,B). PCB effects on EFS responses alone and in combination with various drug treatments is summarized in Table 3. We also assessed the lowest observable effect level (LOEL), or the stimuli at which the contractile response was significantly greater than the first applied stimuli as well as the effective concentration 50% (EC50) (Table 4). For EFS, the LOEL was higher (10 Hz) in the 1 mg/kg/d PCB male and female mice compared to all other treatment groups which had a LOEL of 5 Hz (Table 3). The EC50s for the 1 mg/kg/d PCB exposure groups also reflected this shift where male mice shifted from 6.9 Hz in the control group to 9.8 Hz, and female mice shifted from 8.94 Hz to 11.48 Hz (Table 4). PCB effects on EFS response alone or following treatment with various pharmacological agents is summarized in Table 4. We next examined receptor-mediated bladder contractility with stepwise additive addition of cholinergic agonist, carbachol (10^{-8} to 10^{-5} M).

While there was no PCB effect on contractility in male mice at any of the concentrations of carbachol (Fig. 1C) and the EC50 was relatively unchanged (Table 4), the sensitivity to carbachol as assessed by LOEL was lower in the 0.1 mg/kg/d male mice at 10^{-4} M carbachol compared to all other treatment groups which had a LOEL of $3x10^{-4}$ M carbachol (Table 4). Conversely, in female mice there was no PCB effect to LOEL with carbachol, but PCB effects were observed for contractile strength and EC50 (Fig. 1D, Table 4). There was a significant increase in contractile response to 10^{-3} M carbachol in the 1 mg/kg/d PCB group and at when compared to the sex-matched control group at each concentration of carbachol (Fig. 1D). There was also a significant increase in contractile response to $3x10^{-4}$ and 10^{-4} M carbachol in the 6 mg/kg/d PCB group when compared to the sex-matched control group at each concentration of carbachol (Fig. 1D), and the 6 mg/kg/d PCB group had a lower EC50 indicating increased sensitivity to carbachol (Table 4). We also examined receptor-mediated contractility with the addition of 1 M ATP. There was no PCB effect observed in male or female mice (Fig. 1E,F). Together these results illustrate sex and dose specific effects of developmental PCB exposure on bladder strength and sensitivity to contractile stimuli, with EFS generally decreasing contractile strength while cholinergic stimuli increased strength or sensitivity to contract.

Developmental PCB exposure does not impact overall bladder nerve density at 6 weeks of age

Previously, we observed reductions in bladder contractility in response to nerve evoked stimulation. Here, we assess whether changes in nerve density occurred. Changes to bladder nerve density, or disruption of nerve signaling between the brain and bladder, can lead to lower urinary tract symptoms including neurogenic bladder (Tennyson and Chermansky, 2018; Truzzi et al., 2021). Decreased bladder nerve density is observed in cases of detrusor underactivity, a

urodynamic condition where the bladder demonstrated diminished contractile force (Jhang et al., 2021). Axonal density in the human bladder smooth muscle has been reported to decrease with age (Gilpin et al., 1986) and decrease autonomic innervation in bladders following bladder outlet obstruction (Gosling et al., 1986). We previously demonstrated male mice with the same developmental exposure paradigm had increased nerve density in the bladder at 4 weeks of age (Keil Stietz et al., 2021a). Here, we sought to determine if bladder nerve density is altered in ~P42 mice following developmental exposure to the MARBLES PCB mix in the maternal diet throughout gestation and lactation at 0, 0.1, 1, or 6 mg/kg/d. Using a combination of epithelial (CDH1), pan-nerve (βIII-tubulin), and nuclei (DAPI) markers (Figure 2), we found that PCBs do not impact the nerve density in the muscle (Fig. 2C,F), stroma (Fig. 2D,G), or suburothelium (Fig. 2E,H).

Developmental PCB exposure disrupts expression of calcium pathway and nerve growth factor genes in a sex- and dose-specific manner

Developmental exposure to PCBs can alter both bladder innervation and smooth muscle function. Here, we also sought to determine whether PCBs impacted genes important for smooth muscle contractility and neurogenic regulation. Non dioxin like PCBs, including a majority of the MARBLES mixture, can both bind to and stably open ryanodine receptors and influence RyR expression (Griffin et al., 2024; Sethi et al., 2019; Zheng et al., 2022). In central nervous system neurons, PCB induced RyR activation leads to increased dendritic outgrowth, spine formation, and synaptogenesis via a cAMP response element binding protein (CREB)-dependent mechanism (Keil et al., 2019; Keil Stietz et al., 2021b; Lesiak et al., 2014; Sethi et al., 2018; Wayman et al., 2012; Yang et al., 2009). Notably, RyRs also play a key role in smooth muscle contraction. Here, we aimed to investigate if PCBs could potentially be influencing bladder

contractility via alterations to RNA expression of RyR subtypes (*Ryr1,3*), or RyR-associated signaling pathways including CREB (*Creb1*) and its downstream transcript WNT2 (Wingless-type MMTV integration site family, member 2, *wnt2*). We also examined the expression of neurotrophic factors (Artemin (*Artn)* and nerve growth factor (*Ngf*)),implicated in bladder sensory function and plasticity. Artemin is of especial interest as it can be upregulated in bladder inflammation, modulates bladder response to stimulation, and has been shown to be induced by dioxin (Turco et al., 2021). Notably, one PCB in our mixture is a dioxin like PCB capable of activating the AhR pathway. RT-qPCR was performed on bladders from ~P42 mice following developmental exposure to the MARBLES PCB mix in the maternal diet throughout gestation and lactation at 0, 0.1, 1, or 6 mg/kg/d. In male and female mice, bladders were assessed for PCB effects on RNA abundance compared to the sex-matched vehicle control. No PCB effects were observed in male or female mice for RNA abundance of *Ryr1* (Fig. 3A,B), *Ryr3* (Fig. 3C,D), *Wnt2* (Fig. 3G,H), and *Ngf* (Fig. 3K,L). PCBs increased *Creb1* RNA abundance in female mice at 6 mg/kg/d PCB, and no PCB effects were observed in male mice (Fig. 3E,F). PCBs increased *Artn* RNA abundance in male mice at 1 mg/kg/d PCB, and no PCB effects were observed in female mice (Fig. 3I,J). Together this data suggests that male and female bladder have unique responses to PCBs and implicate *Creb* meditated signaling pathways in female phenotypes and *artemin* in males, but neither change fully overlaps the observed changes in response to EFS and carbachol across all doses suggesting alternative or additional mechanisms may be at play.

PCB effects on bladder contractility in response to EFS is influenced by purinergic signaling in the bladder

Cholinergic and purinergic neurotransmission are central components to the bladder contractile pathway for urinary voiding (Ridlon et al., 2025b). Blockage of cholinergic signaling using

anticholinergics/antimuscarinics is commonly used intervention for lower urinary tract symptoms (Ali et al., 2021). To further assess the PCB effects on contractile strength and sensitivity during electrical field stimulation we sought to determine whether blockade of cholinergic receptors would influence the EFS response. Bladders were stimulated with EFS, then incubated with cholinergic antagonist atropine, and then were stimulated with EFS again. Atropine reduced bladder contractility in all treatment groups, indicating that the pharmacological treatment produced a reduction to EFS similarly in all treatment groups (Fig. 4B). When examining the bladder strength of contraction to EFS following the addition of atropine, there was still a significant reduction in contractile strength in 1 mg/kg/d PCB male bladder at 10, 20 and 50 Hz compared to the sex-matched vehicle control (Fig. 4A), similar to that observed without addition of atropine (Fig. 1). We further assessed the mRNA abundance of the cholinergic receptors (*M1-M5*) we found that there was a significant reduction in both *M2* and *M3* cholinergic receptors at all PCB treatment groups in male bladders while other subtypes were unchanged (Fig. 4C-G). EFS response was reduced in female 1 mg/kg/d PCB bladder at 10 and 20 Hz and was actually increased in 6 mg/kg/d PCB bladder at 20 and 50 Hz when compared to the sex-matched vehicle control group (Fig. 4H), however in females these responses are to be interpreted with caution as we found that atropine had minimal effect in reducing EFS especially among the control animals where there was a significant difference in control versus 1 and 6 mg/kg/d PCB bladders as atropine was more effective in reducing contractility in these PCB bladders than the controls (Fig. 4I). There were no significant differences in *M1-M5* RNA expression in female bladder, there were trends of increased expression of *M2* (overall ANOVA $p=0.044$) and *M4* (overall ANOVA $p=0.064$) noted in the 6 mg/kg/d group compared to the control group (Fig. 4J-N).

We next examined EFS response with purinergic signaling inhibited. In male bladder EFS still evoked a decrease in bladder contractility in 1 mg/kg/d PCB group bladder compared to control in the presence of purinergic receptor antagonist ABMA (Fig. 4O), however ABMA had very little effect in general on changing response to EFS (Fig. 4P) suggesting that purinergic signaling may be playing a very small role in EFS evoked male contractions, conversely ABMA reduced contractility in female bladder of all treatment groups (Fig. 4U) thus the drug is capable of reducing contractility but may do so in a sex specific manner. In female bladders EFS response in the presence of ABMA no longer had any changes seen in the 1 mg/kg/d PCB group and instead there was an increase in contractility in the 6 mg/kg/d PCB group (Fig. 4T). There were no changes in purinergic receptor RNA expression in male or female bladder, however there was a trend for male expression of P2X1 to be reduced in the 1mg/kg/d PCB group compared to the sex-matched control group ($p=0.073$) (Fig. 4Q-S, V-X). When comparing the EFS responses in the presence of cholinergic or purinergic receptor inhibitors (Fig. 4) versus those just to EFS (Fig. 1, Table 3), these data indicate that inhibiting the cholinergic pathway does not alter PCB effects on EFS response. However, these results do indicate that inhibiting the purinergic signaling pathway does prevent the PCB effect, indicating that purinergic signaling is required for the PCB induced decreases in bladder contractility of the female mouse bladder with 1 mg/kg/d PCB exposure.

PCBs increase the efficacy of BK channel opening in female mice at 1 mg/kg/d and changes BK channel subunit distribution in both sexes at 6 mg/kg/d

BK channel opening and closing shapes bladder contractility through maintenance of the resting membrane potential and shaping the current rectification of the repolarization phase (Hayase et al., 2009; Heppner et al., 1997; Herrera et al., 2005). In order to test whether these channels

could influence PCB responses to EFS we examined EFS responses with addition of pharmacological drugs which can either open or inhibit BK channels. Here, bladder contractile strength was assessed with the addition of pharmacological agents NS11021, paxilline, or iberiotoxin, with the effect on contractile strength assessed alone and when compared to EFS without the agent present, and RNA abundance of BK channel subunits in the bladder was quantified using q-PCR (Fig. 5).

Bladders were stimulated with EFS then incubated with BK channel opener, NS11021 and then were stimulated with EFS again. NS11021 was previously reported to decrease bladder contractile strength (Layne et al., 2010). BK opener reduced bladder contractility in all treatment groups, indicating that the pharmacological treatment produced a reduction to EFS evoked contractility, however the 6mg/kg group while reduced, responded the least to the drug (Fig. 5B; percent change in response before vs after NS11021 addition, dashed line would indicate no effect of drug). When examining the bladder strength of contraction following the addition of NS11021 alone, there was an increase in contractile strength in 6mg/kg/d male bladder at 20 and 50 Hz compared to vehicle control (Fig. 5A), and no reductions as see in response to EFS alone (Fig. 1, Table 3). EFS response was reduced in female 1mg/kg bladder at 20 and 50 Hz (Fig. 5C), however in females these responses are likely due to the finding that NS11021 had a greater impact in reducing EFS contraction in the 1mg/kg PCB bladder compared to control bladders in that NS11021 was more effective in reducing contractility in these PCB dose bladders than the controls (Fig. 5D).

Bladders were stimulated with EFS, then incubated with BK channel closer, paxilline, and then were stimulated with EFS again. Paxilline has previously been reported to inhibit BK channels by shifting its gating kinetics to decrease the opening probability (Zhou and Lingle, 2014). In

male bladder EFS still evoked a decrease in bladder contractility in 1 mg/kg/d bladder compared to control in the presence of BK channel antagonist paxilline (Fig. 5E), interestingly paxilline had an effect in all treatment groups in increasing % change in bladder contractility but there was a graded response in that this was seen most robustly at 1-10Hz but less so in the 20 and 50Hz responses (Fig. 5F), this same response was seen in female bladder of all treatment groups (Fig. 5H) thus the drug is capable of increasing contractility but may do so in a hertz specific manner. In female bladders EFS response in the presence of paxilline no longer had any changes seen in any of the PCB groups compared to control (Fig. 5G).

Lastly EFS response was assessed with addition of the BK channel inhibitor iberiotoxin, which is an antagonist of BK channels, however iberiotoxin exhibits differences in efficacy based on BK channel subunits. BK channels comprised of the a alone or α+β1 subunit are stably opened when treated with iberiotoxin, but the α+β4 channels are resistant to iberiotoxin (Meera et al., 2000; Wang et al., 2014). Interestingly in both male and female bladder, when comparing the percent change in EFS response before vs after addition of iberiotoxin, only the 6 mg/kg/d PCB bladders showed an increase in contractility (Fig. 5 J, L), suggesting that the composition of BK channels may differ between treatment groups. When examining EFS response in the presence of iberiotoxin there were no changes in contractility among male bladder (Fig. 5I), however in female bladder there was a significant reduction in the 1 mg/kg/d PCB group compared to the sex-matched vehicle control group (Fig. 5K).

RNA abundance of BK channel pore forming unit BKα (*Kcnma1*) and BKβ regulatory subunits BKβ1-β4 (*Kcnmb1-4*), were quantified from bladders using RT-qPCR. In male mice, *Kcnma1* was increased in the 1 mg/kg/d PCB group versus vehicle control, *Kcnmb1* was trending increased in the 0.1 mg/kg/d PCB group compared to the sex-matched vehicle control groups,

and *Kcnmb2* was increased in the 6 mg/kg/d PCB group compared to the sex-matched vehicle control group, with no PCB effect observed for *Kcnmb3* or *Kcnmb4* (Fig. 5M-Q). In female mice, no PCB effect was observed when compared to the sex-matched vehicle control group (Fig. 5R-V).

When comparing the EFS responses in the presence of BK channel opener and inhibitors (Fig. 5) versus those just to EFS (Fig. 1, Table 3), these data indicate that opening BK channels in male bladder prevents the PCB effects on reduced EFS response. Conversely, blocking BK channels in female bladder prevents the PCB effects on reduced EFS response. Further these results indicate that BK channel abundance is altered in male but not female bladder, indicating that BK channels are important in the PCB induced changes in contractility but in a sex and dose specific manner.

Ryanodine receptors and BK channels mediate PCB effects on contractility in male mice

RyRs embedded in the membrane of the sarcoplasmic reticulum in bladder smooth muscle open during nerve-evoked contractile events and contribute to the increase in intracellular Ca^{2+} (Morimura et al., 2006; Ridlon et al., 2025b). Prior studies demonstrated that application of 10 uM ryanodine to bladders inhibits RyR activity (Herrera et al., 2000; Jiang et al., 2005). Genetic downregulation of RyR's contribution to intracellular influxes leads to a weakened membrane depolarization from nerve-evoked contractile signaling and cholinergic signaling (Hotta et al., 2007). Here, we assessed PCB effects on bladder contractile strength during EFS with the application of ryanodine alone and in combination with the BK channel closer paxilline (Zhou and Lingle, 2014).

