Alterations in \(N\)-methyl-\(d\)-aspartate receptor subunits in primary sensory neurons following acid-induced esophagitis in cats

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Gastroesophageal reflux disease (GERD) is one of the most common gastrointestinal disorders in humans and can be associated with esophagitis and hypersensitivity. Fass and colleagues (9) documented an increase in the expression of NR1 splice variants by inclusion and exclusion of three exons: N1 (exon 2), C1 (exon 21), and C2 (exon 22). Recent studies have demonstrated an increase in the expression of NR1 splice variants as a result of inflammation, further implicating the NMDAR in the development of hypersensitivity following an inflammatory process (10, 47).

Gastroesophageal reflex disease (GERD) is one of the most common gastrointestinal disorders in humans and can be associated with esophagitis and hypersensitivity. Fass and colleagues (9) documented an increase in the expression of NR1 splice variants by inclusion and exclusion of three exons: N1 (exon 2), C1 (exon 21), and C2 (exon 22). Recent studies have demonstrated an increase in the expression of NR1 splice variants as a result of inflammation, further implicating the NMDAR in the development of hypersensitivity following an inflammatory process (10, 47).

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leagues (14) have recently demonstrated a significant correlation between the chemosensitivity of primary afferents and reflex symptoms with the degree of endoscopically observed tissue injuries in GERD patients. A recent study in humans also demonstrates that acute acid induction results in esophageal hypersensitivity that could be prevented by intravenous administration of the NMDAR antagonist ketamine, further suggesting the involvement of NMDAR in gastroesophageal reflex-induced esophageal hypersensitivity (45). Although these receptors have been implicated in the development of hypersensitivity following acid exposure in humans, very little is known about the receptor expression and its subunit composition following chronic acid-induced esophagitis. Understanding these alterations may provide insight into the pathophysiology of GERD and potentially aid in targeting future pharmacological interventions to either prevent or treat pathological GERD.

The present study was undertaken to investigate alterations in the NMDAR subunit expression following acid-induced esophagitis in cats. The expression profiles of NR1 and NR1 splice variants (NR1-1, NR1-2, NR1-3, NR1-4, and NR1a/b) and NR2A subunit were evaluated in the thoracic dorsal root ganglia (DRGs, T1–T3), nodose ganglia (NGs), and the distal esophagus of cats following esophageal acid infusion (25, 46).

**H&E staining of the esophageal tissues.** An independent observer blinded to the groups determined the extent of mucosal damage in the esophagus. The criteria for macroscopic scoring included the presence of hyperemia, edema, erosions, ulcers, and intramural hemorrhage. The tissue sections of 5-μm thickness taken from the proximal, middle, and distal esophagus were stained with H&E and examined for different degrees of epithelial loss (e.g., splitting, erosion, and ulceration), reactive epithelial changes (e.g., basal hyperplasia and vascular alteration including edema, congestion, bleeding, vessel lesions) and inflammation (i.e., the presence of polymorphonuclear leukocytes, lymphocytes).

**EPO staining of the esophageal tissues.** Eosinophil infiltration was studied by microscopic examination after staining for EPO using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma). The sections were incubated initially with 10 mM cyanide buffer and rinsed in PBS, followed by incubation with peroxidase substrate DAB for 10 min at room temperature. The slides were rinsed thoroughly with water and coverslipped before examination under the microscope.

**METHODS**

All protocols were approved by the Animal Care and Use Committee at the Medical College of Wisconsin and are in accordance with the International Association of Pain policies on the use of laboratory animals.

**Surgery.** Cats of either sex weighing 2–4 kg were anesthetized by intraperitoneal injection of ketamine (10 mg/kg) and xylazine (5 mg/kg). Prior to surgery, all animals received the antibiotic (Baytril, 5 mg/kg im) to prevent infection and following surgery daily for 7 days. A small incision was made in the ventral side of the neck to expose the cervical esophagus and an infusion catheter was implanted 4–5 cm below the upper esophageal sphincter. The other end of the catheter was externalized subcutaneously through the dorsal aspect of the neck and sealed with a plastic plug for later access. The incision was sutured in layers using 4-0 silk and animals were observed postsurgery until complete recovery from anesthesia. In this study we have used ketamine only at the time of surgery to implant the infusion catheter into the esophagus. After surgery the animals were kept under observation until full recovery and all the experimental protocols were carried out 3–4 wk after implantation of the infusion catheter. The acid and PBS were infused into the animals through the infusion catheter under fully awake condition. For extraction of the esophagus, DRGs, and NGs, all cats were deeply anesthetized with pentobarbital sodium (Nembutal, 20 mg/kg ip) and were perfused transcardially with either PBS (pH 7.4, for gene expression, Western blot, and enzyme assay) or 4% paraformaldehyde (for histology and immunohistochemistry).

