Peripheral versus central modulation of gastric vagal pathways by metabotropic glutamate receptor 5

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Am J Physiol Gastrointest Liver Physiol 292: G501–G511, 2007. First published October 19, 2006; doi:10.1152/ajpgi.00353.2006.—Metabotropic glutamate receptors (mGluR) are classified into group I, II, and III mGluR. Group I (mGluR1, mGluR5) are excitatory, whereas group II and III are inhibitory. mGluR5 antagonism potently reduces triggering of transient lower esophageal sphincter relaxations and gastroesophageal reflux. Transient lower esophageal sphincter relaxations are mediated via a vagal pathway and initiated by distension of the proximal stomach. Here, we determined the site of action of mGluR5 in gastric vagal pathways by investigating peripheral responses of ferret gastroesophageal vagal afferents to graded mechanical stimuli in vitro and central responses of nucleus tractus solitarius (NTS) neurons with gastric input in vivo in the presence or absence of the mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP). mGluR5 were also identified immunohistochemically in the nodose ganglia and NTS after extrinsic vagal inputs had been traced from the proximal stomach. Gastroesophageal vagal afferents were classified as mucosal, tension, or tension-mucosal (TM) receptors. MPEP (1–10 μM) inhibited responses to circumferential tension of tension and TM receptors. Responses to mucosal tension of mucosal and TM receptors were unaffected. MPEP (0.001–10 nmol icv) had no major effect on the majority of NTS neurons excited by gastric distension or on NTS neurons inhibited by distension. mGluR5 labeling was abundant in gastric vagal afferent neurons and sparse in fibers within NTS vagal subnuclei. We conclude that mGluR5 play a prominent role at gastric vagal afferent endings to distension peripherally and/or centrally, suggesting that it may be rich in molecular targets for inhibiting triggering of transient LES relaxations. Agonists of GABAA, cannabinoid, and -opioid receptors act at various points along vagal and CNS pathways to potently inhibit transient LES relaxations (3, 22–24, 36). Importantly, these actions may be associated with reduced reflux and reflux symptoms in gastroesophageal reflux disease patients (8, 44). However, the side effect profiles of some of these agonists make them less attractive for clinical development.

Recent data show that antagonism of the metabotropic glutamate receptor 5 (mGluR5) potently inhibits transient LES relaxation in conscious ferret (12) and dog models (20) by up to 90%. Glutamate receptors are many and varied in function; in addition to the well-recognized fast synaptic transmission at ionotropic glutamate receptors (iGluR), glutamate also mediates slow synaptic neurotransmission and modulation within the CNS at post- and presynaptic mGluR (11, 13, 14). These diverse effects at mGluR are mediated by group I mGluR (mGluR1 and -5), which cause slow depolarization via positive coupling to phospholipase C, and group II (mGluR2 and -3) and III mGluR (mGluR4, -6, -7, and -8), which cause slow hyperpolarization via negative coupling to adenylyl cyclase and via altered calcium and potassium currents [see review by Cartmell and Schoepf (6)].

Anatomical evidence shows that gastric vagal afferents innervating the ferret proximal stomach express mGluR5 and actively transport mGluR5 to their peripheral endings (34). These afferents also project centrally to the dorsal vagal complex where they synapse preferentially with neurons of the nucleus tractus solitarius (NTS) (39). mGluR5 expression has also been reported in the rat NTS (16, 17), although data from species known to display transient LES relaxations are lacking. Functional data indicate that agonists of inhibitory group II and III mGluR reduce mecanosensitivity of peripheral gastroesophageal vagal afferent endings (34). Transmission of signals from vagal afferent terminals in the NTS is principally glutamatergic, via both excitatory iGluR and mGluR (11), and is modulated presynaptically by a number of inhibitory mGluR (7, 13, 14, 25). The possibility therefore exists that mGluR5 antagonists reduce transient LES relaxations by affecting responses of vagal afferent endings to distension peripherally and/or transmission of vagal signals centrally. However, there is currently no information on whether a peripheral, central, or dual site of action of mGluR5 antagonists may be respon-
sible for these effects on triggering of transient LES relax-

tation.

We tested the hypothesis that the noncompetitive mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) would act at peripheral and central mGluR5 to reduce vagal signals triggered by mechanical stimuli and gastric distension, respec-
tively in ferrets, a species that displays transient LES relax-
ations and gastroesophageal reflex.

MATERIALS AND METHODS

Animal Preparation

Experiments were performed on adult ferrets. All experiments were performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes and with the approval of the Animal Ethics Committees of the Institute of Medical and Veterinary Science (Adelaide, Australia) and the University of Ad-\nelaide. Animals had free access to water and a standard carnivore diet and were fasted overnight before experimentation.