Bladders were stimulated with EFS, then incubated with ryanodine at a concentration of ryanodine previously reported to promote RyR closing (Herrera et al., 2000; Jiang et al., 2005), and then were stimulated with EFS again. 10 μM ryanodine was not sufficient to decrease bladder contractile strength during EFS in vehicle control mice, the % change in contractility was actually increased in the presence of ryanodine and PCBs did not impact the change in EFS response from ryanodine (Fig. 6B,D). There was no significant difference in bladder contractility between treatment groups when EFS was applied in the presence of ryanodine in male mice (Fig. 6A). In female mice there was a decrease in 0.1 mg/kg/d and 1 mg/kg/d PCB groups at 10 Hz and 50 Hz, and all three PCB groups at 20 Hz, when compared to the sex-matched vehicle control group at each respective Hz stimulation.

Next, the bladders were incubated with paxilline (to inhibit BK channels) and ryanodine together, then were stimulated with EFS again. Again, the EFS response was increased in the presence of the drugs in both sexes (Fig. 6F,H). In the presence of paxilline and ryanodine, the 6 mg/kg/d bladders from male mice had significantly reduced contractility in response to 10 Hz stimulation (Fig. 6E). While in female mice a decrease in contractility at the 0.1 mg/kg/d and 1 mg/kg/d PCB groups was observed at the 10, 20 and 50 Hz (Fig. 6G). Together, these results suggest that in this setting 10 μM ryanodine acts to increase contractility. Further, it demonstrates that when ryanodine dependent activity is increased in male mice, no PCB effect on EFS evoked responses is seen and suggests ryanodine receptors and BK channels are important in mediating PCB effects on diminished responses to contractility (Fig. 1, Table 3).

PCBs increase spontaneous contractile activity when treated with paxilline in male mice at 1 mg/kg/d and masculinizes the spontaneous contractile activity in female mice

Spontaneous myogenic contractions describe transient, non-voiding contractile events during the bladder filling phase of the micturition cycle. Fragments of the sarcoplasmic reticulum with embedded RyRs localize near BK channels (Ohi et al., 2001). The functional coupling of RyR-mediated Ca^{2+} release from the sarcoplasmic reticulum with the activation of BK channels is known as transient BK channel currents and acts as negative-feedback regulation of bladder smooth muscle membrane potential (Herrera et al., 2001; Ohi et al., 2001). Here, spontaneous contractile activity was assessed at baseline and after ryanodine treatment, paxilline treatment, or a combination of ryanodine and paxilline treatment (Fig. 7). In vehicle control mice, the bladders have no spontaneous contractile activity at baseline or with ryanodine treatment (Fig. 7B,C). In male vehicle control mice, paxilline increases spontaneous contractile activity above baseline, but this is not observed in female mice (Fig. 7B,C). Conversely, ryanodine with paxilline returns spontaneous contractile activity back to baseline in male vehicle control mice, but it increases spontaneous contractile activity above baseline in female vehicle control mice (Fig. 7B,C). With the addition of PCB exposure, 1 mg/kg/d male mice have a stronger increase in spontaneous contractile activity when treated with paxilline than the vehicle control group, and the 6 mg/kg/d male mice are similar to the vehicle control group (Fig. 7B,C). In female mice, PCBs match the drug response to those of male mice with paxilline increasing spontaneous activity in both PCB groups (Fig. 7B,C). In the 1 mg/kg/d group, paxilline alone was greater than the vehicle control group and ryanodine with paxilline was less than the vehicle control group at each respective drug treatment (Fig. 7B,C).

DISCUSSION

Developmental exposure to the MARBLES PCB mixture leads to changes in bladder contractile activity including contractile strength in response to contractile stimuli, sensitivity to contractile stimuli, and responses to drugs which change spontaneous contractile activity in mice with sex- and dose-dependence. We have previously reported that mice at the same age with the same PCB exposure have changes to their urinary voiding physiology (Kennedy et al., 2022). The PCB effects on bladder contractile activity elucidated in this study can help to begin to explain some of the physiological changes in urination.

The bladder contractile response to 60mM KCl was not impacted by PCB exposure when comparing groups to the sex-matched vehicle control groups (Fig. S1), suggesting the overall bladder contractility was not changed by developmental MARBLES PCB exposure. Electrical field stimulation (1-50 Hz) induces frequency-dependent, neurogenic contractions of the bladder, mimicking excitation during micturition and excitatory neurotransmitter release from efferent nerve terminals, which we can inhibit through application of the neurotoxin tetrodotoxin (Ambrogi et al., 2025). The nerves can be bypassed to elicit a neurotransmitter-mediated contractile response, with the cholinergic mimic carbachol stimulating cholinergic signaling via muscarinic acetylcholine receptors, or ATP stimulating purinergic signaling via purine receptors (Fry and McCloskey, 2021; Schneider et al., 2004). Bladder contractile strength and sensitivity to contractile stimuli can result from changes to nerve- or neurotransmitter-mediated signaling, and this can result in phenotypic changes to urinary voiding physiology including incontinence, overactive bladder, and neurogenic bladder (Ridlon et al., 2025b). PCBs influenced both the strength of bladder contraction and sensitivity to contractile stimuli in a sex-, dose-, and stimulus-dependent manner. Further study targeting neurotransmitter receptors and BK channels

revealed that the decrease in contractile strength and sensitivity to EFS in male and female mice at 1 mg/kg/d is sex-dependent, with female mice requiring functional purinergic signaling and male mice requiring functional BK channel activity (Table 3).

While the overall nerve density was not changed from PCB exposure in the suburothelia, stroma, or muscle of the bladder (Fig. 2), PCBs did increase in artemin expression in the bladders of male mice at 6 mg/kg/d PCB (Fig. 3I). This suggests that while total nerve density was not changed by PCBs at 6 weeks of age, and we previously reported sensory nerves positive for calcitonin gene-related protein (CGRP+) nerves are increased by PCB exposure in the suburothelium of mice at 4 weeks of age (Keil Stietz et al., 2021a), PCBs may cause more subtle changes to bladder innervation. Further work to more precisely and specifically investigate subtypes of bladder innervation are an ongoing area of research using genetic and optogenetic approaches (Robilotto et al., 2023; Tran et al., 2025).

Blockage of cholinergic signaling using anticholinergics/antimuscarinics is a commonly used intervention for lower urinary tract symptoms (Ali et al., 2021); However, while these therapeutics are generally well tolerated, they are not always efficacious and can produce off target effects (Ali et al., 2021; Madhuvrata et al., 2012; Oelke et al., 2015; Yu et al., 2020). Exposure to environmental chemicals can influence cholinergic signaling in the bladder via expressional and posttranslational changes to the muscarinic acetylcholine receptors involved in contraction (van Koppen and Kaiser, 2003). Since PCBs increased sensitivity to carbachol in male mice at 0.1 mg/kg/d PCB and increased contractile strength response in female mice at 1 mg/kg/d and 6 mg/kg/d PCB, and female mice at 1 mg/kg/d and 6 mg/kg/d PCB had a reduced response to the anticholinergic drug atropine, this could implicate environmental chemical exposure in changes to tolerance in anticholinergic drugs in patients undergoing treatment for

LUTS. Further research is required to be able to more clearly elucidate how PCBs and other environmental chemicals impact cholinergic signaling in the bladder and potentially interfere with medical treatment of LUTS. Additionally, we have previously reported PCBs impact urinary voiding physiology of mice in an age-dependent manner (Ridlon et al., 2025a), and cholinergic receptor expression of the *M3* subtype is decreased in male mice over the course of aging (Kamei et al., 2018). Thus, this work here supports further study on PCB effects on bladder contractile pathways, both their molecular expression and functional outcomes, in an age-dependent manner.

At greater intermembrane charge of the bladder smooth muscle or calcium ion binding, the probability of BK channel opening increases (Gonzalez-Perez and Lingle, 2019). NS11021, a drug that decreases bladder contractile strength through increasing BK channel opening (Layne et al., 2010), was effective in male and female mice with an even stronger decrease in contractile strength for female mice with 1 mg/kg/d PCB exposure. (Fig. 5B,D). This PCB effect suggests 1 mg/kg/d female mice have increased efficacy of NS11021 treatment either from greater BK channel expression and/or BK channel opening has a more potent effect due to upstream effects on mounting a contractile response from nerve-evoked contraction. Since no PCB effect was observed for BKα RNA abundance in female mice at any PCB exposure (Fig 5R), this makes an increase in BK channel expression unlikely to explain the 1 mg/kg/d PCB effect on NS11021 efficacy. In male mice, PCBs did not impact NS11021 efficacy (Fig. 5B), but did increase RNA abundance of BKα at 1 mg/kg/d PCB (Fig. 5M). This suggests that in male mice, PCBs did not impact NS11021 efficacy at decreasing bladder contractile strength during EFS, but 1 mg/kg/d PCB exposure increases BKα expression.

One way BK channel opening probability is regulated is through association with beta regulatory subunits (β1-β4) (Gonzalez-Perez and Lingle, 2019). In mice, rats, and humans, β1-β4 are

present in the bladder, but only β1 and β4 are present in the urinary bladder smooth muscle (Chen and Petkov, 2009; Hristov et al., 2011b). β1 prolongs opening bursts and slows closing kinetics, while β4 slows gating kinetics and biphasically modulates calcium ion sensitivity (Brenner et al., 2000a; Gonzalez-Perez and Lingle, 2019). In addition to augmenting the opening kinetics and sensitivity, β regulatory subunits can also confer resistance to pharmacological agents. BK channel subunits comprised of the α alone or α+β1 subunit are stably opened when treated with iberiotoxin, but the α+β4 channels are resistant to iberiotoxin (Meera et al., 2000; Wang et al., 2014). Here, iberiotoxin was not effective in male or female mice with vehicle control PCB exposure (Fig. 5J,L). To the authors knowledge, it has not been previously reported that male and female mouse bladders are resistant to iberiotoxin increase in bladder contractile strength at any age. Changes to BK channel regulation demonstrates a potential mechanism through which species differences in bladder contractile activity are different across species. With the addition of PCB exposure, male and female mice with 6 mg/kg/d PCB exposure lost their resistance to iberiotoxin (Fig. 5J,L) suggesting PCBs decreases the distribution of β4 regulatory subunits across BK channels to a sufficient level which iberiotoxin could increase contractile strength during EFS. This highlights the importance that while gross expression of BK channels and their regulatory subunits can be changed by PCB exposure, the distribution of the β regulatory subunits is also important enough to change iberiotoxin sensitivity absent of expressional changes. When comparing the EFS responses in the presence of BK channel opener and inhibitors (Fig. 5) versus those just to EFS (Fig. 1), these data indicate that opening BK channels in male bladder prevents the PCB effects on reduced EFS response. Conversely, blocking BK channels in female bladder prevents the PCB effects on reduced EFS response. Further these results indicate that BK channel abundance is altered in male but not female

bladder, indicating that BK channels are important in the PCB induced changes in contractility but in a sex and dose specific manner.

While the expressional and functional profiles of ryanodine receptor subtypes have not been thoroughly characterized in the urinary bladder smooth muscle of mice or humans, characterization in the smooth muscles of other tissues and species can elucidate what may be occurring. In the vascular smooth muscle of mice, RyR3 KO increased the strength and frequency of transient calcium release, and increased BK channel opening frequency, and functionally increased perfusion pressure in arterial beds (Matsuki et al., 2018). In the vascular smooth muscle of pregnant sheep uterine arteries, pregnancy increased the association of BK channels with β1 subunits, and enhanced the co-localization of RyR1 and RyR2 with the β1 subunits (Song et al., 2021). Then, RyR1 and RyR2 knockdowns (but not RyR3 knockdowns) decreased BKβ1 (but not BKα) expression and association with BKα, decreased calcium ion spark frequency, suppressed BK channel potassium efflux frequency and amplitude, and increased pressure-dependent myogenic tone (Song et al., 2021). Here we observed no PCB effect on RyR expression (Fig. 3), and increased BK channel subunits in male mice in a dose-dependent manner (Fig. 5). PCBs changed bladder contractile strength when treated with drugs that target RyR alone or in combination with BK channels (Fig. 7) and when stimulated with EFS (Fig. 5 and Fig. 6). These results, in combination with targeting BK channels alone, suggest PCBs impact ryanodine receptor activity in bladder smooth muscle during nerve-evoked contractions and coupling with BK channels during spontaneous myogenic contractions.

This study has further implications for understanding murine spontaneous myogenic activity regardless of PCB exposure. Previous work has assessed the effects of ryanodine on RyR activity in other species or tissue types (Herrera et al., 2000; Kushnir et al., 2010), but the effects of 10

μM ryanodine in the mouse bladder has not been previously assessed to the author's knowledge. Additionally, decreased activity of ryanodine receptors decreases BK channel currents (Hotta et al., 2007), and in mice paxilline increases spontaneous contractions (White et al., 2015). Here, in the vehicle control group of male mice, ryanodine did not increase spontaneous contractile activity, which was increased by paxilline alone and reduced with the addition of ryanodine (Fig. 7B). Conversely, in female mice, neither ryanodine nor paxilline alone were sufficient to increase spontaneous contractile activity, but together these drugs did increase spontaneous contractile activity (Fig. 7C). Together these results highlight sex-differences in the underlying spontaneous contractile activity of the murine bladder. Then with the addition of PCB exposure, sex- and dose-dependent effects of PCBs are observed with the increased response to paxilline in male mice at 1 mg/kg/d, and female mice had a masculinized response to the drug combinations. Overall, this work highlights how sex-differences in the bladder contractility of mice can result in different responses to environmental chemical exposure including PCBs.

While the *ex vivo* bladder contractility assay utilized in this study has use in probing different components of the contractile pathway, and the ability to control the conditions of bladder contractions in the *ex vivo* bladder contractility assay is a strength of this approach, but this approach is unable to investigate other conditions that are important for urinary physiology including blood flow, mechanical forces beyond the bladder, and central nervous system control (Kullmann et al., 2014). This study supports further work in studying PCB effects on other related systems involved in urinary physiology beyond the bladder. Additionally, this work supports further investigation of PCB effects on bladder smooth muscle intracellular calcium dynamics in response to contractile stimuli, bladder relaxation, and formation of micromotions during bladder filling.

DECLARATIONS

Funding

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

TABLES

Primary Antibodies	**Target**	**Source**	**Company**	**Catalog Number**	**Dilution**
Anti-TUBB3	Beta-III tubulin	Mouse	Abcam	Ab78078	1:250
Anti-CDH1	E-cadherin	Rabbit	Cell Signaling	3195s	1:250
Secondary Antibodies					
Anti-mouse Alexa Fluor 488	Beta-III tubulin antibody	Donkey	Jackson ImmunoResearch	715-585-150	1:250
Anti-rabbit Alexa fluor 594	Cleaved caspase 3, E-cadherin antibody	Goat	Jackson ImmunoResearch	111-516-045	1:250

Table 1. Primary and fluorescent secondary antibodies used for immunohistochemistry.

