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Estradiol modulates visceral hyperalgesia by increasing thoracolumbar spinal GluN2B subunit activity in female rats

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Abstract

Background—We previously reported estrogen modulates spinal NMDA receptor processing of colorectal pain through changes in spinal GluN1 subunit phosphorylation/expression. The purpose of the present study was to investigate whether spinal GluN2B containing NMDA receptors are involved in estrogen modulation of visceral pain processing.

Methods—Behavioral, molecular and immunocytochemical techniques were used to determine spinal GluN2B expression/phosphorylation and function 48 hrs following subcutaneous injection of estradiol (E2) or vehicle (safflower oil, Saff oil) in ovariectomized rats in the absence or presence of colonic inflammation induced by mustard oil.

Key results—E2 increased the magnitude of the visceromotor response (VMR) to colorectal distention compared to Saff oil in non-inflamed rats. Intrathecal injection of the GluN2B subunit antagonist, Ro 25-6981, had no effect on the VMR in non-inflamed E2 or Saff oil rats. Colonic inflammation induced visceral hyperalgesia in E2, but not Saff oil rats. Visceral hyperalgesia in E2 rats was blocked by intrathecal GluN2B subunit selective antagonists. In inflamed rats, E2 increased GluN2B protein and gene expression in the thoracolumbar (TL), but not lumbosacral (LS), dorsal spinal cord. Immunocytochemical labeling showed a significant increase of GluN2B subunit in the superficial dorsal horn of E2 rats compared to Saff oil rats.

Conclusions and inferences—These data support the hypothesis that estrogen increases spinal processing of colonic inflammation-induced visceral hyperalgesia by increasing NMDA receptor activity. Specifically, an increase in the activity of GluN2B containing NMDA receptors in the TL spinal cord by estrogen underlies visceral hypersensitivity in the presence of colonic inflammation.

Keywords

visceral nociception; gonadal hormones; spinal cord; pain; NMDA receptor; estradiol

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Introduction

Chronic abdominal pain is a common complaint in patients seeking clinical intervention. Some forms of abdominal pain, such as irritable bowel syndrome, occur more frequently and with greater severity in women than in men (1–6), but the molecular basis for the sex difference in abdominal pain is not fully understood, preventing the development of successful drug intervention.

N-methyl-D-aspartate (NMDA) receptors are heterotetramers composed of two GluN1 and two GluN2 or 3 subunits: (GluN1/GluN2A-D or GluN3A). NMDA receptor activity is subject to changes in receptor abundance, distribution (membrane vs. cytosol, postsynaptic vs. extrasynaptic), phosphorylation status and subunit composition by ischemia, inflammation, aging, or sex hormones (7–10). In the brain, estrogen increases hippocampal spine and synapse density and NMDA receptor binding (11;12). Estrogen enhances NMDA receptor-mediated hypothalamic neuron excitability and the sensitivity of NMDA receptors to glutamate (13;14), possibly by regulating NMDA receptor expression or phosphorylation (15–19).

However, the effect of estrogen on NMDA receptor modulation of visceral nociceptive processing in the spinal cord is not fully understood. Estrogen receptors colocalize with NMDA receptors in dorsal horn neurons providing the anatomical compartmentalization necessary for estrogen modulation of NMDA receptor activity (20). Ovariectomized rats with E2 replacement showed a decreased potency of the NMDA receptor antagonist APV to attenuate the visceromotor response (VMR) to colorectal distention as compared with rats with Saff oil. Meanwhile, there was a corresponding enhancement of spinal GluN1 phosphorylation/expression in E2 rats (20), suggesting increases in GluN1 subunit phosphorylation/expression contribute to E2- facilitation of spinal nociceptive processing. This is further supported by the greater potency of spinal APV in male rats (with lower circulating E2) compared to intact females (21).

Several lines of evidence suggest spinal GluN2B containing NMDA receptors may also play important roles in mediating nociceptive signal transmission. GluN2B subunits are localized to the superficial dorsal horn (laminae I–II) (22). Hindpaw inflammation increased spinal GluN2B expression and phosphorylation, and bath application of a GluN2B selective antagonist significantly attenuated the magnitude of NMDAR-EPSCs in spinal cord slices from inflamed animals (7;23).

Colonic inflammation in neonates or in adult animal results in central sensitization and visceral hypersensitivity (24–27). Recent studies demonstrated increased phosphorylation and/or expression of GluN2B in the central nervous system in several visceral pain models that may be influenced by short-term estrogen replacement (27–29). However, it is not clear whether longer-term estrogen treatment modulates visceral hypersensitivity through changes in spinal GluN2B containing NMDA receptor expression/function. In the present study we aimed to determine the role of GluN2B subunits in different spinal segments in estradiol modulation of visceral nociceptive processing, in an effort to better understand hypersensitive bowel conditions that show higher prevalence in women.

Materials and methods

Female Sprague-Dawley rats (220–250g) were purchased from Harlan (Frederick, MD). All protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee and conform to the guide for use of laboratory animals by the International Association for the Study of Pain.

Surgical preparation

Rats were anesthetized with a subcutaneous (s.c.) injection containing 55 mg/kg ketamine, 5.5 mg/kg xylazine, 1.1 mg/kg acepromazine and ovariectomized. If necessary electromyogram (EMG) electrodes (teflon-coated stainless steel wire, Cooner wire, Chatsworth, CA) were stitched into the external oblique muscle and an intrathecal (i.t.) catheter made from 32 g polyethylene tubing (Micor Inc., Allison Park, PA) was implanted via an incision in the atlanto-occipital membrane as previously described (21). The catheter and electrodes were exteriorized at the back of the neck. Rats were subsequently singly housed on a 12 h-12 h light-dark cycle with free access to food and water.

Ten to 14 days after surgery, the ovariectomized rats were injected with either safflower oil (Saff oil; 100 μ l, s.c.) or 17- β -estradiol (E2; 50 μ g in 100 μ l Saff oil, s.c.) and tested 48 hrs later (30).

Visceromotor response recording

Rats with Saff oil or E2 replacement were fasted for 18–24 hrs prior to testing to facilitate balloon placement. A 5–6 cm distention balloon was placed into the descending colon while the rat was lightly sedated with isoflurane. Rats were placed in acrylic holders and given 30 min to recover. The visceromotor response (VMR) to colorectal distention (three trials of four 20s distentions to 80 mmHg with three minute interstimulus intervals) was recorded. The EMG was rectified and the area under the curve (AUC) for the 20 s prior to each distention was subtracted from the AUC during the 20 s distention to derive the VMR.

