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Cyclophosphamide induces NR2B phosphorylation-dependent facilitation on spinal reflex potentiation

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¹Department of Urology, China Medical University Hospital and ²Department of Physiology, College of Medicine, China Medical University, Taichung; ³Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung; and ⁴Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan

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Chang CH, Peng HY, Wu HC, Lai CY, Hsieh MC, Lin TB. Cyclophosphamide induces NR2B phosphorylation-dependent facilitation on spinal reflex potentiation. *Am J Physiol Renal Physiol* 300: F692–F699, 2011. First published November 24, 2010; doi:10.1152/ajprenal.00531.2010.—It is well-established that cyclophosphamide (CYP) can sensitize the pelvic afferent nerve arising from the urinary bladder and therefore induce suprapubic pain. To test the possibility that CYP might mediate the development of visceral hyperreflexia/hyperalgesia by facilitating spinal activity-dependent neural plasticity, we compared the pelvic-urethra reflex activity and spinal *N*-methyl-D-aspartate receptor NR2B subunit (NR2B) phosphorylation in rats treated with vehicle solution and CYP. Compared with vehicle solution, when accompanied by upregulation of phosphorylated NR2B expression in the lumbosacral (L₆–S₂) dorsal horn, CYP increased the evoked spikes in spinal reflex potentiation induced by repetitive stimulation (1 stimulation/1 s). Moreover, intraperitoneal pretreatments with *N*^G-nitro-L-arginine methyl ester and roscovitine, nitric oxide synthase and cyclin-dependent protein kinase 5 (Cdk5) antagonists, respectively, overwrote CYP-enhanced reflex potentiation and NR2B phosphorylation. When compared with the untreated group, the treatment with small-interfering RNA of NR2B, which decreased the expression of NR2B expression, abolished CYP-dependent reflex facilitation and spinal NR2B phosphorylation. These results suggested that CYP might facilitate spinal reflex potentiation mediated by *N*-methyl-D-aspartate receptors and participate in the development of visceral hyperreflexia/hyperalgesia through nitric oxide- and Cdk5-dependent NR2B phosphorylation at the lumbosacral dorsal horn.

pelvic nerve; urethra; visceral pain; cyclin-dependent protein kinase 5; nitric oxide; *N*-methyl-D-aspartate receptor NR2B subunit

CHRONIC PATHOLOGICAL CONDITIONS such as inflammation or irritation of visceral organs can induce changes in sensory pathway properties, leading to visceral hyperreflexia and/or hyperalgesia (37). For example, because of the urotoxicity of its metabolite acrolein, patients that receive cyclophosphamide (CYP) as an anti-neoplastic agent are known to develop interstitial cystitis, characterized by hematuria (38) and suprapubic pain (12). In vivo animal studies have demonstrated that intraperitoneal administration of CYP induces cystitis symptoms, including irritative voiding, gross hematuria, and lower abdominal pain that resemble interstitial cystitis (33). There-

fore, CYP-induced cystitis has been used as an animal model for bladder-related pain and inflammation (5, 33, 42).

In the lumbosacral dorsal horn, glutamatergic *N*-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission has been implicated in the processing of afferent nociceptive input from the lower urinary tract (4). Spinal administration of NMDAR agonists dose-dependently facilitates both visceromotor reflexes and pressor responses to noxious pelvic stimulation (13). Conversely, pharmacological blockage of NMDARs using selective antagonists inhibits nociceptive responses caused by visceral irritation (43). These results suggest that spinal NMDARs contribute to visceral nociception/hyperalgesia. Moreover, phosphorylation of *N*-methyl-D-aspartate receptor NR2B subunit (NR2B) tyrosine residues in NMDARs has been noted as an important determinant of NMDAR-mediated current (20), which defines the role of NMDARs in pain-related neural plasticity (3, 17, 18, 22).

Our laboratory has recently documented that the acute activation of nociceptive TRPV/TRPA-expressing afferent fibers, fulfilled by instilling irritants in the uterus (23, 32) and descending colon (22, 25, 26, 29), has the potential to facilitate spinal reflex potentiation (SRP) (6, 41), a spinal NMDAR-mediated form of pain-related neural plasticity (15). However, the impact of chronic noxious stimulation/inflammation on SRP is unclear. We set out to study the role of chronic inflammation in pain-related neural plasticity by investigating the impact of chronic CYP injection on SRP. In addition, the role of NR2B in CYP-induced modification of SRP was explored using selective pharmacological agents and genetic knockdown. Moreover, in light of the fact that nitric oxide (NO)-dependent PKG activation (7) and cyclin-dependent protein kinase (Cdk)-dependent ERK phosphorylation (28) are known to regulate NR2B phosphorylation and therefore modulate NMDAR-mediated neural plasticity, we employed pharmacological antagonists of NO and Cdk5 to explore the participation of intracellular NO and Cdk5 in the NR2B-mediated modulation of SRP caused by CYP.

MATERIALS AND METHODS

Animal preparations. This study was reviewed and approved by the Institutional Review Board of National Chung-Hsing University in Taichung, Taiwan. Two hundred and forty-three adult female Sprague Dawley rats (200–300 g) were used throughout this study. In CYP-induced cystitis, there are 75 animals used to establish baseline reflex activity; 33 received vehicle solution (Veh, ip bolus) and 55 received CYP (150 mg/kg ip bolus) pretreatment 2 days before experiments. In small-interfering (si) RNA experiments, there are each 40 rats that

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received intrathecal negative control (siVeh) or siRNA of NR2B (siNR2B) injection 2 days before experiments.

Reflex activity recordings. On the day of experiment, animals were anesthetized (1.2 g/kg ip urethane), and a catheter was inserted through a slit made at the atlanto-occipital membrane to the lumbosacral spinal cord (L₆–S₂). Wire electromyogram electrodes were placed intra-abdominally in the external urethra sphincter to pick up external urethra sphincter electromyogram activity. The left pelvic nerve was carefully dissected and transected. The central stump of the transected nerve was then mounted on a pair of electrodes for stimulation. Two paradigms used were test stimulation (TS, 1 stimulation/30 s, lasting for 30 min) for evoking baseline reflex activity and repetitive stimulation (RS, 1 stimulation/1 s, lasting for 30 min) for inducing reflex potentiation. Square wave pulses with a constant intensity causing a single action potential under TS were used throughout each experiment. Intrathecal agents were injected either 5 min following or 1 min before stimulation started.

Drug applications. Drugs used in this study were as follows: CYP, an anti-neoplastic agent (150 mg/kg ip, 2 days before experiments), 4-hydroxy-1-[2-(4-hydroxyphenoxy)ethyl]-4-(4-methylbenzyl)piperidine (Co-101244, a selective NR2B antagonist, 100 nM, 10 μ l it), N^G-nitro-L-arginine-methyl ester (L-NAME), a NO synthetase inhibitor (100 μ M, 10 μ l it and 0.2 ml 2 times/day ip), roscovitine (ROS, a Cdk5 inhibitor, 100 nM, 10 μ l it and 0.2 ml 2 times/day ip), protoporphyrin IX [PPIX; a soluble guanylate cyclase (sGC) activator, 100 μ M, 10 μ l it], and N-methyl-D-aspartate (NMDA, a selective glutamate NMDA receptor agonist, 10 μ M, 10 μ l it). Drugs were dissolved in artificial cerebrospinal fluid or DMSO and applied in a final DMSO concentration of <1%. A solution of identical volume to the tested agents was dispensed to serve as the vehicle.