Gene Name	NM sequence	Forward Primer	Reverse Primer	Annealing Temperature (C°)
Ryr1	NM_009109.2	ATTACAGAGCAGCCCGAGGAT	AGAACCTTCCGCTTGACAAACT	60
Ryr3	NM_001319156.1	GGCCAAGAACATCAGAGTGACTAA	TCACTTCTGCCCTGTCAGTTTC	64
Creb1	NM_001037726.1	GGTGTAGTTTGACGCGGTGTG	TACAAGCTCCTCCGTCACTGC	62
Wnt2	NM_023653.5	ACTGCAACACCCTGGACAGAG	GCGTAAACAAAGGCCGATTCCC	64
Artn	NM_001284191.1	ATGTCGGAACCACTGACCAAC	CATTTCTGGATTCTCCCAGAGG	60
Ngf	NM_013609.3	TCCCTTTCAACAGGACTCACC	GGCTGTGGTCTTATCTCCAACC	61
M2	NM_203491.3	TGGCTAGCCTTGGACTATGTTG	GTGGTCCGCTTAACTGGGTAG	62
M3	NM_033269.4	GCCCAGAAGAGTATGGATGACC	GTCTTGTCCACCGAGGAGTTG	62
M4	NM_007699.2	CTCTGAGCGGAGGGAGGTATG	ATGAGGGTGCTTGCTTGTGTC	60
M5	NM_205783.2	TCCTCTCTGAGCCCACCATC	CACAGAATCGGAACCTTGGAG	62
P2X1	NM_008771.3	CAGGGCACTTTGTAGCTGAGG	CACACTTGCTGTGGGTGTTTG	60
P2X2	NM_153400.4	TTTGGCCCAACTTTGATCTC	TGGTTACTGAAGAGCGCAGA	63
P2X3	NM_145526.2	TTAGGAGAGCCCAGGGAAAT	GTGAGGCAAGGAAAAATCCA	60
Kcnma1	NM_001253358.1	CAGCTCAGGATATGGGGAAG	GCCTCACCATGAGAACAACA	60
Kcnmb1	NM_031169.4	AGGAGTACCCGGTCCATTTC	TGTGAGGCCTGGCATTATTAG	62
Kcnmb2	NM_028231.2	ACAGCTCCAATGTGCTGTTCC	CCGTTGGATCCTCTCACAAAG	62
Kcnmb3	NM_001195074.1	GAGGAAGTCTGGCCACAAGG	TTAGCCTAACCAAGCCAACAATC	62
Kcnmb4	NM_021452.1	ACTGCCTGTATTCCATGTGCTG	CCGACTTCTTTGAGGGTTTCC	62
Ppia	NM_008907.2	CATACAGGTCCTGGCATCTTG	TCATGCCTTCTTTCACCTTCC	60

Table 2. RT-qPCR Primer information

Male	[mg/kg/d PCB]				Female	[mg/kg/d PCB]		
Treatment	**0.1**	**1**	**6**		**Treatment**	**0.1**	**1**	**6**
EFS alone	N.C.	↓ (10,20)	N.C.		EFS alone	N.C.	↓ (20)	N.C.
+ Atropine	-	↓ (10,20,50)	N.C.		+ Atropine	-	↓ (10,20)	↑ (20,50)
+ ABMA	-	↓ (20,50)	N.C.		+ ABMA	-	N.C.	↑ (20,50)
+ NS11021	-	N.C.	↑ (20,50)		+ NS11021	-	↓ (20,50)	N.C.
+ Paxilline	-	↓ (10,20)	N.C.		+ Paxilline	-	N.C.	N.C.
+ Iberiotoxin	-	N.C.	N.C.		+ Iberiotoxin	-	↓ (10,20,50)	N.C.
+ Ryanodine	N.C.	N.C.	N.C.		+ Ryanodine	↓ (10,20,50)	↓ (20)	↓ (20)
+ Ryanodine and Paxilline	N.C.	N.C.	↓ (10)		+ Ryanodine and Paxilline	↓ (10,20,50)	↓ (10,20,50)	↓ (10,20,50)

Table 3. Summary table of PCB effect on EFS response with various drug treatments. Arrows represent statistical difference from the sex-matched vehicle control group at the corresponding treatment. Values in parenthesis refer to the Hz levels where the significance is observed. N.C.: No change. Treatment groups which were not tested are represented with -.

Male	**Electrical field stimulation [Hz]**		**Carbachol [M]**	
[mg/kg/d MARBLES PCB]	**LOEL**	**EC50**	**LOEL**	**EC50**
0	5	6.9	3×10^{-4}	3.5×10^{-2}
0.1	5	6.98	10^{-4}	3.5×10^{-2}
1	10	9.8	3×10^{-4}	3.9×10^{-2}
6	5	7.14	3×10^{-4}	3.4×10^{-2}
Female	**Electrical field stimulation [Hz]**		**Carbachol [log(M)]**	
[mg/kg/d MARBLES PCB]	**LOEL**	**EC50**	**LOEL**	**EC50**
0	5	8.94	3×10^{-4}	3.7×10^{-2}
0.1	5	9.12	3×10^{-4}	3.2×10^{-2}
1	10	11.48	3×10^{-4}	3.6×10^{-2}
6	5	8.04	3×10^{-4}	1.4×10^{-1}

Table 4. Lowest observed effect levels of different contractile stimuli . Abbreviations: Hertz (Hz), Effective concentration 50% (EC50), lowest observed effect level (LOEL), polychlorinated biphenyl (PCB). Red text indicates a value that is different from the sex-matched vehicle control group.

FIGURES

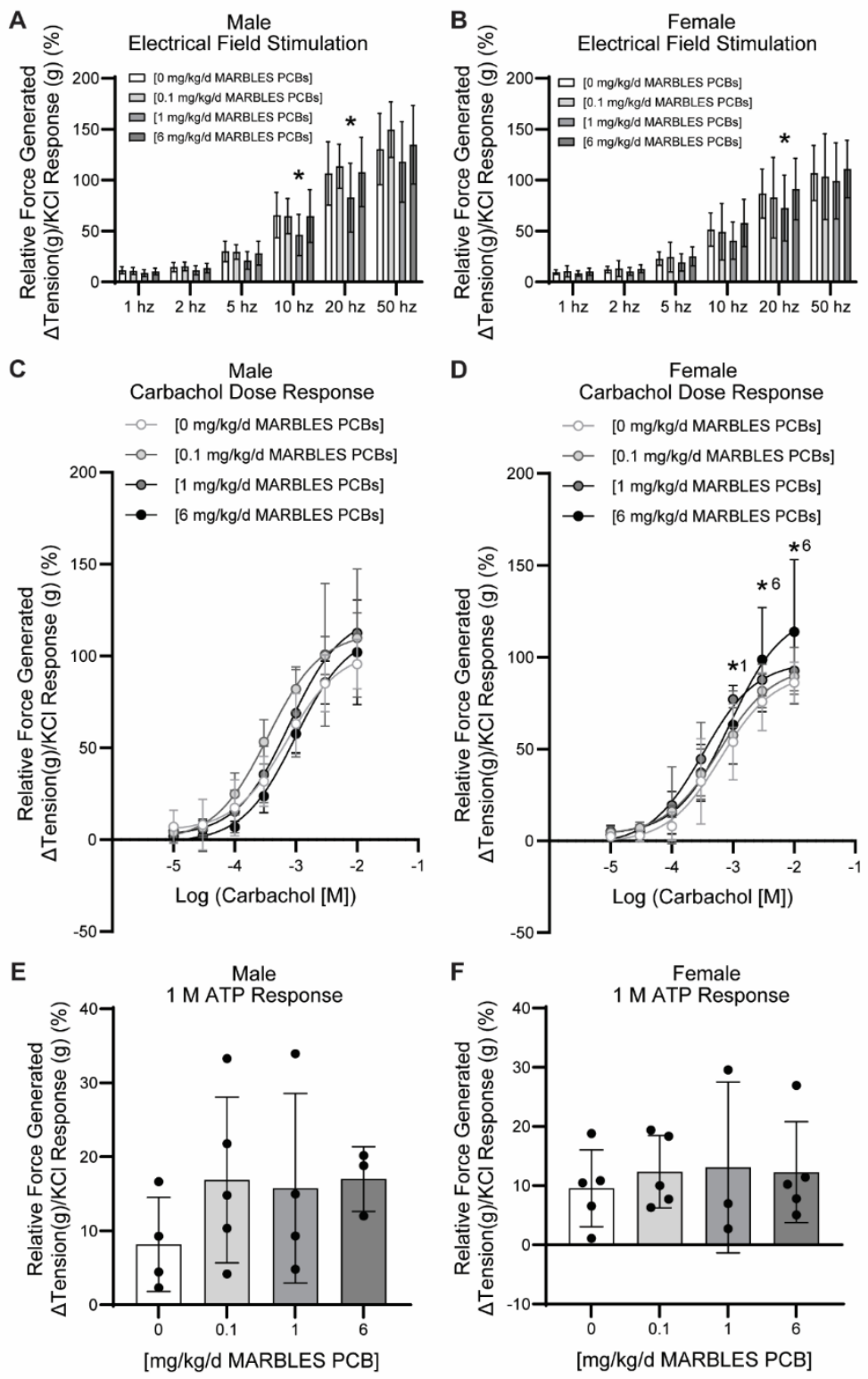
A
Male
Electrical Field Stimulation
Relative Force Generated
ΔTension(g)/KCl Response (g) (%)
[0 mg/kg/d MARBLES PCBs]
[0.1 mg/kg/d MARBLES PCBs]
[1 mg/kg/d MARBLES PCBs]
[6 mg/kg/d MARBLES PCBs]
1 hz
2 hz
5 hz
10 hz
20 hz
50 hz
B
Female
Electrical Field Stimulation
C
Male
Carbachol Dose Response
Log (Carbachol [M])
D
Female
Carbachol Dose Response
E
Male
1 M ATP Response
[mg/kg/d MARBLES PCB]
F
Female
1 M ATP Response

Figure 1. Developmental exposure to MARBLES PCBs results in sex- and dose-dependent changes to contractile strength in response to contractile stimuli during an *ex vivo* tissue bath assay. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age, with bladder strips bladder strips placed into *ex vivo* tissue baths immediately following euthanasia. Baseline tension was subtracted from all responses and then each response normalized to baseline-subtracted KCl response and expressed as %. Quantification of contractility in response to (A,B) electrical field stimulation (1–50 Hz), (C,D) cholinergic agonist, carbachol (stepwise addition of 1×10^{-8} M to 1×10^{-5} M), and (E,F) ATP (1 M) Results are means ± SE, (A,B) n = 6-36 mice/group, (C,D) n = 4-8 mice/group, (A,B) n = 3-5 mice/group. *Indicates significant difference from sex-matched vehicle control group at the same stimulation, $p \leq 0.05$ as determined by 2-way Anova and Dunnett's multiple comparisons test (A-D), or by one-way ANOVA and post-hoc multiple comparisons test (E,F).

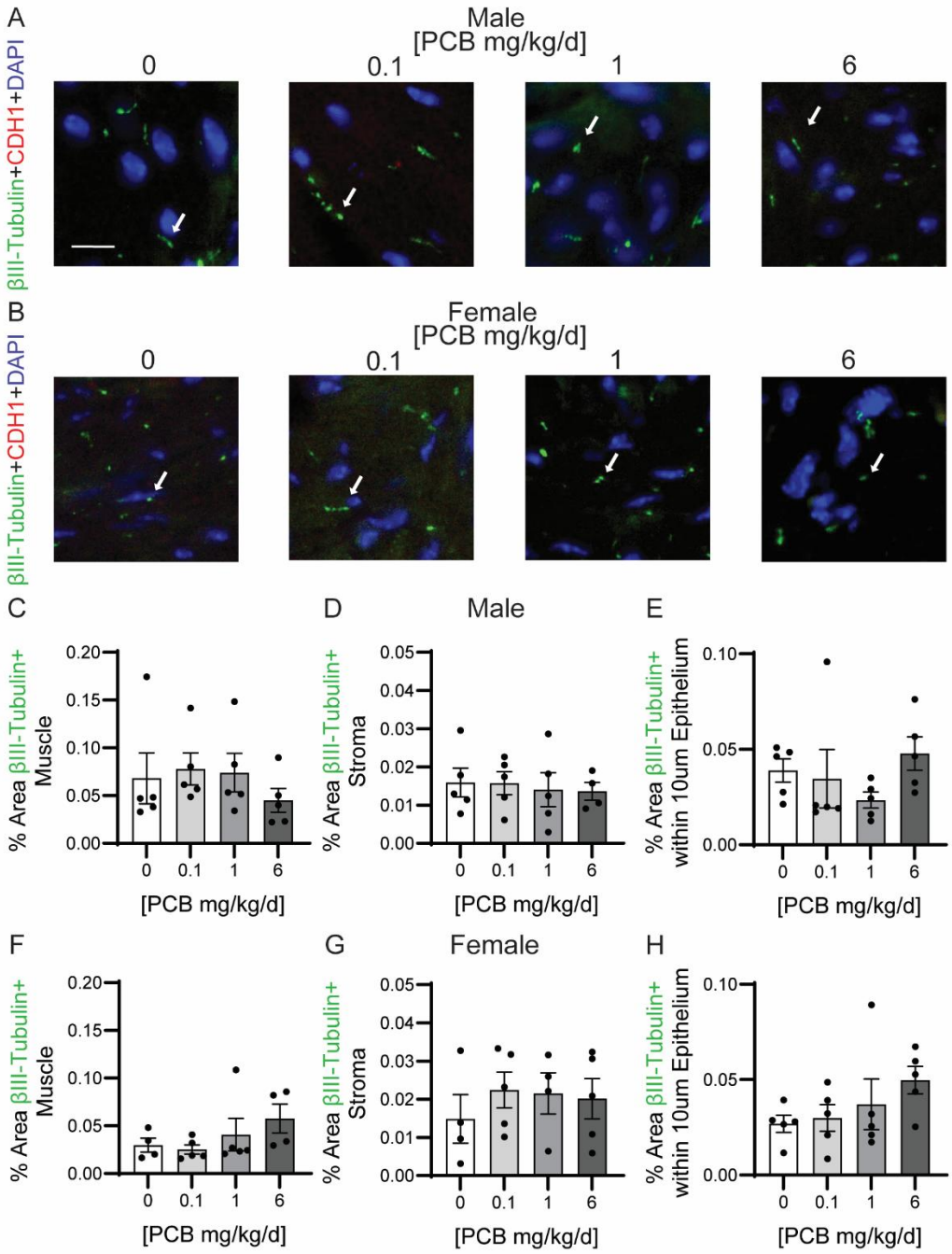

Figure 2. Developmental exposure to MARBLES PCBs does not result in measurable changes in nerve density in the bladder at the levels of the suburothelium, stroma, or muscle in male or female mice. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for nerve density in the bladder. (A, B) Representative images of

muscle with 10 μm scale bar. Nerve density was assessed at the (C, F) muscle, (D, G) stroma, and (E, H) suburothelium. Results are mean ± SEM. n=4-5 animals derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match vehicle control group as determined by one-way ANOVA and post-hoc multiple comparisons test.