In vitro ferret gastroesophagealafferent preparation. This prepa-
ration has been described in detail previously (30–34). Briefly, after anesthesia and exsanguination, thoracic organs were removed and a gastroesophageal flat-sheet preparation was dissected with attached vagal nerves. This was placed in one chamber of a two-chamber organ bath bathed in a modified Krebs solution composed of (in mM) 118.1 NaCl, 4.7 KCl, 25.1 NaHCO3, 1.3 NaH2PO4, 1.2 MgSO4, 1.5 CaCl2, 1.0 citric acid, and 11.1 glucose, bubbled with 95% O2-5% CO2, pH 7.4. The vagus nerves were led into an adjacent chamber through a small hole, bathed in mineral oil, and dissected into strands for single-fiber electrophysiological recordings.

Characterization of gastroesophageal vagal afferent properties. In the ferret three distinct types of afferents could be identified according to mechanical responsiveness: those responding to circular tension applied via a hook to the edge of the preparation, but not to low-intensity mucosal stroking with von Frey hairs (tension receptors); those responding only to mucosal stroking (mucosal receptors); and those responding to both classes of stimuli (tension/mucosal receptors); and those responding only to mucosal stroking (mucosal receptors).

Afferent fiber was determined by mechanical stimulation throughout the preparation with a brush then more accurately with a blunt glass rod. Accurate quantification of mechanical responsibility of mucosal afferents was measured to movement of von Frey hairs at a rate of 5 mm/s across the receptive field, to which the mean response to the middle 8 of 10 standard strokes given at 1-s intervals was recorded. Tension sensitivity was measured in response to graded forces applied via a cantilever system in the range of 0.5–7 g as a step maintained for 1 min, with the response measured as mean discharge evoked over this period. The tension-response curves were produced by randomly applying weights to the cantilever system in the range of 0.5–7 g. A recovery period of at least 1 min was allowed between each tension stimulus.

Effect of MPEP on mechanical sensitivity of vagal afferents. After mechanical sensitivity of the gastroesophageal vagal afferents had been established, the effect of the noncompetitive mGluR5 antagonist MPEP on mechanical sensitivity was determined in full. MPEP (1 μM) was added to the superfusing Krebs solution and was allowed to equilibrate for 20 min, after which time the tension-response and stroke-response curves were redetermined. This equilibration period was observed so as to ensure penetration of the drug into all layers of the tissue. This procedure was repeated for MPEP at increasingly higher concentrations (3 and 10 μM). The concentrations of MPEP used were as reported at mGluR5 in other in vitro studies and at a concentration that minimized allosteric interactions with mGluR4 (27). Time control experiments were also performed in which there was no significant change in the mechanical response over a compar-

able duration.

In vivo electrophysiological recordings. Ferrets were deeply anes-
thetized with pentobarbitone sodium (50 mg/kg ip). The right carotid artery and vein were cannulated for blood pressure recordings and administration of intravenous anesthetic, respectively; supplemental doses of pentobarbitone were administered as required to abolish the hindlimb pinch-withdrawal reflex. Body temperature was maintained at 37–39°C via a heating pad. A tracheostomy tube was inserted, and a multilumen manometric assembly (4.0 mm outer diameter; Dentsleeve, Wayville, Australia) was introduced into the cervical esophagus and advanced so the tip lay within the proximal stomach. A laparotomy was performed and the location of the assembly tip was confirmed; the assembly was then secured at the neck and a drainage cannula was introduced into the stomach via the pylorus. The gut was closed off immediately distal to the drainage cannula with a ligature, and the laparotomy and neck were sutured closed.

The ferret was placed prone in a stereotactic apparatus, and the brain stem accessed by removing the atlantooccipital membrane and dura mater. A partial occipital craniotomy was performed to allow access to the rostral brain stem and agar-soaked (1.7%) cotton wool was applied to the surround to isolate the recording site. A brain stem stabilizing platform was then positioned at the medulla surface by using a micromanipulator. Artificial cerebrospinal fluid composed of (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 26 NaHCO3, 10 n-glucose, and 4.2 CaCl2, bubbled with 95% O2-5% CO2, pH 7.4 (21), was applied to the brain stem as required to maintain cerebro-
spinal fluid volume throughout the experiment.

