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Bladder overactivity induced by psychological stress in female mice is associated with enhanced bladder contractility

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Abstract

Aims: To investigates the effects of water avoidance stress on voiding behaviour and functional bladder responses in mice.

Main Methods: Mice in the Stress group were exposed to water avoidance stress (WAS) for 1hr/day for 10 days, Controls were age-matched and housed normally. Voiding behaviour was measured periodically throughout the stress protocol and bladders were isolated 24-h after final stress exposure to measure bladder compliance, spontaneous phasic activity, contractile responses, and release of urothelial mediators.

Key Findings: Repeated stress exposure induced a significant increase in plasma corticosterone levels in the WAS group compared to control. An overactive bladder phenotype was observed in WAS mice, causing a significant increase in the number of voiding events observed from as early as day-3, and a 7-fold increase following 10-days' stress. This increase in voiding frequency was associated with a significant decrease in void size, an increase in the number of small voids, but no change in total voided volume. Bladders from stressed mice showed a significant increase in the maximum responses to the muscarinic agonist carbachol ($p<0.01$), in addition to enhanced pressure responses to the purinergic agonists ATP ($p<0.05$) and αβ-mATP ($p<0.05$), and non-receptor mediated contractions to KCl ($p<0.05$) compared to controls. Nerve-mediated bladder contractions to electric field stimulation were not significantly affected by stress, nor were spontaneous phasic contractions or release of urothelial ATP and acetylcholine.

Significance: Repeated exposure to water avoidance stress produced an overactive bladder phenotype, confirmed by increased voiding frequency, and associated with enhanced bladder contractile responses.

Keywords: psychological stress; bladder; water avoidance stress; urinary frequency

Introduction

Overactive bladder (OAB) is defined by the International Continence Society as urinary urgency, with or without urge incontinence, usually accompanied by frequency and nocturia, in the absence of urinary tract infection or other obvious pathology (Haylen et al. , 2010). In many instances the origins of OAB remains idiopathic (Leron et al. , 2018). Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic disorder which shares common symptoms with OAB, however, is distinguished by the presence of pain on bladder filling in the absence of infection (Castro-Diaz et al. , 2014, Hanno et al. , 2010). IC/BPS is associated with local inflammation in the bladder wall, which together with sensitization of afferent mechanisms is likely to contribute to the pain experienced, however the origins of these changes remain unknown.

Psychological stress can impact several visceral functions with pathological consequences (Meerveld and Johnson, 2018, Roohafza et al. , 2016), and a growing body of clinical evidence exists linking bladders disorders such as OAB and IC/BPS with psychological stress and stress disorders including anxiety and depression and post-traumatic stress disorder (Bradley et al. , 2014, Fan et al. , 2008, Lai et al. , 2015, Lai et al. , 2016, Rothrock et al. , 2001a, Rothrock et al. , 2001b, Sanford and Rodriguez, 2017, Zhang et al. , 2013). Stress appears to greatly influence the development of bladder symptoms, or worsens symptom severity in both adults and children (Braga et al. , 2019, Minassian et al. , 2013). Lai et al observed that OAB patients reported psychological stress levels as high as those reported by IC/BPS patients, with both groups experiencing significantly higher stress levels compared to healthy controls (Lai et al., 2015). One in 5 female veterans report OAB post-deployment, a prevalence much greater than expected for their age group based on data from the general population; with anxiety, depression and prior sexual assault found to influence the natural progression of OAB in female veterans (Bradley et al. , 2017, Bradley et al., 2014). Bladder dysfunction has also been observed amongst sexual abuse survivors, with storage and voiding symptoms more common in abuse survivors compared to controls (Davila et al. , 2003).

Experimental findings from studies using rodent models of psychological stress have shown that stress exposure can play a causal role in the development of bladder dysfunction (Merrill et al. , 2013, Pierce et al. , 2018). Water avoidance stress has been reported to increase bladder frequency, while decreasing mechanical pain thresholds in rats causing bladder hyperalgesia (Lee et al. , 2015, Matos et al. , 2017). Neonatal maternal separation influences voiding and visceromotor changes associated with water avoidance stress in mice (Pierce et al. , 2016),

strengthening the connection between adverse early childhood events and bladder disorders such as IC/BPS.

Normal bladder function requires involvement of local afferent and efferent systems, with sympathetic bladder relaxation via beta-adrenoceptor stimulation, limiting the increase in intravesical pressure during bladder filling. To initiate voiding, acetylcholine and ATP co-released from parasympathetic nerves instigate detrusor contraction via M_3 muscarinic and P_2X_1 purinergic receptors respectively (Sellers et al. , 2000, West et al. , 2018). The urothelium while acting as a barrier also plays an important sensory and signalling role, releasing numerous chemical mediators including ATP, acetylcholine, PGE_2 and nitric oxide, that contribute to regulating bladder function (Birder et al. , 2012, Sellers et al., 2000). A number of urothelial mediators also influence detrusor contractility (Sellers et al. , 2018). The impact of psychological stress on these bladder mechanisms has not been explored. Therefore, the aim of this study was to investigate the impact of repeated water avoidance stress on voiding behaviour in female mice and determine the changes in bladder function contributing to altered voiding.