Western blotting. After the experimental procedures were finished, animals were decapitated, and the dorsal half of the left spinal cord segments from L₆–S₂ was dissected. Protein samples (20 μ g) were separated on SDS-PAGE (8 and 12%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% nonfat milk and probed with antibodies against phosphorylated NR2B (pNR2B, 1:1,000), total NR2B (tNR2B, 1:1,000), and β -actin (1:5,000). The blots were incubated with horseradish peroxidase-conjugated antibody (1:2,000) for 1 h at room temperature and visualized with enhanced chemiluminescence solution (5 min) followed by film exposure (2 min). Densitometric analysis of the WB membranes was done with Science Lab 2003 (Fuji).

siRNA of NMDA receptor NR2B subunit. The protocol for siRNA of the NMDA NR2B subunit was adapted from our previous study (27). The 19 nucleotide duplex and 2 unpaired nucleotide overhangs of the 3'-end were as follows: negative control, 5'-UUCUCCGAACGUGU-CACGUTT-3' and 3'-ACGUGACACGUUCGGAGAATT-5'; NR2B, 5'-AGCUCGUUCCAAAAGAGCTT-3' and 3'-UUUCGAG-CAAGGGUUUUCUTT-5'.

Chemically synthetic double-strand RNA against the negative control and the NR2B receptor were synthesized by MDBio (Taipei, Taiwan). Specific or nonspecific siRNAs (5 μ g) were complexed with 0.45% of 100 mM PEI. RNA-polymer complexes were allowed 10 min to form at room temperature before the injection. Five to seven microliters of the final solution were intrathecally injected to the L₆–S₂ spinal cord of adult rats one time a day for 5 days. Animals were allowed 2 days to recover and then were used for experiments.

Data analysis. The electromyogram activity was recorded using a sampling rate of 20,000 Hz with a conventional band-pass filter setting (30–3,000 Hz). The number of spikes was computed as the mean from the last three stimulations. Comparisons across different stimulation paradigms as well as all drug- and vehicle-treated groups were determined using one-way, repeated-measures ANOVA, followed by a post hoc test (SigmaStat 2.0; Systa Software, San Jose,

CA). In all cases, a difference of $P < 0.05$ was considered as a statistically significant difference.

RESULTS

CYP facilitated reflex potentiation and NR2B phosphorylation. To investigate the impact of CYP on SRP, we intraperitoneally injected Veh or CYP in animals and then evoked reflex activity 2 days later using TS (1 stimulation/30 s) and RS (1 stimulation/1 s). Because TS evoked baseline reflex activity with single action potentials (Veh + TS and CYP + TS; Fig. 1, A and B), RS-induced SRP in both the Veh and CYP groups (Veh + RS and CYP + RS, respectively) was characterized by a progressive increase in firing that peaked at ~100 s following stimulation onset and plateaued until the end of stimulation. Moreover, CYP facilitated RS-induced SRP by further increasing spike count when compared with the Veh group ($P < 0.01$ vs. Veh + RS, $n = 7$; Fig. 1, B and C). Intrathecal administration of Co-101244, a selective NR2B antagonist, at 1 min before stimulation (Veh + RS + Co and CYP + RS + Co; Fig. 1, A and C) prevented RS-induced SRP ($P < 0.01$ vs. Veh + RS, $n = 7$) and partially reversed CYP-dependent facilitation by decreasing the evoked spike (CYP + Co + RS, $P < 0.01$ vs. Veh + RS + Co, $n = 7$). The involvement of NR2B in CYP-dependent SRP facilitation was then investigated using Western blotting analysis. Immunoblotting showed that, compared with TS (Veh + TS and CYP + TS; Fig. 1D), pNR2B expression in the lumbosacral dorsal horn was significantly increased by RS in both Veh and CYP treatment (Veh + RS and CYP + RS, $P < 0.01$ vs. Veh + TS, $n = 4$), although the amounts of the tNR2B-to- β -actin ratio under various treatments exhibited no statistical difference ($P > 0.05$, $n = 4$). In addition, with the Veh + RS group, pNR2B expression but not the tNR2B-to- β -actin ratio was significantly increased in CYP + RS animals ($P < 0.05$ vs. Veh + RS, $n = 4$).

Cdk5 and NO involvement in RS-induced reflex potentiation and NR2B phosphorylation. We next examined the involvement of NO and Cdk5 in RS-induced SRP by antagonizing each pharmacologically. We found that, compared with animals that received TS and RS, respectively, intrathecal application of L-NAME and ROS, selective nitric oxide synthase (NOS) and Cdk5 inhibitors, respectively, at 5 min after the start of stimulation failed to affect TS-evoked baseline reflex activity (TS + L-NAME and TS + ROS, $P > 0.05$ vs. TS, $n = 7$; Fig. 2A) and RS-induced SRP (RS + L-NAME and RS + ROS, $P > 0.05$ vs. RS, $n = 7$; Fig. 2, A and D). In contrast, pretreatment with L-NAME and ROS at 1 min before stimulation attenuated RS-induced SRP by decreasing the mean spike count (L-NAME + RS and ROS + RS, $P < 0.01$ vs. RS, $n = 7$; Fig. 2, B and D) while exhibiting no effects on TS-evoked baseline reflex activity (L-NAME + TS and ROS + TS, $P > 0.05$ vs. TS, $n = 7$; Fig. 2, B and C). Moreover, pretreatment with L-NAME and ROS (L-NAME + RS and ROS + RS, respectively; Fig. 2E) significantly reduced RS-induced phosphorylation of NR2B in the lumbosacral dorsal horn without affecting the tNR2B-to- β -actin ratio compared with animals that received RS only ($P < 0.05$ vs. RS, $n = 4$), whereas posttreatment application had no effect (data not shown). We next tested the involvement of NO-dependent sGC activation in RS-induced SRP through intrathecal administration of PPIX, a sGC activator, after RS-induced SRP was attenuated by