**Experimental protocol.** Two groups of cats were used (n = 9 in each group). One group was given 0.1 N HCl (pH 1.2) under awake conditions through the infusion catheter over 30 min (1 ml/min) for 7 consecutive days (Acid-7D) whereas the control group received 0.1 M PBS (pH 7.4) for 7 days at a similar rate (PBS-7D). The esophageal tissues from both groups of cats were used for hematoxylin and eosin (H&E) staining, eosinophil peroxidase (EPO) staining, and quantitative estimation of myeloperoxidase (MPO) and EPO. Furthermore, the NMDAR expression at the mRNA and protein levels in the thoracic DRGs (T1–T3) nodose ganglia, and distal esophagus was compared between both groups of animals.

To detect the degree of tissue inflammation, EPO and MPO contents of esophageal tissues from the acid-treated cats (Acid-7D) were assayed and compared with those of the PBS-treated cats (PBS-7D). MPO is a constitutive enzyme found in the intracellular granules of neutrophils and can be used as a marker for inflammation. Similarly, EPO is a marker for infiltrating eosinophils in the esophagus. The tissues were kept frozen (−70°C) until used for extraction. The tissue samples were placed in plastic test tubes (17 × 100 mm), and 1 ml of cetyltrimethylammonium chloride (0.5% in distilled water) was added to each sample and homogenized with a polytron tissue homogenizer for 15 s. The homogenates were centrifuged at 10,000 g for 10 min at 4°C and the clear supernatants were used for the assay. The extract was diluted 1/10 in 50 mM HEPES, pH 8.0 (EPO dilution buffer) or 10 mM citrate buffer pH 5.0 (MPO dilution buffer). For MPO assay, the substrate O-phenylene diamine (3 × 10−5 mol) was prepared in 50 mM HEPES, pH 8.0, along with 6 mM KBr and 8 mM H2O2. The appearance of the yellow color compound over time was measured with a spectrophotometer to determine the MPO and EPO contents of the tissues. The color development was measured as an absorbance at 460 nm for MPO over a period of 5 min. The MPO activity of the tissue samples was calculated on the basis of the change in absorbance per minute. The change in absorbance for 1 μmol H2O2 split is 1.13 × 10−2 and the MPO content of the samples was calculated on the basis of the following formula: 1 unit of MPO = 1 μmol H2O2 split. For EPO content, the color development was measured at 490 nm and the intensity of the color development was directly proportional the EPO content of the tissues.

**NR1 splice variant gene expression in the thoracic DRGs, NGs, and distal esophagus.** RT-PCR analysis was performed in neuronal tissues (DRGs, NGs) and the distal esophagus to examine mRNA expression level of NMDAR subunits in experimental and control animals. We used semiquantitative RT-PCR analysis for studying the expression pattern of NR1 splice variants instead of real-time PCR because the sizes of the amplification products were larger, in the range of 200–400 bp (as designed based on the available rat sequences). Animals in each group were euthanized with Nembutal (20 mg/kg ip), and the DRGs (T1–T3), NGs, and distal esophagus were removed. Total RNA was extracted by use of Aurum total RNA fatty and fibrous tissue kit from Bio-Rad (Hercules, CA). Each RNA preparation was subjected to DNase treatment to remove the contaminated genomic DNA and reverse transcription was performed by using SuperScript first-strand synthesis kit (Invitrogen, Carlsbad, CA). For each tissue sample, we carried out the RT reaction without the enzyme.
subjected both rat and human NR2A sequences to blast search against NR1 antibody for Western blots and immunohistochemistry that could highly conserved between these two species. Moreover, we have used human show 90% sequence homology; therefore the sequences are ing a distinct difference in NR2 subunit sequences between cat and PCR amplification with use of cat brain stem cDNA, thereby indicat- none of the subunits, namely NR2B, NR2C, and NR2D, exhibited number of PCR cycles required to obtain this threshold. Initially we carried out PCR reaction using rat and cat brain stem cDNA thereby indicating the possibility of conserved sequences between cat and other available sequences in the GenBank. For the present study we designed the NR2A primers from this conserved sequence regions. The primer sets have been designed using Beacon Designer program (Premier BioSoft International, Palo Alto, CA). Therefore, we examined the effect of chronic esophageal acid exposure on NR1 and NR2A subunit gene expression in cat and NR1 and NR2A primers designed for real-time PCR are shown in Table 1.

