Experimental colitis modulates the functional properties of NMDA receptors in dorsal root ganglia neurons

Jichang Li,1,4* James A. McRoberts,1,4* Helena S. Ennes,1,4 Marcello Trevisani,6 Paola Nicoletti,6 Yash Mittal, and Emeran A. Mayer1,2,3,4,5

Departments of 1Medicine, 2Physiology, and 3Psychology; 4Center for Neurovisceral Sciences and Women’s Health, and 5Brain Research Institute, Geffen School of Medicine, University of California, Los Angeles, California; and 6Department of Critical Care Medicine and Surgery, University of Florence, Florence, Italy

Submitted 28 February 2006; accepted in final form 23 March 2006

Li, Jichang, James A. McRoberts, Helena S. Ennes, Marcello Trevisani, Paola Nicoletti, Yash Mittal, and Emeran A. Mayer. Experimental colitis modulates the functional properties of NMDA receptors in dorsal root ganglia neurons. Am J Physiol Gastrointest Liver Physiol 291: G219–G228, 2006. First published March 24, 2006; doi:10.1152/ajpgi.00097.2006.—N-methyl-D-aspartate (NMDA) receptors (NMDARs) on spinal afferent neurons regulate the peripheral and central release of neuropeptides involved in the development of hyperalgesia. We examined the effect of experimental colitis on the molecular and functional properties of NMDARs on these neurons. Lumbosacral dorsal root ganglia (DRG) were collected from adult rats 5 days after the induction of colitis for whole cell patch-clamp recording, Western blot analysis, and quantitative RT-PCR. Compared with neurons from control rats, those taken from animals with colitis had a threefold higher density of NMDA currents in both retrograde-labeled, colon-specific, and unlabeled DRG neurons. Increased current densities were not observed in DRG neurons taken from thoracic spinal levels. There was no significant change in increased current densities were not observed in DRG neurons taken retrograde-labeled, colon-specific, and unlabeled DRG neurons. Inflammation-induced changes in the activity of NMDARs could enhance the release of SP and CGRP from both peripheral and central nerve terminals, thereby influencing the course of inflammation and contributing to the development and maintenance of central sensitization.

NMDARs, a particular type of ionotropic glutamate receptor, have unique functional properties such as permeability to Ca2+, slow desensitization, and voltage-dependent Mg2+ inhibition that play a crucial role in neuronal plasticity (9). NMDARs are heteromeric assemblies composed of two NR1 subunits in combination with two or three NR2 and/or NR3 subunits (7, 37). There is only one NR1 gene, which can be expressed as eight different splice variants, four NR2 subunits, designated as NR2A–NR2D, and two NR3 subunits, whose function is still uncertain because they do not make functional NMDARs when combined with NR1. Studies of recombinant receptors containing NR1 and the four different NR2 subunits have shown that single-channel conductivity, glutamate and glycine affinity, and voltage-dependent inhibition by extracellular Mg2+ are strongly influenced by the composition of the NR2 subunits (7, 37). In addition, there are a few subunit-selective inhibitors such as the NR2B-selective antagonist ifenprodil (50). NMDARs containing NR2A or NR2B subunits generate “high-conductance” channel openings with high sensitivity to voltage-dependent inhibition by Mg2+, whereas NR2C- or NR2D-containing receptors give rise to “low-conductance” openings with a lower sensitivity to extracellular Mg2+. In cultured dorsal root ganglia (DRG) neurons innervating the rat colon, we (27) have previously shown that the

* J. Li and J. A. McRoberts contributed equally to this work.
Address for reprint requests and other correspondence: J. A. McRoberts, Univ. of California, Warren Hall, Rm. 14-103, 900 Veteran Ave., Los Angeles, CA 90095 (e-mail: mcrobert@ucla.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
NR2B-selective antagonist ifenprodil could inhibit most of the NMDA current, suggesting that nearly all of the functional NMDARs expressed on DRG neurons were NR2B-containing receptors.

In the present study, we tested the hypothesis that experimental colitis leads to an upregulation of NMDAR channel activity using whole cell patch-clamp recording and molecular techniques. Specifically, we wanted to determine if such an upregulation involves the increased expression of the channel, a change in the subunit composition, a modification of subunit properties by protein phosphorylation, or a combination of these mechanisms.