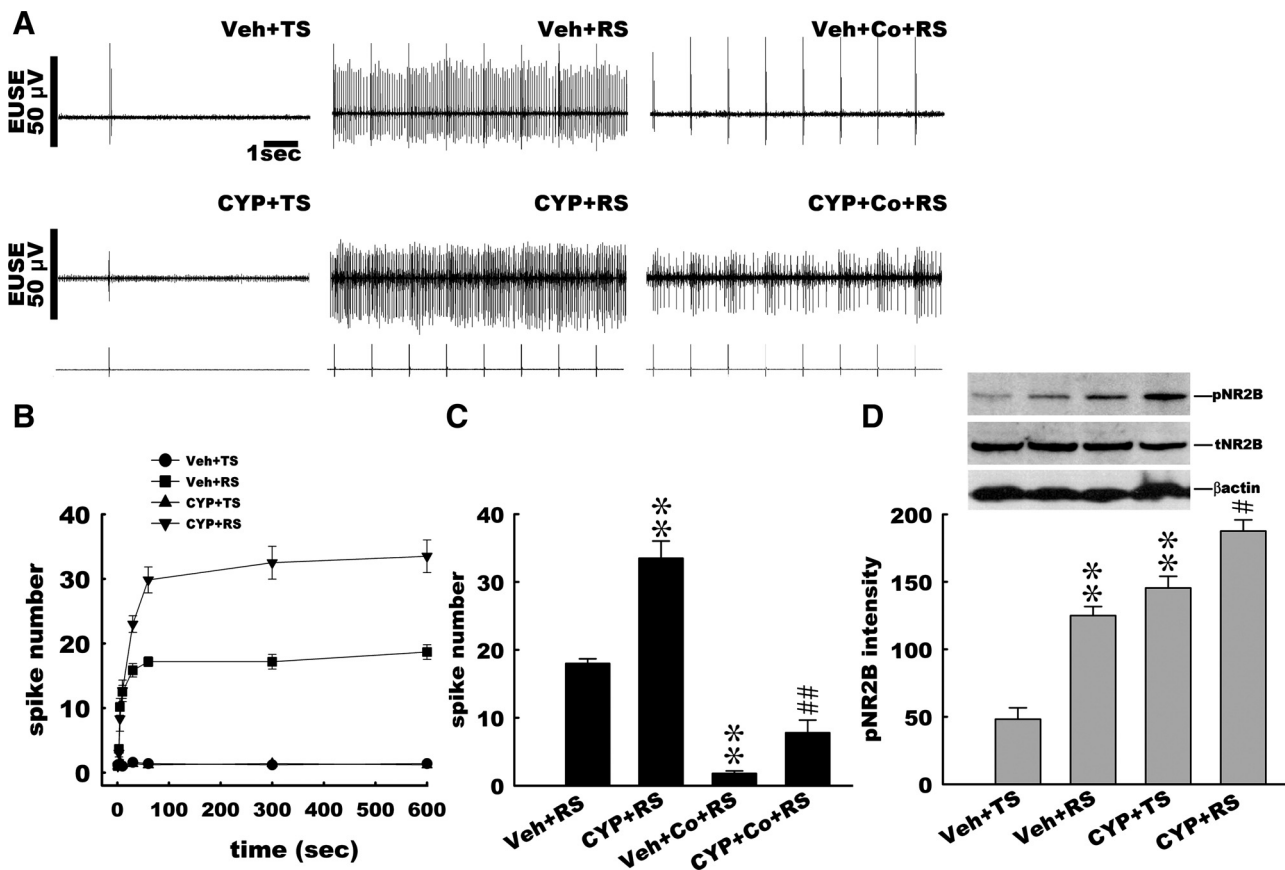


Fig. 1. Cyclophosphamide (CYP) facilitated repetitive stimulation (RS)-induced reflex potentiation and upregulated *N*-methyl-D-aspartate receptor NR2B subunit (NR2B) phosphorylation in the lumbosacral (L₆–S₂) dorsal horn. *A*: tracings showed increased external urethra sphincter electromyogram (EUSE) activity by electric shocks. Single pulses of test stimulation (TS, 1 stimulation/30 s) evoked baseline reflex activity with single action potentials in rats treated with vehicle solution (Veh + TS) and CYP (CYP + TS). The reflex potentiation induced by RS (1 stimulation/1 s) was characterized by an increasing firing in both groups (Veh + RS and CYP + RS) that was antagonized by intrathecal Co-101244 administrations at 1 min before stimulation (Veh + Co + RS and CYP + Co + RS). Ticks at the bottom of the tracing indicate the shocks. *B*: summary data showed the mean spike count of TS-evoked baseline reflex and RS-induced reflex potentiation in rats treated with vehicle solution (Veh + TS and Veh + RS) and CYP (CYP + TS and CYP + RS). *C*: also shown were the average spike counts from the last 3 shocks of stimulation in vehicle solution- and CYP-treated animals without (Veh + RS and CYP + RS) and with (Veh + Co + RS and CYP + Co + RS) intrathecal Co-101244 administration ($n = 7$). *D*: compared with TS (Veh + TS and CYP + TS), RS significantly increased phosphorylated NR2B (pNR2B) expression in both animals treated with vehicle solution (Veh + RS) and CYP (CYP + RS), despite no statistically significant between-group difference in the level of the total NR2B (tNR2B)-to- β -actin ratio ($P > 0.05$, $n = 4$). In addition, compared with the Veh + RS group, pNR2B but not the tNR2B-to- β -actin ratio was significantly increased in CYP + RS animals. *B* and *C*: ** $P < 0.01$ vs. Veh + RS. ### $P < 0.01$ vs. Veh + RS + Co. *D*: ** $P < 0.01$ vs. Veh + TS. # $P < 0.05$ vs. Veh + RS ($n = 4$).

L-NAME pretreatment. Electrophysiological tracings show that intrathecal PPIX reversed L-NAME-elicited SRP attenuation (L-NAME + RS + PPIX, $P < 0.01$ vs. L-NAME + RS, $n = 7$; Fig. 2, *B* and *D*). On the other hand, Cdk-dependent NR2B activation was also studied using a selective NMDAR agonist. We observed that intrathecal NMDA also reversed the ROS-dependent attenuation of RS-induced SRP (ROS + RS + NMDA, $P < 0.01$ vs. ROS + RS, $n = 7$).

Roles of NO and Cdk5 in CYP-dependent facilitation of reflex potentiation. The involvement of NO and Cdk5 in NR2B-mediated SRP facilitation caused by CYP was then elucidated by daily intraperitoneal injection of L-NAME and ROS in CYP-treated animals. We found that, compared with animals receiving saline injection, intraperitoneal pretreatments of L-NAME and ROS did not affect TS-evoked baseline reflex activity and RS-induced SRP (data not shown). On the other hand, compared with vehicle solution [Fig. 3*A* (Veh) and Fig. 3*D* (Veh + RS)], CYP facilitated RS-induced SRP (CYP + RS, $P < 0.05$ vs. Veh + RS; Fig. 3, *B* and *D*) and upregulated

pNR2B expression without affecting the tNR2B-to- β -actin ratio in the lumbosacral dorsal horn ($P < 0.01$ vs. Veh; Fig. 3*A*). Peritoneal pretreatments of L-NAME and ROS, while exhibiting no effects on TS-evoked baseline reflex activity (L-NAME + CYP + TS and ROS + CYP + TS, $P > 0.05$ vs. CYP + TS, $n = 7$; Fig. 3, *B* and *C*), prevented CYP-dependent reflex facilitation (L-NAME + CYP + RS and ROS + CYP + RS, $P < 0.01$ vs. CYP + RS, $n = 7$; Fig. 3, *B* and *D*) and NR2B phosphorylation (L-NAME + CYP and ROS + CYP, $P < 0.01$ for CYP, $n = 4$; Fig. 3*A*).

siNR2B prevention of CYP-dependent facilitation and NR2B phosphorylation. We next knocked down NR2B expression in the lumbosacral spinal cord by daily intrathecal injection of mis-sense (siVeh) or NR2B siRNA (siNR2B) for 5 days. Compared with the siVeh group, tNR2B protein expression in siNR2B animals was significantly reduced compared with β -actin (tNR2B, $P < 0.01$ vs. siVeh, $n = 4$; Fig. 4*A*), indicating that this treatment indeed knocked down spinal NR2B expression. In the siVeh group (Fig. 4*B*), CYP administration