Materials and Methods

Water Avoidance Stress Model

All procedures were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and with the approval of the University of Queensland Animal Ethics Committee. Adult female C57Bl/6J (12-14 weeks in age; n=7 in each group) were used in this study and housed under environmentally controlled conditions, with 12-hour light-dark cycles, with access to food and water ab libitum. Mice were randomly allocated into two experimental groups: Control or Water Avoidance Stress (WAS).

WAS is commonly used in rodents to induce a stress response and the protocol used in this study is as previously described (Sun et al. , 2013). Mice in the WAS group were placed individually on a central pedestal surrounded by water for 1 hour/day for 10 consecutive days. Each WAS exposure was observed by the researcher and mice that jumped off the podium into the water were given the chance to climb back on the pedestal, while those that failed to do so were placed back on the pedestal. Following each stress exposure mice were returned to their normal housing. The control group consisted of age-matched mice housed under normal conditions and were not exposed to water avoidance stress protocols. Control animals were only removed from normal housing during the 10-day experimental protocol for voiding pattern analysis.

Voiding Pattern Analysis

Voiding pattern analysis (VPA) is a semi-quantitative method used to assess voiding behaviour in rodents. It was carried out as previously described to determine the impact of water avoidance stress on urinary frequency, total voided volume, average void size and number of small voids (West et al., 2018, West et al. , 2020). VPA was performed prior to (baseline) and at intervals (1, 3, 5, 7 and 10 days) during and following the WAS protocol. Briefly, standard mouse cages were lined with 'Filtech' hardened ashless filter paper, Quantitative 2um grade 225. The mice were placed individually into the lined cage for 4 hours at the beginning of the light cycle, with access to food and drinking water. Faecal pellets were collected; dried, counted and weighed to assess changes in faecal output. VPA filter papers were collected and urine spots detected using a Molecular Imager ChemiDoc XRS ultraviolet transilluminator (#720BR1293 BioRad, California USA). The papers were photographed, digitized, and then analyzed using Image J software, to measure size (surface area) and number of voids.

Isolated Whole Bladder Preparation

An isolated whole bladder preparation was used for functional bladder studies as previously described (West et al., 2018, West et al., 2020). Mice were sacrificed by cervical dislocation 24 hours following final water avoidance stress exposure, at which time a venous blood sample was taken, and plasma corticosterone levels quantified using the Corticosterone Competitive ELISA (Invitrogen) according to the manufacturer's instructions. Blood samples were collected in the morning to avoid variation due to diurnal changes in corticosterone levels. The bladder was then isolated and a three-way catheter was inserted through the urethra into the bladder. The urethra and ureters were ligated and the bladder was placed into a bath of gassed (95%O_2/5% CO_2) Krebs-bicarbonate solution (composition in mM: NaCl 118, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KCl 4.7, KH_2PO_4 1.15, and D-glucose 11.7) at 37ºC. The three-way catheter was attached to an infusion pump filled with Krebs-Bicarbonate solution to allow bladder filling, a pressure transducer and an outflow syringe to collect intraluminal fluid and allow bladder emptying. Intravesical pressure was measured using a pressure transducer (GlobalTown Microtech, Sarasota, FL) connected to a PC via a PowerLab data acquisition system (AD Instruments, Sydney, Australia), using LabChart 7 software (AD Instruments). Following equilibration, bladder distensions were performed by intravesical infusion of saline at a rate of 30 μL/min to 40 mmHg to assess viability, and to 20 mmHg for all further distensions.

The inner lining of the bladder, the urothelium is known to release numerous signalling mediators during bladder filling including ATP and acetylcholine. To determine the effect of WAS on urothelial mediator release, following distension to 20 mmHg, intraluminal fluid was collected via the catheter and samples stored at -80ºC until analysis of ATP and acetylcholine levels. Quantification of ATP and Ach was carried out using the ATP Determination Kit (Molecular Probes), and the Acetylcholine Amplex Red Assay Kit (Molecular Probes) respectively. The assays were performed according to manufacturer instructions, with luminescence and fluorescence (excitation 540, emission 590 nm) measured, using a Modulus micro-plate reader (Promega).

Following bladder distension to 20 mmHg, bladders were allowed to equilibrate for approximately 60 minutes, during which time spontaneous phasic activity was measured as

frequency of spontaneous contractions, and recorded as the number of contractions per minute, and also the amplitude measured as the change in intravesical pressure from the trough to peak of the contractions.

The effect of WAS on nerve-evoked contractile bladder responses was assessed by electric field stimulation (EFS). The bladder was electrically stimulated (0.1ms pulse-width, 50 V) for 5 seconds, every 100 seconds at 1-20 Hz. Bladders were stimulated at each frequency until 3 consistent response were obtained and contractions were measured as increase in intravesical pressure from baseline. EFS was repeated at 20 Hz in the absence and presence of atropine (1 μM) and αβ-methylene ATP to remove cholinergic and purinergic components, respectively. Application of tetrodotoxin (0.1 μM), abolished responses to EFS, confirming the neurogenic origins of the pressure responses observed.