**MATERIALS AND METHODS**

**Rat model of experimental colitis.** All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the investigators’ institutions. Experimental colitis was induced by an intracolonic instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 45 mg/kg in Krebs-Ringer solution lacking MgCl2 and containing 0.1% BSA, 5 mM ethylene-diaminetetraacetic acid (pH 7.4 with NaOH, ~300 mosmol/kg). Bath solution contained 138 mM NaCl, 5 mM KCl, 0.5 mM CaCl2, 10 mM HEPES, 10 mM glucose, 5 mM strychnine, 0.5 mM tetrodotoxin, and 1 mM diethyl-1-aminocaproic acid (pH 7.4 with NaOH, ~300 mosmol/kg). Tight seals (1–10 GΩ) from visualized small- and medium-sized cells (20–40 μm in diameter) were obtained by applying negative pressure. The membrane was disrupted with additional suction, and the whole cell configuration was obtained. Series resistance was monitored throughout the experiments. Cells were voltage clamped at −60 mV unless otherwise indicated.

**Drug delivery.** NMDA and glycine were prepared as 100 mM stock solutions. Test solutions were prepared daily by diluting stock solutions to the desired concentrations in extracellular solution. Ifenprodil was prepared as a 100-mM stock solution dissolved in DMSO and added to extracellular solution to the desired concentrations. DMSO was added such that all solutions contained equivalent amounts of vehicle. Test compounds were rapidly perfused onto the patched cells using a gravity-fed multibarrel perfusion system (SF-77B Perfusion Fast-Step, Warner Instruments; Hamden, CT) controlled by a Labmaster board using pCLAMP 8.0 software (Axon Instruments). The tip of the multibarrel was positioned 200–300 μm from the neuron under study. When a test solution was not being applied, the neuron was continuously perfused with extracellular solution. Reagents were purchased from Sigma.

**RT-PCR.** Total RNA was isolated from lumbar DRG (L1, L2, L6, and S1) using the TRIzol reagent kit (Invitrogen), treated with RNase-free DNase (Ambion; Austin, TX), and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) using random hexamers following the manufacturer’s instructions. “RT” reactions were treated identically except that reverse transcriptase was omitted from the reaction mixture. Quantitative real-time PCR for NR2 subunits was performed using an Applied Biosystems GeneAmp 7500 sequence detection system and SYBR green reagents (Applied Biosystems). Primer pairs and PCR conditions have been previously described in detail (34). The homogeneity of the reaction product was verified by melting temperature and polyacrylamide gel electrophoresis.

Semiquantitative RT-PCR was used to evaluate changes in the relative expression of NR1 splice variants. Specific PCR primer pairs for the COOH-terminal splice variants NR1-1, NR1-2, and NR1-4 were adapted from Winkler et al. (52), which use a common forward primer and different reverse primers overlapping the splice sites. A new common forward primer and reverse primers for NR1-3 and NR1-4 were designed using Primer3 software (42). The sequence and predicted size for each primer pair is shown in Table 1 together with primer pairs for detecting the NH2-terminal splice site (NR1a/b) and the housekeeping gene GAPDH. The primer pair for the NH2-terminal splice variant gave two products depending on the inclusion (NR1b) or excision (NR1a) of exon 5. PCR was performed using HotStar Taq polymerase (Qiagen; Valencia, CA) and the following cycling conditions: after an initial denaturation/activation for 15 min at 95°C, the amplification profile consisted of 1 min at 94°C, 1-min annealing, and 1 min at 72°C for 32–40 cycles, where the last extension step lasted 10 min. The annealing temperature for the NR1 splice variants was 60°C and that for GAPDH was 55°C. The PCR products were separated on 2% agarose LE (Ambion) containing 0.5 μg/ml ethidium bromide. Gels were photographed under ultraviolet illumination, the
image was digitized, and the intensities of the bands were measured by densitometry (Bio-Rad).

