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Social Stress Induces Changes in Urinary Bladder Function, Bladder NGF Content and Generalized Bladder Inflammation in Mice

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Running head: Social-stress induction of bladder dysfunction

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Abstract

Social stress may play a role in urinary bladder dysfunction in humans, but the underlying mechanisms are unknown. Here, we explored changes in bladder function caused by social stress using mouse models of stress and increasing stress.
In the stress paradigm, individual submissive FVB mice were exposed to C57BL/6 aggressor mice directly/indirectly for 1 hour per day for 2 or 4 weeks. Increased stress was induced by continuous, direct/indirect exposure of FVB mice to aggressor mice for 2 weeks. Stressed FVB mice exhibited non-voiding bladder contractions and a decrease in both micturition interval (increased voiding frequency) and bladder capacity compared with control animals. ELISA assays demonstrated a significant increase in histamine protein expression with no change in NGF protein expression in urinary bladder compared to controls. Unlike stressed mice, mice exposed to an increased stress paradigm exhibited increased bladder capacities and intermicturition intervals (decreased voiding frequency). Histamine and NGF protein expression were both significantly increased with increased stress compared to control bladders. The change in bladder function from increased voiding frequency to decreased voiding frequency with increased stress intensity suggests that changes in social stress-induced urinary bladder dysfunction are context and duration dependent. In addition, changes in the bladder inflammatory milieu with social stress may be important contributors to changes in urinary bladder function.

Key words: NGF, stress, inflammation, voiding frequency, cystometry

INTRODUCTION

Stress affects urinary bladder function and has been reported to exacerbate signs/symptoms of urinary bladder dysfunction in overactive bladder (OAB), interstitial cystitis/bladder pain syndrome (IC/BPS), bladder outlet obstruction, and spinal cord injury-induced bladder dysfunction. Clinicians have long surmised that stress plays a role in bladder dysfunction, especially in children, but the underlying mechanisms have remained obscure. Treatment options for stress-induced bladder dysfunction include behavioral modification and anticholinergic medications (38). However, these interventions merely treat symptoms and have a variable success rate. The frequent ineffectiveness of these treatments stems from a failure to understand how stress affects the mechanics of human urine storage and emptying, and highlights the need for relevant pre-clinical animal models for testing treatments for stress-induced bladder dysfunction.

Animal models based on intravesical instillation of inflammatory agents or surgical ligations of the bladder outlet have recently been created to study OAB (20, 31). However, these models are less effective in mirroring human bladder function disorders in children than in adults and have limited validity in studies of stress-related bladder dysfunction. To overcome this problem, researchers have developed a number of rodent stress models that mimic human bladder dysfunction. In 1973, Desjardins et al. (12) reported wide differences in the micturition patterns of dominant versus submissive mice during direct and barrier exposure. An alternative mouse model, the chronic water-avoidance stress paradigm demonstrated enhanced bladder nociceptive responses as well as alterations in micturition frequency, interval and volume (26). Most recently, Chang et al. (7) reported that social stress leads to remodeling and hypertrophy of the bladder in addition to abnormal voiding patterns. Findings from murine models of social

stress appear to closely correlate with the symptoms of stress-induced bladder overactivity/underactivity observed in children (Mingin, personal observation).

Nerve Growth Factor (NGF), a potent neurotrophin that exerts pleiotropic effects in the peripheral and central nervous system has been shown to regulate sensory and sympathetic neuronal development and maintenance (22) and play a role in painful somatic and visceral inflammation (1,8,17,27 and 32). Notably, NGF contributes to inflammation of the bladder, colon and lung (11, 16 and 35), and there is increasing evidence that NGF plays a role in increased voiding frequency (8, 19, 14, 29, 21, 30 and 44). Recently, increased NGF expression in the urinary bladder was demonstrated in rats exposed to a repeated variate stress (RVS) paradigm (28). Additional inflammatory changes in the urinary bladder were also demonstrated with the RVS model including increases in histamine, chemokine and myeloperoxidase expression, suggesting that markers of inflammation might be useful biochemical indicators of the effects of social stress (2).

In the present study, we examined the role that social stress plays in bladder function using a stress and increased stress paradigm in mice. In this study the difference between the stress and increased stress protocols relates to the duration of time that a submissive mouse is exposed to an aggressor mouse via a permeable barrier allowing for visual and olfactory contact. We demonstrate that social stress produced a significant increase in histamine protein expression in urinary bladder from both the stressed and increased stress groups. In addition we observed a significant increase in NGF protein expression in urinary bladder from the increased stress group. Notably, changes in bladder function depended on the stress paradigm: stress induced decreased bladder capacity and increased voiding frequency, whereas increased stress produced an elevation in bladder capacity and a decrease in voiding frequency.

Collectively, our findings reveal that changes in bladder function in response to stress are context and duration dependent.

MATERIAL AND METHODS

Animal Care and Use. Male C57BL/6 mice and FVB mice (Charles River Canada, St. Constant PQ) (20-30 g) were used in these studies. Mice had free access to food and water while housed in animal facilities. All procedures that involved animals were performed in accordance with the Institutional Animal Care and Use Committee at UVM and were consistent with the Guide for the Care and Use of Laboratory Animals (8th edition).

Social stress protocol. On consecutive days submissive six-week-old FVB mice were placed in direct contact with C57BL/6 retired breeder mice (aggressor mouse) (Charles River Canada, St. Constant PQ) for a period of five minutes or until the C57BL/6 mice initiated aggressive behavior (e.g., biting sufficient to break the skin). After the five-minute period, or demonstrated aggressive behavior a clear plastic barrier with small holes allowing for olfactory stimulation was placed in the cage separating the FVB mouse from the aggressor mouse for a total time of one hr. The FVB mice were then returned to their own cages for 23 hrs. The process was repeated the next day with a different aggressor mouse. Age matched control FVB mice were placed in barrier cages without exposure to aggressor mice for a total of 1 hr. before being returned to their own cage. The submissive mice were exposed to the stress protocol for either two weeks or four weeks.

Increased social stress protocol. Submissive six-week-old FVB mice were placed in direct contact with C57BL/6 retired breeder (aggressor mouse) for five minutes or until the aggressor mouse initiated traumatic aggressive behavior. After the five-minute period, a clear plastic barrier with small holes allowing for olfactory stimulation was

placed separating the FVB mouse from the aggressor mouse for 23 hours. The process was repeated the next day with a different aggressor. The submissive mice were exposed to this protocol for a two week period.

Assessment of additional mouse strain in stress protocols

Both the stress protocol and increased stress protocols were repeated as above substituting six-week-old C57BL/6 mice for six-week-old FVB submissive mice. The six – week-old C57/BL/6 mice were exposed to C57/BL/6 retired breeder aggressor mice.