Intrathecal antagonist

In non-mustard oil rats, after recording the baseline VMR, the GluN2B selective antagonists Ro 25-6981 and Co 101244 (Tocris, Minneapolis, MN) were administered intrathecally in a volume of 20 μ l (10% DMSO was used as the vehicle) followed by a flush with 5 μ l saline. The effect of the drug was examined starting at 30, 90, 150, 210 min following i.t. injection. The doses of both drugs were adopted from previous studies (31–33).

Colonic inflammation

Following the baseline VMR recording, rats were sedated with isoflurane, the distention balloon removed and mustard oil (0.25 ml, 2% in mineral oil) injected into the descending colon with a gavage needle while being slowly withdrawn. The distention balloon was replaced. This dose of mustard oil was reported to increase plasma extravasation in the colon that is considered a sign of colonic inflammation (34). Control rats received colonic instillation of mineral oil. Recordings commenced at the time indicated following intracolonic mustard oil or mineral oil. As appropriate, the GluN2B receptor antagonists

were injected 15 min prior to mustard oil and recordings commenced 30 min later. Recordings ended approximately 4 hours following colonic inflammation.

Immunohistochemistry

Rats were euthanized with pentobarbital sodium (150 mg/kg, i.p.) at 3.5 hr post colonic mustard oil and perfused through the heart with saline followed by 500 ml 4% paraformaldehyde in 0.1M phosphate buffer (PB) at 4°C. The thoracolumbar (TL, T13-L2) and lumbosacral (LS, L6-S2) spinal cord tissue were removed and post-fixed overnight in fresh fixative at 4°C and then transferred to 30% sucrose in PB for 24–48 hrs. Frozen sections (30 µm) were cut on a cryostat. For quantitative analysis, sections were immunohistochemically labeled using goat anti-GluN2B (1:1000, Santa Cruz, Dallas, TX) and biotinylated donkey-anti goat IgG (GARF, 1:2000, Jackson Immuno Research Laboratories, Inc., West Grove, PA) and ABC (Vector Laboratories, Inc., Burlingame, CA). Sections were subsequently incubated in 0.02% diaminobenzidine tetrahydrochloride (DAB) with 2.5% nickel sulfate and 0.003% H₂O₂ in 0.175 M sodium acetate buffer. Data presented are the average of the number of positive stained cells per section on one side of the spinal cord. Data were analyzed using the student's *t*-test, *p* < 0.05 was considered significant. For illustrative purposes only, sections were labeled using Alexa Fluor 488 for immunofluorescence images.

Immunoprecipitation and Western blot

Separate rats from those in the VMR recordings were used for western blots. Non-inflamed rats were euthanized with CO₂ at 48 hr post Saff oil or estrogen replacement. Inflamed rats were sacrificed 3.5 hr post colonic mustard oil. The spinal cord was removed by pressure ejection with ice cold saline. The LS and TL spinal cord segments were isolated and the ventral part of the spinal cord removed. The tissue was sonicated in RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 1 mM Na₃VO₄, 1mM NaF, and protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenate was centrifuged at 14000 rpm for 15 min at 4°C. The protein concentration in the supernatant was determined using the Bradford method. Due to the poor quality of p-GluN2B antibodies commercially available at the time of the experiment, protein samples from the spinal cord were immunoprecipitated with a GluN2B antibody followed by immunoblotting analysis. Phosphorylated GluN2B was detected using a pan anti-phosphotyrosine antibody. Briefly, anti-GluN2B antibody (Santa Cruz, Dallas, TX) was used in overnight immunoprecipitation. Precipitated proteins were separated on a 7.5% SDS-PAGE gel and blotted to nitrocellulose membrane (Thermo Scientific, Pittsburgh, PA). The blot was incubated with the primary antibody (mouse anti- phosphotyrosine, 1:500, Santa Cruz, Dallas, TX) overnight and secondary antibody (Goat anti-mouse, Jackson ImmunoResearch, West Grove, PA) for one hour. The antigen-antibody complexes were visualized by Enhanced Chemiluminescence (ECL, Thermo Scientific, Pittsburgh, PA). The immunoreactive band density was analyzed using NIH Image J software. The membrane was stripped and reprobed with goat anti-GluN2B antibody (1:800, Santa Cruz, Dallas, TX) for total GluN2B analysis. Total GluN2B expression was also analyzed from samples before immunoprecipitation.

Real-time RT-PCR

Total RNA was isolated from the dorsal spinal cord using the Absolutely RNA Miniprep Kit (Stratgene, CA) and reverse transcribed cDNAs were prepared using SuperScript II Reverse Transcriptase kit (Invitrogen/Life Technologies, Grand Island, NY). For real-time PCR, primer sets for GluN2B (forward primer: 5'-GGCATTGCTATCCAAAAGGA; reverse primer: 5'-GCTGCCCCCAACATATAGAA) were used (Sigma-Aldrich, St. Louis, IL). β -actin was used as housekeeping gene (forward primer: GGTCCACACCCGCCACCAG, reverse primer: 5'-CAGGTCCAGACGCAGGATGG, Sigma-aldrich, St. Louis, IL). Real-time PCR reactions were carried out using SYBRgreen qPCR Master Mix (Fermentas/Thermo Scientific, Pittsburgh, PA). C_T values were obtained using Eppendorf Mastercycler Real-plex machine (Eppendorf, Germany). The efficiency of PCR was calculated from the slope of the standard curve and was found within the range of 90–110%. The relative expression of GluN2B mRNA was normalized to the level of β -actin mRNA from the same cDNA and expressed as fold of control using the C_t method.

Statistics

Data were analyzed using Prism6 software. The data are expressed as mean $\pm$ SEM. Data were analyzed using one-way or two-way ANOVA followed by the Student-Newman-Keuls Method or t-test when appropriate. Normalized data were analyzed using nonparametric equivalent tests (Kruskal-Wallis or Friedman repeated measure 1 way ANOVA followed by Dunn's multiple comparison test). $p < 0.05$ was considered significant.