Intravesical pressure responses to pharmacological agents were also assessed by addition of cumulative concentrations of the muscarinic agonist carbachol, the purinergic agonists ATP (10 mM) and αβ-methylene ATP (10 μM) and relaxations to the beta-adrenoceptor agonist isoprenaline following precontraction with carbachol (1 μM). Non-receptor mediated contractile bladder responses were also assessed using KCl (60 mM). All contraction and relaxation responses were measured as change in pressure from baseline.

Statistical Analysis

All experiments were randomized, with seven mice per experimental group and each experimental protocol started on a different day. All graphical analyses and statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, USA). Data is presented as mean ± standard error of the mean (SEM). Significance levels were defined as $p<0.05$ (*). All curve analysis was undertaken using non-linear regression analysis to construct concentration-responses, and used to compare curves between groups and generate EC_{50} or IC_{50} values. Data were analysed using unpaired Student's t-test (Control vs WAS) or repeated measures two-way ANOVA with Tukey-Kramer multiple comparisons test (for time course and EFS data).

Results

Effect of WAS on Animal Parameters and Voiding Behaviour

Animal body weight and bladder weight were not significantly affected by WAS (Table 1). Voiding pattern analysis was performed to assess changes in voiding behaviour following stress exposure with representative image of VPA filter papers shown for control and WAS animals at day 5 (Figure 1A and B). Exposure to WAS resulted in a significant increase in urinary frequency as early as day 3 (Figure 1C), a change that was associated with a decrease in the average void size and an increase in the number of small voids (Figure 1D&F). There was no difference to total voided volume between the WAS and Control groups (Figure 1E), indicating that the increase in voiding frequency in the WAS group was not due to increased urine production. No change in faecal output was observed following WAS (Figure 1G-H). A significant increase in plasma corticosterone was observed in the WAS group following the 10-day stress exposure compared to unstressed control mice (Figure 2B), indicating that a hormonal stress response was present.

Table 1: Baseline animal parameters and whole bladder responses to carbachol and isoprenaline in control and WAS mice. Data is presented as mean ± S.E.M. (n=7) analysed using unpaired Student's test (**p<0.01 vs Control).

	Control	**WAS**
	Animal Parameters	
Body weight (g)	18.2±0.48	18.5±0.67
Bladder weight (mg)	21.7±1.08	20.78±0.53
	Whole Bladder Responses	
Carbachol		
pEC_{50}	5.42±0.11	5.41±0.07
Maximal response (mmHg)	24.5±2.06	32.4±1.71 **
Isoprenaline		
pIC_{50}	6.87±0.12	6.71±0.16
Maximal response (% decrease)	121.6±8.5	135.4±15.1