Measurement of urinary bladder NGF and histamine protein content by ELISAs

Whole bladder was put in T-PER Tissue Protein Extraction Reagent (Thermo SCIENTIFIC, Waltham, Massachusetts, USA) with an added protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Total bladder proteins were then extracted by using MP Biomedicals LYSING MATRIX D (MP Biomedicals, Santa Ana, California, USA) and the protein concentration was measured by using Coomassie Plus (Bradford) Assay Kit (Thermo SCIENTIFIC, Waltham, Massachusetts, USA). This extract was then used for ELISA assays. Histamine protein content was measured using Histamine EIA Kit from (Oxford Biomedical Research) with a detection range from 2.5 to 50.0 ng/ml. The histamine standard curve was calculated using a 4-PL curve-fit (R^2 = 0.9975). Mouse NGF content was measured by using ChemiKine Nerve Growth Factor Sandwich ELISA kit from (Millipore) with a detection range of 10 to 1,000 pg/ml. The mouse NGF standard curve was generated using a 2-PL curve-fit (R^2 = 0.9961). Samples did not fall below the minimum detection limits of either assays. Measured histamine and NGF contents were normalized to total protein concentration.

Quantitative RT-PCR for NGF and histidine decarboxylase (HDC)

There is no specific gene coding for histamine; therefore, we substituted histidine decarboxylase, the enzyme which catalyses the reaction producing histamine from histidine. Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test'B', Friendswood, TX, USA) as previously described. 2 µg of RNA per sample was used to synthesize complementary DNA using a mix of random hexamer and oligo dT primers with M-MLV reverse transcriptase (Promega Corp.) in a 25-µl final reaction volume. The quantitative PCR standards for all transcripts were prepared with the amplified cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically. Complementary DNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB, Cleveland, OH, USA) and 300 nM of each primer in a final 25 µl reaction volume. HDC primer sequences are as follow: upper: 5'-GGATTCTGGGTCAAGGACAAG-3'; lower: 5'-GTCCGTGGCTGCACCAGAG-3'. L32 primer sequences are upper: 5'-AGTCGCCGTGCCTACCAT-3'; lower: 5'-GCCTGCTGCCTTCCTTG-3'. NGF and L32 primer sequences were previously published (39).

The real-time quantitative PCR was performed (Applied Biosystems 7500 Fast real-time PCR system, Foster City, CA, USA) using the following standard conditions: (i) serial heating at 94°C for 2 min; (ii) amplification over 45 cycles at 94°C for 15 s and 60-65°C depending on primer sets for 30 s. The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60 to 95°C. A single DNA melting profile was

observed under these dissociation assay conditions demonstrating amplification of a single unique product free of primer dimers or other anomalous products.

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. Data were analyzed at the termination of each assay using sequence detection software (Sequence Detection Software, version 1.3.1; Applied Biosystems, Norwalk, CT, USA). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number and the threshold cycle was determined by the software as the amplification cycle at which the ΔRn first intersects the established baseline. All data are expressed as the relative quantity of the gene of interest normalized to the relative quantity of the ribosomal protein reference gene, L32.

Open voiding cystometry in conscious, unrestrained mice. Open voiding cystometry in conscious, unrestrained mice was conducted on all animals three days post stress exposure. In anesthetized FVB/C57BL/6 mice and controls, the urinary bladder was exposed through a lower midline abdominal incision under general anesthesia (isoflurane 2-3%). A saline filled PE-10 cannula with the end flared by heat was inserted into the dome of the bladder and secured with a 6-0-nylon purse string suture. The distal end of the cannula was sealed. Muscle and skin layers were closed separately using absorbable and non-absorbable sutures, respectively. The distal part of the cannula was placed in the subcutaneous space and the mice were returned to normal caging for 72 hrs. too ensure complete recovery. Postoperative analgesics were given for a period of 48 hrs. The mice were anesthetized and the cannula was exteriorized. Mice were placed conscious and unrestrained in recording cages with a balance and pan for urine collection and measurement placed below the cage. Intravesical pressure changes were

recorded using a Small Animal Cystometry System (Med Associates, Inc., St Albans, VT). The cannula was exteriorized and connected to one port of a pressure transducer; the other port of the pressure transducer was connected to a syringe pump. Room temperature saline was infused at a rate of 10 µl/min in order to elicit repetitive urinary bladder contractions. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25 to 30 min. Voided saline was collected to determine void volume. Inter-contraction interval, maximal voiding pressure, pressure threshold for voiding and baseline resting pressure were measured. The number of non-voiding urinary bladder contractions (NVC) per voiding cycle, maximal NVC pressure and frequency of NVC were assessed. For these studies, NVCs were defined as rhythmic intravesical pressure rises (greater than 5 cm H_2O from baseline pressure) without a release of fluid from the urethra. Mice were excluded from studies when adverse events occurred such as ≥ 20% reduction in body weight post-surgery, lethargy, pain or distress not relieved by our IACUC-approved regimen of post-operative analgesics or a significant postoperative adverse event. In the present studies, 3 mice were excluded due to iatrogenic bladder outlet obstruction. Behavioral movements such as grooming, standing, ambulation and defecation also rendered bladder pressure recordings during these events unusable and these were excluded from analysis. In the majority of animals the experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements. Mice were euthanized at the conclusion of the study by isoflurane (5%) and thoracotomy and the bladder was harvested and weighed.

Statistical analyses. All values represent mean ± standard error of the mean (S.E.M.). Data were compared with analysis of variance (ANOVA). Animals, processed and analyzed on the same day, were tested as a block in the analysis of variance. When F

ratios exceeded the critical value ($p \leq 0.05$) (two-tailed test), the Newman-Keul's post hoc test was used to compare group means.

RESULTS

Mice evaluated. Fifty-eight (n=58) mice out of a total of 64 were studied. Five (5) mice were discounted due to bladder obstruction (n=3) or unstable cystometric recordings (n=2). One animal died post survival surgery of unknown causes.

Effects of social stress on urinary bladder function in mice*.* To determine whether exposure to stress results in changes in urinary bladder function, we performed conscious urodynamic studies on both stressed, increased stressed and control mice.

