Glucocorticoid mediates water avoidance stress-sensitized colon-bladder cross-talk via RSK2/PSD-95/NR2B in rats

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Peng HY, Hsieh MC, Lai CY, Chen GD, Huang YP, Lin TB. Glucocorticoid mediates water avoidance stress-sensitized colon-bladder cross-talk via RSK2/PSD-95/NR2B in rats. Am J Physiol Endocrinol Metab 303: E1094–E1106, 2012; doi:10.1152/ajpendo.00235.2012.—Unexpected environmental and social stimuli could trigger stress. Although coping with stress is essential for survival, long-term stress impacts visceral functions, and therefore, it plays a role in the development of pelvic pain syndromes (IBS) have a significantly higher prevalence of bowel and lower urinary tract (LUT) disorders. For example, clinical observations have established a link between the bowel and LUT hyperactivity, the possibility that RSK2 could participate in the cross-talk between the bowel and the LUT by exerting its effects on the PSD-95/NR2B cascade needs to be elucidated.

Stressful life events can contribute to alterations in gastrointestinal (27) and LUT (1) function. After exposure to stressful situations, patients with IBS display an intensification of symptoms and a heightened sensitivity to pain (11, 15). More than 60% of interstitial cystitis (IC) patients report symptom exacerbation caused by stress (26, 47), and clinical investigations have shown that acute stress increases bladder urgency that resembles overactive bladder syndrome in these individuals (31). Nevertheless, whether psychological stress also impacts the cross-talk between the bowel and LUT to modulate the visceral functions is presently unclear.

Persistent activation of the hypothalamic-pituitary-adrenal (HPA) axis, one of the distinguishing characteristics of chronic stress (47), involves the release of corticosterone (CORT) from the adrenal cortex (21). By activating intracellular mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (21), CORT initiates genomic events that lead to long-lasting neurophysiological changes in the peripheral and central nervous systems (5). Increased levels of CORT have been demonstrated to enhance RSK activation that regulates Bcl-2-associated death protein in neurons (34) and the Na+/H+...
exchanger in ventricular myocytes (13). Therefore, whether stress-enhanced CORT release can modify glutamate-mediated colon-bladder cross-talk via the spinal RSK2-related cascade is an interesting question.

In this study, we examined the impact of psychological stress on colon-bladder cross-talk in rats by recording cystometry in response to the intracolonic administration of mustard oil (MO). Bilateral adrenalectomy (ADX WAS) and pharmacological antagonism of MRs/GRs were performed to elucidate the role of CORT. Finally, the participation of the spinal RSK2/PSD-95/NR2B cascade in the stress-related sensitization of colon-bladder interaction was investigated.

MATERIALS AND METHODS

Animals. The study used adult female Wistar-Kyoto rats (250–275 g) maintained on a normal light-dark cycle and provided them with food and water ad libitum. All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain (55) and the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. This study was reviewed and approved by the Institutional Review Board of National Chung-Hsing University, Taichung, Taiwan. Rats were randomly submitted to either water avoidance stress (WAS) or sham stress (WAsham). The stress procedures were performed between 8 and 10 AM to minimize the effect of avoidance stress (WAS) or sham stress (WAsham). The stress procedures were performed between 8 and 10 AM to minimize the effect of circadian rhythm. As described by Bradesi and colleagues (7, 8), rats were weighed and placed on a block (8 × 8 × 10 cm) affixed to the center of a Plexiglas cage (25 × 25 × 45 cm) either filled with fresh room temperature water (25°C) to within 1 cm of the top of the block (WAS) or kept empty (WAsham) for 1 h daily for 10 consecutive days. Because fecal pellet output was used to estimate the motility of distal colon as a validation procedure (5), the counts of the fecal pellet at the end of each stress session were recorded and averaged as an index for colonic motility. In some animals, the lumbar sacral spinal segment was dissected 30 min before stress sessions or 60 min after MO or corn oil (CO) instillation for Western blotting, RT-PCR, immunoprecipitation, and immunohistochemical analysis. Because pilot studies showed no statistical difference in the protein and mRNA expression between the L6-S1 and L6-S2 dorsal horn samples, we dissected that L6-S1 for protein and mRNA analyses in this study for these segments were easy to be identified.

Intrathecal and intracolonic catheters. On the day of kaempferol administration, implantation of intrathecal cannula was performed as described in our previous study (42). A PE-10 polyethylene silastic tube was inserted through a slit made at the atlanto-occipital membrane and passed caudally to the T13 vertebrae to reach the lumbo-sacral spinal cord (L6-S1). The outer part of the catheter was plugged and immobilized onto the skin at the closure of the wound. When possible, the catheter position was verified in each animal by postmortem examination of the spinal cord. A PE-50 polyethylene tubing was inserted into the descending colon (4 cm from the anus) for the dispensing of MO or CO, and it was held in place by taping the tube to the tail.