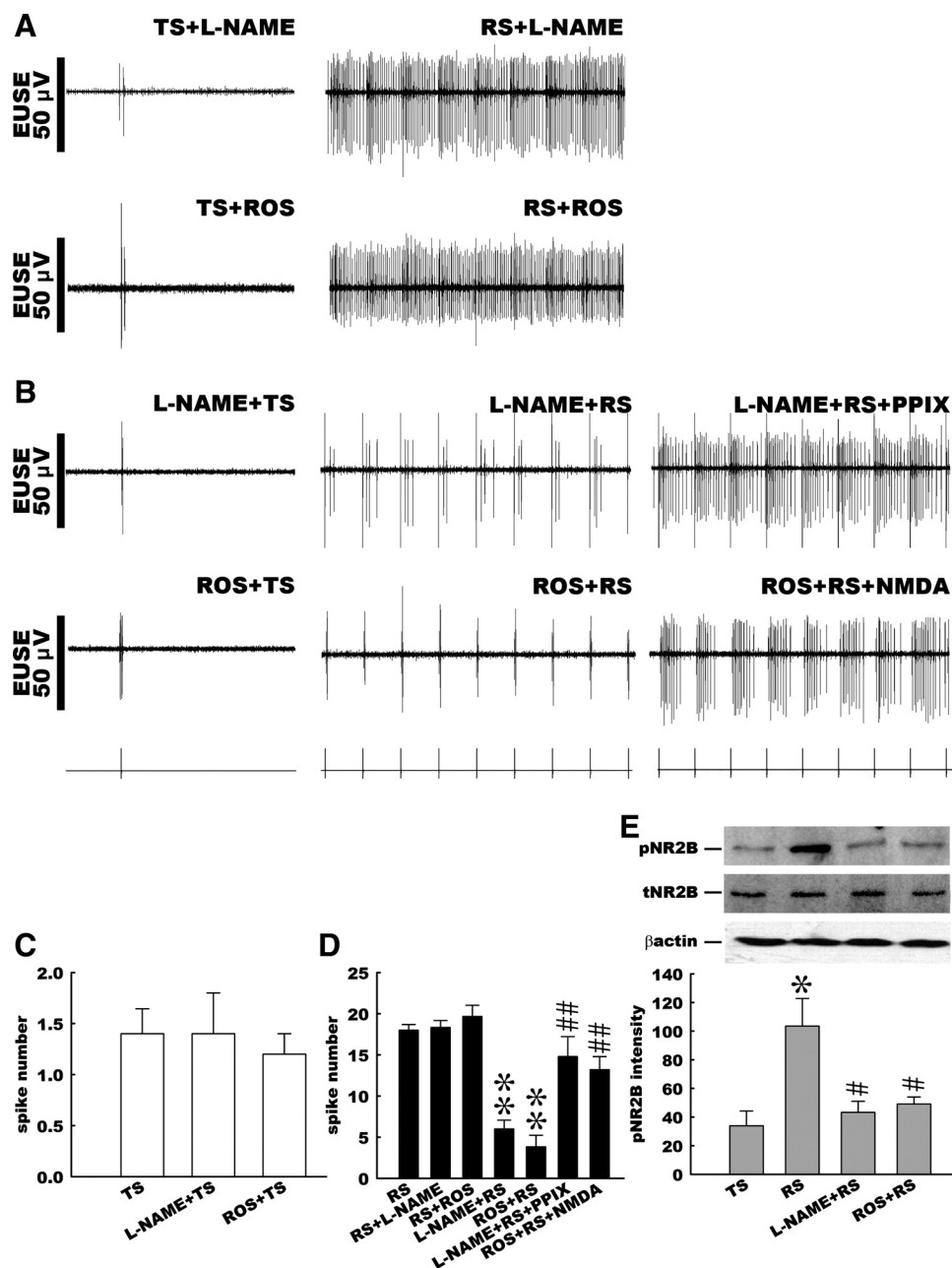


Fig. 2. NOS and cyclin-dependent protein kinase 5 (Cdk5) participated in the reflex potentiation induced by RS. *A*: tracings show increased EUSE activity by electric shocks. In both animals receiving it N^G -nitro-L-arginine methyl ester (L-NAME) and roscovitine (ROS), administered at 5 min following the start of stimulation, single pulses of TS were used to evoke baseline reflex activity with single action potentials (TS + L-NAME and TS + ROS), whereas RS was used to induce reflex potentiation. *B*: although the TS-evoked baseline reflex activity was not affected (L-NAME + TS and ROS + TS), the RS-induced reflex potentiation was antagonized by L-NAME and ROS injections 1 min before stimulation (L-NAME + RS and ROS + RS). Intrathecal protoporphyrin IX (PPIX) and *N*-methyl-D-aspartate (NMDA) overwrote the antagonism caused by L-NAME (L-NAME + RS + PPIX) and ROS (ROS + RS + NMDA), respectively. Ticks at the bottom of the tracing indicate the shocks. *C*: mean spike count averaged from the last 3 shocks of TS in animals receiving vehicle solution (TS), pretreated with L-NAME (L-NAME + TS) and with pretreated ROS (ROS + TS). *D*: averaged spike count evoked by RS in animals receiving vehicle solution (RS), L-NAME (RS + L-NAME), and ROS (RS + ROS) at 5 min following the start of stimulation as well as pretreated with L-NAME and ROS 1 min before stimulation without (L-NAME + RS and ROS + RS) and with (L-NAME + RS + PPIX) PPIX or NMDA (ROS + RS + NMDA, $n = 7$). *E*: in naïve animals, RS significantly increased phosphorylated NR2B (pNR2B) expression but not the tNR2B-to- β -actin ratio in the lumbosacral (L₆-S₂) dorsal horn compared with TS. Pretreatments of L-NAME and ROS both overwrote the RS-induced pNR2B expression (L-NAME + RS and ROS + RS) despite the tNR2B-to- β -actin ratio exhibiting no between-group statistical significance. *B* and *D*: $**P < 0.01$ vs. RS. *E*: $*P < 0.05$ vs. TS. $\#P < 0.05$ vs. RS. $\#\#\#P < 0.01$ vs. L-NAME + RS and vs. ROS + RS.

statistically enhanced NR2B phosphorylation (CYP + RS, $P < 0.05$ vs. RS) compared with animals that received RS only. In animals of the siNR2B group treated with only RS, NR2B phosphorylation was significantly lower than that in the siVeh group (RS in siNR2B, $P < 0.01$ vs. RS in siVeh). In the siNR2B group, there was also a statistically significant decrease in NR2B phosphorylation in CYP-treated animals compared with those in the siVeh group (CYP + RS in siNR2B, $P < 0.05$ vs. CYP + RS in siVeh). On the other hand, siNR2B attenuated the RS-induced SRP (siNR2B, $P < 0.01$ vs. siVeh, $n = 7$; Fig. 5, *A* and *C*) compared with siVeh. Nevertheless, these treatments failed to affect the baseline reflex activity evoked by the TS (Fig. 5, *A* and *B*). Moreover, compared with siVeh (siVeh + CYP), siNR2B significantly overwrote the CYP-dependent facilitation of

SRP (siNR2B + CYP, $P < 0.01$ vs. siVeh + CYP, $n = 7$; Fig. 5, *A* and *C*).