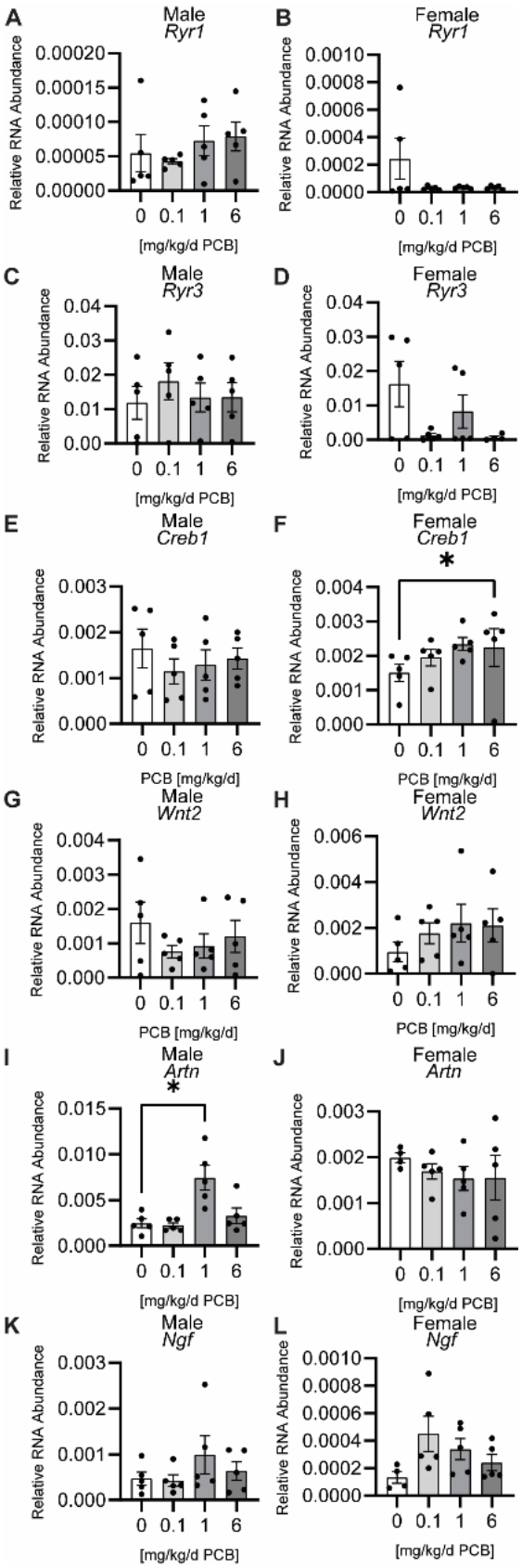
A
Male
Ryr1
Relative RNA Abundance
0.00020
0.00015
0.00010
0.00005
0.00000
0 0.1 1 6
[mg/kg/d PCB]
B
Female
Ryr1
Relative RNA Abundance
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
0 0.1 1 6
[mg/kg/d PCB]
C
Male
Ryr3
Relative RNA Abundance
0.04
0.03
0.02
0.01
0.00
0 0.1 1 6
[mg/kg/d PCB]
D
Female
Ryr3
Relative RNA Abundance
0.04
0.03
0.02
0.01
0.00
0 0.1 1 6
[mg/kg/d PCB]
E
Male
Creb1
Relative RNA Abundance
0.003
0.002
0.001
0.000
0 0.1 1 6
PCB [mg/kg/d]
F
Female
Creb1
*
Relative RNA Abundance
0.004
0.003
0.002
0.001
0.000
0 0.1 1 6
PCB [mg/kg/d]
G
Male
Wnt2
Relative RNA Abundance
0.004
0.003
0.002
0.001
0.000
0 0.1 1 6
PCB [mg/kg/d]
H
Female
Wnt2
Relative RNA Abundance
0.006
0.004
0.002
0.000
0 0.1 1 6
PCB [mg/kg/d]
I
Male
Artn
*
Relative RNA Abundance
0.015
0.010
0.005
0.000
0 0.1 1 6
[mg/kg/d PCB]
J
Female
Artn
Relative RNA Abundance
0.003
0.002
0.001
0.000
0 0.1 1 6
[mg/kg/d PCB]
K
Male
Ngf
Relative RNA Abundance
0.003
0.002
0.001
0.000
0 0.1 1 6
[mg/kg/d PCB]
L
Female
Ngf
Relative RNA Abundance
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
0 0.1 1 6
[mg/kg/d PCB]

Figure 3. Developmental exposure to MARBLES PCBs resulted in sex- and dose-dependent increases in RNA abundances for genes related to nerve outgrowth. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for RNA abundances in the bladder. Bladder RNA abundances for (A,B) *Ryr1,* (C,D) *Ryr3*, (E,F) *Creb1*, (G,H) *Wnt2*, (I,J) *Artn*, and (K,L) *Ngf* were assessed for PCB effects. Results are mean ± SEM. n=4-5 animals derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match vehicle control group as determined by one-way ANOVA and post-hoc multiple comparisons test for parametric data with equal standard distributions (C,E,G,H,K,L), Brown-Forsythe ANOVA and post-hoc multiple comparisons test for parametric data with inequal standard distributions (B,I,J), or Kruskal-Wallis and Dunn's multiple comparisons test for nonparametric data (A,D,F).

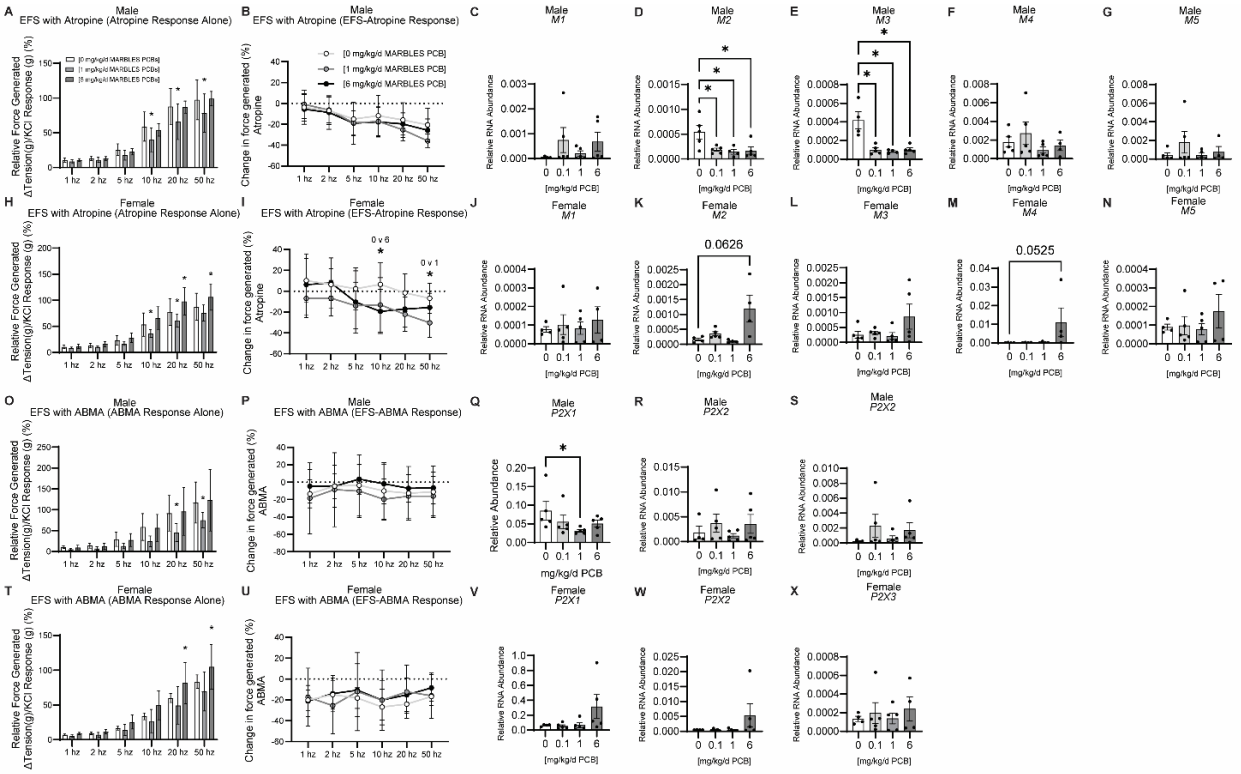

Figure 4. Developmental exposure to MARBLES PCBs resulted in sex- and dose-dependent changes to bladder contractile response in the presence of antagonists of cholinergic and purinergic receptors, and expression of their respective receptor isoforms. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for bladder contractile strength and RNA abundances in the bladder. Bladder contractile responses are reported first (A,H,O,T) as contractile response to EFS, then (B,I,P,U) as percent change in EFS response of the drug during EFS compared to EFS alone, with the dashed line at 0% indicating no change in EFS response from the drug. (C-G,J-N) RNA abundances of muscarinic acetylcholine receptors subtypes and (Q-S,V-X) purinergic receptor subtypes were quantified using RT-qPCR. Results are mean ± SEM. (A,B,H,I,OP,T,U) n=5-11 mice/group, (C-G,J-N,Q-S,V-X) n=4-5 animals/group, derived from at least 3 independent litters per sex and dose group. * Indicates significant difference from sex-matched vehicle control group at the same

stimulation, $p \leq 0.05$ as determined by 2-way Anova and Dunnett's multiple comparisons test (A,H,O,T), mixed effect analysis 2-way Anova and Dunnett's multiple comparisons test (B,I,P,U), one-way ANOVA and post-hoc multiple comparisons test for parametric data with equal standard distributions (D,F,J), Brown-Forsythe ANOVA and post-hoc multiple comparisons test for parametric data with inequal standard distributions (E,K,L,V), or Kruskal-Wallis and Dunn's multiple comparisons test for nonparametric data (C,G,M,N,Q,R,S,W,X).

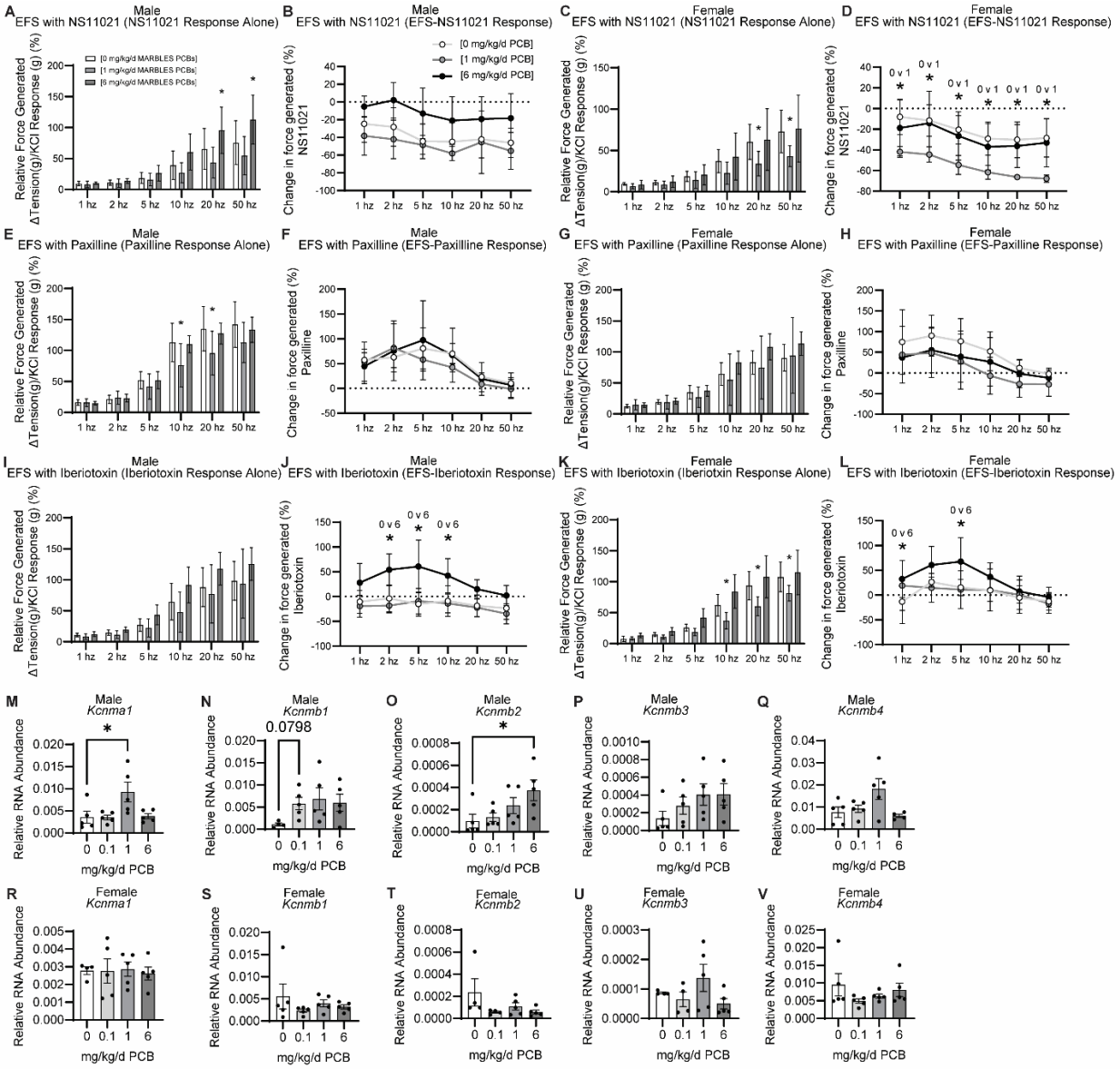

Figure 5. Developmental exposure to MARBLES PCBs resulted in sex- and dose-dependent changes to bladder contractile response in the presence of BK channel openers or closers, and expression of BK channel alpha and beta subunits. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for bladder contractile strength and RNA abundances in the bladder. Bladder contractile responses are reported first (A,C,E,G,I,K) as contractile response to EFS, then (B,D,F,H,J,L) as percent change in EFS response of the drug during EFS compared to EFS alone, with the dashed line at 0% indicating no change in EFS

response from the drug. (M-V) RNA abundances of BK channel subunits was quantified using RT-qPCR. Results are mean ± SEM. (A-L) n=5-7 animals/group, (M-V) n=4-5 animals/group, derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match vehicle control group as determined by 2-way Anova and Dunnett's multiple comparisons test (A,C,E,G,I,K), mixed effect analysis 2-way Anova and Dunnett's multiple comparisons test (B,D,F,H,J,L), one-way ANOVA and post-hoc multiple comparisons test for parametric data with equal standard distributions (O), Brown-Forsythe ANOVA and post-hoc multiple comparisons test for parametric data with inequal standard distributions (Q,M,N,V), or Kruskal-Wallis and Dunn's multiple comparisons test for nonparametric data (P,R,S,T,U).