An isotonic stimulus was delivered to the ferret stomach via gas insufflation. Constant low-flow oxygen was delivered to the stomach via the central lumen of the esophageal manometric assembly and the exhaust path from the drainage cannula was switched between room pressure and resistance pressure (under 10.8 cmH2O) under the control of a triggered solenoid. This isotonic stimulus produced a 6-mmHg rise in intragastric pressure, as recorded from a side-lumen of the manometric assembly. The trigger was generated by a micro-
1401 (CED, Cambridge, UK) under software command from Spike 2 (5.11; CED) using a sequencer script than enabled selection of distension paradigms. In this way a location stimulus (8-s disten-

tion, 4/min) or test stimulus (30-s distension) could be applied to the stomach to drive gastric mechanoreceptors providing input to the NTS.

Glass microelectrodes filled with electrode buffer (0.5 M NaCl and 0.1% Tween 80; 5–10 MΩ) were then advanced in 5-μm steps, from 200 μm below the brain stem surface to a maximum 1,000-μm depth into the NTS at obex by use of stereotaxic coordinates derived from Boissonade et al. (4). A reference electrode was suspended in cere-
brospinal fluid near the recording site. An area bilateral (0.6–1.4 mm) and rostrocaudal (−0.5 to +1.5 mm) to obex was used to locate neurons responsive to whole stomach distension, based on earlier reports from our laboratory (35). Extracellular signals were amplified (Cyto721; World Precision Instruments, Sarasota, FL), scaled (SA-1; JRAK, Melbourne, Australia), filtered (F-1; JRAK), and monitored on an oscilloscope (DL1300A; Yokogawa, Tokyo, Japan). At each 5-μm step a gastric distension was performed and extracellular signals were scrutinized for single neurons that altered discharge frequency in accord with change in gastric pressure.

Extracellular signals were acquired online (micro-1401 + Spike2 software) along with intragastric and blood pressure (Polygraf HR; Synectics Medical, Stockholm, Sweden). Single NTS neurons that responded to gastric distension by a change in discharge frequency ≥30% from basal discharge were included in analyses; these neurons showed no intrinsic cardiac or respiratory frequency modulation and were unaffected by blood pressure change throughout the experiment. NTS neuron responses to gastric distension were calculated in tripli-
cate per time point as the difference between mean discharge fre-

quency during the steady state of increased intragastric pressure (20 s) and basal discharge frequency calculated over the preceding nondis-
tension period (60 s). Because of the variability in discharge fre-

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quency of individual NTS neurons, group data were normalized for comparison as percent changes in discharge frequency.

In three ferrets the extracellular recording site was anatomically confirmed at the end of the recording session by electrodeionjection (Cyto721; 500 nA/10 min) of 5% micro-ruby dye (Invitrogen, Mount Waverley, Australia) previously loaded into the recording electrode. These ferrets were immediately perfused transcardially with heparinized saline at 40°C and then 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4 at 4°C, and the brain stem was removed, fixed overnight in 4% PFA-PBS at 4°C, and then processed into paraffin and sectioned (5 μm). Dye markings were visualized on an Olympus BX-51 microscope equipped with excitation filters for Alexa Fluor546 (AF546; Invitrogen). Recording sites were found to be restricted to less than 10 neuronal perikarya in the section of interest and located within the medial subnucleus of the NTS near obex (4).

Effects of MPEP on distension responses in single NTS neurons.

Once a single NTS neuron responsive to gastric distension was located, three test distensions (30 s) were used to establish basal and distension-evoked responses. MPEP was then administered intracerebroventricularly (icv), in 10-μl volumes directly to the cerebrospinal fluid above the recording electrode as either a single dose (10 nmol) or as cumulative doses (0.001, 0.1, 1, 10 nmol). The concentrations of MPEP used were as shown to be effective centrally at mGluR5 in other in vivo studies (43) and minimized allosteric interactions with mGluR4 (27). None of the MPEP doses used icv significantly altered blood pressure or respiration. Test distensions were repeated in triplicate at 5, 10, 20, 30, and 60 min (single dose) and at 5 and 10 min within dose (cumulative). Any recording that showed loss of neuron discharge due to impalement or loss of action potential amplitude due to drift was excluded from analysis. Single NTS neuron recording was maintained for up to 150 min after single or cumulative dose experiments to measure recovery after MPEP. Time-control experiments were also performed for 12 separate NTS neurons in which there was no significant change in neuron responsiveness over a comparable duration (P = 0.33).