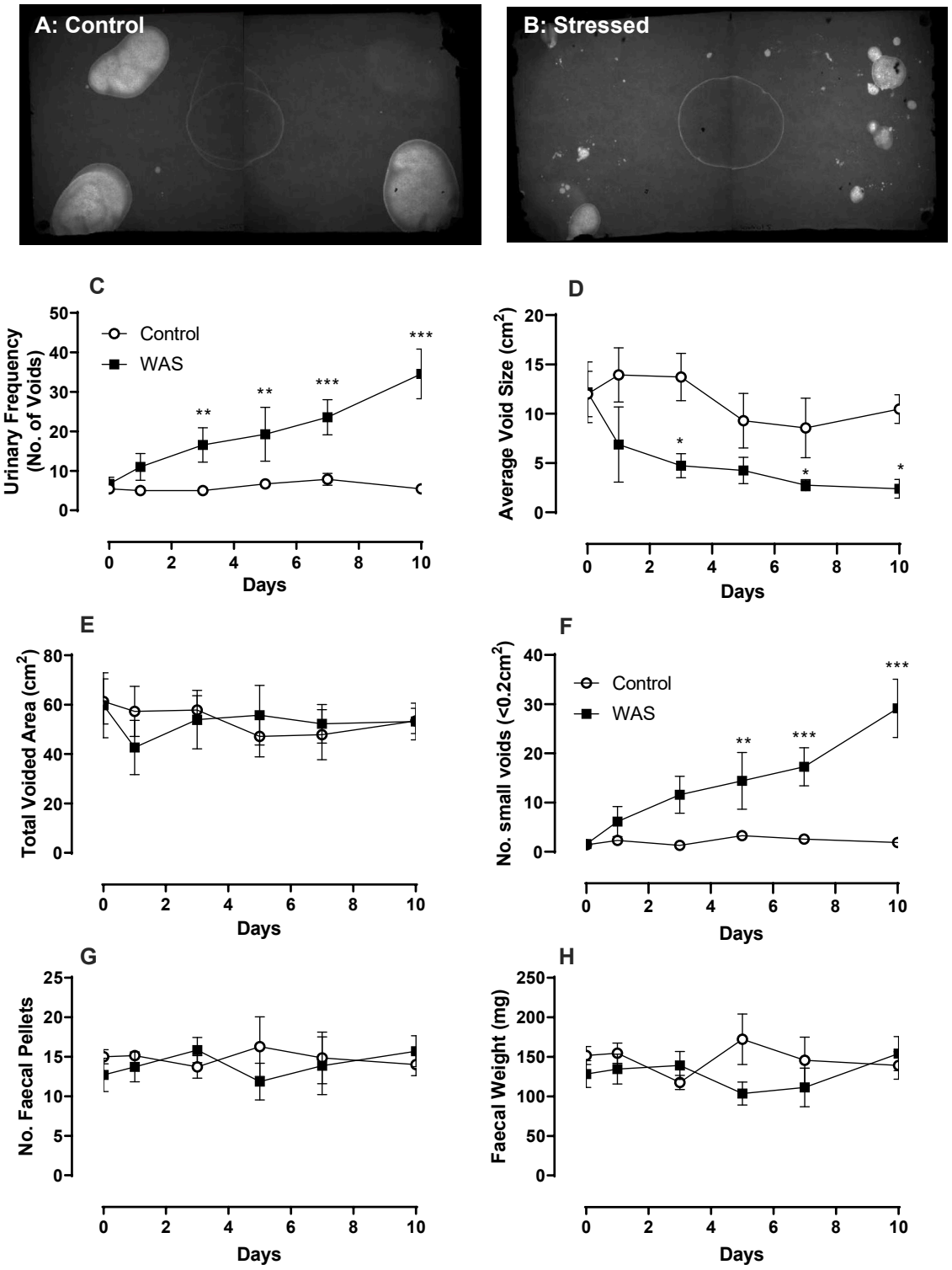

Figure 1: Representative voiding pattern analysis images from A) control and B) WAS animal at day 5. Effect of water avoidance stress on voiding behaviour in female mice measured as C) urinary frequency, D) average voided area, E) total voided area, F) number of small voids, G) number of faecal pellets and H) faecal weight over the 4hr observation period. Data represents

mean ± SEM (n=7) and was analysed using two-way ANOVA with Tukey-Kramer multiple comparisons test.