Stressed. Whereas age-matched controls exhibited similar urinary bladder function (Fig. 1 A), mice exposed to the stress protocol for 2 weeks exhibited significantly reduced intermicturition intervals (increased voiding frequency) and bladder capacities (Fig. 1B; Table 1). They also showed an increase in baseline and micturition threshold pressure with no change in maximum voiding pressure (Table 2). Moreover, non-voiding contractions NVCs were increased (5.8 NVCs per 100 s) in mice exposed to the 2-week stress protocol compared with the control group (1.2 NVCs per 100 s). In mice exposed to the stress protocol for 4 weeks, intermicturition interval and bladder capacity tended to be lower than those in controls, but these differences did not reach statistical significance. The recovery of baseline resting pressure, micturition threshold pressure and maximal voiding pressures at 4 weeks were similar to controls (Table 2).

Increased stress. Exposure of mice to the increased stress protocol produced changes in bladder function that were qualitatively different from those exposed to the stress protocol. Unlike the case with stress, which decreased intermicturition interval (increased voiding frequency) and bladder capacity, mice exposed to increased stress exhibited significantly increased intermicturition intervals (reduced voiding frequency) and increased bladder capacities compared with age-matched controls (Fig.1C;Table 1). No

differences in baseline resting pressure, micturition threshold pressure or maximal voiding pressures were observed between mice exposed to the increased stress protocol and control mice (Table 2).

Effects of social stress on mouse body and urinary bladder weight.

Urinary bladder weight. The weight of bladders from the FVB mice exposed to the stress protocol for 2 weeks (0.018 ± 0.002 g), were not significantly different from control mice (0.018 ± 0.002 g). The weight of bladders from the FVB mice exposed to the increased stress protocol, were not significantly increased (0.023 ± 0.002 g). The weight of bladders from C57BL/6 mice exposed to the stress protocol for 2 weeks (0.062 ± 0.002 g) were significantly ($p \leq 0.01$) increased compared to that of bladders from control mice (0.044 ± 0.001 g). In contrast, the weight of bladders, were unchanged in C57Bl/6 mice exposed to increased stress.

Body weight. The body weights of the FVB mice exposed to both the stress and increased stress protocol (25 ± 0.63 g) were similar to that of the control mice. The body weights of C57BL/6 mice were significantly ($p \leq 0.01$) increased at the end of both stress (27 ± 0.16 g) and increased (22 ± 0.13 g) stress protocols compared to those of control mice (14 ± 0.14 g).

Effects of social stress on NGF and histamine protein expression in mouse urinary bladders

Previous studies (19, 20, 21, 33 and 39) have demonstrated that urinary bladder NGF expression is increased with increased voiding frequency. Given that voiding frequency increased in mice exposed to stress but decreased in mice exposed to increased stress, we hypothesized that NGF expression would be selectively increased in the bladders of stressed mice. We evaluated both NGF and histamine protein expression utilizing

ELISA assays. For these experiments, we utilized 17 additional FVB mice; 5 for controls and 6 in each experimental group (stress and increased stress). Mice were exposed to the previously described stress and increased stress protocols and for consistent comparison underwent tube implantation prior to being euthanized. We observed no change in NGF protein expression in the stressed bladders when compared to controls. In the increased stressed bladders, we observed a significant ($p \leq 0.05$) increase in NGF protein content in urinary bladder. (Fig.2A). Histamine protein expression was significantly increased in both the stressed and increased stressed bladders when compared to controls (Fig.2B).

To demonstrate that elevations in protein expression were not related to post implantation surgery, we repeated the above experiments to determine NGF protein expression in urinary bladder without tube implantation. Fifteen additional mice were distributed equally (n=5 in each) among the control group the stressed and increased stress groups. NGF protein expression was significantly ($p \leq 0.05$) increased in urinary bladder of mice (no tube implant) exposed to increased stress but the magnitude of expression is reduced compared to that observed in mice with tube implants (Fig. 2C).

Effects of Social stress on NGF and HDC (histamine) transcript expression

Twenty-one additional FVB mice, divided equally (n=7 in each) into control, stressed and increased stressed groups were used for quantitative RT- PCR. These mice were exposed to the previously described stress and increased stress protocols and for consistent comparison underwent tube implantation prior to being euthanized. In each group, the urinary bladder was separated into both the urothelium and detrusor using a dissecting microscope, fine forceps and iris scissors.

We observed a decrease in NGF transcript expression in detrusor and urothelial tissues in both stress and increased stress bladders when compared to controls (Fig. 3A) Urothelial and detrusor HDC transcripts expression were also decreased when compared to controls (Fig. 3B).

DISCUSSION

In this study we expand upon the current knowledge of the effects of social stress on bladder function. Functionally, stressed mice (2 weeks) exhibit decreased bladder capacity and intermicturition intervals (increased voiding frequency) in addition to the presence of non-voiding contractions. At four weeks, baseline and micturition threshold pressures, which were increased at 2 weeks with this paradigm, had completely normalized, whereas bladder capacity and intermicturition interval were not different from control animals. Mice exposed to increased stress had increased bladder capacity leading to prolonged periods between urination. Thus, both stress and increased stress exposure affected voiding frequency; however, these paradigms produced opposing results. Other findings of interest included an increase in histamine protein expression in the stress mice consistent with an inflammatory milieu in the urinary bladder. In mice exposed to increased stress, there was both significantly increased NGF and histamine protein expression in the urinary bladder. Collectively, our results demonstrate differences in urinary bladder function and expression of inflammatory mediators that are dependent on the context and duration of the stress exposure.

Few studies in the literature have described the functional effects of this stress model in the bladder. Our work extends that of Chang et al. (7) who reported functional findings consistent with urinary retention. In contrast, we demonstrate that stress induced both retention and increased voiding frequency (i.e., overactivity). The difference is likely attributable to differences in the intensity of the stress stimulus, as determined by the type and degree. In order to overcome the challenges involved with a social stress model (expense involved due to single cage housing and the need for aggressor males to be periodically exposed to females) water avoidance stress protocols were adopted to characterize the voiding phenotype only one of which was carried out using male mice instead of rats (26). In the study by McGonagle et al.

cystometry revealed increased intermicturition intervals and bladder volumes. This pattern of retention is also similar to that reported by Chang et al. Comparing the intensity of stress in the water avoidance model to our protocol is difficult. These findings are similar to what we see with increased stress. Both the studies by Chang and McGongle are concerned with comparing their modals with the hypertrophy and bladder remodeling seen with partial bladder outlet obstruction. These models do not fully mimic the urologic conditions seen in children thus our model of social stress may have more translational applicability as it is likely that perturbations in micturition reside on a spectrum with overactivity and retention at opposite ends. The underlying mechanisms that trigger this transition are not known but our data suggest that this transition point is not time dependent, but rather influenced by the intensity and duration of stress exposure. This is clearly evident when one considers that the functional micturition pattern did not worsen with a doubling of the exposure time from 2 to 4 weeks in the stress mice, but actually showed evidence of recovery. In contrast, increasing the intensity of the stress through prolonged direct/indirect contact resulted in changes in bladder function and a shift in the spectrum. The intensity threshold for this transition and the physiological mechanism responsible for it warrant further investigation in future studies using the social stress model.