Cystometrical investigation. After urethane anesthesia (1.2 g/kg ip), a midline abdominal incision was made to expose the pelvic viscera. Both ureters were ligated distally and cut proximally to the sites of ligation. The proximal ends of the ureters were drained free within the abdominal cavity. A wide-bore cannula, with a sidearm for pressure measurement, was tied into the lumen of the urinary bladder at the apex of the bladder dome. This bladder cannula was connected via a three-way stopcock to a pressure transducer (P23 ID; Gould-Statham, Quincy, IL) and to a syringe pump for recording intravesical pressure (IVP) and infusing warm saline (37°C) into the bladder, respectively. After the bladder was emptied, we infused saline into the urinary bladder continuously (0.05 ml/min) to induce rhythmic bladder contractions. Three urodynamic parameters were recorded, including 1) bladder contraction amplitude (BCA; the peak IVP during micturition), 2) intercontraction interval (ICI; the latency between 2 micturition contractions, and 3) threshold pressure (TP; the critical value of IVP to induce micturition contraction). The rats were monitored for a corneal reflex and a response to noxious stimulation of the paw throughout the course of the experiment. If responses were present, a supplementary dose of urethane (0.4 g/kg) was given through the venous catheter, which was inserted into the jugular vein before abdominal incision. When the experiments were completed, the animals were euthanized via an intravenous injection of potassium chloride saturation solution.

Adrenalectomy. Bilateral adrenalectomy was performed 7 days before the stress paradigm via a dorsal approach under isoflurane anesthesia (5% induction, 2% maintenance in oxygen; Boxter Guayama). Briefly, a 2-cm dorsal midline skin incision was made at the level of the 13th rib. The adrenal glands, located cranial and medial to the kidney, were removed. The skin incisions were then closed with wound clips. Sham-operated rats were subjected to the same procedure without adrenal gland extirpation. After surgery, isotonic saline (0.9%) was provided ad libitum to the ADX rats, and water was given to naive control and sham-operated animals.

Western blotting. The procedures for Western blotting were adapted from our previous work (43). In brief, the dissected dorsal horn (L6-S1; obtained 30 min before daily stress sessions or 60 min after CO or MO instillation) was homogenized in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EGTA with a complete protease inhibitor cocktail (Roche, Indianapolis, IN). After the addition of Triton X-100 to a final concentration of 1%, the lysates were incubated further for 1 h at 4°C. The supernatant was separated on acrylamide gel and transferred to a polyvinylidene difluoride membrane and then incubated for 1 h at room temperature in either rabbit anti-RSK2 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated RSK2 (Santa Cruz Biotechnology) (51), PSD-95 (Millipore, Billerica, MA), NR2B (Millipore), or phosphorylated NR2B (Millipore). Blots were washed and incubated in peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology), donkey anti-mouse IgG (1:5,000; Santa Cruz Biotechnology), or goat anti-mouse (1:5,000; Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection kit (ECL Plus; Millipore), and then, densitometry analysis of the Western blotting membranes was done with Science Lab 2003 (Fuji, Japan). Results were normalized against β-actin and are presented as means ± SE.

Immunoprecipitation. Rabbit polyclonal PSD-95 antibodies were incubated overnight at 4°C with the extraction of the lumboa-sacral (L6-S1) dorsal horns (obtained 30 min before daily stress sessions or 60 min after CO or MO instillation). The 1:1 slurry protein agarose suspension (Millipore) was added into that immunocomplex protein, and the mixture was incubated for 2–3 h at 4°C. Agarose beads were washed once with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.02% sodium azide), twice with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer with an addition of 300 mM NaCl, and three times with an immunoprecipitation buffer only. Binding proteins were eluted with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95°C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes electrophoretically, and detected using rabbit polyclonal anti-RSK2, PSD-95, and NR2B.