The PCR reactions were performed by use of iQ SYBR Green Supermix (Bio-Rad). We used 5 pmol forward and reverse primers and 2 μl cDNA from each tissue sample as template in a total of 25 μl of the reaction mixture. PCR reaction were carried out in 96-well microtiter plates, and the samples were incubated for 3 min at 95°C and were amplified for 45 cycles of 30 s at 95°C and 30 s at 57.4°C. To amplify the amplification efficiency within each experiment, a serial dilution of cDNA derived from a RNA pool of control tissues was amplified in triplicate in each plate. The specificity of PCR reaction and possibility of primer dimerization were verified by using a melting curve program and no template control for each PCR reaction. Since all PCR reactions were performed with equal efficiencies, relative mRNA expression level of the target gene was directly normalized against the expression level of reference gene rRNA for the same tissue sample. The C_T values for reference gene rRNA was highly reproducible between samples and between PCR reactions. The cDNA preparation with C_T values <35 was considered as specific implication and reactions with C_T values >35 were not included in the study. Results were expressed as relative mRNA expression in terms of C_T values in relation to the amount of reference gene mRNA expression by using the formula 2^(-ΔΔC_T).

Western blot analysis of NR1 subunit protein expression in thoracic DRGs (T1–T3), NGs and distal esophagus. Crude extracts from various cat and rat tissues were prepared by powderizing the tissues in liquid nitrogen and homogenization in ice-cold hypotonic lysis buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford,

Table 1. RT-PCR primers for NR1 and NR1 splice variants

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IL), followed by differential centrifugation first at 1,200 rpm for 30 min and then for another 30 min at 14,000 rpm. The membrane pellet was solubilized for 30 min on ice with RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS containing protease and phosphatase inhibitors). The membrane extracts were centrifuged and assayed for protein content by the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Rockford, IL). Approximately 25 μg of brain tissue extracts from cat and rat and 50 μg of DRG and NG extracts from cat were electrophoresed on 8% SDS-PAGE and transferred onto nitrocellulose membrane. After transfer, the membrane was blocked with 5% nonfat milk and then probed with antibodies to NMDAR1 subunit (mouse anti-NMDAR1, 1:100, BD Biosciences, San Jose, CA). Protein bands were visualized by using a horseradish peroxidase-conjugated secondary antibody (Jackson ImmununoResearch, West Grove, PA) and an enhanced chemiluminescent detection system (Thermo Scientific). The relative changes in the intensity of NR1 subunit expression in various samples were normalized against the intensity of housekeeping gene β-actin for the same tissue sample by use of alpha imager 3400 software. For NR2A subunit protein analysis, two different antibodies from Alomone Labs (Jerusalem, Israel) and Santa Cruz Biotechnology (Santa Cruz, CA) were used. However, none of the antibodies showed specific reactivity with cat tissues; therefore, NR2A subunit protein analysis was not included in this study.