We have also extended the scope of our work to include an examination of the neurotrophin, NGF, in stress-induced urinary bladder dysfunction. Altered NGF content in urinary bladder is associated with urinary bladder inflammation and dysfunction in both rodents and humans and may underlie neurochemical organizational and electrophysiological changes that can affect the micturition reflex pathway (13, 19, 20, 21, 28, 33, 35 and 36). In addition, inflammation induced by noxious chemical or mechanical stimulation in the urinary bladders of rodents increases the expression of NGF mRNA as well as protein throughout the bladder, causing morphological changes

in both bladder sensory and motor neurons (6, 23, 39 and 40). Increases in NGF protein content have also been reported in hypertrophied bladders following spinal cord injury and bladder outlet obstruction where expression correlates with neuronal hypertrophy and detrusor overactivity (41, 43). In contrast to the findings of Schnegelsberg et al. (33), who reported an increase in voiding frequency in a transgenic mouse model of NGF overexpression, we observed an increase in voiding frequency but no increase in NGF in the stress mouse.

NGF has been implicated in altering the expression of membrane ion channels such as the transient receptor potential vanilloid (TRPV) family of channels large conductance, calcium-sensitive potassium (BK) channels, and small conductance calcium-sensitive potassium (SK) channels (18,25 and 29). Thus it is possible that the effects of increased NGF bladder expression are indirect including effects of altered expression and function of BK, SK and TRPV channels which may directly contribute to the demonstrated urinary bladder dysfunction. We have recently demonstrated cystometrically that the TRPV1 antagonist capsaicin decreases bladder overactivity in the stress mouse (Mingin et al., unpublished observation). Although this work is preliminary, it appears that stress may play a role in overactive bladder by way of increased inflammation, or by altering the activity of the TRPV1 ion channel however, additional studies are necessary to address these possibilities.

Of note, our results demonstrate a difference in mice subjected to increased stress where they exhibited a decrease in voiding frequency when compared to stress mice or controls. A significant increase in NGF protein in mice exposed to increased stress was noted when compared to control or stressed mice. The difference between the stress and increased stress group in terms of NGF expression is intriguing. Interesting, preliminary studies in our lab have shown a significant up-regulation of the BK channel in bladders of mice subjected to increased stress (data not shown). As BK

channels are important regulators of bladder smooth muscle activity, it is possible that this channel may play a role in modulating the bladder activity in increased stress (34). Thus, NGF as well as changes in BK channel expression/ function may contribute to changes in bladder function in mice exposed to increased stress. In contrast, the present studies do not implicate NGF expression in the urinary bladder as a contributor to urinary bladder dysfunction in mice exposed to stress.

It is interesting to speculate on the etiology of the increased NGF and histamine expression. The results from the quantitative RT-PCR studies do not demonstrate an increase in either NGF or HDC mRNA suggesting that changes in post transcriptional processing contributes to increased NGF and HDC protein expression in the urinary bladder with increased stress. Reports of upregulated protein expression in the absence of a concomitant increase in mRNA in animal models of stress and inflammation have been previously demonstrated (42). Degranulation of mast cells releases NGF (3) and NGF itself may be a mast cell degranulator (4). However, recent data in a stress model of maternal separation suggests that in the setting of stress, NGF is mast cell derived (4,5). These same authors determined that there is a closer association between mast cells and nerves in the setting of neonatal stress. The mechanisms underlying stress induction of mast cell degranulation and release of NGF are not completely understood. It is possible that this is due to a central mediator such as corticotropin releasing hormone (CRH). Several investigators have shown that stress induced visceral hypersensitivity and perturbations in colonic barrier function induced by mast cell degranulation could be inhibited by CRH antagonists (24, 37). In the present study we did not investigate the contributions of CRH in the stress models used.

This speculation will have to await further studies which are planned to investigate the activity of NGF along the voiding spectrum, in particular blocking the NGF/TrKA receptor interactions or using intravesical anti-inflammatory agents. Future

studies will also investigate the role of various ion channels known to be important in bladder contractility including TRPV1, TRPV4, BK and SK channels. Studies examining bladder afferent nerve activity in both stressed and increased stressed mice in response to urinary bladder distention will also be pursued.

We acknowledge certain limitations in this study, namely that the social stress model can be subjective when assessing the aggressiveness of retired breeder mice. The failure to subject all of the mice to each of the protocols for period of 4 weeks is another limitation. Future experiments are planned to examine the effect of social stress over increased duration as well as examining the durability of these findings in order to determine if they are permanent or transient and normalize at some time-point after exposure. The majority of mice in the study were subjected to tube implantation and although we acknowledge that this may be a confounding source of inflammation, control animals were also subjected to survival surgery. In order to remove this variable we further analyzed bladder samples from animals that were subjected to the stress and increased stress protocols but did not undergo tube implantation. The findings for NGF protein were similar in all of the groups. Yet another limitation is the inability to completely control for other confounding sources of stress, such as variations in handling, different animal care technicians and seasonal effects. The former may be partially responsible for the difference in bladder intermicturition intervals between C57BL6 control mice.

The current findings increase our understanding of the role of social stress in bladder dysfunction, in particular how activation of neurotrophins may underlie the condition. Based on the above, we propose that social stress represents a useful animal model for the study of both urinary bladder hyperactivity as well as bladder underactivity. These findings, using the social stress model, represent a novel foundation for future

studies that will identify potential lower urinary tract targets for treatments for children with stress related bladder disorders.

Perspectives and Significance

Stress may be directly causative or exacerbate the symptoms (i,e., urinary frequency) seen in disorders of the urinary bladder such as IC and OAB. The current studies characterize the effects of social stress on bladder function as well as the possible mechanisms underlying the changes in function with a long term goal of identifying potential targets for pharmacologic intervention. Social stress produces changes in the bladder over a spectrum ranging from increased urinary frequency with reduced bladder capacity to decreased voiding and an increase in bladder capacity (urinary retention). Increased expression of NGF and histamine suggest either an inflammatory component and or upregulation of neurotrophins leading to possible increased membrane channel activation. Future studies will be aimed at exploring the mechanisms underlying social stress induced bladder dysfunction by determining the contributions of inflammatory mediators (e.g., NGF), ion channels (e.g., transient receptor potential channels) as well as exploring the role of central mediators (e.g., CRH).

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Disclosures

No conflicts of interest are declared by the authors.