Quantitative reverse-transcription PCR. The protocols for PCR were adapted from our previous work (39). In brief, lumbar spinal cords (L6-S1; obtained 30 min before daily stress sessions) were quickly removed and completely submerged in a sufficient volume of RNAlater solution (AM7021; Ambion) overnight at 4°C to allow thorough penetration of the tissue and then transferred to −80°C. Total RNA was isolated from the L6-S1 segment of the frozen spinal cords under RNase-free conditions using RNA isolation kits (74106;
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Qiagen, Valenica, CA). Reverse transcription was performed using complementary DNA reverse transcription kits (205311; Qiagen). Real-time PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems). TaqMan Universal PCR Master Mix (2×) and TaqMan gene expression assay probes for target genes were GAPDH (Rn09999916_m1), NR2B (Rn00680474_m1), PSD-95 (Rn00571479_m1), and RSK2 (Rn01419235_m1) (purchased from Applied Biosystems). Reactions (total volume, 20 μl) were performed by incubating at 95°C for 20 s, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. Relative mRNA levels were calculated according to the 2-ΔΔCT method (30). All ΔCT values were normalized to GAPDH.

**RESULTS**

**Fecal pellet output in response to stress.** By measuring the animals’ mean fecal pellet excretion after each stress session, we observed that in contrast to their sham-stressed counterparts (WAsham; Fig. 1A), WAS rats had a significantly higher mean fecal pellet output compared with naïve animals, suggesting that chronic WAS is associated with colon hyperactivity.
Baseline cystometry investigation. Cystometry recording performed after 10 consecutive days of stress sessions demonstrated that continuous saline infusion (0.05 ml/min) provoked rhythmic bladder contractions accompanied by urine voiding in naïve, WAsham, and WAS rats (Fig. 1B). No statistical differences in mean BCA (the peak IVP value during micturition; Fig. 1C), ICI (the latency between 2 micturition contractions; Fig. 1D), or TP (the critical value of IVP to induce micturition contraction; Fig. 1E) were evident between the naïve and WAsham or WAS groups. This result indicates that, although it induced colon hyperactivity, WAS did not provoke alteration in baseline bladder function.

Stress-sensitized colon-bladder cross-talk. When compared with CO (naïve + CO, WAsham + CO, and WAS + CO; Fig. 2, A and B), intracolonic MO (3%) instillation failed to affect the urodynamic parameters tested after 10 days of stress sessions in naïve and WAsham animals (naïve + MO and WAsham + MO, respectively). These parameters included BCA (data not shown), ICI, and TP. Nevertheless, MO instillation provoked bladder hyperactivity dramatically, as indicated by significantly decreased ICI and TP in the WAS group. Moreover, MO instillation resulted in a slight but not significant decrease in BCA in WAS animals (data not shown). Furthermore, in WAS animals, MO at concentrations of 1, 3, and 5% decreased ICI and TP statistically in a dose-dependent manner (WAS + MO; Fig. 2C); only at a concentration of 5% MO were these parameters significantly decreased in WAsham animals (WAsham + MO, 5%). This result provides evidence that psychological stress could sensitize the colon-bladder interactions.

WAS enhances plasma CORT level. To test whether stress-sensitized colon-bladder cross-talk is attributable to increased levels of adrenal hormones, we first measured the plasma CORT concentration after 10 days of stress session (i.e., day 11). In contrast to the WAsham counterparts, WAS animals had a significantly higher mean plasma CORT concentration compared with the naïve group (Fig. 3A), indicating that chronic stress exposure did increase plasma level of CORT.

Adrenalectomy antagonizes stress-related colon hyperactivity. The role of the adrenal hormone was investigated further using ADX WAS performed 7 days before the stress paradigm. By measuring the animals’ mean fecal pellet excretion after each stress session, we observed that, as opposed to the sham operation (ADX WAS; Fig. 3B), ADX WAS significantly decreased the mean fecal pellet output of WAS animals compared with those subjected to no surgery (WAS). This result suggests adrenal hormones are necessary for stress-induced colon hyperactivity.

Adrenalectomy prevents stress-related colon-bladder cross-talk. ADX WAS (ADX WAS + MO; Fig. 3C and D), but not sham operation (ADX WAS + MO), attenuated stress-sensitized colon-bladder cross-talk (WAS + MO), as evidenced by statistical increases in ICI and TP, suggesting that the adrenal hormone participates in chronic stress-sensitized colon-bladder cross-talk.

MO does not affect stress-enhanced CORT level. To test whether intracolonic MO instillation could also provoke the release of CORT that participates in stress-associated sensitization, we compared the circulatory CORT levels in WAS animals 30 min after CO or MO instillation. In WAS animals,
intracolonic instillation of CO or MO (3%) did not affect the mean plasma CORT concentration (WAS + CO and WAS + MO, respectively; Fig. 3E) compared with that of animals without agent administration (WAS). These results indicate that acute intracolonic MO instillation does not alter plasma CORT levels.