Western blot analysis. Two-day-old cultures of DRG neurons from spinal levels L1, L2, L6, and S1 were scraped into RIPA buffer (50 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 0.5% Na deoxycholate, and 0.1% SDS; pH 7.5) containing protease inhibitors (1 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 0.5% Na deoxycholate, and 0.1% SDS; pH 7.5) containing protease inhibitors (1 mM PMSF and 1× complete protease inhibitor cocktail, Roche Diagnostics; Mannheim, Germany), vortexed, and set on ice for 30 min before centrifugation at 10,000 g for 30 min to remove cellular debris. NuPAGE SDS sample buffer (Invitrogen) with reducing agent Diagnostics; Mannheim, Germany), vortexed, and set on ice for 30 min before centrifugation at 10,000 g for 30 min to remove cellular debris. NuPAGE SDS sample buffer (Invitrogen) with reducing agent Diagnostics; Mannheim, Germany), vortexed, and set on ice for 30 min before centrifugation at 10,000 g for 30 min to remove cellular debris. NuPAGE SDS sample buffer (Invitrogen) with reducing agent.

Table 1. PCR primers for NRI splice variants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Exon</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRI-1</td>
<td>5' - CGG CTT CCGTCTGCTCGAGCGCTGTCG - 3'</td>
<td>+21, +22</td>
<td>467</td>
</tr>
<tr>
<td>NRI-1 reverse</td>
<td>5' - TGGGATGGTACTGCGTGATCTC - 3'</td>
<td>-21, +22</td>
<td>355</td>
</tr>
<tr>
<td>NRI-3</td>
<td>5' - GAT GT ACG TGC TTG CG AG TGT TT C - 3'</td>
<td>+21, -22</td>
<td>471</td>
</tr>
<tr>
<td>NRI-4 reverse</td>
<td>5' - GAT GT ACG TGC TTG CG AG TGT TT C - 3'</td>
<td>-21, -22</td>
<td>357</td>
</tr>
</tbody>
</table>

The eight different NRI splice variants are denoted as either NRIa or NRIb to indicate the NH2-terminal splice variant or as NRI-1 to NRI-4 to indicate the COOH-terminal variants. The specific exons included or excised from each variant are shown along with the sequence of the forward and reverse primers and the predicted sizes of the PCR products.

Effect of colonic inflammation on NMDAR-mediated currents in DRG neurons. In a nominally Mg2+-free extracellular solution, rapid perfusion of 250 µM NMDA combined with 10 µM glycine induced inward currents in 72% (41 of 57) of DRG neurons taken from spinal levels innervating the distal colon (L1, L2, L6, and S1) of TNBS-treated rats with colitis compared with 69% (9 of 13) of neurons from saline-treated control rats and 70% (61 of 87) of neurons from untreated rats. Inward currents induced by NMDA and glycine (Fig. 1, a) exhibited an initial transient that monophasically declined at a significantly higher rate in neurons from colitis rats compared with those from control rats [time constant values of 2,083 ± 125 ms (n = 12) and 1,693 ± 110 ms (n = 20) for neurons from colitis and control rats, respectively, P < 0.04]. In addition to the faster decay kinetics, NMDAR currents were larger in DRG neurons from rats with colitis compared with those from control rats [maximal currents of 1,693 ± 125 pA (n = 12) vs. 1,436 ± 110 pA (n = 20) for neurons from colitis and control rats, respectively, P < 0.03]. When whole cell capacitance was used to estimate cell size, there was a 2.9-fold increase in the current density in neurons from rats with colitis compared with either saline-treated or untreated rats (Fig. 1, right). This effect was more pronounced in DRG neurons retrogradely labeled from the distal colon (3.1-fold) but also occurred in randomly selected, unlabeled neurons, the majority of which would be expected by chance to innervate somatic tissues. The increase in current density in neurons from TNBS-treated rats was not due to an increase in the size of neurons, because whole cell capacitances were not statistically different between randomly selected and retrograde-labeled neurons from untreated, saline-treated, or TNBS-treated rats (Table 2). These values were similar to our previously reported values in untreated rats (43.2 ± 1.6 and 40.8 ± 1.1 pS for random and labeled, respectively) (27). On the other hand, in DRG neurons taken from thoracic spinal levels not innervating the distal colon (T3–T8), NMDA current densities were not statistically differ-
ent between TNBS- and vehicle-treated rats (Fig. 1). Thus inflammation of the distal colon leads to an increase in the density NMDA currents in all small- to medium-sized neurons from DRG innervating the colon but not those isolated from the distant DRG.

