行政院國家科學委員會補助

大專學生參與專題研究計畫研究成果報告

＊ ********* ＊＊ ＊
＊ 計 畫 結腸內灌注芥子油引起之跨臟器尿道反射敏感效應，是 ＊
＊ ： 否會經由內生性 ephrinB2 使 Src 激酶依賴型 NR2B 的 ＊
＊ 名 稱 酪胺酸磷酸化引起。 ＊
＊ ********* ＊＊ ＊

執行計畫學生： 陳建錞
學生計畫編號： NSC 98-2815-C-040-052-B
研 究 期 間 ： 98 年 07 月 01 日至 99 年 02 月 28 日止，計 8 個月
指 導 教 授 ： 林則彬

處理方式： 本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

執 行 單 位： 中山醫學大學醫學檢驗暨生物技術學系(所)

中華民國 99 年 06 月 04 日

一、計畫成果報告

計畫名稱：結腸內灌注芥子油引起之跨臟器尿道反射敏感效應，是否會經由內生性 ephrinB2 使 Src 激酶依賴型 NR2B 的酪胺酸磷酸化引起

中文摘要：

近年來，ephrin 及其接受器 (Eph receptor) 被發現在神經可塑性 (synaptic plasticity)上和神經病理性疼痛 (neuropathy pain) 之訊息傳遞扮演重要的角色。在過去的研究中，我們發現，透過椎管內注射方式，施予 ephrinB 或使 EphB 接受器被活化，在腰薦椎 (lumbosacral spinal cord) 之脊髓背角 (spinal dorsal horn) 可以引發脊髓反射增益效應 (spinal reflex potentiation, SRP)，然而 SRP 的出現已被證實與臟器疼痛的發生與維持有著直間或間接的關係。本研究，我們想透過由降結腸 (descending colon) 灌注芥子油 (mustard oil, MO) 誘發疼痛反應，並記錄 SRP 的變化，以探討跨臟器敏化作用 (cross organ sensitization, COS) 的成因，初步結果指出，透過透過椎管內注射方式，給予 EphB 接受器抑制劑 (EphB1-Fc, EphB2-Fc chimeras)、Src 抑制劑 (PP2) 和 NMDAR 拮抗劑 (APV)，可以抑制由芥子油誘發的跨臟器敏化作用。因此，推測芥子油灌注後可能增加了內生性 ephrinB 的釋放及活化了 EphB 接受器，且與 ephrinB2/EphB/Src/NMDAR 訊息傳遞路徑有關，導致 NMDAR 從細胞質移置 (trafficking) 細胞膜，誘發甚至是維持 SRP 和跨臟器敏化作用。另一方面，若抑制 ephrin 的釋放或是阻斷 Eph 接受器，可能是一種治療跨臟器轉移疼痛的方法 (viscero-visceral referred pain)。我們想進一步利用干擾 RNA (siRNA) 神經注射技術以及基因剔除小鼠 (knockout mice)，降低甚至去除 Eph 接受器或 ephrin 基因表現，研究其與臟器疼痛的關聯性。希望能提供臟器疼痛或跨臟器轉移疼痛的機轉研究或治療新的方針。

關鍵字(至少五個)