Results

Effect of GluN2B selective antagonist on visceromotor response in non-inflamed rats

Studies by our lab and others indicate transient visceral stimuli activate spinal NMDA receptors (35–40) and spinal GluN1 subunit phosphorylation/expression contributes to changes in NMDA receptor activity and visceral hypersensitivity induced by estrogen (20;21). To investigate the role of spinal GluN2B containing NMDA receptors in estrogen modulation of colorectal distention induced visceral pain behavior, the GluN2B subunit selective antagonist, Ro 25-6981, was intrathecally administered to Saff oil or E2-treated rats. Consistent with our previous findings, the baseline response of the E2 rats (55.6 ± 4.1) was significantly greater compared with the Saff oil rats (34.5 ± 3.3 ; t-test, $p = 0.0011$). I.t. vehicle (10% DMSO) did not affect the VMR in either Saff oil or E2 rats compared to baseline (prior to DMSO) (One way RM ANOVA, $p > 0.05$ for each cohort). In Saff oil rats, no difference in the VMR was observed between Ro 25-6981 and vehicle groups (Two way RM ANOVA, $p > 0.05$; Figure 1A). Similarly, in E2 rats, there was no difference in the magnitude of the VMR between the i.t. vehicle and Ro 25-6981 groups (Two way RM ANOVA, $p > 0.05$; Figure 1B). These data suggest GluN2B containing NMDA receptors do not mediate the visceromotor response to transient colorectal stimuli under normal conditions.

Effect of GluN2B selective antagonists on colonic inflammation-induced hyperalgesia

Colonic inflammation induced visceral hyperalgesia in E2, but not Saff oil replacement rats—We previously reported that mustard oil-induced visceral hyperalgesia was significantly greater in E2 rats compared to Saff oil rats (34). Consistent with our previous observation, mustard oil increased the magnitude of the VMR (visceral hyperalgesia) compared to baseline in E2 rats starting 90 min post inflammation and the hypersensitivity persisted beyond the end of the 4 hr recording period (Friedman ANOVA, $p < 0.01$; Dunn's test as indicated in Figure 2A). In contrast, the visceromotor response in Saff oil rats was not significantly changed during the 4 hr recording period following mustard oil injection (Friedman ANOVA, $p > 0.05$), although a slight increase in response was observed when the peak response (regardless of time) from each rat was calculated (34). Area under the curve analysis for the post mustard oil time points revealed E2 significantly increased the magnitude of the VMR during the 4 hr recording period compared to Saff oil (t-test, $p < 0.0001$; Figure 2B). Considering that intracolonic mustard oil did not induce visceral hyperalgesia following short term (4 hr) E2 replacement (41), genomic actions of estrogen may be involved in mediating visceral hypersensitivity following colonic inflammation.

GluN2B containing NMDA receptors are involved in colonic inflammation induced visceral hyperalgesia—To investigate whether GluN2B containing NMDA receptors are involved in inflammatory visceral pain processing, the GluN2B subunit selective antagonist Ro 25-6981 was injected intrathecally 15 min prior to colonic inflammation by mustard oil in both E2 and Saff oil replacement rats. In E2 rats, pretreatment with 30 μ g Ro 25-6981 or vehicle did not affect mustard oil-induced visceral hyperalgesia (Friedman ANOVA: 30 μ g Ro, $p < 0.05$ vs. baseline; DMSO, $p < 0.0001$ vs. baseline; Dunn's test as indicated in Figure 2C). In contrast, 80 μ g Ro 25-6981 blocked mustard oil-induced facilitation of the VMR (Friedman ANOVA, $p > 0.05$). Area under the curve analysis for the post mustard oil time points revealed the higher dose significantly attenuated the visceral hyperalgesia (1 way ANOVA, $p < 0.05$; Figure 2D). This result was confirmed by intrathecal administration of another GluN2B selective antagonist Co 101244 (100nmol), which also blocked the visceral hyperalgesia (Friedman ANOVA, $p > 0.05$; Figure 2C). In contrast, i.t. Ro 25-6981 had no effect on the visceromotor response in Saff oil rats following intracolonic mustard oil (Friedman ANOVA, $p > 0.05$; Figures 2E, F).

Spinal mechanisms contributing to estrogen modulation of visceral pain and hypersensitivity

Changes of GluN2B expression in the TL and LS spinal cord by estrogen in the absence of colonic inflammation—Changes in protein phosphorylation/expression usually serve as a substrate for changes in receptor function. To explore a possible role of GluN2B containing NMDA receptors in estrogen modulation of visceral nociception, GluN2B phosphorylation and expression in the LS spinal cord was first compared between E2 and Saff oil rats in the absence of colonic inflammation. E2 increased GluN2B expression in the LS spinal cord compared to Saff oil in non-inflamed rats (t-test, $p < 0.05$; Figure 3A).

Consistent with the minimal role of the TL spinal cord in transient visceral pain (42), E2 replacement in non-inflamed rats did not change the phosphorylation or expression of GluN2B in these spinal segments (All groups, t-test, $p>0.05$; Figure 3B).

Changes of spinal GluN2B expression by estrogen in rats with colonic inflammation—In the presence of colonic inflammation, no difference in GluN2B phosphorylation or expression in the LS spinal cord was observed between E2 and Saff oil rats (t-test, $p>0.05$; Figure 4A). In addition, there was no difference in GluN2B gene expression between E2 and Saff oil rats (t-test, $p>0.05$; Figure 4B). These data suggest GluN2B containing NMDA receptors in LS spinal cord are not contributing to the difference in the visceromotor response in Saff oil and E2 rats following colonic inflammation.

Although the TL spinal cord does not contribute to transient visceral nociceptive processing, several lines of evidence suggest these spinal segments contribute to visceral hypersensitivity following visceral inflammation (42;43). A previous study from our lab indicated female rats had more GluN1 expression than males in the TL spinal cord following colonic inflammation, suggesting gonadal hormones may modulate NMDA receptor activity at these spinal segments in rats with colonic inflammation (21). In the present study, we observed an increase in immunoprecipitated GluN2B protein level and pGluN2B/GluN2B ratio in the TL spinal cord in E2 rats with colonic inflammation compared with Saff oil rats (t-test, $p<0.05$; Figure 5A). The increase of GluN2B expression in E2 rats was confirmed by western blot from the total protein lysate (t-test $p<0.05$; Figure 5B). Consistent with the increase in protein expression induced by colonic inflammation, there was a concomitant increase in GluN2B gene expression in the TL spinal cord in E2 rats (t-test, $p<0.05$; Figure 5C).