DISCUSSION

Interstitial cystitis is a chronic inflammatory condition of the urinary bladder that affects an estimated one million people in the United States. Administration of CYP, which is an anti-neoplastic agent metabolized by the liver to produce the bladder irritant acrolein, is known to induce cystitis in rodents and humans (14). We injected CYP intraperitoneally to develop mild but chronic bladder inflammation to explore its possible effects on SRP modulation. Compared with vehicle solution injection, CYP administration facilitated SRP. This finding supports the notion that CYP administration induces

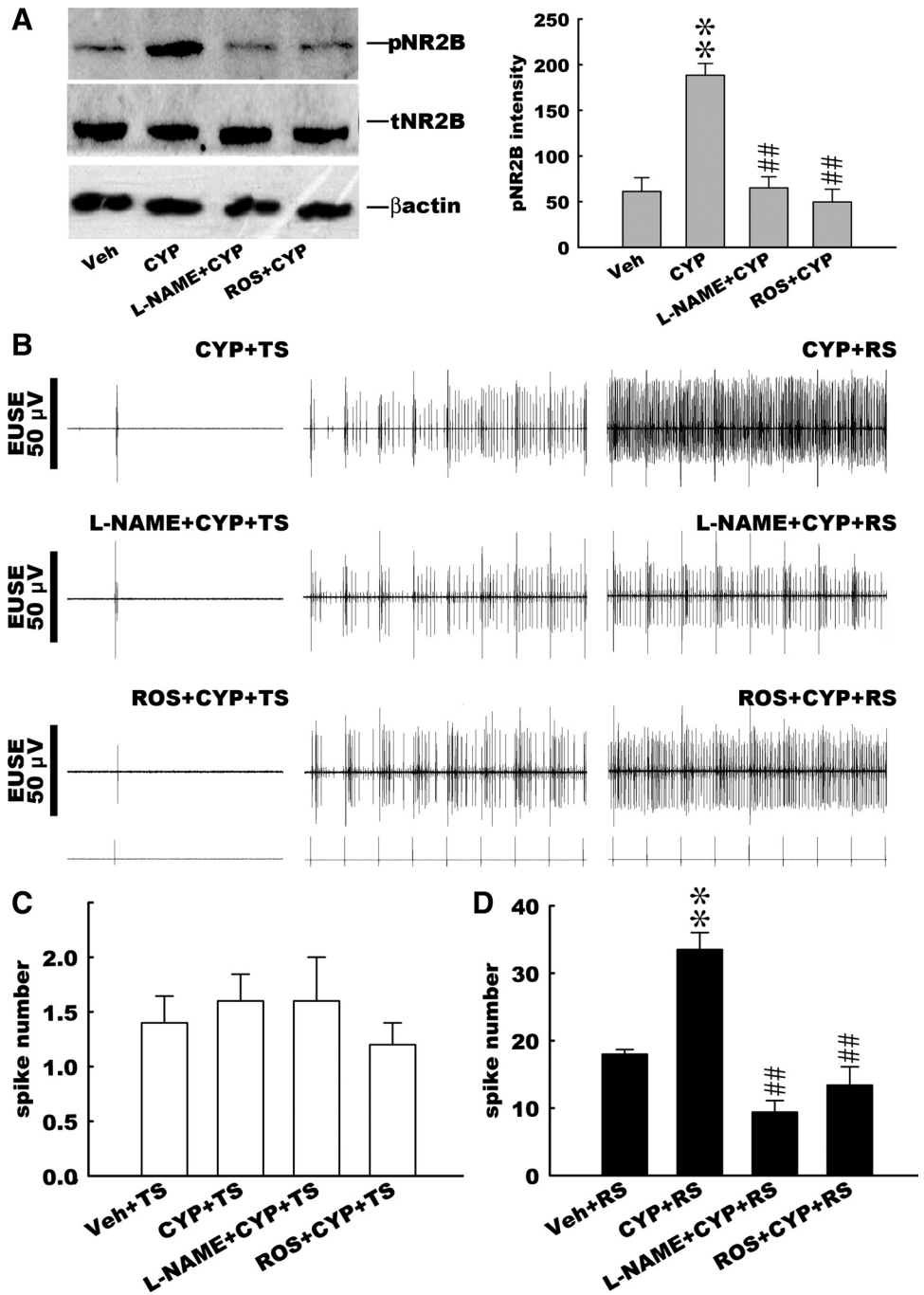


Fig. 3. NOS and Cdk5 participated in CYP-dependent facilitation of reflex potentiation. **A**: compared with vehicle solution (Veh), CYP significantly increased pNR2B expression without affecting the tNR2B-to- β -actin ratio in the lumbosacral (L₆-S₂) dorsal horn (CYP). Intraperitoneal pretreatments of L-NAME and ROS both overwrote the RS-induced pNR2B expression (L-NAME + RS and ROS + RS) despite no statistically significant between-group difference in the levels of the tNR2B-to- β -actin ratio. **B**: tracings show the increased EUSE activity by electric shocks. Compared with animals receiving CYP only (CYP + RS), ip pretreatments of L-NAME (L-NAME + CYP + RS) and ROS (ROS + CYP + RS) both antagonized CYP-dependent facilitation of reflex potentiation. However, these agents exhibited no effect on the TS-evoked baseline reflex activity (L-NAME + CYP + TS and ROS + CYP + TS). **C**: no statistical significance was revealed in the mean spike count evoked by the TS between animals treated with vehicle solution (Veh + TS) and CYP (CYP + TS) as well as those treated with CYP plus L-NAME (L-NAME + CYP + TS) or ROS (ROS + CYP + TS). **D**: compared with the vehicle solution-treated group (Veh + RS), CYP statistically increased the mean spike count evoked by RS (CYP + RS) that was overwrote by it L-NAME (L-NAME + CYP + RS) and ROS (ROS + CYP + RS) injections. **B** and **D**: $**P < 0.05$ vs. Veh + RS. $##P < 0.01$ vs. CYP + RS ($n = 7$). **A**: $**P < 0.01$ vs. Veh. $##P < 0.01$ for CYP ($n = 4$).

visceral pain in the lower urinary tract (5). Because SRP has been known to serve as a form of neural plasticity that relates to pelvic visceral pain (24, 27, 30), our results prove an extended role of CYP-induced inflammation in hyperreflexia/hyperalgesia development following bladder inflammation.

Glutamatergic NMDARs are known to underlie nociception at the spinal cord level (2). Among the NMDAR NR1-3 subunits, the NR2 subunit is of great interest, since it is essential for calcium gating and thus defines the electrophysiological properties of the NMDAR (22). In addition, there are four genes that encode NR2 subunits (NR2A-D), and studies have revealed that NR2B-containing NMDARs play crucial roles in activity-dependent neural plasticity

induction (3, 17, 18) because NR2B phosphorylation has been described as an important determinant for NMDAR-mediated current (20). In the present study, chronic CYP administration facilitated SRP and simultaneously enhanced spinal NR2B phosphorylation. Genetic knockdown of NR2B expression using specific siRNA attenuated CYP-induced facilitation and NR2B phosphorylation. These findings agree well with studies showing that, in rats with an intact spinal cord, administration of NMDAR antagonists strongly decreases spinal c-Fos expression caused by bladder irritation (4) and noxious colorectal distension (43), suggesting that NMDARs are probably involved in visceral nociception resulting from CYP-induced cystitis in rats. Moreover, to-