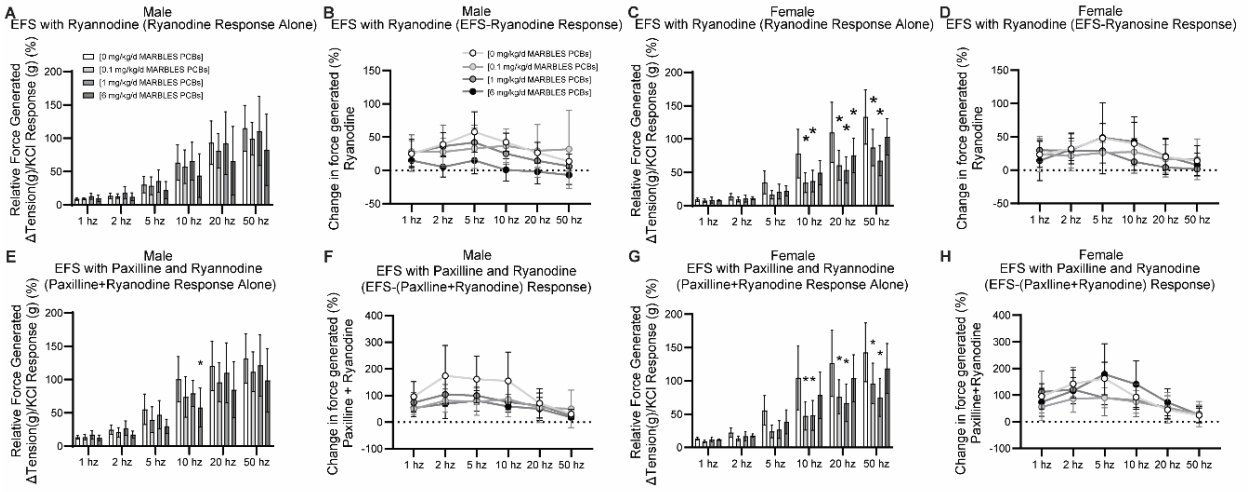

Figure 6. Developmental exposure to MARBLES PCBs resulted in sex- and dose-dependent changes to bladder contractile response in the presence ryanodine alone or in combination with paxilline. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for bladder contractile strength. Bladder contractile responses are reported first as contractile response to EFS with (A,C) ryanodine or (E,G) paxilline and ryanodine treatment, then (B,D,F,H,J,L) as percent change in EFS response of the drug during EFS compared to EFS alone with the dashed line at 0% indicating no change in EFS response from the drug. Results are mean ± SEM. n=4-6 animals/group derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match vehicle control group as determined by 2-way Anova and Dunnett's multiple comparisons test (A,C,E,G) and mixed effect analysis 2-way Anova and Dunnett's multiple comparisons test (B,D,F,H).

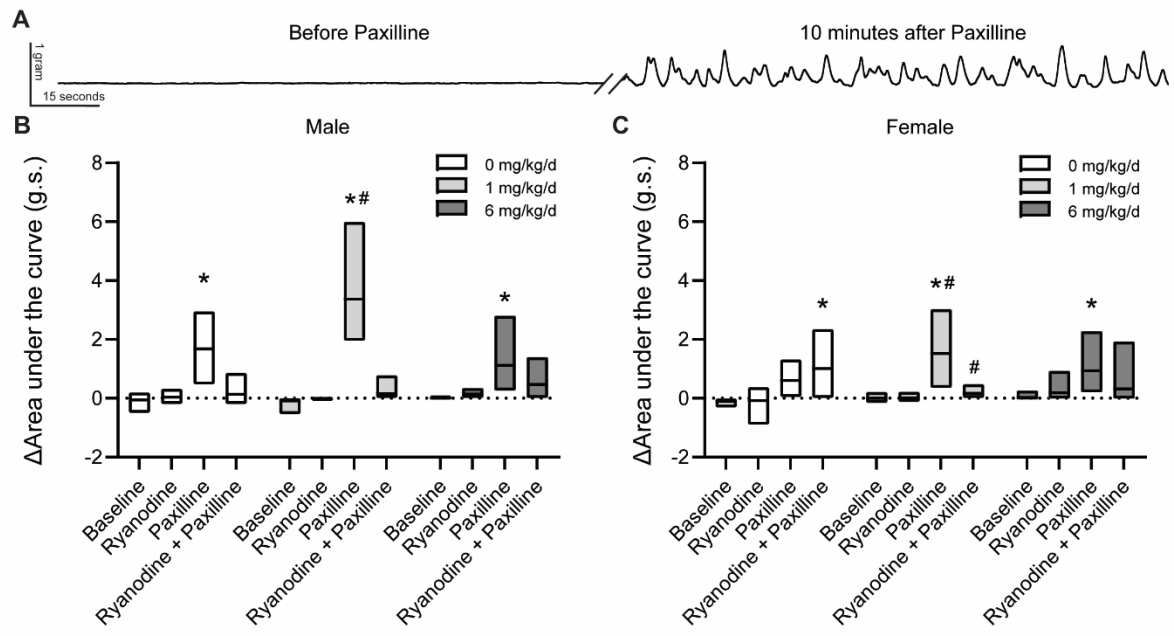

Figure 7. Developmental exposure to MARBLES PCBs resulted in sex- and dose-dependent changes in spontaneous contractile activity when treated with ryanodine and/or paxilline. (A) Representative traces of the spontaneous contractile activity of a male, vehicle control mouse immediately prior to application of paxilline, followed by 10 minutes after the application of paxilline. (B) Male and (C) female change in spontaneous contractile activity by each drug treatment condition. Spontaneous contractile activity was quantified using the difference in area under the curve between these two time points for each drug condition assessed. Results are mean ± SEM. n=5-9 animals/group derived from at least 3 independent litters per sex and dose group. * Indicates $p \leq 0.05$ as determined by 2-way Anova and Dunnett's multiple comparisons test when comparing to baseline within the PCB group (drug effect). # Indicates $p \leq 0.05$ as determined by 2-way Anova and Dunnett's multiple comparisons test when comparing to same drug treatment in the sex-matched vehicle control group (PCB effect).

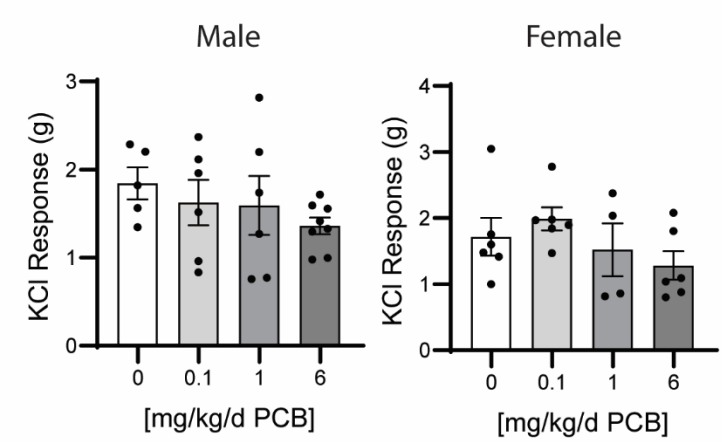

Figure Supplemental 1. Developmental exposure to MARBLES PCBs did not impact bladder contractile response to KCl. Results are mean ± SEM. n=4-8 animals derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match vehicle control group as determined by one-way ANOVA and post-hoc multiple comparisons test.

CHAPTER IV: STEROID HORMONE RECEPTOR EXPRESSION IS IMPACTED IN A SEX-, DOSE-, AND TISSUE SPECIFIC MANNER IN YOUNG ADULT MICE WITH DEVELOPMENTAL EXPOSURE TO HUMAN-RELEVANT POLYCHLORINATED BIPHENYLS

ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent organic pollutants categorized as endocrine disrupting chemicals. Exposure to PCBs, especially during developmentally critical time points, are risk factors for adverse health outcomes including neurodevelopmental disorders. Other health outcomes, including PCB effects on the lower urinary tract, and how it is impacted by the endocrine system, are less well understood. Altered steroidogenesis and steroid hormone receptor expression, as well as puberty onset, are of interest to evaluate PCB contributions to endocrine disruption. We have previously characterized behavioral and urinary voiding physiology endpoints in a mouse model of developmental exposure (*in utero* and lactational) to a human-relevant mixture of PCBs. Here we expand characterization of this model to include PCB effects on serum steroid concentrations, tissue specific receptor expression, and puberty onset. The MARBLES PCBs mixture (Markers of Autism Risk in Babies Learning Early Signs) had no effect on puberty onset in male or female mice. Sex steroid serum levels (estradiol, estrogen, progesterone, and testosterone) as assessed by LC-MS/MS were not altered by PCBs in male, or female mice. Further in female mice there were also no changes regardless of the stage of estrous. Steroid hormone receptors for progesterone (*Pgr*) and estrogen (*Esr1* and *Esr2*) were assessed with RT-qPCR in bladder, cortex, and hippocampus tissues. Sex-, dose-, and tissue-specific effects on RNA abundance were observed following PCB exposure, primarily in the middle dose group of 1 mg/kg/d PCB. Overall, these results indicate developmental exposure to human-relevant PCBs impact sex steroid hormone receptor expression in mice with sex-, dose-, and tissue specific effects. Previous studies using this PCB exposure model in mice have reported changes to neuromodulation, behavior, and urinary physiology, and the PCB effects observed in this study may contribute to those effects. Together, this study is the first to assess

endocrine disruption by the MARBLES PCB mixture and supports a broader effort to understand the adverse health outcomes of the environmental pollutant PCBs.

INTRODUCTION

Exogenous chemicals can impact the endocrine system through changes to circulating sex steroid hormone levels, expression changes to sex steroid hormone receptors, which alone or together can lead to phenotypic outcomes including precocious or delayed puberty onset and behavioral deficits (Adili et al., 2025; Gore et al., 2015; Hilz and Gore, 2022a; Philippat et al., 2017; Streifer and Gore, 2021). The environmental toxicant polychlorinated biphenyls (PCBs) are one such chemical implicated across several studies to impact these outcomes with general scientific consensus (*ATSDR's Toxicological Profiles*, 2002; Buha Djordjevic et al., 2020; Gore et al., 2015), however outcomes manifest with varying severity or penetrance likely due to human population segmentation, laboratory model differences, and PCB exposure paradigms (Butler et al., 2023; Conley et al., 2016; Den Hond et al., 2011; Goncharov et al., 2009; Grandjean et al., 2012; Ohtani et al., 2018; Ptak et al., 2006; Sechman et al., 2016; Wójtowicz et al., 2000). Studies focused on determining the effects of PCBs on endocrine disruption and puberty onset in humans are challenged by variability in the timing, frequency, quantity, and composition of PCBs present; The toxicokinetics of PCBs and an individual's sensitivity to PCB toxicity is further influenced by lifestyle, stress, genetics, and exposure to other xenobiotics (Quinn and Wania, 2012; Ritter et al., 2011). These challenges underscore the importance of utilizing animal models and using consistent terminology and methodology in investigation of the effects of PCBs and other known endocrine disruptors on growth and development. Here we utilize a human-relevant mixture of PCBs. We employ a dietary, exposure to the dam, resulting in the offspring mice receiving a developmental exposure (*in utero* and lactational) that mimics sources of PCB exposure in humans.

We have previously reported physiological and molecular outcomes of PCB exposure in developmentally exposed mice at 4 weeks of age (Keil Stietz et al., 2021a; Kennedy et al., 2021), 6 weeks of age (Kennedy et al., 2022; Spiegelhoff et al., 2023; H. Wang et al., 2026b), and 12 weeks of age (Ridlon et al., 2025a). We have also assessed effects following adulthood exposure in male mice (K. Wang et al., 2026) and female mice (Lavery et al., 2023). These studies were primarily focused on changes to the lower urinary tract and urinary voiding physiology. At 6 weeks of age, the same age assessed in this study, mice have sex- and dose- dependent changes to urinary voiding physiology including an increase in intravesical pressure in the 0.1 and 1 mg/kg/d PCB male mice, increased frequency of urinary voiding event while anesthetized in 0.1 and 6 mg/kg/d female mice, and more frequent, drop-like voids in both sexes (Kennedy et al., 2022). These PCB effects on the lower urinary tract occurred without signs of overt PCB toxicity (no changes in body mass, bladder mass, bladder muscle thickness, or epithelial composition) (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2022; Ridlon et al., 2025a). Since urinary function in male mice can be altered by changes in growth or morphology of the prostate, we also assessed PCB effects on this organ. Contrary to mouse models of bladder outlet obstruction which present with increased prostate mass, following developmental MARBLES PCB exposure offspring at 6 weeks of age have ventral prostate mass that is decreased in the 1 mg/kg/d PCB dose group compared to the 0.1 mg/kg/d and 6 mg/kg/d PCB dose groups, but not to vehicle control (Spiegelhoff et al., 2023). Offspring at 12 weeks of age also had ventral prostate masses of both the 1 mg/kg/d and 6 mg/kg/d PCB groups significantly decreased compared to the 0.1 mg/kg/d PCB group (Ridlon et al., 2025a). Together, these studies indicate that dose-specific effects occur in prostate mass following developmental MARBLES PCB exposure, with reduced masses at middle and higher doses versus low doses.

Other signs of endocrine disruption following developmental exposure to MARBLES PCBs include decreased anogenital distance in postnatal day 7 male mice at the 0.1, 1 and 6 mg/kg/d PCB groups when compared to the sex-matched vehicle control group (Matelski et al., 2020). Following observations of the MARBLES PCB mixture's effects on endocrine-sensitive endpoints, as well as alterations to neurodevelopment, and social and grooming behaviors (Keil Stietz et al., 2021b; Matelski et al., 2020; Sethi et al., 2021), we aimed to assess the effects of developmental MARBLES PCBs exposure on key steroid hormone concentrations, expression of their respective classical receptors in the bladder and brain, and gross endocrine disruption as assessed by puberty onset.

PCBs effects on the endocrine system, especially regarding sex-steroid hormones and their respective receptors, are of interest because the endocrine system alone and in combination with other organ systems including the central nervous system and urogenital system are relevant for human health. The Agency for Toxic Substances and Disease Registry (ATSDR) within the United States Centers for Disease Control and Prevention (CDC) recognizes the human health risks of PCBs as an endocrine disrupting chemical, with a focus on the ability for PCBs to mimic estrogens and block their endogenous activities (*Toxicological Profile for Polychlorinated Biphenyls (PCBs)*, 2000). Exposure to PCBs is associated with impacts on circulating hormone levels of sex-steroid hormones in humans. Native American men (Mohawk tribe) with increased blood serum levels of PCBs (congeners 74, 99, 153, and 206) correlated with decreased testosterone concentrations (Goncharov et al., 2009). Women undergoing invitro fertilization for infertility with increased blood serum PCB levels had decreased estradiol (PCB138, PCB153 and PCB180), decreased follicle stimulating hormone (PCB 118 and PCB138), and decreased luteinizing hormone (PCB138, PCB153 and PCB180) (Butler et al., 2023).

The different molecular and physiological outcomes of PCB exposure on different endocrine dependent hormones illustrate the complex nature of PCBs as endocrine disruptors. Estrogen and progesterone receptors are expressed in the bladder and across regions of the brain including the cortex and hippocampus of both humans and rodents (Brinton et al., 2008; Mitra et al., 2003; Schumacher et al., 2014; Torres-Reveron et al., 2020). In the bladder, progesterone receptors are expressed in the epithelium of the mouse bladder and the interstitial cells in the human bladder (Blakeman et al., 2000; Gevaert et al., 2016a), while estrogen receptors are expressed in the epithelium of the rat (Mäkelä et al., 2000) and human bladder (Teng et al., 2008). While estrogenic activity has not been previously assessed for impacts on progesterone or estrogen receptor expression in the bladder in the absence of cancers to the knowledge of the author, other studies evaluate these effects in other tissues or cell-based models. Progesterone receptor expression is in part regulated by estrogenic activity (For review, see Pu et al., 2023). Estrogens induce progesterone expression in the hippocampus of ovariectomized rats (Camacho-Arroyo et al., 1998; Guerra-Araiza et al., 2002) and hippocampus of ovariectomized rabbits (Camacho-Arroyo et al., 1996), with no effect in the cortex of ovariectomized female and gonadectomized male rats (Guerra-Araiza et al., 2002) or the hippocampus and cortex of male mice (Meffre et al., 2013). Estrogen receptors can also be upregulated by estrogenic activity in the hippocampus and cortex of male mice (Meffre et al., 2013). Other impacts such as disease or exogenous chemical exposure can influence expression of estrogen and progesterone receptors including upregulation of estrogen receptors in myocardial tissue in human aortic stenosis (Nordmeyer et al., 2004), xenoestrogens upregulate estrogen and progesterone receptors in an NCBI the gene expression dataset (GSE5200) (Warrier et al., 2024), and there is an association of downregulation of progesterone receptors in endometrial cancer tissue is associated with higher clinical grade and

poorer prognosis (Arnett-Mansfield et al., 2001). Thus, the physiological and behavioral impacts of xenoestrogens and other toxicants on human health are of interest to identify exposure risk factors and identify opportunities to intervene on their biological pathways.