Immunocytochemical staining with GluN2B antiserum confirmed the changes in protein expression in the spinal cord of rats with colonic inflammation. Four hours following mustard oil injection GluN2B positive staining was observed in the spinal dorsal horn in both Saff oil and E2 rats. There was no difference in the number of GluN2B positive cells in the LS superficial (189 ± 16 /section in E2 rats vs. 195 ± 8 /section in Saff oil rats) or deep dorsal horn (516 ± 38 in E2 rats vs. 513 ± 20 in Saff oil rats; t-test, $p>0.05$ for both superficial and deep cells; Figure 6). In the TL spinal cord, there were a greater number of GluN2B immunoreactive cells in the superficial dorsal horn in E2 rats (201 ± 5 in E2 rats vs. 176 ± 10 in Saff oil rats; t-test, $p<0.05$). E2 rats also tended to have more cells expressing GluN2B in the deep TL dorsal horn compared with Saff oil rats (289 ± 17 in E2 rats vs. 243 ± 24 in Saff oil rats), though the difference was not statistically significant (t-test, $p>0.05$; Figure 6).

Discussion

The major findings of the present study are: 1) spinal GluN2B containing NMDA receptors are not involved in acute colonic nociceptive processing in ovariectomized rats with or without E2 replacement; 2) forty-eight hrs following E2 replacement, colonic inflammation with mustard oil induced visceral hyperalgesia that was inhibited by spinal GluN2B subunit antagonists; 3) there were no differences in GluN2B subunit expression in the LS spinal cord of inflamed Saff oil and E2 rats. However, GluN2B subunit expression in the TL spinal

cord was upregulated in inflamed E2 rats. These data suggest increased GluN2B expression and function in the TL spinal cord may underlie the colonic inflammation-induced visceral hypersensitivity to colorectal distention in rats with elevated circulating E2.

The function of NMDA receptors in nociceptive signal processing has been widely investigated. NMDA receptor activation contributes to spinal neuron hyperexcitability (central sensitization) and behavioral hyperalgesia following somatic tissue inflammatory or neuropathic pain, but not acute pain (44–48). In contrast, spinal NMDA receptors contribute to the signaling of both noxious and nonnoxious, transient and prolonged visceral stimuli (36;49;50). Using the colorectal distention pain model in the rat, we previously reported that an intrathecally administered NMDA receptor antagonist, APV, dose-dependently attenuated the visceromotor response in male and female rats (20;21;40), suggesting NMDA receptors are activated by transient noxious visceral stimuli. Moreover, APV was more potent in Saff oil rats than rats with E2 replacement, suggesting E2 modulates NMDA receptor activity. Using Western blot we previously reported that E2 significantly increased GluN1 protein expression in the LS spinal cord compared to Saff oil rats (20). In addition, colorectal distention significantly increased GluN1 phosphorylation in E2 but not Saff oil rats, suggesting that an increase in GluN1 subunit phosphorylation and expression in the LS spinal cord may contribute to estradiol induced increases in NMDA receptor activity in non-inflamed rats.

Functional NMDA receptors consist of two obligatory GluN1 subunits and two GluN2/3 subunits. Accumulating lines of evidence suggest GluN2B containing NMDA receptors play important roles in sensory signal processing under inflammatory, neuropathic or cancer pain conditions (51–53). A rapid and prolonged increase of GluN2B subunit phosphorylation was observed in the lumbar spinal cord following hindpaw inflammation and an intrathecally injected tyrosine kinase inhibitor decreased inflammation-induced GluN2B phosphorylation and delayed the onset of mechanical hyperalgesia (7). Using a colonic anaphylaxia model, a recent study showed GluN2B subunit expression was significantly increased in the anterior cingulate cortex (ACC) in rats. Accordingly, administration of Ro 25-6981 or GluN2B siRNA to the ACC suppressed the VMR in visceral hypersensitive, but not normal rats (28).

The GluN2B subunit activity is also modulated by E2. In the hippocampus, E2 preferentially increased GluN2B subunit mRNA, the number of GluN2B binding sites, and the synaptic localization of GluN2B containing receptors. These changes in GluN2B expression/distribution seem to be functional, since E2 potentiation of LTP was prevented by the GluN2B selective antagonist, Ro 25-6981 (54;55).

In the LS spinal cord, E2 modulation of GluN2B expression/phosphorylation and visceral sensitivity is dependent on several conditions. In OVx rats, short term E2 replacement (several hours) increased GluN2B phosphorylation in rats with and without colonic inflammation (29;41). In contrast, in the current study in noninflamed rats with longer term E2 replacement (48 hrs), there was an increase in GluN2B expression, though not phosphorylation compared to Saff oil treated rats. However, Ro 25-6981 had no effect on the colorectal distention induced visceromotor response in either Saff oil or E2 rats, suggesting changes in GluN2B subunit expression in the LS spinal cord might not be

sufficient to account for the E2-induced facilitation of visceral nociceptive processing from a normal colon.

Surprisingly, in long term E2 replacement rats that showed colonic inflammation-induced visceral hypersensitivity that was blocked by intrathecally injected GluN2B selective antagonists, there was no change in the level of GluN2B phosphorylation or expression in the LS spinal cord compared to rats without E2 replacement. This seemingly negative finding in the LS spinal cord does not exclude the possibility that GluN2B containing NMDA receptors in these spinal segments could still contribute to colonic inflammation induced visceral hyperalgesia in E2 rats. It has been reported that E2 induced an increase in the GluN2B component of NMDAR-mediated EPSCs in the CA1 region of the hippocampus without changing GluN2B subunit phosphorylation or protein levels (56). A possible explanation for this phenomenon is that the effects of E2 are due to the recruitment of GluN2B-containing NMDARs from extrasynaptic sites to synapses rather than from increased expression of NMDARs or changes in their phosphorylation state. Extrasynaptic NMDARs account for over 35% of total surface NMDAR receptors and may preferentially contain the GluN2B subunit (57–59). E2 could affect the localization of NMDARs by increasing CamKII activity. It was shown that inhibition of CamKII results in translocation of GluN2B from synaptic sites to extrasynaptic locations, with no change in GluN2A localization (60). This change was not able to be detected using the current protein extracting protocol.