**Immunostaining of NMDAR subunits in DRGs, NGs, and distal esophagus.** Cats from both the groups (Acid-7D and PBS-7D) were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and the chest was opened by midsternal incision. Animals were perfused transcardially with cold phosphate buffer solution followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Bilateral thoracic (T1–T3) DRGs, NGs, and esophagi were collected and incubated in 4% paraformaldehyde overnight at 4°C. Tissue samples were cleaned of DRGs, NGs, and esophagi were collected and incubated in 4% chest was opened by midsternal incision. Animals were perfused against the intensity of housekeeping gene with cold phosphate buffer solution followed by 4% C. In contrast there was no evidence of inflammation in the esophagus of the PBS-7D group (Fig. 1A). In the Acid-7D group, severe mucosal damage (high-grade esophagitis or ulcerative esophagitis) was characterized by extensive epithelial cell loss with erosion/ulceration, edema, and granulation tissue replacing the mucosa with extensive infiltration of neutrophils, eosinophils, and polymorphonuclear leukocytes and lymphocytes. To confirm eosinophilic infiltration in the esophagus, we stained the esophageal tissues for eosinophilic peroxidase. An extensive eosinophilic infiltration was observed around the mucosa and submucosa in the Acid-7D group demonstrated a significant increase in MPO content compared with PBS-7D (Fig. 1C, P < 0.01). These cats showed 35-fold increase in MPO units (35 ± 13.07 MPO units/mg tissue) compared with PBS-7D cats (1.306 ± 0.615 MPO units/mg tissue). Similarly, quantitative estimation of EPO as measured by ELISA absorbance at 490
NM showed a significant increase in the acid-treated group compared with PBS-treated controls (*P<0.01, Fig. 1C).

NR1 splice variant gene expression in DRGs, NGs, and esophagus. To examine the specificity of amplified PCR products of cat cDNAs using rat specific NR1 splice variant primers, we carried out PCR reactions of these primer sets using cat brain stem cDNA as a template and compared amplified products with those obtained from the PCR with rat brain stem cDNA. With cat brain stem cDNA, distinct PCR amplified products were obtained for both NH2-terminal variants NR1-a and NR1-b and COOH-terminal variants NR1-1, NR1-3, and NR1-4. The sizes of the PCR products were comparable to that obtained from the PCR reaction with rat brain stem cDNA (Fig. 2) and also to the predicted sizes (Table 1). However, no PCR amplification was observed for NR1-2 in cat brain stem tissue, indicating either very poor expression of NR1-2 splice variant in cat or a gene sequence different from that of the rat sequence.

After evaluating the specificity of the amplified products, we used these NR1 primer sets for PCR amplification with cDNA preparations from DRGs (T3), NGs, and distal esophagus from PBS and acid-treated cats. Primers used for the amplification of six splice variants are shown in Table 1. In DRGs of PBS-infused cats, out of six NR1 splice variants, NR1-a (+exon 5)
and NR1-2 (–exon 21, +exon 22) failed to show amplification (Fig. 3A). The COOH-terminal splice variant NR1-4 showed the strongest expression. Acid infusion resulted in significant increase in the expression of NR1-b and NR1-4 variants (Fig. 3B, *P < 0.05, **P < 0.001 vs. PBS group). Interestingly, the NR1 mRNA expression (with primers designed outside the region of exons 5, 21, and 22) also significantly increased in DRGs from Acid-7D cats compared with PBS-7D (P < 0.05 vs. PBS). There appear to be distinct differences in the expression profile of NR1 splice variant mRNAs in NGs compared with DRGs (Fig. 4A). The major splice variants in NGs from PBS-7D cats were NR1-a, NR1-b, NR1-1, NR1-3, and NR1-4. No amplification was observed for NR1-2. Interestingly, acid exposure resulted in a significant increase of NR1 (P < 0.05 vs. PBS-infused cat) and a marked downregulation of NR1-4 splice variants compared with PBS control, although the difference was not statistically significant (Fig. 4B). In esophageal tissues from PBS-7D cats, we failed to detect the expression of any of the six splice variants and found only a very low expression of NR1 (Fig. 5A). However, in esophageal tissues...
from Acid-7D cats a significantly high expression was observed for NR1 and NR1-4 (Fig. 5B, *P* < 0.05 vs. PBS-7D).