骨盆腔疼痛；尿道；大腸急躁症；結腸；N-甲基-D-天門冬胺酸

Abstract

Recently, the role of EphB receptor (EphBR) tyrosine kinase and their ephrinB ligands in spinal pain-related neural plasticity has been identified. To test whether Src-family non-receptor tyrosine kinase-dependent glutamatergic N-methyl-d-aspartate receptor (NMDAR) NR2B subunit phosphorylation underlies lumbosacral spinal EphBR activation to mediate cross-organ sensitization between the colon and the urethra, external urethra sphincter electromyogram activity evoked by pelvic nerve stimulation and protein expression in the lumbosacral (L6-S2) dorsal horn were studied before and after intracolonic mustard oil (MO) instillation. We found MO instillation produced colon-urethra reflex sensitization along with an upregulation of endogenous ephrinB2 expression as well as phosphorylation of EphB 1/2, Src-family kinase, and NR2B tyrosine residues. Intrathecal immunoglobulin fusion protein of EphB1 and EphB2 as well as PP2 reversed the reflex sensitization and NR2B phosphorylation caused by MO. All these results suggest that EphBR-ephrinB interactions, which provoke Src-family kinase-dependent NMDAR NR2B phosphorylation at the lumbosacral spinal cord level, are involved in cross-organ sensitization, contributing to the development of viscero-visceral referred pain between the bowel and the urethra.

Keywords

pelvic pain syndrome; urethra; irritable bowel syndrome; colon; NMDA

MATERIALS AND METHODS

Animal Preparations

Female Sprague-Dawley rats (*n* =160; 205–290g) were used in this experiment, which was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University (Taiwan). Rats were anesthetized with urethane (1.2 g/kg ip). A PE-50 intracolonic catheter was inserted into the descending colon (4 cm from the anus), and another PE-10 catheter was inserted through a slit made at the atlanto-occipital membrane and passed caudally to the T13 vertebrae (L6–S2 spinal cord) for the dispensing of test agents. The right pelvic nerve was dissected and mounted on a pair of wire electrodes for stimulation. Oligo-/single-unit action potentials in external urethra sphincter electromyogram (EUSE) activity were recorded by a pair of epoxy-coated copper wire electrodes placed ~1–2 mm lateral to the urethra and were continuously recorded on a recording system (MP30, Biopac, Santa Barbara, CA). Single shocks at a fixed suprathreshold strength were repeated at 1 stimulation/30 s (test stimulation; TS) and given through the stimulation electrodes. The protocols for assessing the effects of different kinds of reagents on reflex activity were the following.

Protocol 1: colon irritation. MO (0.5%, 0.1 ml) or corn oil (CO) was instilled into the lumen of the descending colon 1 min before TS onset through an intracolonic catheter to induce acute colon irritation. The excitability of reflex activity was evaluated by applying the TS to the pelvic afferent nerve that was continuously recorded from TS
starting for 120 min.

Protocol 2: intracolonic pharmacological tests. Lidocaine (0.5%, 0.1 ml) was instilled into the descending colon via the intracolonic catheter 10 min before TS started.

Protocol 3: intrathecal pharmacological tests. EphB1-Fc (10 μg, 10 μl), EphB2-Fc (10 μg, 10 μl), PP2 (50 μM, 10 μl), and D-2-amino-5-phosphonovalerate (APV; 10 μM, 10 μl) were injected via the intrathecal catheter 10 min before TS started.

Application of Drugs

Drugs administered included NMDA (10 μM, 10 μl it, Sigma), a selective glutamatergic NMDAR agonist (APV, 10 μM, 10 μl it, Sigma), a glutamatergic NMDA receptor antagonist; ephrinB2-Fc chimera (5 μg/rat it, Sigma), EphB receptor ligand; EphB1-Fc chimera (10 μg /rat it, Sigma), EphB1-selective

antagonist; EphB2-Fc chimera (10 μg /rat it, Sigma), EphB2-selective antagonist; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolol[3,4-d]pyrimidine (PP2; 50 μM, 10 μl it, Tocris) a Src-family kinase inhibitor; lidocaine hydrochloride (0.5%, 0.1 ml, intracolonic, AstraZeneca), a nerve conduction blocker; MO (0.5% diluted in CO, 0.1 ml, intracolonic, Sigma), a component causing acute colon irritation; and CO (intracolonic, Sigma), a vehicle control for MO. In all cases, solvent solutions of identical volume to tested agents were dispensed to serve as the vehicle control.