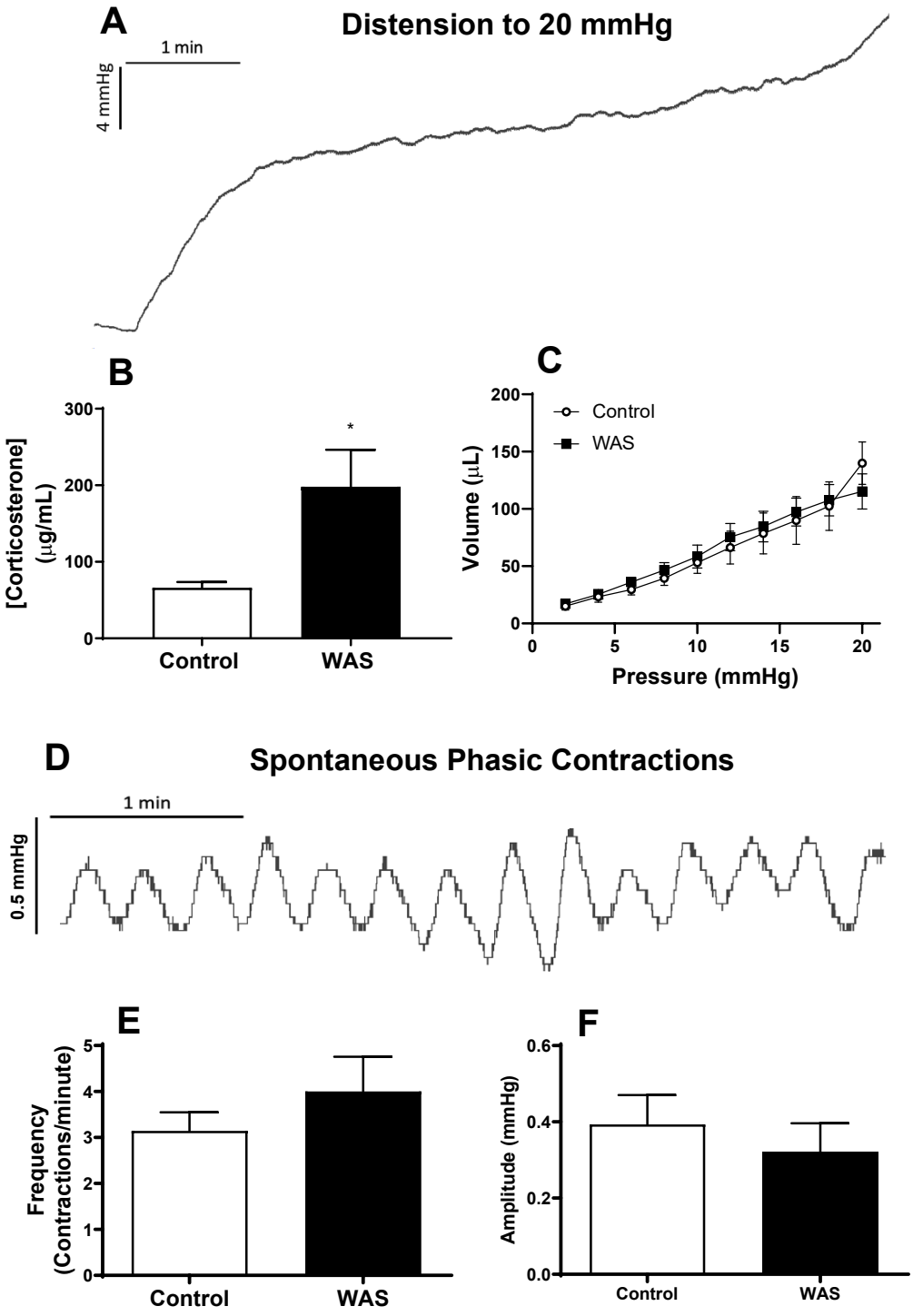

Figure 2: Representative trace showing A) bladder distension to 20 mmHg and D) spontaneous phasic contractions during accommodation in controls. Effect of WAS on B) plasma

corticosterone levels at the time of sacrifice, C) the volume pressure relationship during filling of isolated bladders, and E) the frequency and F) amplitude of spontaneous phasic contractions recorded before the addition of any drugs. Data represents mean ± SEM of n= 7 independent experiments and was analysed using two-way ANOVA (C) or unpaired Student's t-test (B, E & F).

Effect of WAS on Bladder Compliance, Contractile Responses and Urothelial Function

Bladder compliance measured as the volume-pressure relationship during bladder distension to 20mmHg (Figure 2A) remained unchanged following repeated WAS (Figure 2C). Spontaneous phasic contractions were observed in all bladders during accommodation (Figure 2D), and these were not significantly altered following WAS (Figure 2E&F).

Intravesical pressure increased in a concentration-dependent manner following the addition of the muscarinic agonist carbachol (Figure 3A&B), with the maximum responses increased significantly in the WAS group (Figure 3B, $p<0.01$), while the pEC_{50} values remained unchanged (Table 1). Similarly, the contractile response to purinergic stimulation with ATP and αβmATP (Figure 3 C&D), and non-receptor mediated bladder contractions to KCl were also significantly enhanced by WAS exposure (Figure 3E&F, $p<0.05$). Bladder relaxation to the beta-adrenoceptor agonist isoprenaline following precontraction to carbachol (1μM) was not significantly affected by WAS (Figure 3G&H). The phasic elements of the carbachol (1 μM) pressure response were also measured (Figure 4A), with a significant increase both the frequency ($p<0.001$) and amplitude ($p<0.05$) of the phasic component observed in the WAS group (Figure 4 B&C).