**Quantitative estimation of NR1 and NR2A subunit gene expression in NGs, DRGs, and esophagus.** The quantitative NR1 mRNA expression in DRGs, NGs, and distal esophagus for PBS- and acid-treated cats as determined by real-time PCR is shown in Fig. 6A. For PBS-7D cats, the relative expression of NR1 was the highest in the NGs followed by DRGs and the lowest in the esophagus. The quantitative analysis of the expression profile of NR1 subunits in the DRGs, NGs, and esophagus exhibited a significant upregulation of NR1 subunits in these tissues after acid infusion (esophagus, *P* < 0.05; DRGs, *P* < 0.001; NGs, *P* < 0.001 vs. PBS-7D). The sizes of the amplified products were in the range of 200 to 100 bp (Table 1). In real-time PCR, the highest expression of NR2A subunit was observed in NGs followed by esophagus and DRG, respectively (Fig. 6B). However, in the acid-treated group, a significant downregulation of NR2A subunit gene expression was observed in esophagus and DRG (Fig. 6B, *P* < 0.05 vs. PBS-7D).

**Western blot analysis of NR1 subunit polypeptide expression in NGs and DRGs.** In Western blot, we analyzed two protein extracts each from both rat and cat brain stems using rat specific NR1 antibody (Fig. 7A). We have used a monoclonal antibody raised against NMDA-NR1 subunit and generated by using a fusion protein encoding NMDA-NR1 amino acid residues 660–811, representing the intracellular loop between the transmembrane region III and IV not encompassing any splice variant regions. A specific polypeptide band of 120 kDa was identified in cat brain stem, and the size was comparable to the expression pattern in rat tissue extracts, thereby indicating the sequence homology between cat and rat NR1 subunits of NMDAR. By use of this rabbit anti-NR1 antibody, a significant increase in polypeptide level of the NR1 subunit was detected in DRGs and NGs from acid-treated group compared with PBS controls (Fig. 7, B and C, *P* < 0.05).

**Immunohistochemical analysis of NR1 immunoreactivities in NGs and DRGs.** Immunohistochemical analysis of NR1 staining demonstrated a significant increase in the intensity of NR1 expression in DRGs from Acid-7D cats compared with PBS-7D (Figs. 8A and 9A). The majority of these NR1 expressed cells also showed a high expression of SP indicating NR1 expression was mostly in SP expressing peptidergic C fibers. In DRGs, immunostaining with NR1 antibody preabsorbed with blocking peptide failed to show significant immunoreactivities, indicating the specificity of the reaction (Fig. 8B). The immunostaining using normal rabbit serum as primary antibody also failed to show any specific immunoreactivites in DRG sections (Fig. 8B). In NGs, a marked increase in the intensity of NR1 staining was observed after esophageal acid exposure in Acid-7D group compared with control; however, this was not statistically significant (Figs. 8C and 9B). Unlike DRGs, less than 50% of the NR1 expressing cells in NGs exhibited coexpression of SP, indicating the involvement of other neurotransmitters or cell types in NMDAR-mediated signaling in NGs (Fig. 8C).

**DISCUSSION**

We have shown in the present study that repetitive acid exposure in cats results in tissue damage and inflammatory cell infiltration in the esophagus. This model mimics the findings reported in humans with high-grade esophagitis characterized by severe epithelial injury with erosions, ulceration, and infiltration of polymorphonuclear cells (neutrophil, eosinophils, and lymphocytes). This inflammation is confined primarily in
the mucosa, lamina propria, and muscularis mucosa (18, 21, 24). Our results also demonstrated a significant increase in the NR1 subunit of the NMDA gene receptor and overall down-regulation of NR2A mRNA in the DRGs, NGs, and esophagus in cats following esophageal acid exposure. These findings are in accordance with our Western blot and immunohistochemical data exhibiting a significant increase in the intensity of NR1 protein immunoreactivity in the DRGs and NGs in the Acid-7D group compared with PBS-7D. To our knowledge this is the first study demonstrating alterations in NMDAR subunit expression as a result of acid-induced esophagitis.