Data recording and analysis. In vitro and in vivo experiments, single neurons were discriminated offline by use of Spike2 on a personal computer laptop (IBM T40) based on action potential shape, duration, and amplitude. Peristimulus time histograms and instantaneous frequency plots were generated from these neurons by Spike2 analysis. Differences between in vitro stimulus-response relationships were assessed by two-way ANOVA; differences within the in vivo stimulus-response relationship and between basal activities of afferent receptor types in vitro were assessed by one-way ANOVA with Dunnett’s multiple-comparison test for differences at individual MPEP doses (Prism 3.02; Graphpad, San Diego, CA). A P value of <0.05 was considered significant, and results are expressed as means ± SE of the number of animals (n).

Drugs

MPEP stock solutions were kept frozen as aliquots and thawed for use on the day of experimentation either undiluted (in vivo experiments) or diluted to final concentration in Krebs (in vitro). MPEP was obtained from AstraZeneca (Möln达尔, Sweden) or Sigma (Australia).

Retrograde tracing. This protocol has been described in detail previously (33, 34, 40). Briefly, ferrets were anesthetized with isoflurane (2.5% in O2) and a total of 20 μl of 0.5% cholera toxin B subunit conjugated to Alexa Fluor 555 (CTB-AF555; Invitrogen) injected suberosally in 2- to 5-μl aliquots into the anterior and posterior surfaces of the proximal stomach. After 4–6 days, ferrets were anesthetized with pentobarbital (60 mg/kg ip) and perfused transcardially with heparinized saline at 40°C and then 4% PFA in 0.1 M PBS, pH 7.4 at 4°C, and the brain stem and nodose ganglia were removed, fixed overnight in 4% PFA-PBS at 4°C, and then cryoprotected in 30% sucrose at 4°C for 24–48 h. Frozen transverse sections at 20 μm were then cut serially through the rostrocaudal axis of the nodose ganglia and brain stem.

Immunohistochemistry. As previously described in detail (34), mGluR5 immunolabel was detected by a rabbit anti- rat mGluR5 polyclonal primary IgG raised against COOH-terminal sequence LIIRDYTQSSSL (445872; Calbiochem, EMD Biosciences, San Diego, CA). Primary IgG was visualized by a goat anti-rabbit secondary IgG conjugated to Alexa Fluor 488. Sections were air dried (room temperature for 10 min) and rinsed in PBS + 0.2% Triton X-100 (PBS-T, Sigma-Aldrich; pH 7.4) to facilitate antibody penetration. Sections were then treated with 10% normal goat serum in PBS-T to saturate nonspecific binding sites. Following incubation overnight with the primary IgG (1:2–400 in 10% normal goat serum + PBS-T), unbound antibody was removed with PBS-T, and sections were incubated with the relevant secondary IgG (1:200 in PBS-T) and washed again. Sections were drained and mounted with ProLong Antifade (Invitrogen). Slides where the primary IgG was preadsorbed with mGluR5 immunizing peptide (AUSPEP, Parkville, Australia) served as negative controls.

Before use in ferret tissues, mGluR5 primary IgG was tested in the sacral dorsal horn of the rat spinal cord to confirm immunohistochemical target identification in lamina I and II (1). Immunolabel for mGluR5 was cytoplasmic, perinuclear, and membrane bound in somata and membrane bound in dendritic projections and was absent in sections where the primary IgG were preadsorbed with immunizing peptide (data not illustrated).

Visualization and Quantification

High-power epifluorescent images were obtained on a conventional epifluorescence microscope (BX-51, Olympus, Australia) equipped with excitation filters for rhodamine (CTB-AF555) and fluorescein (Alexa Fluor 488, mGluR5) and images acquired on a Photometrics CoolSnapfx monochrome digital camera (Roper Scientific, Tucson, AZ). A differential interference contrast (Nomarski DIC) stage was used for imaging total neurons in the nodose ganglia. Percent retrogradely traced and single and dual-labeled neurons were calculated using UTHSCSA Image Tool version 3.0 (University of Texas, Health Sciences Center, San Antonio, TX); labeled neurons were counted only for “central profile counts,” indicative of completely labeled cells, whereas cell fragments were ignored. Brain stem sections were dual imaged in fluorescence and differential interference contrast and overlaid to align cytarchical features of NTS subnuclei described in ferret (4) with mGluR5 immunolabel and retrograde tracer. Fluorescence images from nodose ganglia and brain stem were then imported unmodified into V + + imaging software version 4.0 (Digital Optics, Auckland, New Zealand), pseudocolored, and merged for composite images; luminance intensity was not adjusted.