REFERENCES

1. Allen SJ, Dawbarn D. Clinical relevance of the neurotrophins and their receptors. Clin Sci 110:175-191, 2006.
2. Arms L, Girard BM, Vizzard MA. Expression and function of CXL12/CXR4 in rat urinary bladder with cyclophosphamide-induced cystitis. Am J Physiol Renal Physiol 298:F589-600, 2010.
3. Barbara G, Wang B, Stanghellini V. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterologyn 132:26-27, 2007.
4. Barreau F, Cartier C, Ferrier L, Fioramonti J, Bueno L. Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. Gastroenterology 127:524-34, 2004.
5. Barreau F, Cartier C, Leveque M. Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin – releasing factor and nerve growth factor interplay. J Physiol 580:347-356, 2007.
6. Bjorling DE, Jacobsen HE, Blum JR, Shih A, Beckman M, Wang ZY, Uehling DT. Intravesical escherichia coli lipopolysaccharide stimulates an increase in bladder nerve growth factor. BJU Int 87: 697-702, 2001.
7. Chang A, Butler S, Silwoski J, Valentino R, Canning D, Zderic S. Social stress in mice induces voiding dysfunction and bladder wall remodeling. Am. J Physiol Renal Physiol 297: F1101-1108, 2009.
8. Chuang YC, Fraser MO, Yu Y, Chancellor MB, De Groat WC, Yoshimura N. The role of bladder afferent pathways in bladder hyperactivity induced by the intravesical administration of nerve growth factor. J Urol 165: 975-979, 2001.

9. Clemow DB, Steers WD, McCarty R, Tuttle JB. Altered regulation of bladder nerve growth factor and neurally mediated hyperactive voiding. Am J Physiol Regul Integr Comp Physiol 275:R1279-R1286, 1998.
10. Corrow KA, Vizzard MA. Phosphorylation of extracellular signal-related kinases (pERK) in urinary bladder in rats with cyclophosphamide (CYP)-induced cystitis. Am J Physiol Regul Integr Comp Physiol 293(1):R125134, 2007.
11. Delafoy L, Gelot A, Aridid D, Eschalier A, Bertrand C, Doherty AM, Diop L. Interactive involvement of brain derived neurotrophic factor, nerve growth factor, and calcitonin gene related peptide in colonic hypersensitivity in the rat. Gut 55; 940-945, 2006.
12. Desjardins C, Maruniak JA, Bronson FH. Social rank in house mice: differentiation revealed by ultraviolet visualization of urinary marking patterns. Science 182: 939-941, 1973.
13. Dmitrieva N, McMahon SB. Senitization of visceral afferents by nerve growth factor in the adult rat. Pain 66:87-97, 1996.
14. Dmitrieva N, Shelton D, Rice AS, McMahon SB. The role of nerve growth factor in a model of visceral inflammation. Neuroscience 78:449-459, 1997.
15. Dopont MC, Spitsbergen JM, Kim KB, Tuttle JB,Steers WD. Histological and neurotrophic changes triggered by varying models of bladder inflammation. J Urol 166:2119-2120, 2001.
16. Freund-Michel V, Frossard N. The nerve growth factor and its receptors in airway inflammatory disease. Pharmacol Ther 117:52-76, 2008.
17. Frossard N, Freund V, Advenier C. Nerve growth factor and its receptors in asthma and inflammation. Eur J pharmacol 500: 453-465, 2004.

18. Girard BM, Merrill l, Malley S, Vizzard MA. Increased TRPV4 expression in urinary bladder ans lumbosacral dorsal root ganglia in mice with chronic over expression of NGF in urothelium. J Mol Neurosci 51(2):602-14, 2013.
19. Guerios SD, Wang ZY, Bjorling DE. Nerve growth factor mediates peripheral mechanical hypersensitivity that accompanies experimental cystitis in mice. Neurosci let 392:193-197, 2006.
20. Homan T, Tsuzuki T, Dogishi K, Shirakawa H, Oyama T, Nakagawa T, Kaneko S. A novel mouse model of chronic inflammatory and overactive bladder by a single intravesical injection of hydrogen peroxide. J Pharmacol Sci 121(4):327-337, 2013.
21. Hu VY, Zavara P, Dattilio A, Redman TL, Allen SJ, Dawbarn D, Stoemer RP, Vizzard MA. Decrease in bladder over activity with REN1820 in rats with cyclophosphamide induced cystitis. J Urol 173:1016-1021, 2005.
22. Huang EJ, Reichardt LF. Neurtrophins: Roles in neuronal development and function. Ann Rev Neurosci 24:677-736, 2001.
23. Klinger MB, Vizzard, MA. Role of p75NTR in female rat urinary bladder with cyclophosphamide-induced cystitis. Am J Physiol Renal Physiol 295:F1778-1789, 2008.
24. Larauche MH, Bradesi S, million M. Corticotropin releasing factor type1 receptors mediate the visceral hyperalgesia induced by repeated psychological stress in rats. Am J Physiol Gastrointest Liver Physiol 294:G1033-40, 2008.
25. McDowell TS, Wang ZY, Singh R, Bjorling D. CB1 cannabinoid receptor agonist prevents NGF-induced sensitization of TRPv1 in sensory neurons. Neurosci Lett 13(551):34-8, 2013

26. McGongle E, Smith A, Butler S, Siliwoski J, Valentino R, Canning D, Zderic SA. Water avoidance stress results in an altered voiding phenotype in male mice. Neurourol Urodyn 31(7) :1185-9, 2012.
27. Mcmahon SB, Dmitrieva N, Koltzenburg M. Visceral pain. Br J Anaesth 75: 132-144, 1995.
28. Merrill L, Malley S, Vizzard MA. Repeated variate stress in male rats induces increased voiding frequency, somatic sensitivity, and urinary bladder nerve growth factor expression. Am J Physiol Regul Integr Comp Physiol 305: R147-156, 2013.
29. Mills CD, Nguyen T, Tanga FY, Zhong C, Gauvin DM, Mikusa J, Gomez EJ, Salyers AK, Bannon AW. Characterization of nerve growth factor-induced mechanical and thermal hypersensitivity in rats. Eur J Pain 17(4):469-79, 2013
30. Oddiah D, Anand P, McMahon SB, Rattray M. Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder. Neuroreport 9:1455-1458, 1998.
31. Parsons BA, Drake MJ. Animal models in overactive bladder research. Handb Exp Pharmacol 202:15-34, 2011.
32. Pezet S, McMahon SB. Neurotrophins: Mediators and modulators of pain. Ann Rev Neurosci 29:507-538, 2006.
33. Schnegelsberg B, Sun TT, Cain G, Bhattacharya A, Nunn PA, Ford, A, Vizzard MA, Cockayne DA. Overexpression of NGF in mouse urothelium leads to neuronal hyper innervation, pelvic sensitivity and changes in urinary bladder function. Am J Physiol Regul Integr Comp Physiol 298: R534-R547, 2010.
34. Sprossmann F, pankert P, Sausbier U, Wirth A, Zhou XB, madlung J, Z H, Bucurenciu I, Jakob A, Lamkemeyer T, Neuhuber W, Offermanns S, Shipston MJ, Jorth M, Nordheim A, Ruth P, Sausbier M. Inducible knockout mutagenesis reveals compensatory mechanisms eliceteed by constitutive BK channel deficiency in overactive murine bladder. FEBS Journal 276:1680-1697, 2009.