Clinical observations suggest that acid exposure and acid-induced esophagitis may sensitize primary sensory neurons leading to wind-up or sensitization of secondary neurons in the spinal cord and/or brain stem (11, 32, 35, 38, 39). In humans, secondary allodynia of the chest wall and proximal esophagus has been reported following acute acid infusion in the distal esophagus. Furthermore, this hypersensitivity is attenuated by intravenous administration of the NMDAR antagonist ketamine, suggesting that the NMDAR plays an important role in the development of acid-induced hypersensitivity (45). Although studies have documented that ketamine attenuates pain by modulating ionic influx through the NMDA channel and reducing NR1 phosphorylation (42), it requires frequent repeated administration of the drug to maintain analgesia. This is primarily due to short-acting nature and rapid metabolism of the drug by hepatic microsomal enzymes (41). In the present study, ketamine was used in combination with xylazine acutely only one time to induce anesthesia during surgical procedure (see METHODS). It is very unlikely that such acute use of the drug will leave a long-lasting effect on expressions of NR1 and NR2 subunits. Despite the fact that both experimental (i.e., chronic acid infused) and control (i.e., PBS infused) cats received ketamine and xylazine as anesthetics during the surgical procedure, the expressions of NMDAR subunits are significantly different in acid-treated cats, suggesting that altered expression observed is due to acid and not the influence of ketamine used during surgery.

Several immunohistochemical and molecular studies have investigated NMDAR expression in DRG cell bodies and in vagal afferent neurons projecting from the stomach and duodenum (6, 8, 26, 29, 40). In rats, for example, experimental colitis with TNBS (trinitrobenzene sulfonic acid) results in NMDAR subunit phosphorylation and upregulation in the colon and DRGs (25). Since TNBS-induced colitis results in hypersensitivity even after the inflammation has resolved, it can be speculated that these alterations in NMDAR play an important role in hypersensitivity following inflammation (33).

Fig. 6. Real-time RT-PCR for NR1 and NR2A subunits in thoracic T1–T3 DRGs, NGs, and distal esophagus (ESO) from acid- and PBS-treated cats. A: cDNAs were amplified by using primers for NR1 or for the reference rRNA. B: cDNAs were amplified using primers for NR2A or for the reference rRNA. \( \Delta C_t \), difference between the number of cycles necessary to detect the PCR products of the experimental genes and the reference gene. Ordinate value \( 2^{-\Delta C_t} \) corresponds the amount of NR1 or NR2A subunit mRNA relative to the reference gene in the tissue sample. Values are expressed as means \( \pm SE \) of 3 real-time PCR determinations. The expression of NR1 subunit significantly increased, whereas NR2A subunit expression significantly decreased in DRGs and esophagus of acid-treated cats compared with PBS-treated cats (*\( P < 0.05 \) vs. PBS-treated cats).

Fig. 7. Effect of chronic acid treatment on the polypeptide level of NR1 subunit. A: Western blot analysis of tissue extracts from both cat and rat brain stems (BS) exhibited a 120-kDa band for NR1 subunit indicating complete homology of NR1 subunit proteins in these 2 different species. B: representative Western blot showing NR1 immunoreactivities in NG and DRG extracts from both acid- and PBS-treated groups. The intensity of NR1 immunoreactivity for different tissues was normalized against the intensity of \( \beta \)-actin expression for the same tissue. C: the relative changes in the NR1 expression were quantified with AlphaImage 3400 software. Results were normalized and the data were expressed as means \( \pm SE \) (*\( P < 0.05 \)).
Fig. 8. Photomicrograph of immunoreactivities of NR1 subunit and substance P (SP) in the thoracic T3 DRG and NG. A: immunostained sections (20 μm) of DRG from acid- and PBS-treated cats are represented at left and merged images of NR1 and SP stained sections are shown at right. The majority of (>90%) NR1-positive cells exhibited high expression of SP in the acid-treated cats compared with PBS-treated cats. Arrows indicate some of the small- and medium-diameter cells coexpressing NR1 and SP. B: in DRG, immunostaining with blocking peptide preabsorbed NR1 antibody failed to show significant immunoreactivities, indicating the specificity of the reaction (left). The immunostaining using normal rabbit serum as primary antibody also failed to show any specific immunoreactivities (right). C: immunostained sections (20 μm) of NG from acid- and PBS-treated cats are represented at left and merged images of NR1 and SP stained sections are shown at right. In NGs, <50% of NR1-positive cells exhibited SP immunostaining. Arrowheads indicate the NR1-positive cells not expressing SP and arrows indicate cells coexpressing NR1 and SP. Scale bar, 100 μm.
In the NGs, the NH2-terminal splice variant NR1-a (between rats and cats. We have demonstrated the expression of highlighting the differences in NR1 splice variant isoforms PCR amplification in the DRG, NGs, and esophageal tissues, NMDAR subunit expression in visceral organs has been doc-
soma has not been shown (29). The complexity and diversity of well as in esophageal tissues from the control cats. In contrast, observed significantly high expression in the DRGs and NGs as
rat-specific primer sequences for NR2A subunit gene, we also showed expression along with the NR1-b, NR1-1, NR1-3, and NR1-4 splices. We did not observe any expression of the NR1 splice variants in the PBS-treated cat esophagus. However, in the acid-treated cats, there was a distinct upregulation of NR1-4 in the esophagus as well as in the DRGs. Interestingly, there was a trend toward downregulation of NR1-4 in the NGs, indicating a distinct difference in the pattern of NR1 splice variant expression among the tissues.