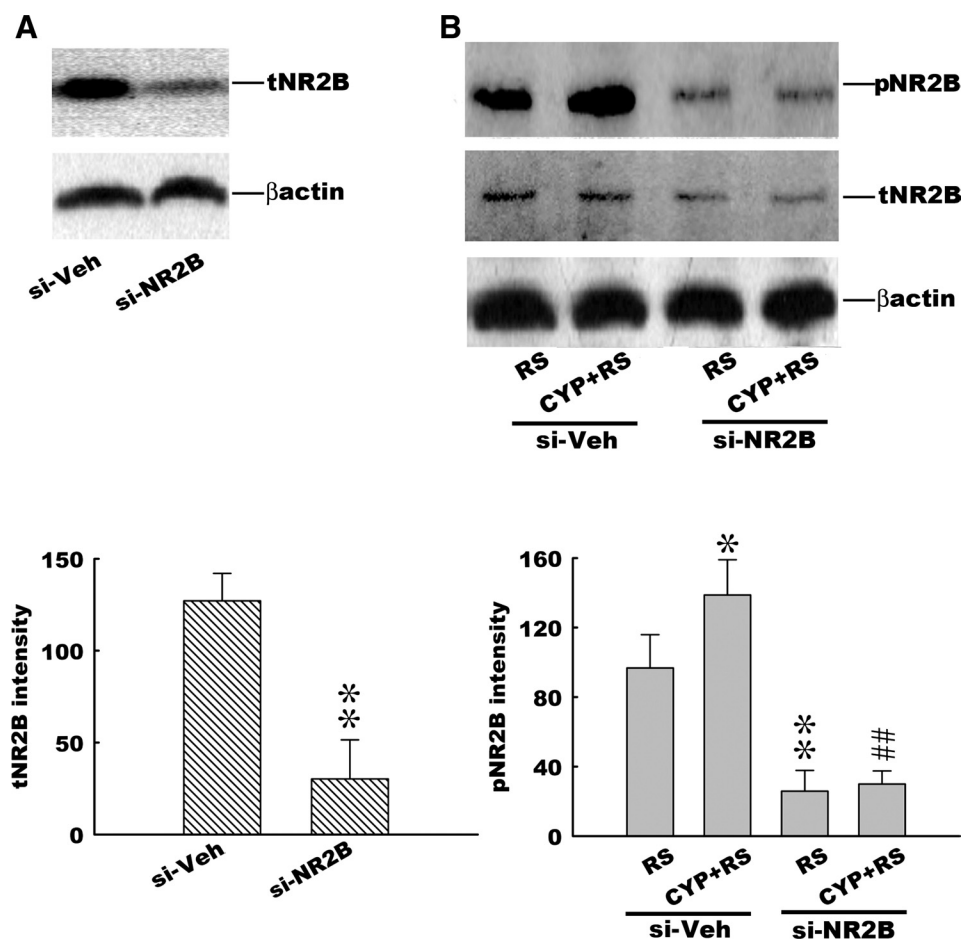


Fig. 4. Small-interfering (si) RNA of NR2B blocked CYP-dependent NR2B phosphorylation. **A:** compared with vehicle control (siVeh), siRNA of NR2B significantly decreased the expression of NR2B in the lumbosacral (L_6 – S_2) dorsal horn (siNR2B) compared with β -actin. **B:** the CYP-dependent pNR2B expression was attenuated in siNR2B compared with siVeh animals. In the vehicle control group (siVeh), CYP resulted in a statistically significant increase in NR2B phosphorylation (CYP + RS) compared with animals receiving only RS. siNR2B significantly decreased NR2B phosphorylation in animals receiving only RS (in siNR2B) compared with siVeh. There was a statistically remarkable decrease in the NR2B phosphorylation caused by CYP in the siNR2B group (CYP + RS in siNR2B) compared with the vehicle control group (CYP + RS in siVeh). **A:** ** $P < 0.01$ vs. siVeh. **B:** * $P < 0.05$ vs. RS. ** $P < 0.01$ vs. RS in siVeh. ### $P < 0.05$ vs. CYP + RS in siVeh.

gether with the results showing that NR2B phosphorylation caused by nerve injury-induced neuropathic pain is prevented by NR2B knockout (1) and knockdown (39), our data suggest that NR2B phosphorylation at the dorsal horn is essential for neuropathic pain maintenance and/or hyperalgesia.

Our previous work has shown that acute irritation of TRPV1-expressing afferent fibers coming from the descending colon may activate spinal Cdk5 activity, which induces subsequent NR2B phosphorylation, resulting in cross-organ SRP sensitization. In the current study, administration of ROS, a Cdk5 inhibitor, attenuated CYP-induced SRP facilitation and pNR2B expression, suggesting that Cdk5-dependent NR2B phosphorylation is involved in CYP-induced SRP facilitation. On the other hand, NO-dependent guanine cyclase activation has also been implicated in SRP (7). In the present study, pharmacological blockage of NOS using a selective inhibitor, L-NAME, also attenuated CYP-induced SRP facilitation and NR2B phosphorylation, suggesting a role for NO in NR2B-dependent SRP facilitation caused by CYP. This finding is in line with the idea that spinal NO plays roles in the modulation of pain-related central sensitization (10). To our knowledge, no studies have shown interaction between cascades depending on NO and Cdk5 in activity-dependent neural plasticity development. We suspect that CYP might activate different intracellular pathways to induce NR2B phosphorylation, mediating subsequent SRP facilitation. Studies investigating CYP-in-

duced cystitis have shown multiple pathological changes in the lower urinary tract. For example, in vivo histological modifications have been documented (40). Considerable secondary neurological responses, including changes in neurochemical (21, 33–35) and electrophysiological properties (42), have also been noted. Yoshimura and de Groat also demonstrated that, in primary sensory fibers coming from the urinary bladder, the initial stimulating effects of CYP are mediated by the release of inflammatory mediators such as prostaglandin, serotonin, histamine, ATP, and nerve growth factor to afferent terminals. CYP could also produce delayed effects that alter ion channel properties in bladder afferent neurons (42).

In this study, we pharmacologically antagonized NOS and Cdk5 using acute intrathecal injections when we tested their roles in the RS-induced reflex potentiation, whereas daily intraperitoneal administrations were used when we explored their participation in CYP-dependent facilitation. Although these experiments shed light on the involvement of NOS and Cdk5 in different conditions, further studies are required to elucidate the proper doses of these agents administered through different routes that will yield comparable bioavailability. Moreover, studies investigating leukemic cell apoptosis (36) and macrophage NO production (9) have demonstrated that, in addition to Cdk5, ROS inhibits Cdk1, Cdk2, and Cdk7 as well. In macrophages, both Cdk5 and Cdk7 were shown to inhibit lipopolysaccharide-induced NO production by suppressing the nuclear factor- κ B (NF- κ B) family (9). Previous work has