In addition to the LS spinal cord, the TL spinal segments contribute to visceral hypersensitivity following colonic inflammation. The colorectum is dually innervated by pelvic nerve afferents projecting through the L6-S2 DRG to the LS spinal cord and lumbar splanchnic afferents projecting through the T13-L2 DRG to the TL spinal cord (42;43;61–63). The LS spinal cord mediates the visceromotor response to colorectal distention in the absence of colonic inflammation (42;43;64). Colonic inflammation changes the relative contribution of the LS and TL segments in colorectal nociceptive processing. Increased activity in the TL dorsal horn is associated with increased colorectal sensitivity following colonic inflammation (42;43). In contrast to no detectable changes in the TL spinal cord under non-inflammatory conditions, an increase in phosphorylated GluN2B level as well as GluN2B mRNA and protein expression in the TL spinal cord after inflammation was observed in E2 rats compared with Saff oil rats in the current study. Accordingly, Ro 25–6981 attenuated visceral hyperalgesia in E2 rats following inflammation, but had no effect in Saff oil rats, suggesting an increase in GluN2B containing NMDA receptor activity in the TL spinal cord following colonic inflammation could account for visceral hyperalgesia in the presence of estrogen.

In addition to expression at postsynaptic sites of the spinal cord, GluN2B subunit is also present in dorsal root ganglia, central terminals of primary afferents and in the colonic myenteric plexus (65–67). In the spinal cord, presynaptic NMDA receptor activation potentiates nociception through increased release of neurotransmitters such as substance P, BDNF and glutamate (68–70). Intrathecally injected GluN2B antagonists could have acted on both pre- and postsynaptic sites to attenuate visceral hypersensitivity.

Electrophysiological recordings in the spinal cord slices could be carried out to address this issue (71).

In summary, our data suggest that estrogen facilitates colonic inflammation induced visceral hyperalgesia by increasing GluN2B containing NMDA receptor activity in the TL spinal cord. Interestingly, an increase in GluN2B expression in supraspinal sites have also been reported in response to colonic irritation (28;72). Given that visceral hypersensitivity is one of the characteristic symptoms in IBS, therapeutic drugs targeting GluN2B containing NMDA receptors in the CNS may provide additive effect in the management of IBS.

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YJ and RT designed the research. YJ performed the behavioral and molecular biological experiments. GB guided the RT-PCR study. DC participated in tissue processing. YJ and RT analyzed the data and wrote the paper. We would like to thank Ms. Sangeeta Pandya and Ms. Jane Karpowicz for providing technical support. This work was supported by National institutes of Health Grant NS 37424 (RT) and T32DE007309 (YJ).

Abbreviations

E2	17- β -estradiol
LS	lumbosacral
OVx	Ovariectomized
Saff oil	safflower oil
TL	thoracolumbar

Reference List

1. Chang L, Heitkemper MM. Gender differences in irritable bowel syndrome. *Gastroenterology*. 2002 Nov; 123(5):1686–1701. [PubMed: 12404243]
2. Vob U, Lewerenz A, Nieber K. Treatment of irritable bowel syndrome: sex and gender specific aspects. *Handb Exp Pharmacol*. 2012; (214):473–497. [PubMed: 23027463]
3. Cain KC, Jarrett ME, Burr RL, Rosen S, Hertig VL, Heitkemper MM. Gender differences in gastrointestinal, psychological, and somatic symptoms in irritable bowel syndrome. *Dig Dis Sci*. 2009 Jul; 54(7):1542–1549. [PubMed: 18979200]
4. Hungin AP, Chang L, Locke GR, Dennis EH, Barghout V. Irritable bowel syndrome in the United States: prevalence, symptom patterns and impact. *Aliment Pharmacol Ther*. 2005 Jun 1; 21(11): 1365–1375. [PubMed: 15932367]
5. Chang L, Mayer EA, Labus JS, Schmulson M, Lee OY, Olivas TI, et al. Effect of sex on perception of rectosigmoid stimuli in irritable bowel syndrome. *Am J Physiol Regul Integr Comp Physiol*. 2006 Aug; 291(2):R277–R284. [PubMed: 16574882]
6. Mayer EA, Berman S, Chang L, Naliboff BD. Sex-based differences in gastrointestinal pain. *Eur J Pain*. 2004 Oct; 8(5):451–463. [PubMed: 15324776]
7. Guo W, Zou S, Guan Y, Ikeda T, Tal M, Dubner R, et al. Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *J Neurosci*. 2002 Jul 15; 22(14):6208–6217. [PubMed: 12122079]
8. Zhou Q, Price DD, Caudle RM, Verne GN. Spinal NMDA NR1 Subunit Expression Following Transient TNBS Colitis. *Brain Res*. 2009 Apr 27; 1279:109–120. [PubMed: 19406112]
9. Adams MM, Morrison JH, Gore AC. N-methyl-D-aspartate receptor mRNA levels change during reproductive senescence in the hippocampus of female rats. *Exp Neurol*. 2001 Jul; 170(1):171–179. [PubMed: 11421594]