The differences in the NMDAR subunit distribution among various species are also evident in the present study. Using rat-specific primer sequences for NR2A subunit gene, we observed significantly high expression in the DRGs and NGs as well as in esophageal tissues from the control cats. In contrast, NR2A expression either at the gene or protein level in rat DRG soma has not been shown (29). The complexity and diversity of NMDAR subunit expression in visceral organs has been doc-
umented in recent studies. Following carrageenan-induced hind paw inflammation, NR1 serine phosphorylation and NR2B suppression has been observed in the dorsal horn neu-
rons (3). The long-lasting decrease in NR2B expression in this injury model is in sharp contrast to the findings in the TNBS-induced colitis model, in which inflammation resulted in up-regulation and persistent phosphorylation of NR2 subunit expression in the DRG neurons (25). In the present study, esophagitis following acid infusion resulted in significant up-regulation of NR1 protein and mRNA with a significant down-regulation of NR2A mRNA in the DRGs, NGs, and esophagus. These data indicate that NMDAR are highly dynamic in their subunit composition during the development, maintenance, and recovery from a pathological condition.

This differential subunit expression pattern of NMDAR is also an important factor in regulating function and neuronal plasticity under pathological conditions. For example, rapid calcium-mediated signaling through NR1/NR2A in contrast to slower signaling through NR1/NR2B may activate different downstream signaling and gene expression patterns under various pathological conditions. Therefore, experimental findings of the NMDAR expression and activation in various animal models of inflammation need careful interpretation and may be important for the development of targeted pharmacological inventions.

Overexpression of N1 and C1 splices and serine phosphor-
ylation within the C1 cassette have been observed 14 days following inflammation of the rat colon with TNBS. In con-
trast, rats without inflammation fail to show expression of N1 and C1 splice variants (10, 47). In the present study, 7 days of acid infusion resulted in the expression of NR1-4 splice vari-
ants in esophageal tissues, whereas PBS-treated cats failed to show any NR1 splice variant. It is likely that in the esophagus, NMDAR have lower levels of constitutively expressed NR1 subunits and alternative splicing under normal physiological states with a subsequent upregulation of NR1 splice variants in the pathological state.

In a recent study, repeated long-term application of an NMDAR agonist resulted in a rapid loss of functional NR1-
NR2A channels at the surface of HEK-293 cells (44). This use-dependent receptor downregulation was prevented by application of tyrosine phosphatase inhibitors, indicating that a tyrosine-based signaling mechanism is involved in NMDAR subunit modulation and expression. An overall downregulation of NR2A subunit in the present study is in accordance with previously reported suppression of NR2 subunits in rats following carrageenan-induced hind paw inflammation (3).

Since currently there is no reliable, reproducible, and quant-
tifiable animal model to measure the esophageal pain, the major limitation of the present study is the absence of behavioral testing to determine the esophageal hypersensitivity following repeated acid infusion. However, on the basis of pre-
viously reported human psychophysical studies and electro-
physiological recordings from spinal and brain stem neurons of cats it can be speculated that acid exposure results in altered esophageal sensations (15, 31, 32, 35, 38, 39). Human study has also documented that NMDAR antagonist ketamine pre-
vents esophageal hypersensitivity following acute infusion of acid (45). Therefore, our present findings indicate that altered NMDAR expression following acid exposure may contribute to the esophageal hypersensitivity.
GRANTS
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