RU-38486 antagonizes stress-related colon hyperactivity. By measuring the animals’ mean fecal pellet excretion after each stress session, we observed that neither vehicle solution (Veh + WAS; Fig. 3F) nor RU-28318 (a selective mineralocorticoid receptor antagonist, RU-28318 + WAS) but daily subcutaneous administration of RU-38486 (a selective gluco-
corticoid receptor antagonist, RU-38486 + WAS, 30 min before each stress exposure) statistically decreased mean fecal pellet output compared with WAS animals administered no drugs (WAS), suggesting a role of glucocorticoid in chronic stress-related colon hyperactivity.

**RU-38486 prevents stress-related colon-bladder cross-talk.** Baseline cystometry recorded after exposure to the stress paradigm revealed no statistical differences in urodynamic variables, including BCA, ICI, and TP, between drug-free rats and rats pretreated daily with vehicle solution, RU-38486, or RU-28318. Daily administration of RU-38486 (RU-38486 + WAS + MO; Fig. 3, G and H), but not vehicle solution (Veh + WAS + MO) or RU28318 (RU28318 + WAS + MO), attenuated stress-sensitized cross-talk by statistically increasing ICI and TP compared with drug-free animals (WAS + MO). This result suggests that pharmacological antagonism of the GR could prevent stress-sensitized colon-bladder interaction.

**Stress-induced spinal RSK2 expressions.** No statistical differences were evident in the intensity of the RSK2 band (t-RSK2; Fig. 4, A and B) in dorsal horn samples (L6-S1) obtained from naïve, WAsham, and WAS animals on the day before the stress paradigm (day 0). Without affecting the expression of β-actin, WAS time-dependently increased the band intensity of RSK2 on days 4, 7, and 11 of the stress paradigm (i.e., after 3, 6, and 10 days of stress sessions) compared with the pre-stress control (day 0). In contrast, no statistical significance in the band intensity of RSK2 was found at these time points in the naïve or WAsham group. Similarly, in contrast to maintaining a stable level in naïve animals, repeated exposure to WAS, but not sham stress, upregulated RSK2 mRNA expression on days 4, 7, and 11 of the stress paradigm compared with the pre-stress control (Fig. 4C). These findings suggest that increased spinal RSK2 expression is associated with chronic psychological stress.

**Stress-induced spinal PSD-95 and NR2B expression.** Western blotting analysis demonstrated that exposure to daily WAS (Fig. 4, A and B) increased the band intensity of PSD-95 (days 7 and 11) and NR2B (days 4, 7, and 11) in dorsal horn samples in a time-dependent manner compared with pre-stress controls (day 0). Similarly, daily exposure to WAS, but not sham stress, upregulated the level of spinal PSD-95 (days 7 and 11; Fig. 4C) and NR2B (days 4, 7, and 11) mRNA expression. In contrast, the expression of PSD-95 and NR2B protein (Fig. 4, A and B) and mRNA (Fig. 4C) was maintained at a constant level in naïve animals. Together, these results suggest that, in addition to enhancing the level of RSK2, chronic psychological stress also upregulates the expression of PSD-95 and NR2B in the lumbosacral dorsal horn.

**Stress induces colocalized RSK2, PSD-95, and NR2B expression in dorsal horn neurons.** After 10 days of stress sessions, immunohistochemistry analysis of lumbosacral dorsal horn samples (L6-S1) showed increased RSK2 immunofluorescence (Fig. 5A, images a and b, t-RSK2 red) in animals subjected to WAS compared with that in the sham stress controls (WAsham). Double-staining experiments using an antibody selectively against neuronal nuclear antigen (Fig. 5A, image d, NeuN, green) revealed that most RSK2 immunoreactivity (Fig. 5A, image c) was colocalized with NeuN (Fig. 5A, image e), suggesting that WAS induced RSK2 expression in lumbosacral dorsal horn neurons. Moreover, the results of double-staining experiments using antibodies selectively against RSK2, PSD-95, and NR2B demonstrated that RSK2-immunoreactive neurons (Fig. 5B, images a and g, green) colocalized with PSD-95 (Fig. 5B, image h, red) and NR2B (Fig. 5B, image i, red) immunoreactivity in the dorsal horn of WAS rats (Fig. 5B, images c and i, yellow). NR2B-immunoreactive neurons (Fig. 5B, image d, green) also colocalized with PSD-95 (Fig. 5B, image e, red) in the dorsal horn (Fig. 5B, image f, yellow). These findings demonstrate that chronic WAS can provoke RSK2 expression that colocalizes with PSD-95 and NR2B in dorsal horn neurons.