10. Hardingham GE, Bading H. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci.* 2010 Oct; 11(10):682–696. [PubMed: 20842175]
11. Daniel JM, Dohanich GP. Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. *J Neurosci.* 2001; 21(17):6949–6956. [PubMed: 11517282]
12. Gazzaley AH, Weiland NG, McEwen BS, Morrison JH. Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. *J Neurosci.* 1996 Nov 1; 16(21):6830–6838. [PubMed: 8824322]
13. Kow LM, Easton A, Pfaff DW. Acute estrogen potentiates excitatory responses of neurons in rat hypothalamic ventromedial nucleus. *Brain Res.* 2005 May 10; 1043(1–2):124–131. [PubMed: 15862525]
14. Woolley CS, Weiland NG, McEwen BS, Schwartzkroin PA. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. *J Neurosci.* 1997 Mar 1; 17(5):1848–1859. [PubMed: 9030643]
15. Diano S, Naftolin F, Horvath TL. Gonadal steroids target AMPA glutamate receptor-containing neurons in the rat hypothalamus, septum and amygdala: a morphological and biochemical study. *Endocrinology.* 1997 Feb; 138(2):778–789. [PubMed: 9003015]
16. Romeo RD, McCarthy JB, Wang A, Milner TA, McEwen BS. Sex Differences in Hippocampal Estradiol-Induced N-Methyl-D-Aspartic Acid Binding and Ultrastructural Localization of Estrogen Receptor-Alpha. *Neuroendocrinology.* 2005 Nov 4; 81(6):391–399. [PubMed: 16276117]
17. Bi R, Broutman G, Foy MR, Thompson RF, Baudry M. The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. *Proc Natl Acad Sci U S A.* 2000 Mar 28; 97(7):3602–3607. [PubMed: 10725383]
18. Nilsen J, Chen S, Brinton RD. Dual action of estrogen on glutamate-induced calcium signaling: mechanisms requiring interaction between estrogen receptors and src/mitogen activated protein kinase pathway. *Brain Res.* 2002 Mar 15; 930(1–2):216–234. [PubMed: 11879813]
19. Vedder LC, Smith CC, Flannigan AE, McMahon LL. Estradiol-induced increase in novel object recognition requires hippocampal NR2B-containing NMDA receptors. *Hippocampus.* 2013 Jan; 23(1):108–115. [PubMed: 22965452]
20. Tang B, Ji Y, Traub RJ. Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in the rat. *Pain.* 2008; 137:540–549. [PubMed: 18068901]
21. Ji Y, Tang B, Cao DY, Wang G, Traub RJ. Sex differences in spinal processing of transient and inflammatory colorectal stimuli in the rat. *Pain.* 2012 Sep; 153(9):1965–1973. [PubMed: 22819535]
22. Nagy GG, Watanabe M, Fukaya M, Todd AJ. Synaptic distribution of the NR1, NR2A and NR2B subunits of the N-methyl-d-aspartate receptor in the rat lumbar spinal cord revealed with an antigen-unmasking technique. *Eur J Neurosci.* 2004 Dec; 20(12):3301–3312. [PubMed: 15610162]
23. Fan QQ, Li L, Wang WT, Yang X, Suo ZW, Hu XD. Activation of alpha2 adrenoceptors inhibited NMDA receptor-mediated nociceptive transmission in spinal dorsal horn of mice with inflammatory pain. *Neuropharmacology.* 2013 Oct 5; 77C:185–192. [PubMed: 24103367]
24. Coutinho SV, Urban MO, Gebhart GF. Role of glutamate receptors and nitric oxide in the rostral ventromedial medulla in visceral hyperalgesia. *Pain.* 1998 Oct; 78(1):59–69. [PubMed: 9822212]
25. Roza C, Laird JM, Cervero F. Spinal mechanisms underlying persistent pain and referred hyperalgesia in rats with an experimental ureteric stone. *J Neurophysiol.* 1998 Apr; 79(4):1603–1612. [PubMed: 9535931]
26. Al-Chaer ED, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology.* 2000; 119(5):1276–1285. [PubMed: 11054385]
27. Luo XQ, Cai QY, Chen Y, Guo LX, Chen AQ, Wu ZQ, et al. Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord contributes to chronic visceral pain in rats. *Brain Res.* 2014; 1542:167–175. [PubMed: 24125810]

28. Fan J, Wu X, Cao Z, Chen S, Owyang C, Li Y. Up-regulation of anterior cingulate cortex NR2B receptors contributes to visceral pain responses in rats. *Gastroenterology*. 2009 May; 136(5):1732–1740. [PubMed: 19208366]
29. Peng HY, Chen GD, Lai CY, Hsieh MC, Hsu HH, Wu HC, et al. PI3K modulates estrogen-dependent facilitation of colon-to-urethra cross-organ reflex sensitization in ovariectomized female rats. *J Neurochem*. 2010 Apr; 113(1):54–66. [PubMed: 20067569]
30. Ji Y, Murphy AZ, Traub RJ. Estrogen modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in the rat. *J Neurosci*. 2003 May 1; 23(9):3908–3915. [PubMed: 12736360]
31. Peng HY, Chen GD, Tung KC, Lai CY, Hsien MC, Chiu CH, et al. Colon mustard oil instillation induced cross-organ reflex sensitization on the pelvic-urethra reflex activity in rats. *Pain*. 2009 Mar; 142(1–2):75–88. [PubMed: 19167822]
32. Kim Y, Cho HY, Ahn YJ, Kim J, Yoon YW. Effect of NMDA NR2B antagonist on neuropathic pain in two spinal cord injury models. *Pain*. 2012 May; 153(5):1022–1029. [PubMed: 22424878]
33. Qu XX, Cai J, Li MJ, Chi YN, Liao FF, Liu FY, et al. Role of the spinal cord NR2B-containing NMDA receptors in the development of neuropathic pain. *Exp Neurol*. 2009 Feb; 215(2):298–307. [PubMed: 19046970]
34. Ji Y, Tang B, Traub RJ. Estrogen increases and progesterone decreases behavioral and neuronal responses to colorectal distention following colonic inflammation in the rat. *Pain*. 2005; 117:433–442. [PubMed: 16154701]
35. Kolhekar R, Gebhart GF. NMDA and quisqualate modulation of visceral nociception in the rat. *Brain Res*. 1994; 651:215–226. [PubMed: 7922569]
36. Olivar T, Laird JM. Differential effects of N-methyl-D-aspartate receptor blockade on nociceptive somatic and visceral reflexes. *Pain*. 1999 Jan; 79(1):67–73. [PubMed: 9928778]
37. Strigo IA, Duncan GH, Catherine Bushnell M, Boivin M, Wainer I, Rodriguez Rosas ME, et al. The effects of racemic ketamine on painful stimulation of skin and viscera in human subjects. *Pain*. 2005 Feb; 113(3):255–264. [PubMed: 15661431]
38. Zhai QZ, Traub RJ. The NMDA receptor antagonist MK-801 attenuates c-Fos expression in the lumbosacral spinal cord following repetitive noxious and nonnoxious colorectal distention. *Pain*. 1999; 83:321–329. [PubMed: 10534605]
39. Zhang L, Zhang X, Westlund KN. Restoration of spontaneous exploratory behaviors with an intrathecal NMDA receptor antagonist or a PKC inhibitor in rats with acute pancreatitis. *Pharmacol Biochem Behav*. 2004 Jan; 77(1):145–153. [PubMed: 14724052]
40. Ji Y, Traub RJ. Spinal NMDA receptors contribute to neuronal processing of acute noxious and nonnoxious colorectal stimulation in the rat. *J Neurophysiol*. 2001; 86(4):1783–1791. [PubMed: 11600639]
41. Ji Y, Cao DY, Bai G, Traub RJ. Estrogen modulation of spinal GluN2B subunit in a visceral pain model in the rat. *Society for Neuroscience Abstract Viewer*. 2012 2012.
42. Traub RJ. Evidence for thoracolumbar spinal cord processing of inflammatory, but not acute colonic pain. *NeuroReport*. 2000; 11:2113–2116. [PubMed: 10923654]
43. Wang G, Tang B, Traub RJ. Differential processing of noxious colonic input by thoracolumbar and lumbosacral dorsal horn neurons in the rat. *J Neurophysiol*. 2005; 94:3788–3794. [PubMed: 16093344]
44. Dickenson AH, Sullivan AF. Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in the rat. *Brain Res*. 1990; 506(1):31–39. [PubMed: 1967963]
45. Ren K, Williams GM, Hylden JL, Ruda MA, Dubner R. The intrathecal administration of excitatory amino acid receptor antagonists selectively attenuated carrageenan-induced behavioral hyperalgesia in rats. *Eur J Pharmacol*. 1992 Aug 25; 219(2):235–243. [PubMed: 1358641]
46. Ren K, Hylden JL, Williams GM, Ruda MA, Dubner R. The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal horn neuronal activity in rats with unilateral inflammation. *Pain*. 1992 Sep; 50(3):331–344. [PubMed: 1454389]
47. Woolf CJ, Thompson SWN. The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. *Pain*. 1991; 44:293–299. [PubMed: 1828878]