NMDAR-mediated current characteristics and pharmacology. The current-voltage ($I$-$V$) relationship of NMDA-induced currents was evaluated to verify that the currents measured in DRG neurons from control and colitis animals were due to the same types of ion channels with similar ion permeabilities. As shown in Fig. 2, the $I$-$V$ relationship in the nominally Mg$^{2+}$-free extracellular solution was nearly linear between $-100$ and $+40$ mV for both groups of neurons. The average reversal potential for the NMDA-induced currents was $1.49 \pm 1.19$ mV ($n = 8$) in randomly selected neurons from control rats and $1.27 \pm 0.6$ mV ($n = 7$) in randomly selected neurons from TNBS-treated rats, which was not significantly different. When normalized to maximum current at a holding potential of $-100$ mV, the $I$-$V$ curves were completely superimposed (Fig. 2, inset), indicating that there was no change in the inherent properties of the currents in neurons from rats with TNBS-induced colitis.

The increase in current density could be due to a long-lasting transcription alteration such as an increase in NMDAR subunit expression or a change in the relative expression of the different subunits. To differentiate between these possibilities, we first examined the affinity for the two agonists, NMDA and glycine, in neurons collected from rats with colitis and compared these values with those from untreated animals. As shown in Fig. 3, EC$_{50}$ values for NMDA and glycine were not statistically different between recorded in neurons

<table>
<thead>
<tr>
<th>DRG</th>
<th>Untreated</th>
<th>Saline</th>
<th>TNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbosacral DRG</td>
<td>L1–L2 and L6–S1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>42.8±1.2 (50)</td>
<td>45.1±2.6 (6)</td>
<td>42.9±1.6 (18)</td>
</tr>
<tr>
<td>Labeled</td>
<td>40.8±1.1 (11)</td>
<td>42.6±3.4 (3)</td>
<td>38.1±2.1 (11)</td>
</tr>
<tr>
<td>Thoracic DRG</td>
<td>T3–T8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>44.3±2.1 (19)</td>
<td>43.2±2.2 (17)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE (in pS); numbers in parentheses are numbers of neurons in each group. DRG, dorsal root ganglia.
taken from colitis and control animals (90 vs. 78 μM for NMDA and 1.9 vs. 2.7 μM for glycine in colitis and control animals, respectively). In addition, as shown in Fig. 4, there was no significant difference in voltage-dependent Mg2+ inhibition. At holding potentials of −80 and −100 mV, IC50 values for Mg2+ were 5.4 and 2.5 μM in control neurons and 4.2 and 2.0 μM in neurons from colitis animals, respectively. These observations effectively ruled out an increase in the NR2C or NR2D content of functionally expressed NMDARs in neurons from colitis rats, which would be expected to increase the affinity for glycine and lower the sensitivity to Mg2+.

The NR2B-selective antagonist ifenprodil was much less potent in inhibiting NMDA currents in DRG from colitis animals compared with normal animals (Fig. 5). Curve fitting of the individual sets of data gave IC50 values of 26 and 30 μM for unlabeled and labeled neurons from colitis rats, respectively. The corresponding IC50 values in normal rats were 2.7 and 3.1 μM for random and labeled neurons, respectively. Thus the 10-fold rightward shift in sensitivity occurred in both colon-specific and unlabeled neurons. The 10-fold decrease in ifenprodil sensitivity could suggest a replacement of NR2B subunits with NR2A subunits, which also have a high affinity for glycine and low sensitivity to Mg2+.

Expression of NMDAR subunits. To evaluate a possible increase in NR2A subunit expression, we employed quantitative RT-PCR to measure the expression of all four NR2 subunits in DRG neurons taken from vehicle- and TNBS-treated rats. The results shown in Fig. 6 summarize the findings from three separate experiments with RNA isolated from L1, L2, L6, and S1 DRG pooled from three animals for each condition. As previously shown in untreated rats (34), NR2B and NR2D were predominantly expressed in DRG, with much lower levels of NR2A and NR2C. In DRG from colitis rats, NR2B expression increased significantly by 28 ± 8% compared with control rats (P < 0.05). There was also a trend for an increase in the expression of NR2D (43 ± 17%, P = 0.07). In contrast, there was no change in the expression of NR2A and NR2C, which remained very low, near the detection limit for this method.