PCBs are metabolized by cytochrome P450s into hydroxylated metabolites, and developmental exposure to MARBLES PCBs results a bladder and urine burden of hydroxylated PCB metabolites in mice at 6 weeks of age (H. Wang et al., 2026b). Hydroxylated PCBs, the major metabolite of PCBs, prevents estradiol catalysis through competitive binding to estrogen sulfotransferase (Shevtsov et al., 2003; Wang et al., 2021). Several studies have investigated PCB effects on basal steroid hormone secretion and steroidogenesis *in vitro*. In white and yellowish prehierarchical follicles from hens, PCBs (126 (0.1-100nM) and 153 (0.1-100uM)) had congener specific effects on steroidogenic gene expression and ERα and ERβ gene expression, with both PCB congeners increasing testosterone secretion and diverging effects on estradiol secretion (Sechman et al., 2016). PCB3 (6ng/mL) and its hydroxylated metabolites in porcine theca and granulosa cells decreased progesterone secretion and stimulated the conversion of testosterone to estradiol by cytochrome P-450-dependent aromatase (CYP19) (Ptak et al., 2006). Further study of PCB126 and 153 in porcine theca and granulosa cells demonstrated both PCBs impacted androgen and estrogen secretion concentrations and steroidogenesis, with PCB126 potentially impacting androgen precursor synthesis and PCB153 potentially impacting the aromatization process (Wójtowicz et al., 2000).

METHODS

Animals

All procedures involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Madison Animal Care and Use Committee. Wild type C57BL/6J mice (#000664, Jackson Labs, Bar Harbor, ME), were used in this study. All mice were housed in clear plastic cages containing corn cob bedding and maintained on a 12 h light and dark cycle at 22 ± 2°C. Feed (Diet 2019 (breeder) and 2020x, Teklad, Indianapolis, IN) and water were available ad libitum. Mice were euthanized via carbon dioxide asphyxiation followed by cervical dislocation. Whole blood was immediately collected through aortic puncture and allowed to coagulate in a microcentrifuge tube at room temperature. The blood was then centrifuged at low speed (3,000 RPM for 3 minutes) to allow for serum separation while minimizing red blood cell lysis. Bladders and brains were immediately collected, followed by microdissection of the hippocampus and cortex regions. Samples were immediately frozen upon isolation for further analysis.

Developmental PCB exposures

Mice with developmental (*in utero* and lactational) exposure to human-relevant PCBs were generated for this study. The PCB mixture, termed MARBLES PCB after the cohort of pregnant woman the mixture is derived from (MARLBES, (Markers of Autism Risk in Babies Learning Early Signs) (Hertz-Picciotto et al., 2018), and this MARBLES PCB mixture been used in prior mouse studies on neurological and urological function (Keil Stietz et al., 2021a; Kennedy et al., 2022, 2021; Matelski et al., 2020; Panesar et al., 2020; Ridlon et al., 2025a; Sethi et al., 2021; Spiegelhoff et al., 2023; H. Wang et al., 2026a; K. Wang et al., 2026). The MARBLES PCB

mixture contains each of the following PCBs at the specified percentage: 28 (48.2%), 11 (24.3%), 118 (4.9%), 101 (4.5%), 52 (4.5%), 153 (3.1%), 180 (2.8%), 149 (2.1%) 138 (1.7%), 84 (1.5%), 135 (1.3%), and 95 (1.2%). PCBs were synthesized and authenticated by the Synthesis Core of the University of Iowa Superfund Research Program and through Accustandard previously described (Li et al., 2018; Sethi et al., 2019). Briefly, female nulliparous adult mice were orally dosed daily with peanut butter (Spectrum Organic Products, LLC, Melville, NY, Trader Joe's, Monrovia, CA) spiked with vegetable oil for the vehicle control group, or vegetable oil with MARBLES PCBs dissolved in it for the different PCB exposure groups. Dosing started two weeks prior to mating and continued through gestation and lactation until pups were weaned at postnatal day (P) 21. The 0.1, 1, or 6 mg/kg body weight/day doses and dosing paradigm were chosen because it results in exposed offspring bearing detectable levels of PCBs in bladder and other organs at levels relevant to human health and lead to varied phenotypes in offspring (Kania-Korwel et al., 2017; Keil Stietz et al., 2021a; Sethi et al., 2021; H. Wang et al., 2026a; Yang et al., 2009). After weaning, male and female offspring were group housed with same sex and dose littermates, aged to ~6 weeks of age without additionally exposure to PCBs.

Puberty onset

Pubertal onset as determined by male preputial separation and female vaginal opening was assessed as described previously (Hoffmann, 2018). Briefly, restrained male mice were positioned supine and the skin around the penis was pushed towards the abdomen. Presence or absence of preputial separation was determined by visual identification of an exposed *glans penis*. Restrained female mice were positioned supine and the vulva was examined. Presence or absence of vaginal opening was determined by visual identification of separation of vulva and

opening to the vagina. Mice were assessed daily following weaning (P21) until puberty onset was determined.

Estrus cycle stage identification

Estrus cycle stage was determined by vaginal cytology, with collection and assessment following best practices as outlined previously (Cora et al., 2015). Briefly, restrained female mice were positioned supine and pipette tip containing ~15 µL of sterile water was placed into the vaginal opening. The vaginal wall was gently flushed 2-4 times, then the contents were smeared onto a glass microscope slide. Slides were then stained (Hema 3 Stat Pack, 123-869, Fischer Scientific) following manufacturer instructions. Once dried, slides were imaged at 10x magnification to assess cellular morphology. Images of vaginal cytology were assessed by a blinded individual, and results were segmented into either estrus/proestrus or diestrus/metestrus based on the predominance of leukocytes to align with cyclic fluctuations in estradiol and estrone (Nilsson et al., 2015).

Serum hormone levels

Steroid hormone analysis was adapted from methods previously described (Kenealy et al., 2017; Skalski et al., 2024). Briefly, internal standard was added to mouse serum samples (100-200 µl) and then they were extracted twice using methyl tert-butyl ether followed by dichloromethane. Samples were derivatized using dansyl chloride and then analyzed by LC-MS/MS (Sciex 6500+). Individual calibration curves were constructed for each analyte with at least 8 points. The linearity was $r > 0.9990$ and the curve fit was linear with 1/x weighting. None of the compounds of interest were detected in blank or double blank samples. Inter-assay coefficient of

variation was determined by a pool of human serum and a pool of mouse serum and ranged from 5.6-9.7%.

RNA isolation and quantification

RNA was isolated using the Cytiva RNAspin Mini RNA Isolation Kit with on-column DNase I digestion according to manufacturer recommendations. RNA concentrations were quantified using a Qubit 4 fluorometer (Thermo Fischer Scientific). cDNA was synthesized from 300 ng of total RNA using the GoScript Reverse Transcription Reaction Kits (Promega). Singleplex quantitative real-time PCR was performed using SSoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad; cat. #1855201). Real-time qPCR primers were designed using primer3 (Kõressaar et al., 2018; Koressaar and Remm, 2007), and sequences are listed in Table S1. Target gene specificity was confirmed using the National Center for Biotechnology Information Primer Basic Local Alignment Search Tool (NCBI Primer-BLAST). PPIA was used as the housekeeping gene, and analyses were conducted with the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistics were run with GraphPad Prism software version 10. Data was analyzed in a structure format appropriate for the data type. Puberty onset data was assessed by 2-way Anova and Dunnett's multiple comparisons test. Serum steroid levels and RNA abundances were analyzed a single time for outliers using the identify outliers function using the ROUT method at Q=1%, then assessed for normality using Shapiro-Wilk and Kolmogorov-Smirnov normality tests, and variance using Brown-Forsythe and Bartlett's tests. Data that did not pass normality were assessed via Kruskal-Wallis test followed by Dunn's multiple comparisons tests. Data that passed

normality and variance tests were assessed via one-way ANOVA followed by Tukey's multiple comparisons test. Data that passed normality and did not pass variance tests were assessed by Brown-Forsythe ANOVA and post-hoc multiple comparisons test. For summary statistics of serum sex hormone levels, samples with non-detectable values were assigned a value ½ the limit of detection (LOD). Results with $P \leq 0.05$ were considered statistically significant. Where $0.05 < P \leq 0.09$ the P value is reported as a numerical value and considered trending. N values for each treatment group and parameter, along with the statistical test run, are indicated in the figure legends.

RESULTS

Developmental MARBLES PCB exposure does not impact puberty onset in male or female mice

Prior studies have correlated increased levels of PCBs both perinatally and at age of puberty with an earlier onset of puberty in boys (Den Hond et al., 2011; Grandjean et al., 2012) and girls (Den Hond et al., 2011). Previous studies in C57 mice have similarly described the expected age of puberty onset without exogenous chemical exposures or stressors at approximately 28 days of age in male mice and 30 days of age in female mice (Hoffmann, 2018; Nelson et al., 1990). Here, developmental exposure to MARBLES PCBs did not change onset of puberty when compared to the sex-matched vehicle control group (Fig. 1).

Key sex-steroid hormones are not impacted by developmental MARBLES PCBs at 6 weeks of age

Sex steroid hormones (androgens, estrogens, and progestins) are important regulators of health, acting through canonical genomic pathways and nongenomic pathways, and endocrine disrupting chemicals can disrupt levels of steroids in the body including blood serum (f (Hampl et al., 2016). Here, we assess the effects of developmental exposure to the MARBLES PCB mixture on serum concentrations of 4 sex steroids (estradiol, estrone, testosterone, and progesterone). In female mice, estrus cycle stage was assessed to assess PCB effects near ovulation (estrus and proestrus) or reproductive quiescence (diestrus and metestrus). No PCB effect was observed when comparing serum concentration levels to the sex- or estrus stage- matched vehicle control group (Table 1).

PCBs have sex-, dose-, and tissue-dependent effects on expression of sex steroid hormone receptors

Endocrine disruption can adversely impact urogenital processes including bladder function of urine storage, micturition, and prostatic enlargement that can cause obstruction (De Falco and Laforgia, 2021; Nicholson et al., 2018; Turco and Vezina, 2023). Estrogen and progesterone receptors are expressed in the bladders of mice and humans (Carley et al., 2003; Gevaert et al., 2016b; Iosif et al., 1981), and these receptors are potentially involved in the pathogenesis of LUTS including benign prostatic hyperplasia-related LUTS (Nicholson et al., 2015; Shao et al., 2014) and interstitial cystitis (Imamov et al., 2007). Here, we assess RNA abundances of sex steroid hormone receptors in the bladders of male and female mice to investigate if PCBs impact expressional abundances as a potential mechanism leading to LUTS. No significant effect of PCB exposure was observed in *Esr1* (Fig. 2A,B) or *Esr2* (Fig. 2G,H). PCBs have an ANOVA effect of p=0.05 in *Pgr* of male mice with no effect in female mice (Fig. 2M,N). Trending increases were observed in male mice at 1 mg/kg/d PCB for *Esr2* (Fig. 2G) and *Pgr* (Fig. 2M) and male mice at 6 mg/kg/d PCB for *Pgr* (Fig. 2M) when compared to the sex-matched vehicle control groups.

Endocrine disruption is also of interest in the brain for investigating the role of chemical exposure on behavior and cognitive processes. Early life exposure to PCBs are associated with neurodevelopmental disorders and deficits in cognition and altered behavior in humans (Berghuis et al., 2018; Boucher et al., 2009; Schantz et al., 2003; Sprowles et al., 2022). In mice, early life exposure to human-relevant PCBs results in sex- and dose-dependent changes to behavioral phenotypes (Sethi et al., 2021; Wilson et al., 2024). Sex steroid hormone receptors are involved in neurodevelopment and behavior (Bernardi and Genazzani, 1999; Vigil et al., 2016), and

endocrine disruption by environmental chemicals can impact the expression of sex steroid hormones and result in cognitive changes (Hilz and Gore, 2022b; Walker and Gore, 2017). Here, we assess RNA abundances of sex steroid hormone receptors in the cortex and hippocampus of male and female mice to investigate if PCBs impact expressional abundances as a potential mechanism of PCB effects on behavior. In the cortex of male mice, PCBs have an ANOVA effect p=0.04 on *Esr1* with a significant decrease in male mice at 1 mg/kg/d PCB when compared to the sex-matched vehicle control group (Fig. 2C). No PCB effects were observed in the cortex of male mice for *Esr2* (Fig. 2C) or *Pgr* (Fig. 2O). In the cortex of female mice, no PCB effect was observed for *Esr1* (Fig. 2D) or *Esr2* (Fig. 2J); However, PCBs did have a significant ANOVA effect p=0.007 on *Pgr* including a significant decrease in female mice at 6 mg/kg/d PCB and a trending decrease at 1 mg/kg/d PCB when compared to the sex matched control groups (Fig. 2P). In the hippocampus of male mice, no PCB effect was observed for any of the three RNA abundances (Fig. 2E,K,Q). In the hippocampus of female mice, PCBs have a trending significant ANOVA effect p=0.06 on *Esr1* without observing any PCB effects at any particular dose group when compared to the sex matched control group (Fig. 2F). A trending increase in *Esr2* was observed at 1 mg/kg/d PCB dose group when compared to the vehicle control group (Fig. 2L). No PCB effect was observed on *Pgr* (Fig. 2R).

DISCUSSION

Exposure to human-relevant PCBs results in numerous adverse health outcomes including neurodevelopmental disorders and lower urinary tract disfunction (Badley et al., 2023; Hertz-Picciotto et al., 2018; Keil Stietz et al., 2021b; Keil-Stietz and Lein, 2023, 2023; Kennedy et al., 2022; Lavery et al., 2023; Matelski et al., 2020; Panesar et al., 2020; Sethi et al., 2021). This study aims to characterize PCB effects on endocrine sensitive endpoints to better contextualize the underlying mechanisms that drive PCB's phenotypic effects. Expression of key sex steroid hormones were quantified in the bladder and brain, and systemic effects on the endocrine system was assessed through serum levels of steroid hormones and puberty onset.

Developmental MARBLES PCB exposure had no observed impact on male mice as assessed by preputial separation, or in female mice as assessed by vaginal opening (Fig. 1). We did not assess estrus cycle cyclicity or reproductive fidelity as these would conflict with desired tissue collection of nulliparous mice at 6 weeks of age to align with previous studies using this PCB exposure paradigm (Hoffmann, 2018).

Serum levels of sex steroid hormones (estradiol, estrogen, progesterone, and testosterone) had no PCB effect in male or female mice (Table 1). Estrus cycle stages were considered for female mice to account for natural fluctuations in estradiol and estrone (Nilsson et al., 2015). Additionally, we wanted to account for PCB effects on steroidogenesis or sequestration throughout the estrus cycle. Although no PCB effects on sex-steroid hormone concentrations were observed in this study, these results do not exempt PCBs from having an effect in a different PCB exposure paradigm, animal model or human study, or co-occurring comorbidity or chemical exposure.