**MO induces dorsal horn RSK2 phosphorylation.** Compared with CO (naïve + CO, WAsham + CO, and WAS + CO; Fig. 6A), intracolonic MO instillation (3%) enhanced dorsal horn RSK2 phosphorylation, as indicated by increases in the intensity of phosphorylated RSK2 (p-RSK2) bands in the naïve, WAsham, and WAS groups (Naïve + MO, WAsham + MO, and WAS + MO, respectively), and it did not affect the levels of total RSK2 (t-RSK2) in these groups. The MO-induced increase in p-RSK2/t-RSK2 was statistically higher in WAS animals than in naïve and WAsham animals (Fig. 6B), suggesting that acute intracolonic MO instillation induces spinal RSK2 phosphorylation.

**Kaempferol antagonizes stress-sensitized colon-bladder cross-talk.** Intrathecal administration of kaempferol, a selective RSK2 activation antagonist (10 μM, 10 μl it, 30 min before MO instillation, WAS + kaempferol + MO; Fig. 6, C and D), but not vehicle solution (WAS + Veh + MO), reversed the MO-induced bladder hyperactivity seen after exposure to stress.
paradigm (WAS + MO) by statistically increasing ICI and TP, whereas kaempferol per se exhibited no effect on urodynamic parameters derived from the cystometry recording (WAS/kaempferol). Moreover, intrathecal kaempferol at concentrations of 1, 10, and 30 μM (WAS/kaempferol, 1, 10, and 30 μM, respectively; Fig. 6E), but not vehicle solution, dose-dependently attenuated the MO-induced decreases in ICI and TP in WAS animals. These results, which were obtained through the pharmacological antagonism of RSK2 activation, provide support for the idea that spinal RSK2 phosphorylation plays a pivotal role in stress-sensitized colon-bladder cross-talk.

Stress-related RSK2-PSD-95 and PSD-95-NR2B interactions. The results of coimmunoprecipitation analyses carried out after 10 days of stress sessions demonstrated that, compared with CO, intracolonic MO instillation (3%) increased the coupling of PSD-95 with RSK2 and NR2B in the PSD-95 antibody-recognized immunoprecipitates of dorsal horn samples (L6-S1) obtained 30 min after instillation (3.12 ± 0.12- and 2.57 ± 0.42-fold above WAS/CO, respectively; Fig. 6F), suggesting that MO instillation in the descending colon of WAS animals induced spinal PSD-95-RSK2 and PSD-95-NR2B interactions. In addition, Western blotting analysis demonstrated that intracolonic MO instillation increased the levels of p-RSK2 and p-NR2B, but not t-RSK2, PSD-95, or t-NR2B expression, in the dorsal horn compared with CO dispensing (Fig. 6, G and H).