Western Blotting

Two hours after intracolonic MO/CO instillation, the spinal cords were obtained after a laminectomy. The right lumbosacral (L6 –S2) dorsal horns were dissected out and were immediately snap-frozen on liquid nitrogen. The protocol for Western blotting has been described elsewhere (22, 23); in brief, the sample was homogenized and the extracts were centrifuged to retain supernatants. The supernatant was separated on an acrylamide gel and transferred to a polyvinylidene difluoride membrane and then were incubated for 1 h at room temperature in either rabbit antiephrinB2 (1:2,000, Santa Cruz Biotechnology), anti-phosphorylated EphB1/2 (pEphB1/2l 1:2,000, Millipore), rabbit anti-phospho-Src antibody (1:1,000, Millipore), or rabbit anti-phosphotyr1336 or anti-phosphotyr1472-NR2B (1:1,000 Millipore). Blots were washed and incubated in peroxidase-conjugated donkey anti rabbit IgG (1:5,000, Santa Cruz Biotechnology) or donkey anti mouse IgG (1:10,000, Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized using an enhanced hemiluminescence detection kit (ECL Plus, Millipore), and then densitometric analysis of the Western blot membranes was done with Science Lab 2003 (Fuji). Results were normalized against β-actin and are presented as the means ± SD.

RNA Extraction and Quantitative RT-PCR

The dissected right lumbosacral spinal dorsal horns (L6 –S2) were placed in RNAlater solution (Applied Biosystems). Total RNA was extracted using RNA extraction kits (GeneMark). Reverse transcription was performed using cDNA reverse transcription kits (Applied Biosystems). cDNA samples underwent absolute quantitative real-time PCR on the OneStep instrument (Applied Biosystems) the default 40-cycle program. TaqMan gene expression assays were purchased from Applied Biosystems (assay Rn_00667869_ml and Rn_00680474_ml). For each amplicon, PCR efficiency was estimated near 1.0

by serial dilutions of cDNA. Relative quantities of mRNA were estimated using the $\triangle\triangle C_T$ method. RT-PCR of the β-actin gene was also performed, and the expression level of β-actin mRNA was used to serve as a normalized control. All the results of RT-PCR are presented as the means ± SD.

Data Analysis

Comparisons across drug- and vehicle-treated groups were determined using one-way, repeated-measures ANOVA, followed by a post hoc test without correction. In all cases, differences of $P < 0.05$ were considered as statistically significant differences.