35. Stanzel RDP, Lourenssen S, Blennerhassett MG. Inflammation causes expression of NGF in epithelial cells of the rat colon. Exp Neurol 211:203-213, 2008.
36. Steers WD, Kolbeck S, Creedon D, Tuttle JB. Nerve growth factor in the urinary bladder of the adult regulates neuronal form and function. J Clin Invest 88:1709-1715, 1991.
37. Tache Y, Perdue MH. Role of peripheral CRF signaling pathways in stress-related alterations of gut motility and mucosal function. Neurogastrenterol Motil 16:137-42, 2004.
38. Van Gool JD, de Jong TPVM, Winkler-Seinstra P, Tamminen-Mobius T, Lax h, Hirche H, NijmanRJM, Hjalmas K, Jodal U, Bachmann H, Hoebeke P, Vande Walle J, Misselwitz J, John U, Bael A. Multi-center randomized controlled trail of cognitive treatment, placebo, oxybutynin, bladder training, and pelvic floor training in children with functional urinary incontinence. Neurourol Urodyn 15:2-6, 2013.
39. Vizzard MA. Changes in urinary bladder neurotrophic factor mRNA and NGF protein following urinary bladder dysfunction. Exp Neurol 161; 273-284, 2000.
40. Vizzard MA. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. J Chem Neuroanat 21:125-138, 2001.
41. Vizzard MA. Increased expression of spinal cord Fos protein induced by bladder stimulation after spinal cord injury. Am J Physiol Regul Integr Comp Physiol 279:R295-R305, 2000.
42. Winston JH, Li Q, Sarna S. Paradoxical regulation of ChAT and nNos expression in animal models of Crohn's colitis and ulcerative colitis. Am J Gastrointest Liver Physiol 305:G295-G302, 2013.

43. Zvara P, Kliment J, Jr, DeRoss AL, Irwin BH, Malley SE, Plante MK and Vizzard MA. Differential expression of bladder neurotrophic factor mRNA in male and female rats after bladder outflow obstruction. J Urol 168:2682-2688, 2002.
44. Zavara P, Vizzard MA. Exogenous overexpression of nerve growth factor in the urinary bladder produces over activity and altered micturition circuitry in the lumbosacral spinal cord. BMC Physiol 7:9, 2007.

Figure Legends

Figure 1. Cystometrogram traces of stress and increased stress protocols in FVB mice. (A) Representative cystometry traces from control mice. (B) Representative cystometry traces following stress (2-week). (C) Representative cystometry traces following increased stress (2 week). IV=infused volume, MP=mean pressure, VV= voided volume. Social stress (B) significantly ($p \leq 0.01$) increased voiding frequency and decreased bladder capacity (total infused saline at the start of micturition; 1.65-fold), and intercontraction intervals (1.65-fold) relative to control measurements (A).

Figure 2. Social stress (2 week) increases NGF and histamine protein in the urinary bladder of mice. (A) NGF protein expression is unchanged in the stress bladder compared to control mice. NGF protein is significantly ($p \leq 0.01$) elevated in the increased stress bladders compared to controls and to the stress bladders (with tube implantation). (B) Histamine protein expression is significantly increased in both stress and increased stress bladders compared to control mice (with tube implantation) ($p \leq 0.01$). (C) NGF is unchanged in the stressed bladders and significantly elevated ($p \leq 0.01$) in increased stress mice compared to controls (without tube implantation).

Figure 3. Social stress decreases histidine decarboxylase (HDC) and NGF transcripts expression in urinary bladder. (A) NGF mRNA is decreased in both urothelium and detrusor in stress and increased stress mice compared to control mice. (B) HDC mRNA is decreased in the urothelium and significantly ($p \leq 0.01$) decreased in the detrusor in stress and increased stress mice compared to control mice and to each other. L32, ribosomal protein used as a reference gene.

Table Legends

Table 1. Intermicturition interval and bladder capacity of mice exposed to social stress at 2- and 4-weeks compared to controls. Intermicturition interval and bladder capacity for mice exposed to increased stress (2-week) compared to controls is also presented. Number of mice (FVB, C57BL/6) in each group is shown. *, $p \leq 0.01$.

Table 2. Micturition pressure (threshold, baseline, maximum) of mice exposed to social stress at 2- and 4-weeks compared to controls. Micturition pressure (threshold, baseline, maximum) of mice exposed to increased stress (2-week) compared to controls is also presented. Number of mice (FVB, C57BL/6) in each group is shown. *, $p \leq 0.01$.

Figure 1.