To determine whether there was a similar change in the NR2B and NR2D expression by cultured DRG neurons, we performed Western blot analysis of total protein extracted from 2-day-old cultures. The relative expression of NR2B and NR2D were measured by scanning densitometry of the immunoblots and normalized for protein as described in MATERIALS AND METHODS. Similar to the RT-PCR results of intact DRG

Fig. 4. Dose-dependent inhibition of NMDAR currents by Mg2+. NMDAR currents were generated by rapid perfusion with 250 μM NMDA and 10 μM Gly at holding potentials of −80 and −100 mV. Concentration-current relationships were established for concentrations of Mg2+ ranging from 0 (nominally Mg2+-free control solution) to 500 μM. The dose-dependent effect of Mg2+ at different holding potentials was determined by expressing the current responses as a percentage of that determined in Mg2+-free buffer. At membrane potentials of −100 and −80 mV, nonlinear regression gave IC50 values of 2.5 μM (95% CI: 1.8–3.2 μM) and 5.4 μM (95% CI: 4.0–7.3 μM) in randomly selected neurons from control rats and 2.0 μM (95% CI: 1.3–3.0 μM) and 4.2 μM (95% CI: 2.9–5.9 μM) in colon-specific (retrograde labeled) from colitis neurons, respectively. F-test comparisons of the curves indicated that there were no significant differences between neurons from rats with colitis compared with those from untreated rats.
neurons, cultured neurons from colitis rats showed a significant 28 ± 9% increase in NR2B expression, whereas there was no apparent change in the expression of NR2D (Fig. 7). We were unable to detect NR2A in Western blots of DRG neurons from either colitis or control rats, in agreement with our earlier report (34).

One previous study (11) has shown that NR1 splice variant expression does not influence the affinity of NR2B-containing NMDARs for ifenprodil, even though amino acids near exon 5 influence ifenprodil sensitivity (35). To formally rule out a change in the NR1 splice variant expression, we used semi-quantitative RT-PCR to compare the levels of expression of the different NH2- and COOH-terminal splice variants. As shown in Fig. 8, DRG neurons from both colitis and control rats predominately expressed the NR1b isoform, which includes exon 5. The ratio of NR1b to NR1a increased from 2.8 ± 0.2 in control to 3.4 ± 0.3 in DRG from rats with colitis (n = 4), but the change was not significant (P = 0.1). On the basis of the intensities of PCR products relative to those for GAPDH, there was a significant decrease in the expression of NR1a (*P < 0.05) with a small but insignificant increase in NR1b (Fig. 8, right). Similar comparisons between control and colitis DRG showed no significant changes in the COOH-terminal splice variants NR1-1, NR1-2, and NR1-4, with a trend for an increase in NR1-3 (P = 0.07). Because these changes in NR1 splice variant expression are small, they are not likely to account for the large change in ifenprodil sensitivity or channel activity.

Evidence for NR2B subunit phosphorylation. To address the issue of whether or not tyrosine phosphorylation of NR2B might underlie the increase in activity and the shift in ifenprodil sensitivity, we first evaluated the effect of the general protein tyrosine phosphatase inhibitor sodium orthovanadate on NMDA currents in DRG neurons isolated from normal rats. This treatment would be expected to cause a time-dependent increase in NR2B phosphorylation. As shown in Fig. 9A, the addition of sodium orthovanadate (100 μM) caused a rapid 2.3-fold increase in NMDAR currents, which peaked after 15 min and then declined to a steady-state level of 1.8-fold. The increase in peak current was markedly inhibited by the prior addition of 1 μM lavendustin, a general tyrosine kinase inhibitor, or by 10 μM PP2, a selective Src family tyrosine kinase inhibitor. To evaluate the effect of tyrosine phosphorylation of NMDARs in DRG neurons on ifenprodil sensitivity, we performed a separate set of experiments in which neurons were pretreated for 45 min with 100 μM sodium orthovanadate before being placed on the stage of the microscope for patch clamping. These neurons were continuously perfused with 100 μM sodium orthovanadate to maintain the steady-state level of phosphorylation. The current density in these neurons was 1.7-fold higher than in control neurons not receiving treatment (Fig. 9B). Ifenprodil sensitivity was tested using a dose of 5 μM ifenprodil, which inhibited 66% of the current in normal neurons but only 26% in neurons taken from rats with colitis (extrapolated from the fitted curves shown in Fig. 5). The results (Fig. 9C) showed that vanadate treatment caused a decrease in ifenprodil sensitivity from 56 ± 2% to 24 ± 5% inhibition. Thus Src family kinase-mediated phosphorylation of NMDARs in DRG neurons caused both an increase in the current density and a shift in ifenprodil sensitivity similar to that observed in neurons taken from rats with colitis.