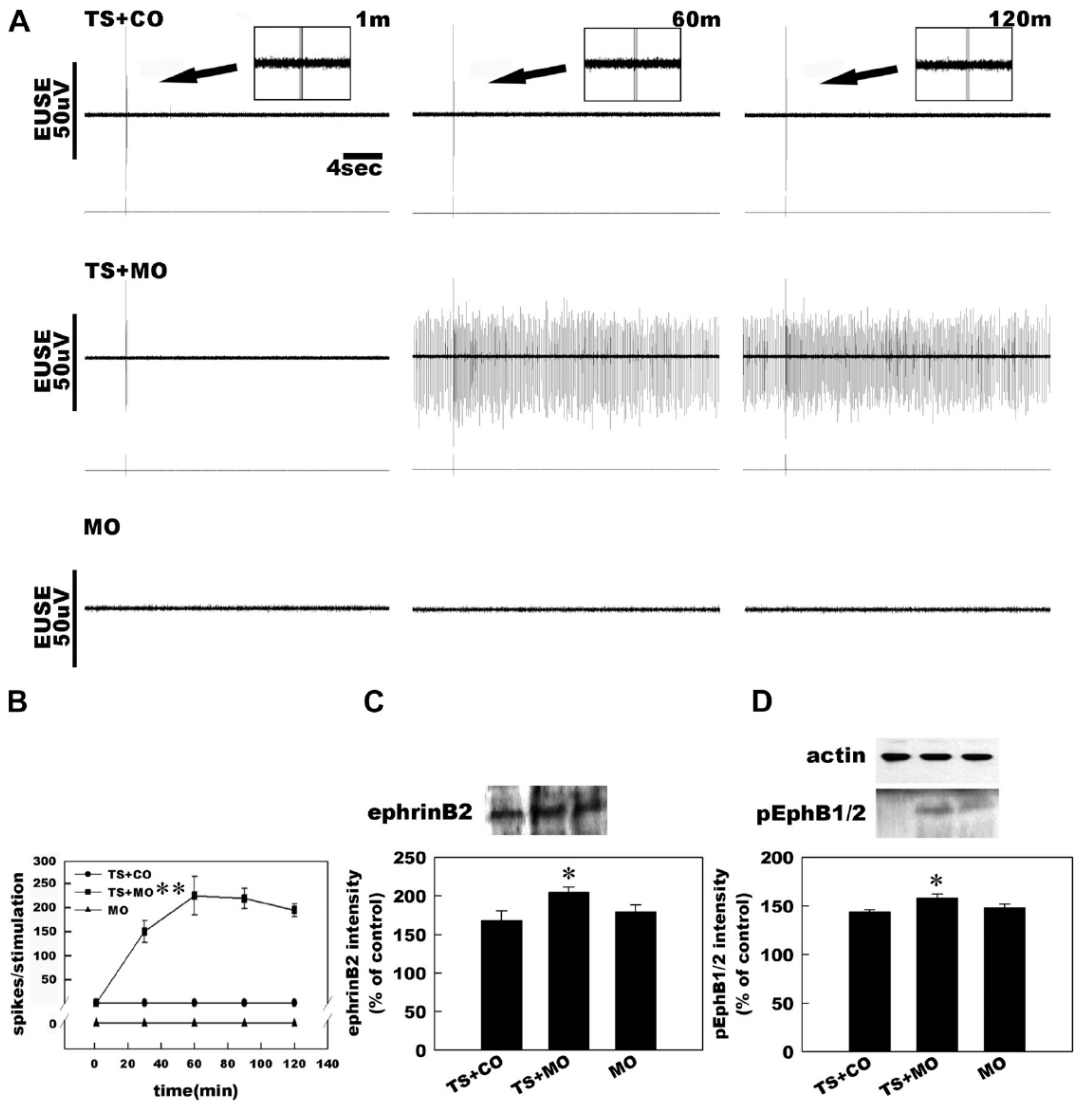

Fig. 1. Cross-organ reflex sensitization caused by intracolonic mustard oil (MO) instillation. A: compared with corn oil (TS+CO), which did not elicit any effects on the baseline reflex activity caused by the test stimulation (TS; single action

potentials are indicated by arrows), MO instillation into the descending colon produced reflex sensitization (TS+MO) of the external urethra electromyogram (EUSE) activity, while it did not induce any spontaneous firing (MO). B: intracolonic MO instillation significantly increased the mean spike numbers evoked by the TS (TS+MO) compared with CO instillation (TS+CO). **P $<$ 0.01 vs. TS+CO; n = 7. C and D: in rats that received TS in associated with colon MO instillation (TS+MO), the expression of endogenous ephrinB2 (% of β-actin) and phosphorylated EphB1/2 (pEphB1/2; % of β-actin) both increased significantly compared with those that received the TS with CO instillation (TS+CO). *P $<$ 0.05 vs. TS+CO; n = 4.