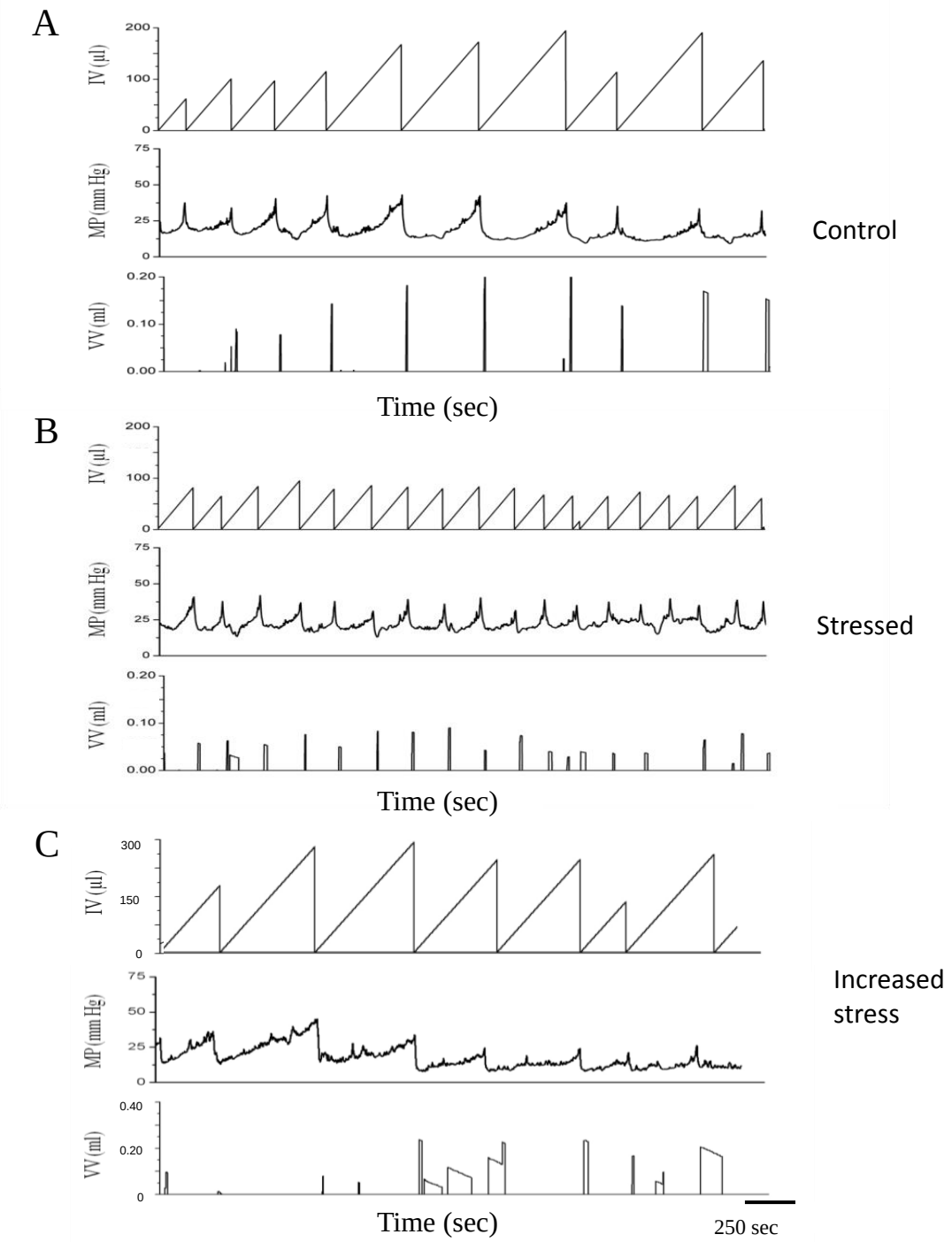
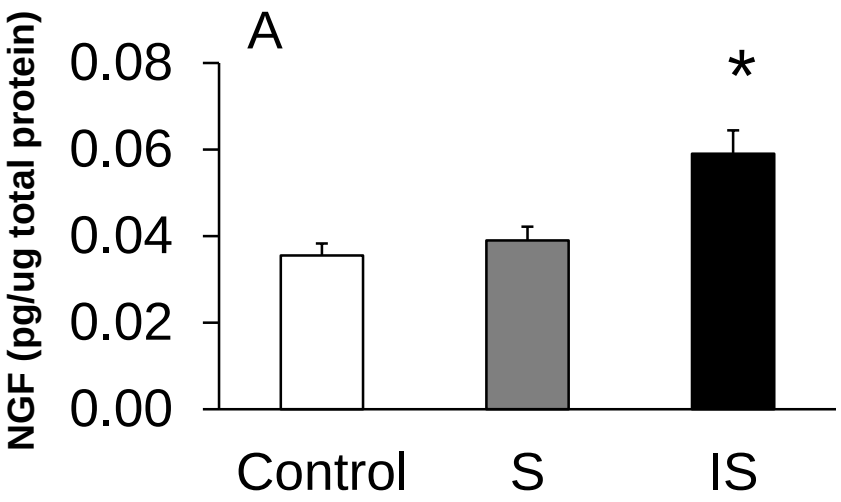
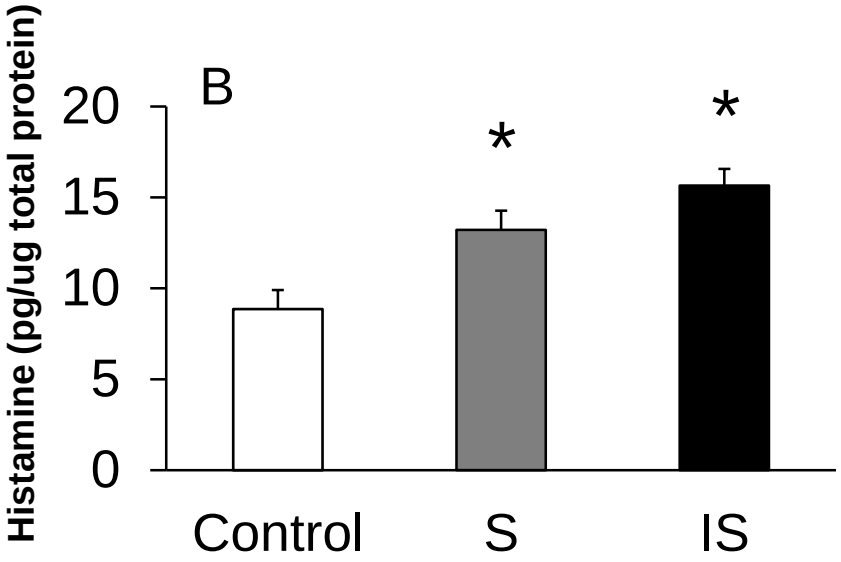

A
IV (µl)
200
100
0
MP (mm Hg)
75
50
25
0
VV (ml)
0.20
0.10
0.00
Control
Time (sec)
B
IV (µl)
200
100
0
MP (mm Hg)
75
50
25
0
VV (ml)
0.20
0.10
0.00
Stressed
Time (sec)
C
IV (µl)
300
150
0
MP (mm Hg)
75
50
25
0
VV (ml)
0.40
0.20
0
Increased stress
Time (sec)
250 sec