To evaluate the possible contribution of enhanced phosphorylation of NMDARs in DRG neurons from rats with colitis, we evaluated the effect of PP2 addition on these neurons. As shown in Fig. 10, the addition of 10 μM PP2 to neurons isolated from control rats had only a small effect (9 ± 2% decrease) on NMDA currents over the 60-min time course of the experiment, which was not statistically different from the normal rundown observed previously with the repeated application of NMDA and glycine (27). In contrast, the addition of 10 μM PP2 to neurons from rats with colitis caused a slow
results in a significant increase in CGRP release into the medium from capsaicin-sensitive primary afferent nerve terminals. The release was Ca\(^{2+}\) dependent and mediated through NMDARs. Because TNBS colitis is associated with peripheral tissue damage including the loss of peripheral nerve terminals, we measured NMDA-mediated CGRP-LI release from spinal cord slices receivingafferent input from the colon. Considerable evidence for a role of spinal release of CGRP (and/or SP) from central terminals of primary afferents after peripheral tissue inflammation and associated hyperalgesia has previously been reported (16, 23, 45). In spinal cord tissue from colitis animals, the NMDA-stimulated release of CGRP-LI was 70% larger than that observed in tissues from vehicle-treated animals, consistent with either facilitated release (including up-regulation or increased sensitivity of NMDARs) and/or increased content of CGRP in nerve terminals in the spinal cord. This increase was almost entirely sensitive to a maximal dose of the NR2B-selective NMDAR antagonist ifenprodil, suggesting selective upregulation of NMDARs containing this subunit.

Consistent with the increase in NMDAR-mediated neuropeptide release, there was a threefold increase in NMDAR-mediated current density in DRG neurons taken from rats with colitis. Pharmacological characterization of currents demonstrated that the increase in current density in DRG neurons taken from rats with colitis was due to the same type of ion channels as in control neurons. Interestingly, the upregulation of NMDAR-mediated currents was observed in DRG neurons innervating the inflamed colon but also in noncolonic neurons from the same spinal cord level, the majority of which would be expected to have innervated somatic tissues. The fact that there was no change in NMDAR currents in neurons isolated

Discussion

Using a validated rat model of experimental colitis and associated visceral hyperalgesia (8, 14, 15), we evaluated the effect of visceral inflammation on the expression and function of NMDARs in primary afferent neurons located in the DRG. We found that the activity of NMDARs increased threefold in DRG neurons that had innervated the inflamed colon and in neurons within the same ganglia but not in neurons isolated from more distant dermatomes. NMDA-induced CGRP release from spinal cord slices receiving input from DRG neurons innervating the colon was also significantly increased in colitis. Electrophysiological and molecular studies identified an up-regulation of NR2B subunit expression as well as persistent tyrosine phosphorylation of NR2B as the likely mechanisms underlying the observed increase in NMDA currents.

Effect of colon inflammation on NMDAR-mediated peptide release and currents. We (36) have previously demonstrated that NMDA application to colon and urinary bladder slices results in a significant increase in CGRP release into the...
from DRG not sending processes to the colon rules out a role for a circulating inflammatory mediator in causing this effect. Cross-sensitization of DRG neurons within the same ganglia has been noted previously by Malykhina et al. (31), who demonstrated that colonic inflammation increased tetrodotoxin-resistant Na\(^+\) and capsaicin-stimulated TRPV1 currents in DRG neurons innervating the urinary bladder. Global upregulation of the mRNA and protein for several tetrodotoxin-sensitive and -resistant Na\(^+\) channels and their corresponding currents has also been documented in all small neurons within the ipsilateral ganglia receiving afferents from the inflamed hind paw, but not on the contralateral side (2). These data suggest that inflammatory mediators or other signals from DRG neurons innervating the inflamed tissue mediate cross-sensitization of neighboring neurons either directly within the ganglia or indirectly through dorsal root reflexes. The nature of these signals and the role of support cells, such as satellite cells in the DRG or glia in the spinal cord, remain to be investigated. Given the relatively low numbers of sensory afferent fibers innervating visceral tissue compared with somatic tissues, cross-sensitization of DRG neurons may contribute to the diffuse nature of visceral pain and its referral to somatic and other visceral tissues within the same dermatome (5).