Spinal administration of kaempferol (WAS/kaempferol/MO), but not vehicle solution (WAS/Veh/MO), reversed...
Fig. 6. Kaempferol prevents bladder hyperactivity following intracolonic MO instillation. A: Western blot analysis of dorsal horn sample (L6-S1) obtained from naïve, WASham, and WAS animals after intracolonic instillation of CO (naïve + CO, WASham + CO, and WAS + CO, respectively) or MO (3%; naïve + MO, WASham + MO, and WAS + MO, respectively). B: the relative density of phosphorylated RSK2 (p-RSK2)/t-RSK2 in these samples was statistically increased after MO treatment compared with after CO instillation (**P < 0.05 vs. naïve + CO, #P < 0.05 vs. WASham + CO, and ++P < 0.01 vs. WAS + CO; all n = 7). The MO-enhanced p-RSK2/t-RSK2 immunoreactivity ratio was higher in stress than in naïve or WASham groups (**P < 0.01 vs. naïve + MO, §§P < 0.01 vs. WASham + MO; both n = 7). C: cystometry recordings from WAS animals that received no drugs (WAS + MO), vehicle (Veh) solution (WAS + Veh + MO), or kaempferol (WAS + kaempferol + MO) following intracolonic MO instillation (3%). The bar under the top trace indicates a period of 2 min. D: in stressed animals, intracolonic MO instillation decreased the mean intercontraction interval and threshold pressure measured by cystometry compared with CO (***P < 0.01 vs. WAS + CO; n = 7). Spinal administration of kaempferol (10 μM, 10 μl, #P < 0.05 vs. WAS + MO, n = 7; +P < 0.05 vs. WAS + Veh + MO), but not Veh solution (P > 0.05 vs. WAS + MO; n = 7), attenuated the MO-induced decrease in intercontraction interval and threshold pressure, whereas kaempferol per se had no statistical effect (WAS + kaempferol, P > 0.05 vs. WAS + MO; n = 7). E: intrathecal administration of kaempferol at concentrations of 1, 10, and 30 μM (WAS + 1 μM kaempferol + MO, WAS + 10 μM kaempferol + MO, and WAS + 30 μM kaempferol + MO, respectively; **P < 0.01 vs. WAS + CO; n = 7). Spinal administration of kaempferol (10 μM, 10 μl, #P < 0.05 vs. WAS + MO, n = 7; +P < 0.05 vs. WAS + Veh + MO), but not Veh solution (P > 0.05 vs. WAS + MO; n = 7), attenuated the MO-induced decrease in intercontraction interval and threshold pressure, whereas kaempferol per se had no statistical effect (WAS + kaempferol, P > 0.05 vs. WAS + MO; n = 7). F: in PSD-95 immunoprecipitates (IP; PSD-95) of dorsal horn samples obtained from WAS animals, intracolonic MO instillation (WAS + MO) increased the immunoreactvity of PSD-95-bound t-RSK2 and t-NR2B (respectively). Spinal administration of kaempferol but not Veh solution reversed the MO-enhanced coupling between PSD-95 and RSK2 and NR2B. G and H: In WAS animals, intracolonic MO instillation statistically upregulated band intensity of p-RSK2 and p-NR2B but not t-RSK2, t-PSD95, t-NR2B, or β-actin in dorsal horn samples compared with CO instillation (***P < 0.01 vs. WAS + CO; n = 7). Rather than Veh solution (P > 0.05, vs. WAS + MO; n = 7), intrathecal kaempferol (θP < 0.05 and #θP < 0.01 vs. WAS + MO, +P < 0.05 and ++P < 0.01 vs. WAS + Veh + MO; n = 7) statistically decreased the MO-enhanced p-RSK2 and p-NR2B expression.
MO-enhanced PSD-95-RSK2 and PSD-95-NR2B coupling in the PSD-95-recognized precipitates (1.56 ± 0.18- and 0.84 ± 0.26-fold above WAS + CO) as well as MO-enhanced pNR2B and pRSK2 expression. Together, these findings support the hypothesis that in animals subjected to chronic WAS, acute MO instillation can induce phosphorylation of the spinal RSK2 and NR2B as well as RSK2-PSD-95 and PSD-95-NR2B interaction.

Adrenalectomy prevents stress-related RSK2, PSD-95, and NR2B expression. Both the results of Western blotting (Fig. 7A) and RT-PCR analysis (Fig. 7B) showed that ADX WAS, but not ADsham WAS, attenuated WAS-induced spinal RSK2, PSD-95, and NR2B protein and mRNA expression in the dorsal horn (L6-S1) compared with that of stressed animals that received no surgery (WAS). These results further support the idea that adrenal hormones participate in stress-provoked spinal RSK2/PSD-95/NR2B expression.

RU-38486 antagonizes stress-related spinal RSK2/PSD-95/NR2B expression. Compared with drug-free animals, the band intensities of RSK2, PSD-95, and NR2B in the dorsal horn samples obtained fromm WAS rats were statistically decreased after daily administration of RU-3846 (RU-38486 + WAS; Fig. 7C). In contrast, administration of RU-28318 (RU-28318 + WAS) or vehicle solution (Veh + WAS) had no effect on the intensity of these bands. RT-PCR demonstrated parallel results that daily RU-38486, but not vehicle solution or RU-28318, statistically decreased RSK2, PSD-95, and NR2B mRNA expression (Fig. 7D). These results indicated that glucocorticoids, but not mineralocorticoids, play an essential role in the stress-related spinal RSK2/PSD-95/NR2B cascade.

DISCUSSION

Symptoms of stress are commonly found in association with clinical disorders affecting gastrointestinal and urogenital functions, and emerging evidence has linked stress to symptoms of bowel or bladder disorders (1, 47). In this study, instillation of MO into the descending colon reflexively decreased the ICI and TP without significantly affecting BCA in animals subjected to daily stress. Considering that MO did not elevate BCA, a phenomenon that could be explained by unchanged flow resistance, the contribution of the urethra to the MO-induced modulation of LUT function may be minor. Moreover,