Figure 2


A
NGF (pg/ug total protein)
0.08
0.06
0.04
0.02
0.00
*
Control
S
IS


B
Histamine (pg/ug total protein)
20
15
10
5
0
*
*
Control
S
IS

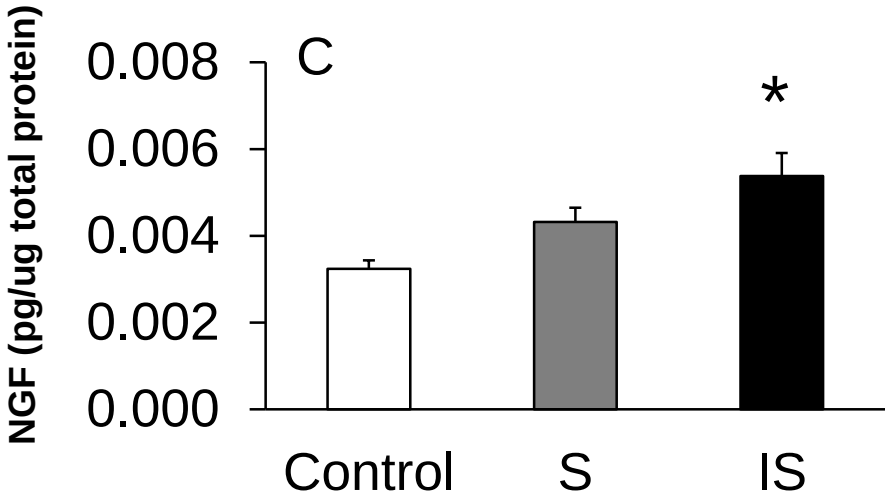
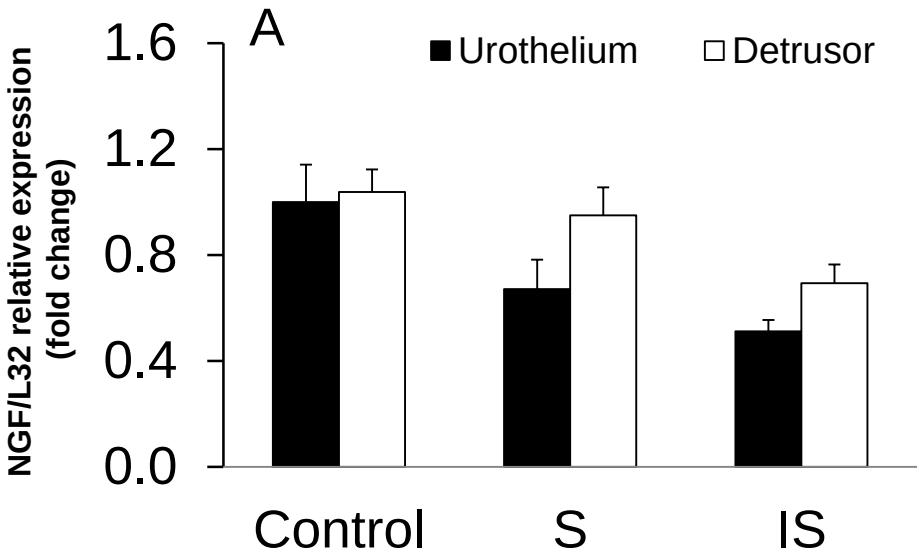
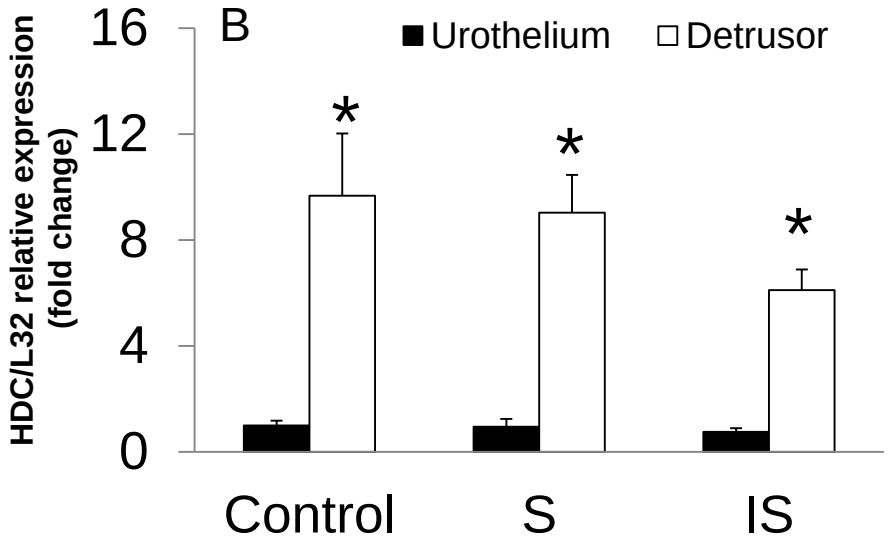

C
NGF (pg/ug total protein)
0.008
0.006
0.004
0.002
0.000
*
Control
S
IS

Figure 3


A
Urothelium
Detrusor
NGF/L32 relative expression (fold change)
1.6
1.2
0.8
0.4
0.0
Control
S
IS


B
Urothelium
Detrusor
HDC/L32 relative expression (fold change)
16
12
8
4
0
*
*
*
Control
S
IS

Strain	Urodynamic Parameter	2-Week Control	2-Week Stress	4-Week Control	4-Week Stress	2-Week Increased Stress Control	2-Week Increased Stress
FVB	Intermicturition Interval (seconds)	321.2±26.4	193.2±10.2 *	311.5±19.5	264.2±18.3 n.s.	395.2±17.3	568.7±24.3 *
	Bladder capacity (µl)	133.9±11.0	80.7±8.5 *	129.5±10.2	110.3±5.2 n.s.	131.6±8.2	236.7±18.2 *
	n =	5	5	6	6	6	6
C57BL/6	Intermicturition Interval (seconds)	284.2±18.4	208.5±12.5 *			272.6±17.5	495.4±19.2 *
	Bladder capacity (µl)	118.5±13.0	87.2±7.2 **			113.8±12.4	205.5±12.4 *
	n =	5	8			5	6

Strain	**Micturition Pressure**	**2-Week *Control***	**2-Week *Stress***	**4-Week *Control***	**4-Week *Stress***	**2-Week Increased Stress *Control***	**2- Week Increased *Stress***
FVB	Threshold	16.1 ± 1.0	24.1 ± 3.3*	20.3 ± 4.0	18.6 ± 1.7	14.8 ± 3.0	16.6 ± 1.5
	Baseline	14.7 ± 0.9	20.7 ± 2.2*	17.0 ± 3.9	15.1 ± 1.4	13.1 ± 2.4	13.0 ± 1.2
	Maximum	28.5 ± 3.0	37.0 ± 2.9	30.4 ± 3.8	33.9 ± 3.1	23.9 ± 3.6	25.5 ± 1.6
	n =	5	5	6	6	6	6
C57BL/6	Threshold	15.4 ± 1.9	16.2 ± 1.3			18.0 ± 3.3	17.6 ± 1.9
	Baseline	12.5 ± 1.0	13.4 ± 0.8			17.3 ± 3.2	15.7 ± 1.5
	Maximum	33.5 ± 5.0	33.0 ± 1.9			29.7 ± 4.2	28.8 ± 1.7
	n =	5	8			5	6