**Colitis induced increased NMDAR subunit expression.** Using molecular techniques, we found a modest increase in the expression of NMDAR subunits with no evidence for a major shift in the relative expression of NR2 subunits or NR1 splice variants. As previously observed in normal rats (34), NR2B and NR2D continued to be the predominant subunits expressed in the DRG. The expression of NR2B increased significantly after the induction of colitis, but the increase was small (28%) compared with the 300% increase in NMDAR current density. These observations agree with the results of the electrophysiological experiments, which found no change in the affinity of the NMDARs for glycine or NMDA or in voltage-dependent Mg\(^{2+}\) inhibition. The relatively low affinity for glycine and the high affinity for Mg\(^{2+}\) suggest that there is little, if any, contribution of the NR2D subunit to NMDAR currents expressed on the cell surface of DRG neurons from animals with or without colitis and that most of the current is mediated by NR2B-containing receptors. However, we also found a 10-fold rightward shift in the dose-dependent inhibition by the NR2B selective antagonist ifenprodil. However, we also found a 10-fold rightward shift in the dose-dependent inhibition by the NR2B selective antagonist ifenprodil. The IC\(_{50}\) was still fivefold less than that reported for inhibition of recombinant NR2A-containing NMDARs (IC\(_{50}\) ∼ 150 μM) (50), although it was similar to that reported for mixed NR2A/NR2B and NR2B/ NR2D receptors (3, 24). However, a shift in cell surface expression of NR2B/NR2D receptors is unlikely because there was no change in agonist or Mg\(^{2+}\) affinity. Mixed receptors containing NR2A and NR2B are also unlikely because NR2A subunits are expressed at very low levels in DRG neurons. Therefore, a more likely hypothesis is that there is a change in

![Fig. 9. Effect of sodium vanadate (VO\(_4\)) on NMDAR currents and ifenprodil sensitivity. A: whole cell patch-clamp recordings were obtained of cultured DRG neurons from untreated rats in Mg\(^{2+}\)-free extracellular solution at a holding potential of −60 mV. NMDAR currents were generated by a 5-s long perfusion with 250 μM NMDA and 10 μM Gly at 5-min intervals. After a stable baseline period, 100 μM sodium vanadate was continuously perfused onto the cell in the presence or absence of 1 μM lavendustin (Lav) or 10 μM PP2. Results are means ± SE of 3–4 determinations for each condition. *Significantly different from baseline current at time 0 and PP2 and Lav conditions at the same time point. B and C: DRG neurons from untreated rats were stimulated with 100 μM sodium vanadate for 45 min in extracellular solution before being patch clamped in the continued presence of 100 μM sodium vanadate. Control neurons were not treated with sodium vanadate. NMDAR currents were measured before and after the addition of 5 μM ifenprodil, and inhibition was expressed relative to the initial current. Values are means ± SE of 6–8 neurons for each condition. *P < 0.05 and **P < 0.0003 compared with control.](http://ajpgi.physiology.org/)

![Fig. 10. Effect of the nonreceptor tyrosine kinase inhibitor PP2 on NMDAR currents in DRG neurons from colitis and control rats. After stable recordings were established in the whole cell patch configuration, NMDAR currents were elicited for 5 s at 5-min intervals by a rapid perfusion of 250 μM NMDA and 10 μM Gly before and after the addition of 10 μM PP2 to the extracellular buffer. Three different conditions were tested: DRG neurons from saline-treated rats, those from rats with colitis, and neurons from untreated rats prestimulated with 100 μM sodium vanadate. In the latter case, currents were measured in the continued presence of 100 μM sodium vanadate. A: currents over time are expressed relative to the initial current at time 0. B: absolute currents immediately before (time 0) and 60 min after PP2. Values are means ± SE of 3–4 neurons for each condition. *Significantly different from the initial current at time 0 (P < 0.05).](http://ajpgi.physiology.org/)
the activation state of pure NR2B receptors that also affects the affinity for ifenprodil.