Fig. 7. ADX WAS and RU-38486 decreases WAS-induced spinal protein/mRNA expressions. A: compared with WAS animals that did not receive surgery (WAS), the band intensities of RSK2, PSD-95, and NR2B (t-RSK2, t-PSD95, and t-NR2B, respectively) in dorsal horn samples (L6-S1) were statistically decreased after ADX WAS (*P < 0.05 and **P < 0.01; n = 7) but not after ADsham WAS (P > 0.05 vs. WAS, n = 7). B: the dorsal horn mRNA levels of RSK2, PSD-95, and NR2B calculated by the 2−△△Ct method (−2Ct) were statistically decreased by ADX WAS (***P < 0.01 vs. WAS; n = 7), but not by sham operation (P > 0.05 vs. WAS; n = 7), compared with surgery-free animals. C: compared with WAS animals, the band intensities of RSK2, PSD-95, and NR2B in the dorsal horn samples (L6-S1) were statistically decreased by RU-3846 (sc daily, RU-38486 + WAS, ***P < 0.01 vs. WAS; n = 7) but not by vehicle solution (Veh + WAS, P > 0.05 vs. WAS; n = 7) or RU-28318 (sc daily, RU-28318 + WAS, **P < 0.01 vs. WAS; n = 7), but neither vehicle solution (P > 0.05 vs. WAS; n = 7) nor RU-28318 (P > 0.05 vs. WAS; n = 7) statistically decreased the mRNA expression of RSK2, PSD-95, and NR2B in the dorsal horn compared with drug-free animals (WAS).
because the infusion rate was kept constant during the experiment, the observed decreases in ICI and TP in the stressed animals could be attributed to an increased excitability of the voiding reflex. In addition, because we administered MO into the lumen of the descending colon rather than into the urinary bladder, the possibility that MO directly established organic pathology in the urinary bladder itself, thereby resulting in bladder hyperactivity, could be ruled out. Considering these findings, we propose that the enhancement in bladder activity demonstrated in this study resembles the symptoms that occur in functional bladder disorders, in which bladder function is altered but without organic consequences. Our results provide evidence that, in addition to directly impacting the function of solitary viscera, chronic psychological stress may also facilitate bowel-bladder interactions and that, in the case of colon irritation, bladder hyperactivity resembling functional bladder disorders can be more easily induced in stressed animals. We suggest that the enhanced colon-bladder cross-talk seen in animals exposed to stress demonstrated in the present study could, at least in part, underlie the high prevalence of chronic pelvic pain syndrome with stress. Our study provides a model available for further investigation of the concurrence of functional bowel and bladder disorders as well as of the impact of stress on visceral functions.

Of the RSK isoforms (RSK1–4) (53), RSK2 has been shown to be clinically relevant to brain development because mutations of RSK2 resulted in a clinical syndrome known as the Coffin-Lowry syndrome (20), and RSK2-deleted mice perform poorly in water maze learning tasks (14). RSK2 is as well implicated in cellular functions (10, 11, 15, 23, 36, 52) and tumorigenesis (12, 33). Although RSK is known to be regulated at the posttranscriptional level by phosphorylation (12, 36), it has also been shown that the expression of RSK protein in the frontal cortex is enhanced by hypoxic preconditioning (44) or traumatic stress (54). In this work, we found that daily stress exposure induced dorsal horn RSK2 expression in the dorsal horn of rats. Moreover, in association with the induction of bladder hyperactivity, acute intracolonic MO instillation induced dorsal horn RSK2 phosphorylation in stressed rats, and pharmacological blockade of spinal RSK2 activation prevented bladder hyperactivity. These data imply that repetitive stress exposure upregulates the expression of spinal RSK2, which could be phosphorylated during acute colonic irritation and may underlie the sensitization of bowel-bladder cross-talk in stressed animals. Our proposal is supported by clinical observations that IBS and chronic pelvic pain arise frequently in patients facing stressful situations, suggesting that psychological stress represents a clear “prime suspect” in these disorders (16). The present results, for the first time, provide evidence that characterizes the impact of psychological stress on the bowel-bladder interaction and that suggests a putative molecular mechanism underlying this phenomenon. However, whether RSK members other than RSK2 could also be involved in the stress-related modulation on bowel-bladder interaction needs further study to be elucidated.

PSD-95, a NMDAR-anchoring protein, has been demonstrated to interact with and, therefore, as a downstream effector of RSK2 (50), modulate the efficacy of glutamatergic neurotransmission in dorsal horn neurons (24). The immunofluorescence images presented in this study illustrated that stress-related dorsal horn RSK2 expression is colocalized with PSD-95. Intracolonic MO instillation upregulated spinal PSD-95 expression and correspondingly enhanced the amount of RSK2-PSD-95 immunocomplexes in WAS animals, implying that PSD-95 could interact with and, therefore, be a downstream effector of spinal RSK2 activation that participates in stress-sensitized colon-bladder cross-talk. This hypothesis is further supported by our finding that administration of kaempferol attenuated MO-induced spinal RSK2-PSD-95 interactions accompanied by ameliorated bladder hyperactivity in WAS animals. Our result is in agreement with an earlier study in which forebrain neurons expressing kinase-dead RSK2 were shown to display postsynaptic density/discs large/zona occludens (PDZ)-dependent reductions in miniature excitatory postsynaptic potential frequency, suggesting that RSK2-PDZ interactions are functionally important for glutamatergic synaptic transmission (50).