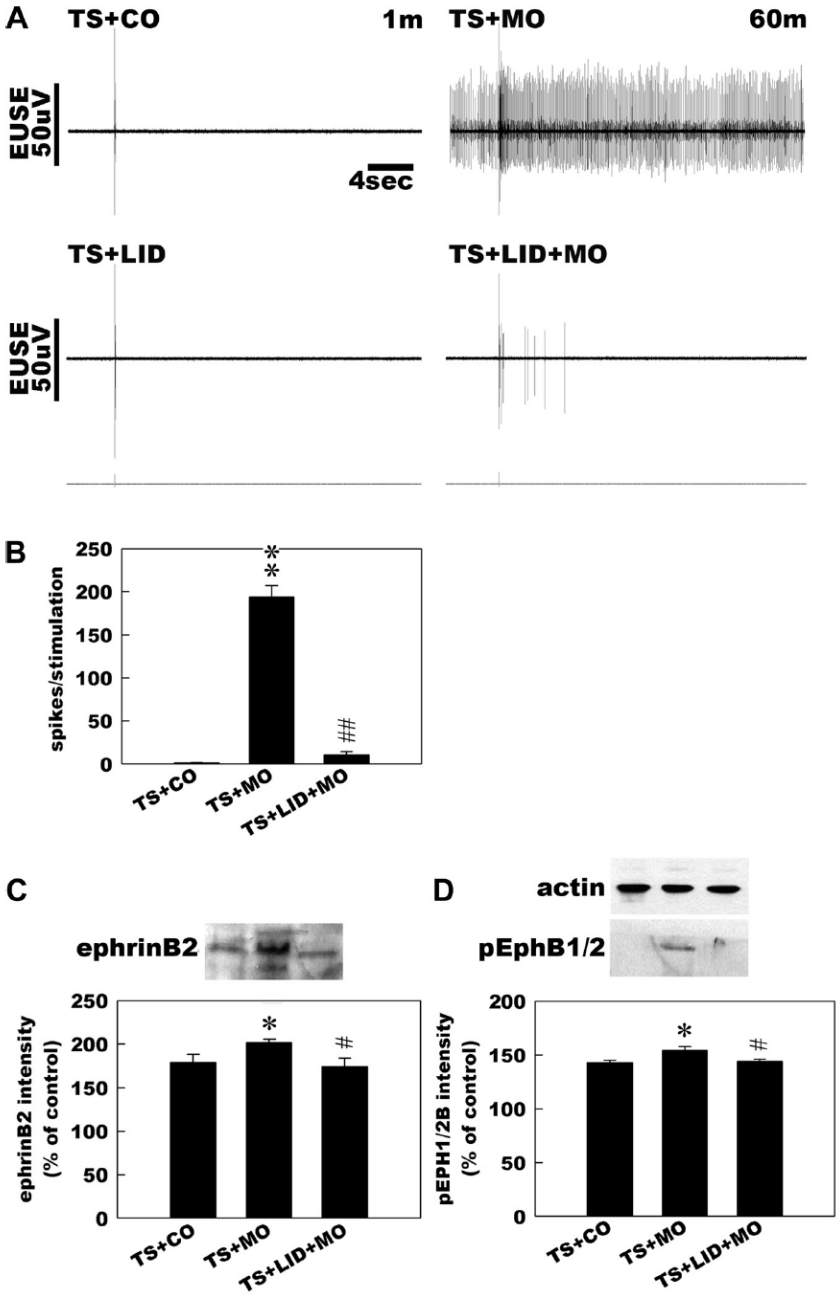

Fig. 2. Intracolonic lidocaine pretreatment abolished the MO-elicited cross-organ sensitization as well as endogenous ephrinB2 and pEphB1/2 expression. A: intracolonic MO instillation induced cross-organ sensitization on TSinduced reflex activity (TS+MO) that is reversed by pretreatment with lidocaine (TS+LID+MO). B: intracolonic MO (TS+MO) but not CO (TS+CO) instillation significantly increased the mean spike number evoked by the TS (**P $<$ 0.01 vs. TS+CO; n = 7) that was reversed by intracolonic pretreatment with lidocaine (TS+LIDC+MO). ##P $<$ 0.01 vs. TS+MO; n 7. C: Western blotting showed that in rats that received TS in associated with intracolonic MO instillation (TS+MO), the expression of ephrinB2 (% of β-actin) and pEphB1/2 (% of β-actin) were both increased significantly compared with those that received TS with CO instillation (TS+CO). *P $<$ 0.05 vs. TS+CO; n = 4. Moreover, intracolonic treatment with lidocaine before MO instillation reversed the MO-elicited increments in the expression levels of ephrinB2 and pEphB1/2 (TS+LID+MO). #P $<$ 0.05 vs. TS+MO; n = 4.