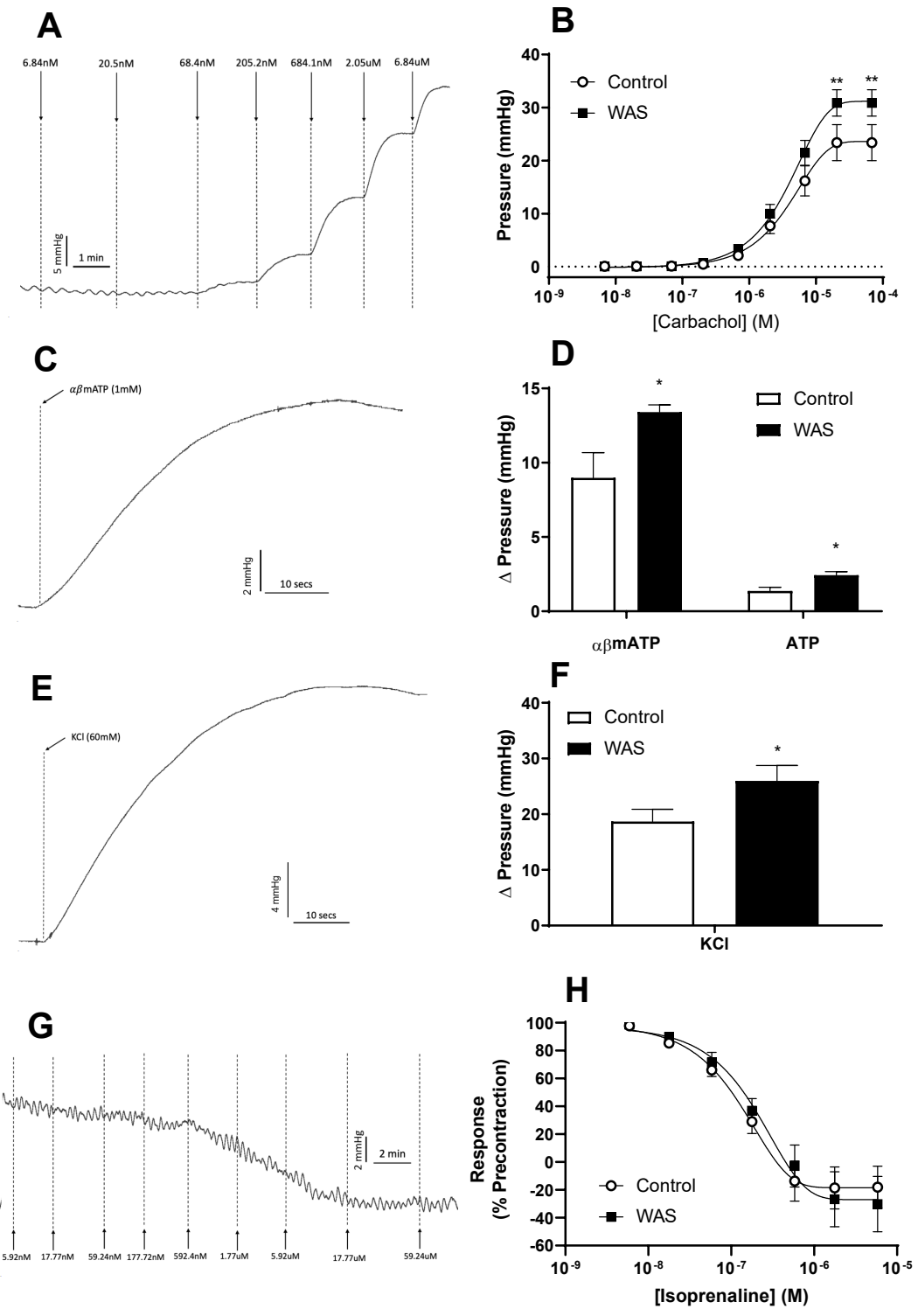

Figure 3: Representative traces showing responses of control isolated whole bladders to A) the muscarinic agonist carbachol, C) αβmATP (10 μM), E) KCl (60mM) and G) the beta-adrenoceptor agonist isoprenaline. Effect of WAS on isolated whole bladder response to B) carbachol, D) αβmATP (10 μM) and ATP (10 mM), F) KCl (60 mM) and H) isoprenaline. Data represent mean ± SEM (n=7) and was analysed using non-linear regression curve fit

analysis (A and D) or unpaired Student's t-test (B and C). (*p<0.05 vs control, **p<0.01 vs control).

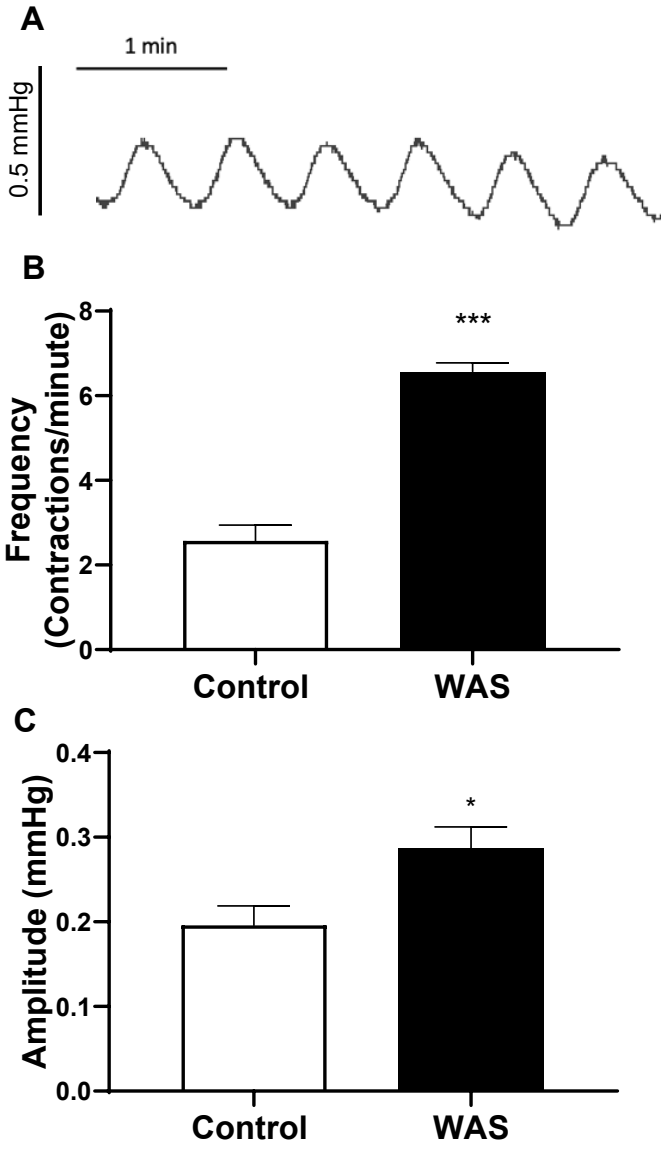

Figure 4: Representative trace showing phasic component of the response to the muscarinic agonist carbachol (1 μM) in isolated bladder from control animal and effect of WAS on the B) frequency and C) amplitude of the phasic contractions. Data represents mean ± SEM (n=7) and was analysed using unpaired Student's t-test (*p<0.05 and ***p<0.001 vs control).

EFS of isolated whole bladders produced a frequency-dependent increase in intravesical pressure in bladders from control animals (Figure 5A&B), and these nerve evoked responses were not significantly affected by WAS. Desensitization of purinergic receptors with αβmATP

decreased the response to EFS by 77.2 ± 2.3%, while the muscarinic antagonist atropine reduced the pressure response to EFS by 22.7 ± 2.3% in control bladders, indicating that the contribution of ATP to neurotransmission was greater than that of acetylcholine in control bladders, and the relative contribution of these neurotransmitters was similar in the WAS group (Figure 5C).