While prior *in vitro* studies have demonstrated PCBs can impact steroid hormone secretion and steroidogenesis in the ovary, further consideration of PCB effects in other systems including neuroendocrine cells in the central nervous system are of further interest. GnRH reproductive axis of the hypothalamus, pituitary, and gonads. GnRH+ and kisspeptin neurons in the hypothalamus are involved in puberty onset and this is estrogen receptor alpha receptor dependent (Mayer et al., 2010). Additionally, PCBs in the brain or bladder may bypass estrogen levels all together by mimicking or antagonizing estrogen binding to estrogen receptors. *In silico* modeling predict hydroxylated PCB metabolites bind to human estrogen receptors (Akinola et al., 2024; Li et al., 2013, p. 201; Ruiz et al., 2016; Waller et al., 1995). Competitive binding assays demonstrate PCB metabolites directly bind to estrogen receptors (Korach et al., 1988; Vakharia and Gierthy, 2000), and PCBs and their metabolites can induce estrogen responsive element gene transcription in cell-based reporter assays (Bonefeld-Jørgensen et al., 2001; Tam et al., 2022; Zhang et al., 2014). PCB 126, a dioxin-like PCB, promotes endometriosis progression in a humanized models through enhancing estrogen receptor beta activity directly leading to epigenetic modifications and immune reprogramming (Park et al., 2025).

PCB effects on estrogen and progesterone receptor RNA abundances in the bladder, cortex, and hippocampus were sex-, dose-, and tissue-specific (Figure 2). Since exposure to exogenous estrogens can upregulate progesterone and estrogen receptor expression (Camacho-Arroyo et al., 1998, 1996; Guerra-Araiza et al., 2002; Meffre et al., 2013; Warrier et al., 2024), and PCBs and their metabolites can directly interact with estrogen receptors to induce estrogen responsive element gene transcription assays (Bonefeld-Jørgensen et al., 2001; Tam et al., 2022; Zhang et al., 2014), and endogenous estrogen levels were not impacted by PCBs in this study (Table 1),

we interpret this data to support PCBs impacting estrogen and progesterone receptor expression through estrogenic activity.

Overall, this study demonstrates that developmental MARBLES PCB exposure impacts expression of steroid hormone receptors regions of the brain important for cognition, while effects on the bladder and systemic endocrine disruption was not observed. While bladder specific effects were not observed, control of urinary voiding physiology and behavior is closely controlled by the central nervous system, and regions of the brain important for urinary voiding including the pontine micturition center are of interest as a future direction of this study. Generally, this work supports further study in endocrine disruption by PCBs with a focus on exposure models that more accurately recapitulate the timing, frequency, quantity, and composition of human PCB exposures. Further study is warranted to focus on the mixture effects of PCB estrogenic activity and investigate of cell- and tissue-specific responses throughout the body. This work highlights the importance of interdisciplinary approaches to branch between neurology, urology, and endocrinology.

DECLARATIONS

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

TABLES

				[mg/kg/d MARBLES PCB via dam]			
Analyte	**Sex**	**Estrus stage**	**LOD (ng/mL)**	**0**	**0.1**	**1**	**6**
Estradiol	Female	DM	0.00125	0.002812±0.0007229	0.007903±0.004559	0.003209±0.0008860	0.002613±0.001432
Estradiol	Female	EP	0.00125	0.002379±0.001754	0.004267±0.001286	0.0378±0.03256	0.003823±0.002019
Estrone	Female	DM	0.00125	0.001153±0.0003250	0.00738±0.003813	0.003534±0.002246	0.001454±0.0002932
Estrone	Female	EP	0.00125	0.001626±0.0001067	0.001806±0.0001350	0.003767±0.002188	0.001753±0.0004275
Progesterone	Female	DM	0.0125	0.921±0.1591	0.6726±0.2012	1.28±0.6268	0.6381±0.3335
Progesterone	Female	EP	0.0125	0.2783±0.1038	3.149±2.447	1.325±0.5276	0.5115±0.1503
Testosterone	Female	DM	0.0125	0.02064±0.009763	0.01233±0.003729	0.5201±0.5050	0.008438±0.002188
Testosterone	Female	EP	0.0125	0.00625±0.000	0.01631±0.007550	0.00625±0.000	1.612±1.606
Estradiol	Male	N/A	0.00125	0.001155±0.0001517	0.00141±0.0003217	0.00145±0.0002790	0.001049±0.0001041
Estrone	Male	N/A	0.00311	0.00555±0.000	0.00555±0.000	0.00555±0.000	0.00555±0.000
Testosterone	Male	N/A	0.0125	1.821±1.066	1.871±0.8417	0.3584±0.1227	0.5975±0.1227

Table 1. Developmental exposure to MARBLES PCBs had no effect on mean serum hormone levels (ng/mL) in male mice, female mice in estrus cycle stages diestrus/metestrus, or female mice in estrus cycle stages estrus/proestrus. Mice were developmentally exposed to PCBs via the dam and blood serum was assess for estradiol, estrone, testosterone, and/or progesterone via LC-MS/MS. Estrus cycle stage of female mice were assessed at time of euthanasia, and samples were pooled into diestrus and metestrus (DM) or estrus and proestrus (EP). Results are mean ± SEM. n=4-5 pools of serum derived from at least 3 independent litters per sex, estrus, and dose group. Samples with non-detectable values were assigned a value ½ the limit of detection (LOD). Groups were assessed for outliers, and their removal did not impact the presence of significant results or lack thereof. * Indicates $p<0.05$ compared to the sex-match control as

determined by one-way ANOVA and post-hoc multiple comparisons test for data with parametric data with equal standard distributions, or as determined by Kruskal-Wallis and Dunn's multiple comparisons test with nonparametric data.

Gene Name	NM sequence	Forward Primer	Reverse Primer	Annealing Temperature (C°)
Esr1	NM_001302531.1	GCTGTGCACCCTGGAAATAAC	CTCGACGACCAATGACCTCTC	64
Esr2	NM_010157.3	CATCCAGAAGATGTGGTGCTG	GCTGCCTGTGGAAGACAAAG	63
Pgr	NM_008829.2	TACGGGTGCGAGGATTCTGAC	TACTCGTTCTCCTGCAGCGAC	63
Ppia	NM_008907.2	CATACAGGTCCTGGCATCTTG	TCATGCCTTCTTTCACCTTCC	60

Table S1. RT-qPCR Primer information

FIGURES

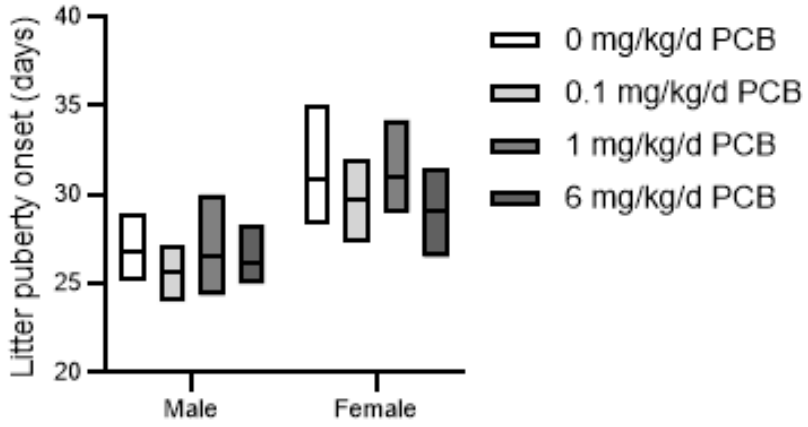

Figure 1. Developmental exposure to MARBLES PCBs had no effect on puberty onset in male or female mice. Mice were developmentally exposed to PCBs via the dam. After weaning (P21) mice were checked daily for puberty onset. Results are line at mean with box covering min to max. n=4-9 independent litter averages per sex and dose group. * indicates significance as determined by two-way ANOVA and Dunnett's multiple comparisons test to compare PCB effect to the sex-matched control group.

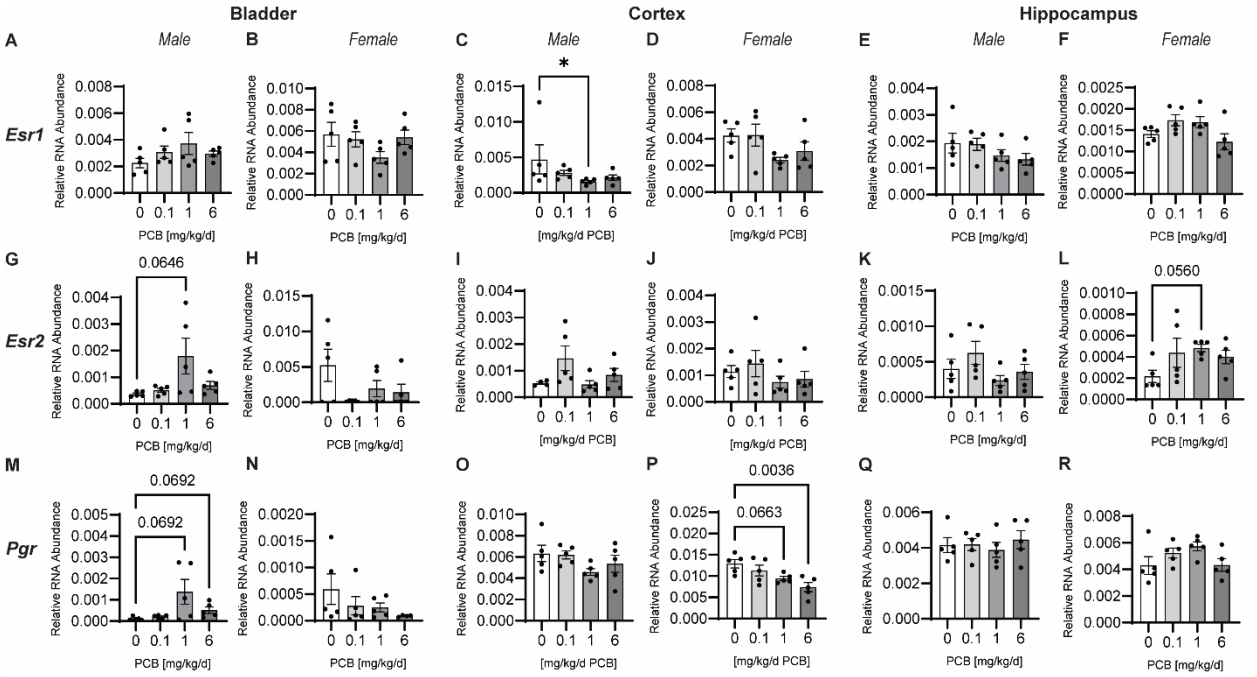

Figure 2. Developmental exposure to MARBLES PCBs drives sex- and dose-dependent changes in the expression of sex steroid hormone receptors in the bladder, hippocampus, and cortex in 6-week-old mice. Mice were developmentally exposed to PCBs via the dam and tissues were assessed at 6 weeks of age for relative RNA abundances. Results are mean ± SEM. n=4-5 animals derived from at least 3 independent litters per sex and dose group. * Indicates $p<0.05$ compared to the sex-match control as determined by one-way ANOVA and post-hoc multiple comparisons test for data with parametric data with equal standard distributions (A,B,D,E,F,J,K,L,O,P,Q,R), Brown-Forsythe ANOVA and post-hoc multiple comparisons test for parametric data with inequal standard distributions (G,I,M,N), or as determined by Kruskal-Wallis and Dunn's multiple comparisons test with nonparametric data (C,H).

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

This chapter has been in part adapted from a published manuscript in Journal of Muscle Research and Cell Motility (2025 Sept 1. doi: 10.1007/s10974-025-09707-w). The manuscript was authored by Monica Ridlon, Julia Tlapa, and Kimberly Keil Stietz. MR proposed and led the writing of the manuscript; MR, JT, KKS reviewed and edited the manuscript; all authors read and approved the final manuscript.

CONCLUSIONS

Defining the role of PCBs in lower urinary tract physiology and function is an important step in developing intervention strategies to improve human health. In this work, we investigated the impacts of developmental exposure (*in utero* and lactational) to a human-relevant mixture of PCBs. Absent of signs of overt toxicity, developmental exposure to the MARBLES mixture of PCBs results in changes to urinary voiding physiology of mice in a time-, sex-, and dose-dependent manner. We extended the age of characterization of urinary voiding physiology from 6 weeks of age to 12 weeks of age, with the addition of a secondary stressor, sensitizing chemical exposure. This work highlighted that developmental exposure to the MARBLES PCB mixture impacts urinary voiding physiology, both during homeostatic conditions and upon challenge of chemical sensitization, and these effects on voiding can converge, diverge, or persist over the course of normal aging. To identify histological and molecular targets and outcomes of PCB exposure capable of augmenting urinary voiding physiology, we investigated bladder innervation and key ion channels and neurotransmitter receptors involved in bladder contractility, which are important regulators of nerve-evoked and myogenic bladder contractions. An *ex vivo* bladder contractile assay revealed PCBs decrease contractile strength and sensitivity when mimicking nerve evoked bladder contraction, change the composition of BK channel regulatory beta subunits, and modulate the efficacy of increasing spontaneous bladder contractions from pharmacological agents, all in a sex- and dose-dependent manner. Then, we expanded the scope of this work to consider the role of PCBs as endocrine disruptors to characterize puberty onset, serum concentrations of steroid hormones, and expression of their respective receptors in the bladder and brain. This work demonstrated that while developmental exposure to MARBLES PCBs does not elicit robust markers of endocrine disruption, it does change the expression of sex

steroid hormone receptors in regions of the brain important for cognition. These studies demonstrate that mice with a similar profile and total burden of PCBs as humans is capable of changing urinary voiding physiology, and we identified several molecular targets of PCB action. These results advance the understanding of early life chemical exposures can not only lead to adverse health outcomes, but also these changes can continue to occur into adulthood. By better understanding the physiological and functional outcomes of PCB exposure, we can better investigate and contextualize the molecular mechanisms that can lead to adverse health outcomes.

FUTURE DIRECTIONS

PCB metabolites directly binding to estrogen receptors and inducing ERE gene transcription

PCBs are metabolized by cytochrome P450s (CYP) enzymes to hydroxylated metabolites (OH-PCBs) (Grimm et al., 2015b). Mice with developmental exposure to MARBLES PCBs increases hepatic CYP expression, with PCBs and OH-PCBs measurable in the blood, urine, and bladder at 6 weeks of age (H. Wang et al., 2026a). Chapter 4 investigated markers of endocrine disruption in mice with developmental exposure to MARBLES PCBs, which included changes in estrogen and progesterone receptor expression in the brain. As a next step in this project, I propose we test if PCBs and OH-PCBs relevant in the developmental exposure paradigm be tested for the ability to bind to estrogen receptors and induce estrogen responsive element (ERE) gene transcription.

Previous studies have demonstrated OH-PCBs are able to directly bind to estrogen receptors including through predictive modeling (Akinola et al., 2024; Li et al., 2013, p. 201; Ruiz et al., 2016; Waller et al., 1995), and competitive binding analysis (Korach et al., 1988; Kuiper et al., 1998; Vakharia and Gierthy, 2000). Using cell-based reporter assays, OH-PCBs are demonstrated to induce ERE-mediated gene transcription (Bonefeld-Jørgensen et al., 2001; Tam et al., 2022; Zhang et al., 2014). Using a similar assay with a luciferase-based reporter, it would be feasible to test not only the relevant PCBs and OH-PCBs present in mice with developmental exposure to MARBLES PCBs. This approach also allows for testing mixtures effects that may amplify the transcriptional effects when the different chemicals are present in combination rather than in isolation. Resolving if MARBLES PCBs induce gene transcription via estrogen receptors is a necessary step to understanding the direct mechanisms involved in the adverse health outcomes.