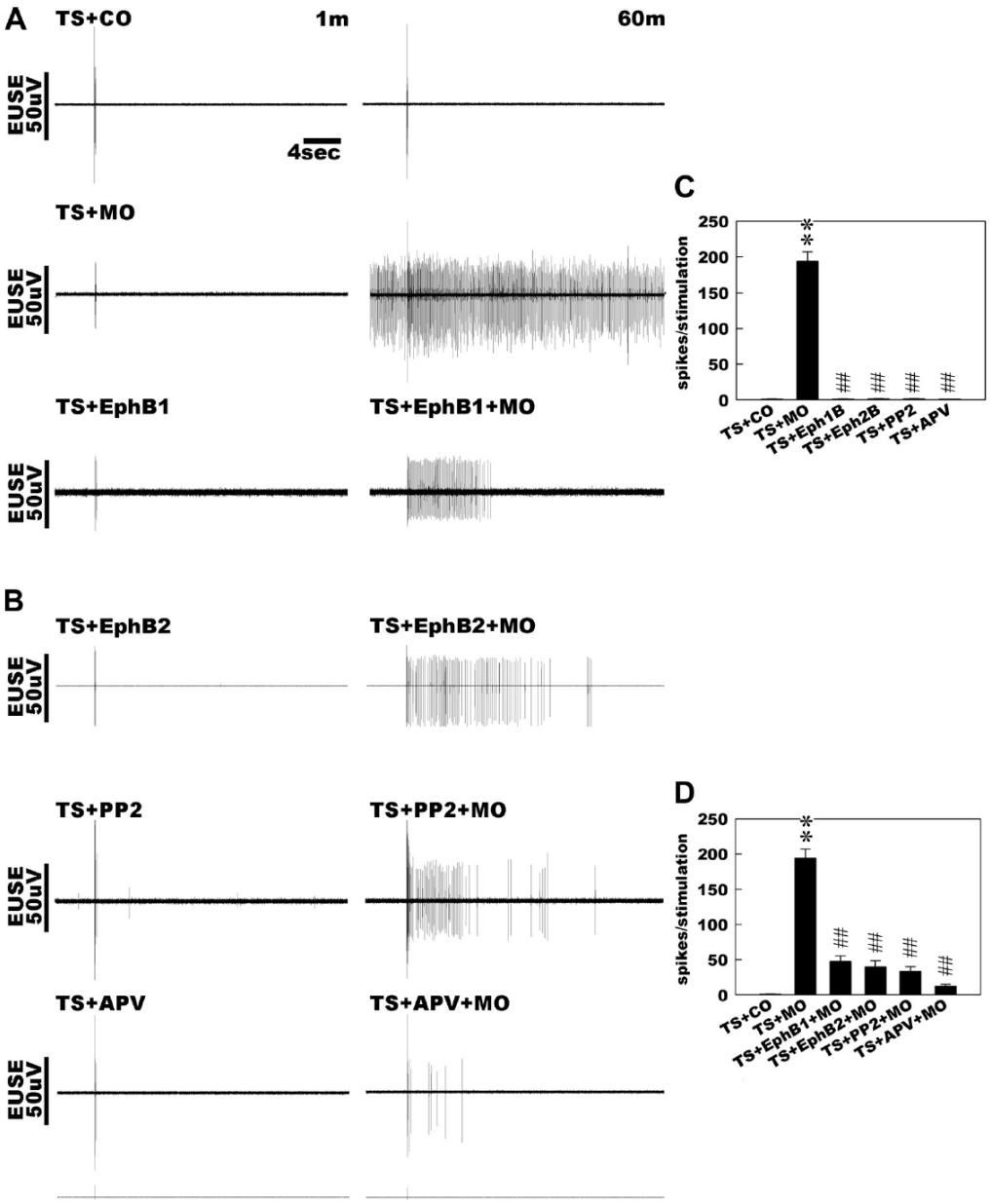

Fig. 3. Src-family kinase downstream of EphB1/2 mediates NMDA-dependent cross-organ sensitization caused by intracolonic MO instillation. A: compared with CO (TS+CO), intracolonic MO instillation (TS+MO) elicited cross-organ sensitization on the TS-induced reflex activity that was attenuated by prior intrathecal administration of Eph1B-Fc and Eph2B-Fc (TS+Eph1B+MO and TS+Eph2B+MO, respectively). B: intrathecal pretreatment with PP2 and D-2-amino-5-phosphonovalerate (APV) each prevented the development of MO-elicited reflex sensitization (TS+PP2+MO and TS+APV+MO, respectively). C: compared with CO instillation (TS+CO), colonic MO instillation (TS+MO; **P $<$ 0.01 vs. TS+CO; n = 7) but not intrathecal EphB1-Fc, EphB2-Fc, PP2, and APV (TS+EphB1, TS+EphB2, TS+PP2, and TS+APV, respectively; ##P $<$

0.05 vs. TS+MO; n = 7) significantly increased the mean spike numbers evoked by each stimulus in electromyogram activity. D: intracolonic MO instillation (TS+MO) but not CO instillation (TS+CO) significantly increased the mean spike numbers evoked by the TS (**P ＜ 0.01 vs. TS+CO; n = 7) which was reversed by intrathecal pretreatment with EphB1-Fc, EphB2-Fc, PP2, and APV (TS+ephB1+MO, TS+ephB2+MO, TS+PP2+MO and TS+APV+MO, respectively; ##P ＜ 0.05 to TS+MO).