PSD-95 is known to interact with postsynaptic NMDARs and link them to the cytoskeleton and signaling molecules to mediate efficacy specificity in signaling cascades (25, 28, 29). In this study, we found that stress-related spinal PSD-95 immunofluorescence colocalized with the NMDAR NR2B subunit. In addition to inducing bladder hyperactivity in stressed animals, intracolonic MO incited NR2B subunit phosphorylation in the dorsal horn, as indicated by an increase in the pNR2B/t-NR2B ratio, and coincidently enhanced the amount of PSD-95-NR2B immunocomplexes in PSD-95 immunoprecipitates. In addition to providing further support for our previous data showing that spinal PSD-95 and subsequent NR2B phosphorylation in dorsal horn neurons are essential for the interactions between the colon and LUT (42, 43), these findings also suggest that spinal PSD-95-NR2B interaction and subsequent NR2B phosphorylation are involved in the sensitization of bowel-bladder cross-talk in animals subjected to chronic psychological stress. This idea is also supported by our results showing that intrathecal administration of kaempferol antagonized MO-provoking bladder hyperactivity and dose-dependently prevented spinal NR2B phosphorylation as well as PSD-95-NR2B coupling in PSD-95 immunocomplexes in stressed rats. Based on these results, we suggest that chronic exposure to psychological stress could upregulate the expression of spinal RSK2 protein, which may then be activated to mediate the sensitization of bowel-bladder cross-talk through the PSD-95/NR2B cascade in the lumbosacral dorsal horn.

One of the distinguishing characteristics of the chronic stress state is a prolonged, elevated, central drive of the HPA axis that results in a persistent increase in CORT release from the adrenal glands (3, 4, 9). In this study, bilateral adrenalectomy before paradigm prevented stress-induced CORT release and MO administration and subsequent NR2B phosphorylation as well as PSD-95-NR2B coupling in PSD-95 immunocomplexes in stressed rats. Based on these results, we suggest that chronic exposure to psychological stress could upregulate the expression of spinal RSK2 protein, which may then be activated to mediate the sensitization of bowel-bladder cross-talk through the PSD-95/NR2B cascade in the lumbosacral dorsal horn.

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trigger the sensitized circuitry to produce bladder hyperactivity, as seen in this study. Moreover, because CORT exerts its effects by binding to and activating MRs or GRs (48), we administered selective GR and MR antagonists daily before each stress session. Compared with bilateral adrenalectomy, RU-38486 prevented stress-associated spinal RSK2, PSD-95, and NR2B protein/mRNA expression as well as MO-induced bladder hyperactivity in stressed animals, whereas RU-28318 failed to affect the stress-related expression of these molecules and colon-bladder cross-talk. These results imply that the glucocorticoid that binds to GR is the major CORT hormone underlying stress-associated colon-bladder hyperactivity. Moreover, the glucocorticoid-mediated sensitization of cross-organ interaction could involve the activation of the spinal RSK2/PSD-95/NR2B cascade. However, the precise mechanism of how CORT participates in the spinal circuitry involved in the stress-sensitized colon-bladder cross-talk needs more study to be elucidated.

The prominent role of stress in the pathophysiology of clinical pain states in functional gastrointestinal (6, 19) and urinary disorders (2, 32, 46) has been well documented. Our results reveal that a stress-enhanced glucocorticoid level is crucial for the sensitization of colon-bladder cross-talk and suggest a possible mechanism underlying the concurrence of functional bowel and LUT disorders. Moreover, pharmacological antagonism of GR ameliorated the stress-associated sensitization of colon-bladder cross-talk. Together, these results suggest that the development of a therapeutic strategy targeting GRs provides a good starting point for the treatment of IBS and/or IC in patients facing stressful situations. Further investigations are warranted to carefully evaluate whether GR antagonists currently used in clinical practice, such as Milfpred and Cyprostat, are beneficial for patients suffering from stress-related pelvic pain. In addition kaempferol, a well-known dietary flavonoid found in edible plants, has been demonstrated to selectively inhibit RSK2 activity (11). Although there is only one agent tested in one site this study, our findings that kaempferol prevented MO-induced sensitization of colon-bladder cross-talk suggest that kaempferol could also be a candidate for the treatment of chronic pelvic pain. However, other actions of this plant-derived chemical need studies to be elucidated.

**REFERENCES**