Linking estrogenic PCB action to bladder function

Endocrine disruption can adversely impact urogenital processes including bladder processes involved in urine storage and micturition, as well as prostatic enlargement that can cause obstruction (De Falco and Laforgia, 2021; Nicholson et al., 2018; Turco and Vezina, 2023). Estrogen receptors are present in the urinary bladder of mice (Carley et al., 2003) and humans (Iosif et al., 1981). ERs are potentially involved in the pathogenesis of LUTS including benign prostatic hyperplasia-related LUTS (Nicholson et al., 2015; Shao et al., 2014) and interstitial cystitis (Imamov et al., 2007), but the mechanisms of action are not clearly understood. The pathogenesis of LUTS is complex, and further research is necessary to more fully elucidate the role of environmental chemical such as PCBs in the etiology of LUTS. It is of great interest to better understand the extent that PCBs can act upon steroid hormone receptors in the bladder to influence voiding physiology, with potential mechanism via increasing big potassium channel expression and decreasing muscarinic acetylcholine receptor expression.

Estrogen receptor-mediated gene transcription may increase BK channel expression in the bladder, which is observed in other tissues and cell lines but has not been tested in the bladder. 14β-Estradiol (E2) increases BK subunit mRNA and/or protein abundance in the vascular smooth muscle of sheep (Hu et al., 2011; Nagar et al., 2005) and neuronal cell lines of mice and humans (Li and Qiu, 2015b; Nishimura et al., 2008). E2 acts via estrogen receptors to increase BK channel expression (Li and Qiu, 2015b; Nishimura et al., 2008). PCBs and other endocrine disrupting compounds including bisphenol A (BPA) can bind to estrogen receptors and trigger its transcriptional activity (Kuiper et al., 1998). BPA increases BK channel expression in a pancreatic beta cells line via estrogen receptors (Martinez-Pinna et al., 2019). The ability for MARBLES to impact BK channel expression was investigated in Chapter 3, where we observed

sex- and dose-dependent increases to certain BK channel subunit expression. Whether this increase in BK channel expression was a result of estrogen receptor-mediated gene transcription is of interest for further study as a mechanism for PCBs and other estrogen-mimicking chemicals to impact bladder function.

Changes in cholinergic signaling in bladder smooth muscle have been observed in patient populations suffering from interstitial cystitis/painful bladder syndrome (Berndt-Paetz et al., 2019) and neurogenic bladder dysfunction (Mukerji et al., 2006). Decreased abundance of one of the bladder muscarinic acetylcholine receptors (M_3 subtype) can reduce the bladder's ability to contract (Aizawa and Igawa, 2017) and overabundance of M3 is correlated with overactive bladder (Birder et al., 2013). Female rats with E2 treatment, have increased maximum micturition volume and decreased M3 expression in the bladder (Matsumoto et al., 2007). In Chapter 3, we observed developmental exposure to MARBLES PCBs can impact RNA abundances of muscarinic acetylcholine receptors in the bladder, including sex- and dose-dependent effects on M3 expression. In Chapter 1, we reviewed treatments for LUTS including those that target muscarinic acetylcholine receptors, which improves LUTS symptom severity primarily by reducing detrusor smooth muscle contraction (Athanasopoulos and Giannitsas, 2011). While antimuscarinic/anticholinergic drugs are generally well tolerated and improve symptom severity, muscarinic receptors are present in tissues outside of the urinary tract, leading to the drugs having side effects including dry mouth, dry eyes, and constipation (Foon and Toozs-Hobson, 2007; Madhuvrata et al., 2012). Thus, further understanding of how environmental chemicals including PCBs interact with muscarinic receptor expression, activity, and pharmacological targeting is of interest for their roles in LUTS and LUTS treatment.

Aging and hormone treatment effects on lower urinary tract function

Hormone levels change through the course of normal aging and upon treatment of hormone therapy (Cappola et al., 2023). Hormone therapy is provided to individuals for a variety of treatments including precocious puberty, peri- and post-menopause, birth control, hypogonadism, hair loss, and prostate enlargement (BPH) (Brabaharan et al. 2022; Jarvis et al. 2015; Oesterling 1994; Yang et al. 2025). Conditions necessitating hormone treatments themselves can be risk factors for LUTS, but if hormone treatment itself impacts LUTS is an ongoing area of study (Al-Zoubi et al. 2022; Baas and Köhler 2016; Baruch et al. 2023; Bianchi et al. 2021; Kathrins et al. 2016; Yaish et al. 2025). In mice, treatment with testosterone and estradiol to mimic the hormonal changes of aging men, results in enlarged prostate and an obstruction phenotype (Nicholson et al. 2012). Voiding physiology changes are observed in male mice after undergoing castration or treatment with finasteride (to reduce circulating testosterone levels) or when given exogenous testosterone (Ruetten et al. 2019). Similarly, treatment with testosterone in female mice leads to a more masculine voiding phenotype, and administration of finasteride also impacts voiding even in female mice (Ruetten et al. 2019). Evidence also suggests that BK channels are important for mediating effects of testosterone on the lower urinary tract. Testosterone decreases guinea pig detrusor excitability via increased BK channel open probability and BK channels are necessary for mediating effects of testosterone on detrusor smooth muscle resting potential (Hristov et al. 2016). In vascular smooth muscle, testosterone causes vasodilation in part through activation of BK channels (Lorigo et al. 2020). Estrogens are also important in lower urinary tract function and disease. Estrogen deficiency during and following menopause can lead to urogenital atrophy increasing the risk of urgency urinary incontinence and urinary tract infection (Fait 2019; Henn 2010; Su and Freeman 2009). A recent

study from the RISE FOR HEALTH study, conducted by the Prevention of Lower Urinary Tract Symptoms (PLUS) Research Consortium, demonstrates the transition from pre- to perimenopause increases the risk for urgency urinary incontinence, and overall bladder health is worsened in postmenopausal women when undergoing hormone treatment (Vaughan et al. 2025). While this study included a variety of hormone therapies including vaginal estrogen and systemic menopausal hormone therapy, a meta-review of 17 studies reported vulvovaginal topical estrogen therapy improves LUTS in postmenopausal women (Porcari et al. 2025). Ovariectomy in mice, to reduce estrogen levels, leads to mice which display an overactive voiding phenotype which is reversed by estrogen supplementation (Zhang et al. 2024). Therefore, future research may benefit from considering aging and hormone treatment effects on lower urinary tract function, and the work of Chapter 4 demonstrates the opportunity for environmental chemicals to impact the endocrine system.

PCB effects on the pontine micturition center

The pontine micturition center (PMC) is a region of the brain, including neurons that make up Barrington's nucleus, recognized for control over normal urinary voiding in rodents and humans (Fowler et al., 2008; Satoh et al., 1978; Verstegen et al., 2019, 2017). Disruption PMC signaling or input from other brain regions can lead to abnormal storage and release of urine (Fowler et al., 2008; Verstegen et al., 2017), and direct stimulation of PMC neurons elicits immediate voiding in awake and anesthetized mice (Verstegen et al., 2019). Neurotoxicants, including PCBs, are of interest for how they impact PMC neuron signaling as a potential mechanism for disruption of brain-bladder signaling that can lead to LUTS. This is an ongoing area of research, and details about how future studies can address this research question are available in Appendix A.

TAKEAWAYS

A classic conundrum in science is understanding the relative influences of nature versus nurture. As a toxicologist, my perspective on this question is how do our bodies exist in the world and how does external stimuli change us? To this end, there are three broad frameworks this thesis fits within. (1) *The regular process of health across life stages*. The only way to escape the influence of time is to cease to exist, and nothing truly remains the same.[1] As the body develops, ages, and performs the functionalities of life, it undergoes systemic changes. Hormonal status, pregnancy, interoception (mind-body connection), are a few of many ways hormonal, psychophysiological, and responses to external stimuli produces intrinsic changes to physiological functionality. (2) *The influence of unintentional chemical exposures*. Polychlorinated biphenyls (PCBs) are chemicals that are no longer intentionally produced for consumer or industrial uses. Yet despite government regulations, everyone is chronically exposed to these chemicals regardless of lifestyle or geological location. While this thesis focuses on PCBs, these chemicals are merely a drop in the ocean of anthropogenic or naturally occurring chemicals that make up a person's exposome. The exposome, the culmination of environmental exposures throughout an individual's lifetime, is unique to each person, and no two people will respond exactly the same to a chemical exposure. Thus, each person is unique in both their exposure profile at any individual point, the culmination of their lifetime circumstances, and how responses to these stressors set the groundwork for future responses. (3) *The opportunities for*

[1] [1]While I do not interrogate this perspective further, for anyone interesting in engaging with this topic further I would recommend the book "Why the World Doesn't Make Sense: An Inquiry into Science, Philosophy, and Perception" by Steve Hagen. While it has been nearly a decade since I first picked up this book, it continues to shape and challenge my world view.

therapeutic interventions. In biology, we continue to expand our understanding of the human body, fueling medical advances and technologies that were otherwise unfathomable in recent decades and centuries. Humanity, despite its many flaws and challenges, continues to expand upon in knowledge and ability. It is my hope that my work can contribute to even the smallest positive impact on the health and wellbeing of people alive today and for future generations. As the frontier of science moves forward, it shines light upon uncertainties, which reframes current understandings and provides opportunities for future trajectories of progress. I believe that by understanding the basic underlying mechanisms and processes of human health and function, we can move forward in understanding pathophysiology at a more fundamental level.

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APPENDIX A

BACKGROUND

Polychlorinated biphenyls (PCBs) are established developmental neurotoxicants that disrupt neuronal Ca^{2+} signaling, impair cognition, and are risk factors for neurodevelopmental disorders. While this thesis has characterized markers of urinary function and physiology, including bladder smooth muscle dynamics and endocrine disruption, a key factor at the forefront of urological research has been omitted. This is the role of the bladder-brain connection. The goal of this project is to use fiber photometry in mice to measure PCB effects on excitatory neuronal activity in the pontine micturition center of the brain, a region within the pons responsible for the initiation of the urinary voiding reflex in mice and humans, during cystometry to concurrently measure bladder function. Utilizing a genotype of mice with Cre recombinase present only in glutamatergic neurons, and then introducing the calcium sensor via AAV injection, then we control for both the location and cell type for fluorescence. Visualization of fluctuations in fluorescence of the calcium sensor acts as a proxy for neural activity within glutamatergic neurons of the PMC during urinary voiding. My contribution to this project has been in the validation of methodology and generation of preliminary data through animal husbandry during animal generation and post-surgery monitoring, as well as optimization of the protocol for post-humorous validation of the fiber photometry readout. This side project has supported the application of an NIH RO1 grant and serves as a future direction for this thesis work.

APPROACH

Female nulliparous wild type mice (C57Bl/6J, Jackson Laboratories, #000664) are dosed daily with vehicle (peanut butter/peanut oil) or MARBLES PCB mix (mixed in peanut butter). After 14 days of PCB or vehicle control exposure, the female wild type mice are mated with VGlut male mice (VGlut2-ires Cre mice, Jackson Laboratories, #016963, homozygous viable) to produce 100% heterozygous offspring mice. Offspring are weaned at 3 weeks of age (last exposure to lactational PCBs) and housed with same sex and dose littermates. Once offspring reach 6 weeks of age, they undergo a stereotactic injection of a cre-dependent GCaMP6 calcium sensor (pAAV.Syn.Flex,GCaMP6s.WPRE.SV40 (AAV1), #100845, Addgene) in the PMC as described (Verstegen et al., 2019, 2017). PMC brain coordinates (from Bregma, in ~30gr mouse) are AP: -5.40, DV: -3.70, ML: 0.68. The optical fibers are implanted at the same time as AAV injection, and are a stainless-steel ferrule 1.25mm, Fiber optic type: 400um, NA 0.66, 4mm length, flat fiber tip purchased from Doric (Doric, MFC_400/430-0.66_4mm_MF1.25_FLT mono fiberoptic cannula). Following surgery, mice are housed for an additional 6 weeks of age to allow time for optimal GCaMP protein expression (Verstegen et al., 2019). At this point, mice are at least 12 weeks of age, and research performed in Chapter 2 supports testing PCB effects on urinary voiding function and physiology at this age with the same exposure paradigm (Ridlon et al., 2025a).

Anesthetized cystometry is performed as previously described in Chapter 2 (Ridlon et al., 2025), while at the same time fiber photometry will be conducted as previously described (Verstegen et al., 2019, 2017). Briefly, fluorescence from the GCaMP6 calcium reporter reflecting calcium influx into the VGlut2 PMC population of nerves and neural activity is recorded using the ThorLabs fiber photometry system to excite, collect and filter GCaMP6 fluorescence, which is

interfaced with LabChart and Matlab software for data acquisition and analysis (Verstegen et al., 2019). Analysis of the fluorescence signal is calculated as relative change in fluorescence will be calculated ((Ft-F0)/F0) with the background signal subtracted (405 nm excited fluorescence subtracted from the 465 nm excited fluorescence) (Verstegen et al., 2019).

Following the procedure, animals will be euthanized and brains collected, fixed, and sectioned. Sections will undergo immunohistochemistry to confirm placement of the canula and expression of the GCaMP in the PMC, which lies adjacent to the locus coeruleus stained by antibodies targeting tyrosine hydroxylase (TH), as visualized in Fig. 1.

FIGURES

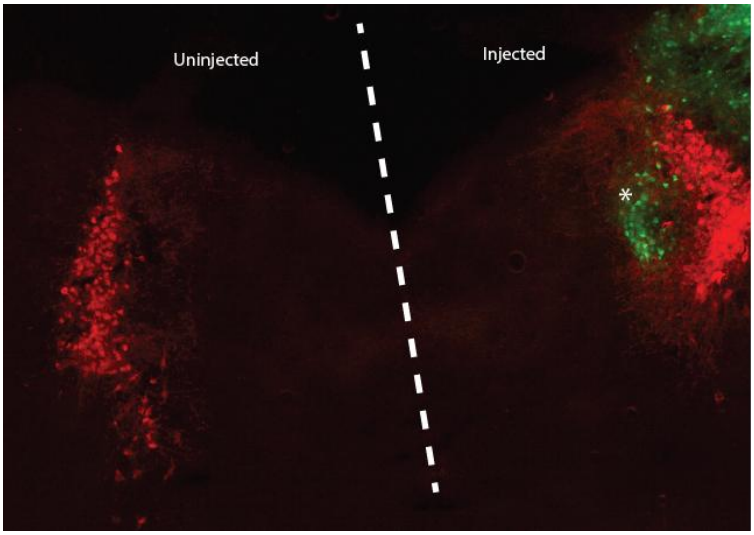

Figure 1. Stereotactic injection of AAV-GCaMP6 (green) into PMC of mouse brain. AAV-GCaMP6 (green) expression in a mouse brain slice 6 weeks after stereotactic injection. Immunohistochemistry was also performed using an antibody targeting tyrosine hydroxylase (TH) to stain TH positive neurons (red) of the locus coeruleus which lies adjacent to the PMC.