48. Zhou HY, Chen SR, Pan HL. Targeting N-methyl-D-aspartate receptors for treatment of neuropathic pain. *Expert Rev Clin Pharmacol*. 2011 May; 4(3):379–388. [PubMed: 21686074]
49. Nishiyama T. Interaction among NMDA receptor-, NMDA glycine site- and AMPA receptor antagonists in spinally mediated analgesia. *Can J Anaesth*. 2000 Jul; 47(7):693–698. [PubMed: 10930211]
50. Haley JE, Sullivan AF, Dickenson AH. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res*. 1990; 518(1–2):218–226. [PubMed: 1975214]
51. Mihara Y, Egashira N, Sada H, Kawashiri T, Ushio S, Yano T, et al. Involvement of spinal NR2B-containing NMDA receptors in oxaliplatin-induced mechanical allodynia in rats. *Mol Pain*. 2011; 7:8. [PubMed: 21247499]
52. Katano T, Nakazawa T, Nakatsuka T, Watanabe M, Yamamoto T, Ito S. Involvement of spinal phosphorylation cascade of Tyr1472-NR2B, Thr286-CaMKII, and Ser831-GluR1 in neuropathic pain. *Neuropharmacology*. 2011 Mar; 60(4):609–616. [PubMed: 21167847]
53. Gu X, Mei F, Liu Y, Zhang R, Zhang J, Ma Z. Intrathecal administration of the cannabinoid 2 receptor agonist JWH015 can attenuate cancer pain and decrease mRNA expression of the 2B subunit of N-methyl-D-aspartic acid. *Anesth Analg*. 2011 Aug; 113(2):405–411. [PubMed: 21519057]
54. Smith CC, Vedder LC, McMahon LL. Estradiol and the relationship between dendritic spines, NR2B containing NMDA receptors, and the magnitude of long-term potentiation at hippocampal CA3-CA1 synapses. *Psychoneuroendocrinology*. 2009 Dec; 34(Suppl 1):S130–S142. [PubMed: 19596521]
55. Smith CC, McMahon LL. Estradiol-induced increase in the magnitude of long-term potentiation is prevented by blocking NR2B-containing receptors. *J Neurosci*. 2006 Aug 16; 26(33):8517–8522. [PubMed: 16914677]
56. Snyder MA, Cooke BM, Woolley CS. Estradiol potentiation of NR2B-dependent EPSCs is not due to changes in NR2B protein expression or phosphorylation. *Hippocampus*. 2011 Apr; 21(4):398–408. [PubMed: 20082293]
57. Tovar KR, Westbrook GL. Mobile NMDA receptors at hippocampal synapses. *Neuron*. 2002 Apr 11; 34(2):255–264. [PubMed: 11970867]
58. Harris AZ, Pettit DL. Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices. *J Physiol*. 2007 Oct 15; 584(Pt 2):509–519. [PubMed: 17717018]
59. Harris AZ, Pettit DL. Recruiting extrasynaptic NMDA receptors augments synaptic signaling. *J Neurophysiol*. 2008 Feb; 99(2):524–533. [PubMed: 18057106]
60. Gardoni F, Mauceri D, Malinverno M, Polli F, Costa C, Tozzi A, et al. Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. *J Neurosci*. 2009 Jan 21; 29(3):669–677. [PubMed: 19158293]
61. Brierley SM, Jones RC III, Gebhart GF, Blackshaw LA. Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology*. 2004 Jul; 127(1):166–178. [PubMed: 15236183]
62. Christianson JA, Traub RJ, Davis BM. Differences in spinal distribution and neurochemical phenotype of colonic afferents in mouse and rat. *J Comp Neurol*. 2005 Nov 30; 494(2):246–259. [PubMed: 16320237]
63. Wang G, Tang B, Traub RJ. Pelvic nerve input mediates descending modulation of homovisceral processing in the thoracolumbar spinal cord of the rat. *Gastroenterology*. 2007 Nov; 133(5):1544–1553. [PubMed: 17916357]
64. Ness TJ, Randich A, Gebhart GF. Further evidence that colorectal distention is a noxious visceral stimulus in rats. *Neurosci Lett*. 1991; 131:113–116. [PubMed: 1791969]
65. Marvizon JC, McRoberts JA, Ennes HS, Song B, Wang X, Jinton L, et al. Two N-methyl-D-aspartate receptors in rat dorsal root ganglia with different subunit composition and localization. *J Comp Neurol*. 2002 May 13; 446(4):325–341. [PubMed: 11954032]
66. Chen W, Walwyn W, Ennes HS, Kim H, McRoberts JA, Marvizon JC. BDNF released during neuropathic pain potentiates NMDA receptors in primary afferent terminals. *Eur J Neurosci*. 2014 May; 39(9):1439–1454. [PubMed: 24611998]