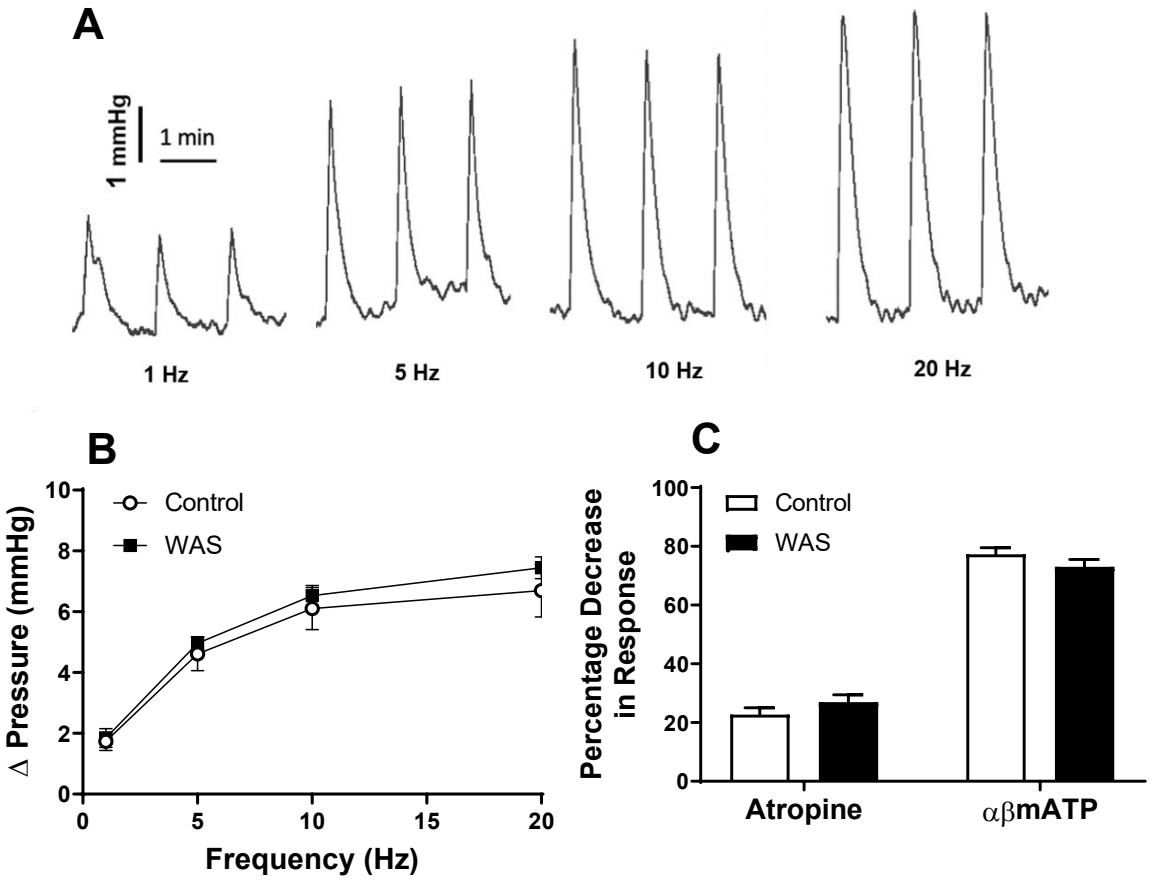

Figure 5: A) Representative frequency response trace in isolated bladder from control animal. B) Nerve-evoked (1-20Hz) pressure response of isolated whole bladders from control and water avoidance stress mice. C) Percentage decrease in nerve evoked pressure response at 20 Hz in the presence of the muscarinic antagonist atropine (1 μM) and following desensitization of purinergic receptors with αβmATP (10 μM) Data represents mean ± SEM (n=7) and was analysed using unpaired Student's t-test (*$p<0.05$ and ***$p<0.001$ vs control)

Total ATP and acetylcholine released into the intraluminal fluid was quantified as a measure of urothelial function, with total release in the control group (ATP 0.97 ± 0.41 pmoles & Ach

0.15 ± 0.05 nmoles) similar to levels in the WAS group (1.36 ± 0.41 pmoles & 0.2 ± 0.05 nmoles), with no significant change observed.

Discussion

While the link between psychological stress and bladder dysfunction has long been established with an abundance of clinical evidence, there is little understanding as to which local bladder mechanisms are altered by stress and contribute to changes in bladder function. This study employed a WAS model to determine the impact of psychological stress on voiding behaviour, detrusor and urothelial function and detrusor control by the efferent innervation.