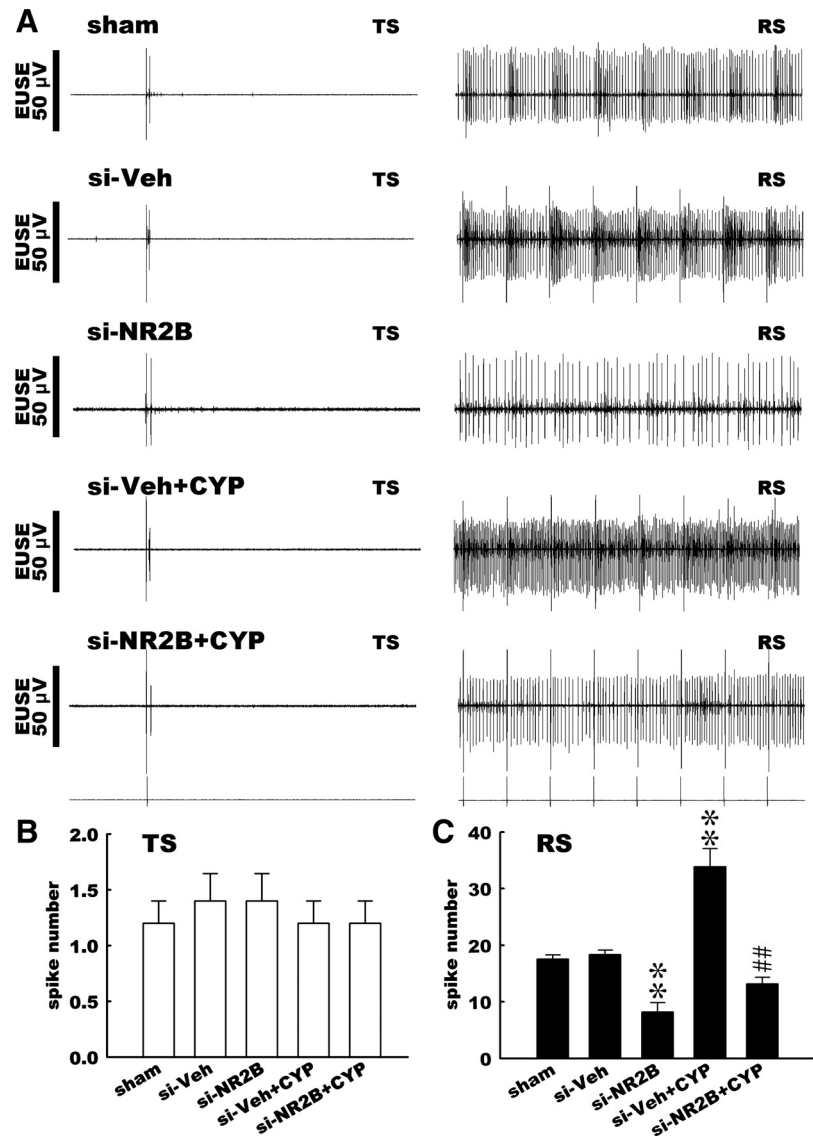


Fig. 5. siRNA of NR2B blocked CYP-dependent facilitation of reflex potentiation. **A:** tracings show increased EUSE activity by electric shocks. TS evoked baseline reflex activity with single action potentials in both animals receiving vehicle control (siVeh) and siRNA NR2B (siNR2B), whereas the reflex potentiation induced by RS was statistically decreased in siNR2B compared with vehicle control. CYP facilitated RS-induced reflex potentiation in the siVeh group but not in the siNR2B group. **B:** no statistical significance was found in the mean spike count evoked by the TS between siVeh and siNR2B and siVeh + CYP and siNR2B + CYP groups. **C:** CYP caused a statistically significant increase in the mean spike count in animals receiving vehicle solution (siVeh + CYP) but not in those receiving siNR2B (siNR2B + CYP). C: ***P* < 0.01 vs. siVeh. ##*P* < 0.01 vs. siVeh + CYP.

demonstrated that the CaMKII-dependent NO pathway is involved in the RS-induced pelvic-urethra reflex potentiation (7), and our unpublished data showed IKK α / β -dependent NF- κ B activation participated in a reflex potentiation. The possibility that whether or not other Cdks, in addition to Cdk5, might have been involved in the induction of SRP is an interesting question to be investigated further. Finally, because ROS has been reported to regulate calcium channel activity (8), whether the ROS could modulate the intracellular calcium signaling to play roles in the SRP, a calcium-dependent form of neural plasticity (7), should be taken into consideration.

The current study notes that, despite a lack of statistical significance in the relationship between the spike counts for TS-evoked baseline reflex activity for Veh and CYP-treated animals, the expression of pNR2B in CYP-treated rats was significantly higher than that for Veh. Although the detailed mechanism for this phenomenon remains unclear, one cause may be the fact that we used single pulses with an intensity that evoked a single action potential (TS-evoked baseline reflex activity) to standardize reflex activity. Although neural excit-

ability in CYP-treated animals enhanced pNR2B expression upregulation in the dorsal horn, the spike count evoked by the TS would not reflect this change, since the threshold intensity could be reduced. This proposal was supported by the fact that the mean threshold intensity for TS to evoke the baseline reflex activity in CYP-treated animals (2.4 ± 0.8 mA) was statistically lower than that for the Veh group (4.8 ± 1.4 mA). However, further experiments are needed to understand the exact causes for such phenomenon and whether it may limit the applicability of SRP to serve as a biological index for investigation into visceral pain in the pelvic region.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