67. Del Valle-Pinero AY, Suckow SK, Zhou Q, Perez FM, Verne GN, Caudle RM. Expression of the N-methyl-D-aspartate receptor NR1 splice variants and NR2 subunit subtypes in the rat colon. *Neuroscience*. 2007 Jun 15; 147(1):164–173. [PubMed: 17509768]
68. Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, et al. Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. *J Neurosci*. 2001 Jun 15; 21(12):4469–4477. [PubMed: 11404434]
69. Liu H, Mantyh PW, Basbaum AI. NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature*. 1997 Apr 17; 386(6626):721–724. [PubMed: 9109489]
70. Yan X, Jiang E, Gao M, Weng HR. Endogenous activation of presynaptic NMDA receptors enhances glutamate release from the primary afferents in the spinal dorsal horn in a rat model of neuropathic pain. *J Physiol*. 2013 Apr 1; 591(Pt 7):2001–2019. [PubMed: 23359671]
71. Chen SR, Hu YM, Chen H, Pan HL. Calcineurin inhibitor induces pain hypersensitivity by potentiating pre- and postsynaptic NMDA receptor activity in spinal cords. *J Physiol*. 2014 Jan 1; 592(Pt 1):215–227. [PubMed: 24081160]
72. Li Y, Zhang X, Liu H, Cao Z, Chen S, Cao B, et al. Phosphorylated CaMKII post-synaptic binding to NR2B subunits in the anterior cingulate cortex mediates visceral pain in visceral hypersensitive rats. *J Neurochem*. 2012 May; 121(4):662–671. [PubMed: 22380661]

Key messages

- Our data suggest increased GluN2B expression and function in the TL spinal cord may underlie the colonic inflammation-induced visceral hypersensitivity to colorectal distention in rats with elevated circulating E2.
- The aim of the present study was to investigate the role of spinal GluN2B containing NMDA receptors in estrogen modulation of visceral nociceptive processing.
- The visceromotor response to colorectal distention was used as a behavioral measurement of visceral sensitivity; the mRNA and protein levels of spinal GluN2B were determined using real time RT-PCR and western blot.
- The major findings of the present study are: 1) spinal GluN2B containing NMDA receptors are not involved in acute colonic nociceptive processing in ovariectomized rats; 2) colonic inflammation with mustard oil induced visceral hyperalgesia in E2 rats that was blocked by spinal GluN2B subunit antagonists; Concomitantly, GluN2B subunit expression in the TL spinal cord was upregulated in inflamed E2 rats.

Figure 1.

The effect of the GluN2B selective antagonist, Ro 25-6981, on the visceromotor response in rats in the absence of colonic inflammation. A: I.t. Ro 25-6981 had no effect on the VMR in Saff oil rats. B: In E2 rats, Ro 25-6981 did not change the magnitude of the VMR. Distention trials began at the indicated time points. Inset: an example showing the visceromotor response to four 80mmHg colorectal distentions.

Figure 2.

The effect of GluN2B selective antagonist, Ro 25-6981, on the visceromotor response in rats with colonic inflammation. A,B. Visceral hyperalgesia was induced in E2 rats that lasted at least 4 hours. In Saff oil rats, colonic administration of mustard oil had no effect on the visceromotor response. Data were normalized to the baseline VMR that was established prior to mustard oil instillation. C,D. In E2 rats, i.t. Ro 25-6981 (80 µg) attenuated the visceral hyperalgesia compared to vehicle. Inset C: there was no visceral hypersensitivity following pretreatment with i.t. Co 101244 (100nmol) compared to baseline (n=4). E,F. In

Saff oil rats, i.t. Ro 25-6981 had no effect on the visceromotor response. Dunn's test following significant ANOVA, *, **, *** $p < 0.05, 0.01, 0.001$ vs. baseline. T-test or ANOVA, #, ## $p < 0.05, 0.01$ vs. vehicle.

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Figure 3.

Changes of GluN2B phosphorylation and expression in the lumbosacral (LS) and thoracolumbar (TL) spinal cord in the absence of colonic inflammation. A. In the LS spinal cord, 48 hours after E2 injection, there was significantly more immunoprecipitated GluN2B protein compared with Saff oil rats (t-test, **p<0.001, n=4-8/group). There was no increase in GluN2B phosphorylation. (B) In the TL spinal cord E2 rats were not different from Saff oil rats in GluN2B protein phosphorylation or expression (t-test, p>0.05; n=4/group).

Figure 4.

Colonic inflammation does not affect GluN2B protein and gene expression in the LS spinal cord. A. E2 rats were not different from Saff oil rats in immunoprecipitated GluN2B protein phosphorylation or expression in the LS spinal cord (t-test, $p > 0.05$; $n = 4/\text{group}$). B. There was no difference in GluN2B gene expression between E2 and Saff oil rats in the LS spinal cord (t-test, $p > 0.05$; $n = 4/\text{group}$).

Figure 5.

Colonic inflammation alters GluN2B expression in the TL spinal cord. A. Colonic inflammation in E2 rats increased immunoprecipitated GluN2B and pGluN2B/GluN2B level as compared with Saff oil rats (** t-test, $p < 0.05$; $n = 4/\text{group}$). B. The increase in GluN2B protein expression in the TL spinal cord was confirmed by western blot of whole protein lysate (* t-test, $p < 0.05$; $n = 7/\text{group}$). C. There was a parallel increase in GluN2B gene expression (* t-test, $p < 0.05$; $n = 4/\text{group}$).

Figure 6.

The distribution of GluN2B immunoreactive neurons in the spinal dorsal horn in the presence of colonic inflammation. A: Representative immunocytochemical staining of LS and TL spinal cord sections with anti-GluN2B antiserum. Scale bar: 100 μ m. B: Dorsal horn laminar quantification (I–II and III–V) of GluN2B immunoreactive cells in the LS and TL spinal segments in Saff oil and E2 rats (* t-test, $p < 0.05$; $n = 4–6$ /group).