A significant increase in plasma corticosterone following repeated exposure to WAS confirmed that the mice used in this study were stressed when voiding pattern analysis and whole bladder preparations were performed. This is consistent with other animal studies which have observed a similar hormonal stress response in the water avoidance model (Hassan et al. , 2014). Here, we observed significant changes in voiding behaviour as early as day 3 of the stress protocol, with an overactive phenotype evident in WAS mice who exhibited a significant in increased urinary frequency. Interestingly, one study also observed an increase in voiding frequency, which increased over time, compared to the control animals (Yoon et al. , 2010), however, they also found that stress increased the volume of urine output compared to the controls, while our study showed no change in urine volume following water avoidance stress, which indicates the increase in voiding frequency in mice was not simply due to changes in urine production. The number of small voids ($<0.2cm^2$) increased significantly following WAS while the average void size decreased. This suggests that voiding interval decreased in the WAS group which has previously been reported in a study that measured frequency, latency to void, voiding interval and volume (Smith et al. , 2011). This finding suggests that WAS mice are urinating a smaller volume, more frequently than the control mice which suggests that the bladder may not be emptying completely on each void or micturition is being activated at a lower bladder volume.

To investigate the local bladder mechanisms that may contribute to the altered voiding phenotype of the stressed mice, functional bladder responses were assessed using an *in vitro* whole-bladder preparation. Bladder compliance, urothelial mediator release, spontaneous phasic contractions were not affected by WAS, nor was bladder relaxation to beta-adrenoceptor stimulation. However, contractile responses to muscarinic (carbachol) and purinergic agonists

(αβmethylene-ATP and ATP) were increased significantly in the WAS group, as was receptor-independent (KCl) detrusor contraction. This suggests an overall increase in general detrusor contractility rather than changes in receptor expression and may be related to calcium-sensitisation as a result of stress induced changes in the Rho-kinase (ROCK) pathway (Frazier et al. , 2008). Detrusor smooth muscle contraction can occur independently of calcium concentration through inhibition of myosin phosphatase by ROCK, a process known as calcium-sensitization which enhances detrusor contraction (Somlyo and Somlyo, 2000, Yoshii et al. , 1999). The ROCK pathway has been shown to be upregulated under pathophysiological conditions in bladder smooth muscle (Zhang and DiSanto, 2011), and has been reported to be enhanced in rodent stress models (Han et al. , 2015, Yoon et al., 2010). The immunohistochemical expression of ROKα was significantly increased in the rat bladder following 14-days of environmental stress compared to controls and was accompanied by an increase in voiding frequency (Yoon et al., 2010). Chronic variable stress also increased RhoA/ROCK expression in the rat bladder, with a decrease in micturition duration, interval and volume also reported (Han et al., 2015). The changes in the ROCK pathway previously described, may explain the overall enhanced bladder contractility in the current study, and may contribute to alterations in voiding behaviour with water avoidance stress.

Interestingly, nerve mediated bladder responses to EFS, were not affected by stress. The normal physiological contraction to void relies on the co-release of neurotransmitters ACh and ATP, acting on M_3 muscarinic receptors and P_2X_1 purinoceptors respectively (Sellers et al., 2000), and the relative contribution of these neurotransmitters was not altered by WAS in the present study. Given the general increase in bladder contractility observed in the WAS group, the absence of a similar changes in nerve mediated contraction suggests that the efferent nerves and/or the process of neurotransmission are affected by stress. A possible explanation would be increased breakdown of neurotransmitters after repeated psychological stress. While this theory has not been well documented previously, one study concluded that acute stress increases ATP diphosphohydrolase activity, contributing to elimination of ATP, and increased availability of extracellular adenosine; while chronic stress resulted in increased ecto-ATPase activity (Fontella et al. , 2004). A_1 adenosine receptors have been implicated in modulating neuromuscular transmission in the bladder, with A_1 agonists shown to inhibit murine bladder contractions in response to EFS (Searl et al. , 2016). A greater rate of neurotransmitter breakdown may explain why nerve-mediated contractions were unchanged despite an increase in bladder contractility. Increased nerve density has previously been linked to overactive

bladder (Arrabal-Polo et al. , 2012), however, our findings would suggest this is not the case in the WAS model given the absence of changes in nerve mediated contraction.

In addition to large increases in tonic contraction to carbachol, lower concentrations induced phasic contractile activity. A sub-maximal carbachol concentration was used to precontract bladders to allow examination of β-adrenoceptor mediated relaxation and the phasic activity during this response was also quantified. The amplitude and frequency of this activity was significantly enhanced in the WAS group. M_2 and M_3 receptor subtypes have been linked to the phasic component of carbachol contractions in guinea pig bladder, with bladder volume also shown to influence the response in the murine bladder (Lagou et al. , 2006, Srinivasan et al. , 2006). It has been suggested that fluctuations in this phasic response may amplify signals to sensory nerves, bringing them above threshold and initiating micturition (Lagou et al., 2006). Given that bladder compliance was not altered by WAS but there was an increase in urinary frequency, it suggests that sensory changes may also be involved in the altered voiding patterns observed.

Conclusion

The results presented here indicate that psychological stress affects bladder function, in terms of increased voiding frequency, as well as enhanced contractile responses. We identified a non-specific increase in detrusor contraction that is offset by changes in the efferent innervation. Furthermore, urothelial release of ATP and ACh was not changed following WAS at the level of distension tested, suggesting these mediators may not be contributing to the alterations in voiding observed.

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