Evidence for tyrosine phosphorylation of NR2B subunits. The present data support the conclusion that tyrosine phosphorylation of NR2B-containing NMDARs in DRG neurons contributes to the increase in activity and decrease in ifenprodil sensitivity. The addition of sodium vanadate, which would be expected to increase the steady-state level of tyrosine phosphorylation, caused an increase in NMDAR channel activity and a decrease in ifenprodil sensitivity in neurons from normal rats similar to that observed in neurons from rats with colitis. Lavendustin, a general tyrosine kinase inhibitor, and PP2, an Src family kinase-selective inhibitor, prevented the effect of vanadate, strongly suggesting a role for one or more members of the Src family of tyrosine kinases.

Src-mediated phosphorylation of NR2B subunits has been reported in the central nervous system. In fact, NR2B is the principal NMDAR subunit that is tyrosine phosphorylated in the brain (38, 43). In the hippocampus, it is involved in long-term potentiation both by increasing channel activity and channel insertion into synapses (29, 43). In the brain and spinal cord, Src family kinases, including Src, Fyn, Lyn, and Yes, are associated with NMDAR complexes, and all have been shown to upregulate NMDAR activity in recombinant expression systems or native membranes (22, 39, 46, 53). Much less is known about Src family kinases in DRG neurons. Src itself has long been known to be expressed by sensory neurons (32); however, it has only recently been shown to mediate interleukin-1β-induced upregulation of preprotachykinin gene expression and enhance SP release (19). In recombinant cells using single-channel recording techniques, Src kinase-mediated phosphorylation of NR2B has been shown to increase open probability without altering channel kinetics (48). Our data are consistent with the concept that Src-mediated phosphorylation has little effect on the kinetic properties of NMDAR channels aside from its effect on activity and ifenprodil sensitivity. Taken together, our results suggest that the increase in NMDAR current in neurons taken from rats with colitis is due in part to an increase in tyrosine phosphorylation of cell surface NR2B-containing NMDARs.

The mechanisms involved in persistent Src family kinase-mediated phosphorylation and activation of NMDARs could involve changes in any one of a number of proteins that have been shown in the central nervous system to regulate the activation or deactivation of Src kinase or its association with the NMDAR complex (21). It is not known whether DRG neurons form NMDAR complexes containing the same or similar components, nor did this study address whether NMDARs on the central and peripheral terminals of DRG neurons in intact tissue are phosphorylated and upregulated after inflammation. It should be noted that our study examined neurons in short-term culture, which could influence the expression, insertion, and/or activity of NMDARs. However, because neurons from both control and TNBS-treated rats were grown and characterized under identical conditions, our results strongly suggest that there is a fundamental change in the activation and/or association of Src family kinases with NMDARs after inflammation.

Possible physiological implications of inflammation-induced upregulation of NMDARs. The findings reported in this study may have several implications for the development and maintenance of visceral hyperalgesia after colon inflammation. On the peripheral terminals of afferent fibers, the activation of NMDARs in healthy animals causes behavioral pain responses (54), sensitizes a subset of primary afferents to colorectal distension (47), and mediates the release of SP and CGRP in the gut (36). SP released in the mucosa can have effects on gut immune cells (49) and vascular permeability, whereas CGRP, a potent vasodilator (18), may enhance mucosal blood flow. Together, the peripheral release of these peptides has been implicated in the development of neurogenic inflammation (13). Thus a facilitated peripheral neuropeptide release after inflammation may influence the duration and severity of the inflammatory response. NMDARs located on the central terminals of C-fibers regulate the release of both peptides as well (28, 30, 33). The interaction of SP with postsynaptic neurokinin-1 receptors plays an important role in the development of hyperalgesia and pathological pain (4, 25). The facilitated central release of SP and CGRP from central terminals may therefore play a role in determining the time course of chronic visceral hyperalgesia after colon inflammation.

In summary, upregulation of NMDARs on primary afferents by NR2B subunit phosphorylation may have important implications for the development and persistence of colon inflammation, for the severity and duration of peripheral and central sensitization, and for the referral of pain to other visceral and somatic structures.

ACKNOWLEDGMENTS

The authors thank Dr. Juan Carlos Marvizoñ for helpful discussions.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-58173 and 1-P50-DK64539-01 (to E. A. Mayer).

REFERENCES