- Abe T, Matsumura S, Katano T, Mabuchi T, Takagi K, Xu L, Yamamoto A, Hattori K, Yagi T, Watanabe Nakazawa T M, Yamamoto T, Mishino M, Nakai Y, Ito S. Fyn kinase-mediated phosphorylation of NMDA receptor NR2B subunit at Tyr1472 is essential for maintenance of neuropathic pain. *Eur J Neurosci* 22: 1445–1454, 2005.
- Ali DW, Salter MW. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Curr Opin Neurobiol* 11: 336–342, 2001.
- Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH, Hvalby O, Kohr G. Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. *J Neurosci* 25: 6907–6910, 2005.
- Birder LA, De Groat WC. The effect of glutamate antagonists on c-fos expression induced in spinal neurons by irritation of the lower urinary tract. *Brain Res* 580: 115–120, 1992.
- Brumovsky PR, Feng B, Xu L, McCarthy CJ, Gebhart GF. Cystitis increase colorectal afferent sensitivity in the mouse. *Am J Physiol Gastrointest Liver Physiol* 297: G1250–G1258, 2009.
- Chang JL, Peng HY, Wu HC, Lu HT, Pan SF, Chen MJ, Lin TB. Acute neurosteroids inhibit the spinal reflex potentiation via GABAergic neurotransmission. *Am J Physiol Renal Physiol* 299: F43–F48, 2010.
- Chen GD, Peng ML, Wang PY, Lee SD, Chang HM, Pan SF, Chen MJ, Tung KC, Lai CY, Lin TB. Calcium/calmodulin-dependent kinase II mediates NO-elicited PKG activation to participate in spinal reflex potentiation in anesthetized rats. *Am J Physiol Regul Integr Comp Physiol* 294: R487–R493, 2008.
- DeStefino NR, Pilato AA, Dittrich M, Cherry Cho S SV, Stiles JR, Meriney SD. (R)-roscovitine prolongs the mean open time of unitary N-type calcium channel current. *Neuroscience* 19: 838–849, 2010.
- Du Jianhai Wei N, Guan T, Xu H, An J, Pritchard KA, Shi Y. Inhibition of CDKs by roscovitine suppressed LPS-induced NO production through inhibiting of NFkB activation and BH4 biosynthesis in macrophage. *Am J Physiol Cell Physiol* 297: C742–C749, 2009.
- Freire MAM, Gimaraes JS, Leal WG, Perera A. Pain modulation by nitric oxide in the spinal cord. *Front Neurosci* 3: 175–181, 2009.
- Häbler HJ, Jänig W, Koltzenburg M. Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. *J Physiol (Lond)* 425: 545–562, 1990.
- Ho N, Koziol JA, Parsons CL. Epidemiology of interstitial cystitis. In: *Interstitial Cystitis*, edited by Sant GR. Philadelphia, PA: 1997, p. 9–16.
- Kolhekar R, Gebhart GF. NMDA and quisqualate modulation of visceral nociception in the rat. *Brain Res* 651: 215–226, 1994.
- Korkmaz A, Topal T, Oter S. Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation. *Cell Biol Toxicol* 23: 303–312, 2007.
- Lin TB. Dynamic pelvic-pudendal reflex plasticity mediated by glutamate in anesthetized rats. *Neuropharmacology* 44: 163–170, 2003.
- Lippincott-Raven. Kolhekar R, Gebhart GF. NMDA and quisqualate modulation of visceral nociception in the rat. *Brain Res* 651: 215–226, 1994.
- Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science* 304: 1021–1024, 2004.
- Massey PV, Johnson BE, Moulton PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI. Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J Neurosci* 24: 7821–7828, 2004.
- Meen M, Coudore-Civiale MA, Parry L, Eschaliere A, Boucher M. Involvement of N-methyl-D-aspartate receptors in nociception in the cyclophosphamide-induced visceral pain model in the conscious rat. *Eur J Pain* 6: 307–314, 2002.
- Moon IS, Apperson ML, Kenedy MB. The major tyrosine-phosphorylated protein in the post-synaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc Natl Acad Sci USA* 91: 3954–3958, 1994.
- Murray E, Malley SE, Qiao LY, Hu VY, Vizzard MA. Cyclophosphamide-induced cystitis alters neurotrophin and receptor tyrosine kinase expression in pelvic ganglia and bladder. *J Urol* 172: 2434–2439, 2004.
- Peng HY, Chang CH, Tsai SJ, Lai CY, Tung KC, Wu HC, Lin TB. Protein Kinase A-dependent spinal α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor mediates capsaicin-induced colon-urethra cross-organ reflex sensitization. *Anesthesiology* 114: 70–83, 2011.
- Peng HY, Chang HM, Chang SY, Tung KC, Lee SD, Chou D, Lai CY, Chiu CH, Chen GD, Lin TB. Orexin-A modulates glutamatergic NMDA-dependent spinal reflex potentiation via inhibition of NR2B subunit. *Am J Physiol Endocrinol Metab* 295: E117–E119, 2008.
- Peng HY, Chang HM, Lee SD, Huang PC, Chen GD, Lai CH, Lai CY, Chiu CH, Tung KC, Lin TB. TRPV1 mediates the uterine capsaicin-induced NMDA NR2B-dependent cross-organ reflex sensitization in anesthetized rats. *Am J Physiol Renal Physiol* 295: F1324–F1335, 2008.
- Peng HY, Chen GD, Lai CY, Hsieh MC, Hsu HH, Wu HC, Lin TB. PI3K modulates estrogen-dependent facilitation of colon-to-urethra cross-organ reflex sensitization in ovariectomized female rats. *J Neurochem* 113: 54–66, 2010.
- Peng HY, Chen GD, Lai CH, Tung KC, Chang JL, Lin TB. Endogenous ephrinB2 mediates colon-urethra cross-organ sensitization via Src kinase-dependent tyrosine phosphorylation of NR2B. *Am J Physiol Renal Physiol* 298: F109–F117, 2010.
- Peng HY, Chen GD, Lee SD, Lai CY, Chiu CH, Cheng CL, Chang YS, Hsien MC, Tung KC, Lin TB. Neuroactive steroids inhibit spinal reflex potentiation by selective enhancing specific spinal GABAA. *Pain* 143: 12–20, 2009.
- Peng HY, Chen GD, Tung KC, Chien YW, Lai CY, Hsieh MC, Chiu CH, Lai CH, Lee SD, Lin TB. Estrogen-dependent facilitation on spinal reflex potentiation involves the Cdk5/ERK1/2/NR2B cascade in anesthetized rats. *Am J Physiol Endocrinol Metab* 297: E416–E426, 2009.
- Peng HY, Chen GD, Tung KC, Lai CY, Hsien MC, Chiu CH, Lu HT, Liao JM, Lee SD, Lin TB. Colon mustard oil instillation induced cross-organ reflex sensitization on the pelvic-urethra reflex activity in rats. *Pain* 142: 75–88, 2009.
- Peng HY, Cheng YW, Lee SD, Ho YC, Chou D, Chen GD, Cheng CL, Hsu TH, Tung KC, Lin TB. Glutamate-mediated spinal reflex potentiation involves ERK 1/2 phosphorylation in anesthetized rats. *Neuropharmacology* 54: 686–698, 2008.
- Peng HY, Huang PC, Liao JM, Tung KC, Lee SD, Cheng CL, Shyu JC, Lai CY, Chen GD, Lin TB. Estrous cycle variation of TRPV1-mediated cross-organ sensitization between uterus and NMDA-dependent pelvic-urethra reflex activity. *Am J Physiol Endocrinol Metab* 295: E559–E568, 2008.
- Petralia RS, Wang YX, Wenthold RJ. The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J Neurosci* 14: 6102–6120, 1994.
- Qiao LY, Gulick MA. Region-specific changes in the phosphorylation of ERK 1/2 and ERK 5 in rat micturition pathways following cyclophosphamide-induced cystitis. *Am J Physiol Regul Integr Comp Physiol* 292: R1375–R1386, 2007.
- Qiao LY, Vizzard MA. Cystitis-induced upregulation of tyrosine kinase (TrkA, TrkB) receptor expression and phosphorylation in rat micturition pathways. *J Comp Neurol* 454: 200–211, 2002.
- Qiao LY, Vizzard MA. Up-regulation of phosphorylated CREB but not c-Jun in bladder afferent neurons in dorsal root ganglia after cystitis. *J Comp Neurol* 466: 262–274, 2004.
- Rogalinska M, Blonski JZ, Komina O, Goralski P, Zolnierczyk JD, Piekarski H, Robak T, Kilianska ZM, Wesierska-Gadek J. R-roscovitine (Seliciclib) Affects CLL Cells More strongly than combination of fludarabine or cladribine with cyclophosphamide: inhibition of CDK7 sensitizes leukemic cell to caspase-dependent apoptosis. *J Cell Biochem* 109: 217–235, 2010.
- Sengupta JN, Gebhart GF. Mechanosensitive properties of pelvic nerve afferent fibres innervating the urinary bladder of the rat. *J Neurophysiol* 72: 2420–2430, 1994.
- Stillwell TJ, Benson RC. Cyclophosphamide-induced cystitis. A review of 100 patients. *Cancer* 61: 451–457, 1988.
- Tao F, Tao YX, Gonzalez JA, Fang M, Mao P, Johns RA. Knockdown of PSD-95/SAP90 delays the development of neuropathic pain in rats. *Neuroreport* 12: 3251–3255, 2001.
- Vizzard MA, Erdman SL, de Groat WC. Increased expression of neuronal nitric oxide synthase in bladder afferent pathway following chronic bladder irritation. *J Comp Neurol* 370: 191–202, 1996.
- Wu HC, Chiu CH, Tung KC, Chen GD, Peng HY, Lin TB. Dopaminergic D2 receptors activate PKA to inhibit spinal pelvic-urethra reflex in rats. *Am J Physiol Renal Physiol* 299: F681–F686, 2010.
- Yoshimura N, de Groat WC. Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. *J Neurosci* 19: 4644–4653, 1999.
- Zhai QZ, Traub RJ. The NMDA receptor antagonist dizocilpine attenuates c-Fos expression in the lumbosacral spinal cord following repetitive noxious and non-noxious colorectal distention. *Pain* 83: 321–329, 1999.