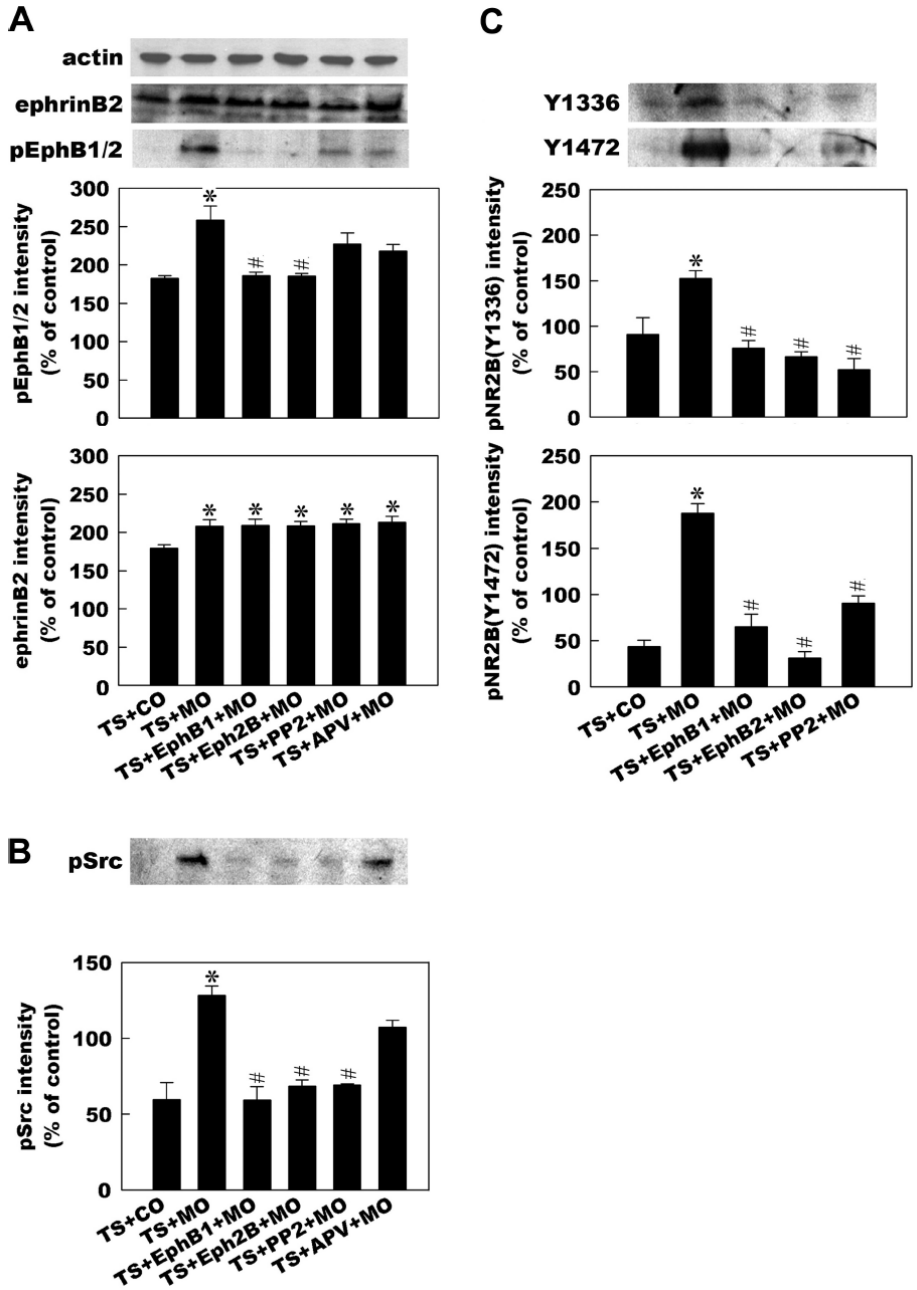

Fig. 4. Endogenous ephrinB2 mediates NMDA-dependent cross-organ sensitization on the TS-induced reflex activity via Src-family kinase-elicited phosphorylation of tyrosin residue of the NMDA NR2B subunit. A: in rats that

received intracolonic MO instillation (TS+MO), the expression of endogenous ephrinB2 (% of β-actin) and pEphB1/2 (% of β-actin) both increased significantly compared with those that received CO instillation (TS+CO). *P $<$ 0.05 vs. TS+CO; n = 4. Prior intrathecal treatment with EpB1-Fc and EphB2-Fc (TS+EphB1+MO and TS+EphB2+MO, respectively) but not PP2 and APV (TS+PP2+MO and TS+APV+MO, respectively) reversed the increments in pEphB1/2. #P $<$ 0.05 vs. TS+MO; n = 4. Moreover, all these agents failed to reverse the upregulation in endogenous ephrinB2 expression caused by CO instillation. *P $<$ 0.05 vs. TS+CO; n = 4. B: expression levels of Src (pSrc; % of β-actin) increased significantly in animals that received intracolonic MO instillation (TS+MO) compared with CO instillation (TS+CO). P $<$ 0.01 vs. TS+CO, n = 7. Intrathecal pretreatment with EpB1-Fc, EphB2-Fc and PP2 (TS+EphB1+MO, TS+EphB2+MO and TS+PP2+MO, respectively; #P • 0.05 vs. TS+MO; n = 7) but not APV (TS+APV+MO; P $<$ 0.05 vs. TS+MO) reversed the increment in pSrc expression caused by MO instillation. C: in rats that received intracolonic MO instillation (TS+MO), phosphorylated Tyr1336 and Tyr1472 (pY1336 and pY1472) both increased significantly compared with rats that received CO instillation (TS+CO). *P $<$ 0.05, n = 4. Prior treatment with EpB1-Fc, EphB2-Fc, PP2, and APV (TS+EphB1+MO, TS+EphB2+MO, and TS+PP2, respectively) reversed the increments in pY1336 and pY1472 expression caused by MO instillation. #P $<$ 0.05; n = 4.