RESULTS

Effect of MPEP on the Mechanical Sensitivity of Gastroesophageal Vagal Afferents

Receptive fields of tension receptors were randomly distributed in the esophagus (n = 4), stomach (n = 2), and LES (n = 1) in this study and showed identical responses to mechanical stimuli and MPEP irrespective of location; mucosal and TM receptor receptive fields were all located within the esophagus (n = 6 and 8, respectively). Receptive fields were small (1–3 mm2) and distinct. Ferret tension receptors and TM receptors responded to circumferential tension in vitro with increasing occurrence of action potentials (see, for example, Fig. 1D) as we have previously described (30–34). mGluR5 antagonism with MPEP (1, 3, and 10 μM) significantly reduced the stimulus response function of tension receptors (P < 0.01, P <
0.001, and \( P < 0.01 \), respectively). This effect was concentration dependent at the lower tension stimuli (0.5–3 g, Fig. 1B). Circumferential tension responses of TM receptors were also significantly reduced by 3 and 10 \( \mu \)M MPEP \( (P < 0.01, \text{Fig. 1CII}) \). Mucosal and TM receptors responded to mucosal stroking with von Frey hairs showing graded increases in numbers of action potentials evoked with increasing von Frey forces (Fig. 1, A and CII). No inhibition of stimulus response to mucosal stroking was seen in the presence of MPEP in either class of afferents. In fact, MPEP significantly increased the response of mucosal stroking was seen in the presence of MPEP in either class of afferents. In fact, MPEP significantly increased the response of mucosal and TM receptors to mucosal stroking at the lowest dose of 1 \( \mu \)M \( (P < 0.05, \text{Fig. 1, A and CII}) \). MPEP (1–10 \( \mu \)M) did not significantly affect the basal discharge of vagal gastroesophageal mucosal or tension receptor afferents; however, basal discharge of TM receptors was significantly reduced in the presence of 3 and 10 \( \mu \)M MPEP \( (P < 0.05, \text{Table 1}) \).

**In Vivo Extracellular Recordings**

A total of 24 responsive single neurons were recorded from the NTS of 32 ferrets; 19 neurons showed increased action potential discharge (excited) during isobaric gastric distension, and 5 neurons showed a decrease in action potential discharge (inhibited) during distension (for examples, see Fig. 2, A and D). Both excited and inhibited responses were graded according to the extent of gastric distension, with 6 mmHg evoking a submaximal response in preliminary studies (data not illustrated). Recording sites of responsive neurons were located in a restricted region of the NTS, extending +1.0 to −1.0 mm rostrocaudal of obex (opening of the fourth ventricle floor), 0.6–1.4 mm bilateral from midline and at 400–1,000 \( \mu \)m depth from the brain stem surface, measured by stereotaxic coordinates (Fig. 3, C and D) and recording site marking (Fig. 3B); neurons
inhibited by gastric distension tended to be located in more ventral regions of the medial subnucleus of the NTS (mnNTS; Figs. 2D and 3D).

**Effect of MPEP on NTS Neurons Excited by Gastric Distension**

The effect of MPEP applied icv was tested on 19 NTS neurons excited by gastric distension: on 5 as a single dose of 10 nmol (Fig. 2) and on 14 as cumulative doses of 0.001–10 nmol (Fig. 3A). At a dose of 10 nmol, MPEP blocked responses of two NTS neurons and significantly attenuated the response of one neuron (Fig. 2, A, B, and E). MPEP was without effect in two neurons excited by distension (Fig. 2, C and E). Neither basal discharge of NTS neurons nor heart rate was affected by MPEP (data not illustrated). Pooled responses from separate cumulative dose-response experiments on 14 neurons excited by gastric distension tested with MPEP (0.001–10 nmol, icv) are shown in Fig. 3A. This demonstrates that MPEP effects were observable at doses greater than 1 nmol icv, even when nonresponders were included. In single or cumulative dose experiments NTS neurons excited or inhibited by gastric distension were reliably recorded up to 150 min; however, NTS neurons blocked or attenuated by MPEP did not recover responsiveness during this time.

**Effect of MPEP on NTS Neurons Inhibited by Gastric Distension**

The effect of MPEP was tested on five NTS neurons inhibited by gastric distension. At a single dose of 10 nmol MPEP did not affect any NTS neuron inhibited by gastric distension (Fig. 2, D and E) and did not significantly alter basal discharge (data not illustrated).

**Retrograde Tracing**

Injection of CTB-AF555 into the proximal gastric muscle resulted in bright cytoplasmic labeling within neurons of nodose ganglia and their primary axons (see Fig. 5A). There was no evidence of selective distribution of tracer in neurons at the rostral, mid, or caudal pole of the ganglion in ferret. Counts in four retrograde traced ferrets showed that 8 ± 1% of nodose ganglion neurons innervated the proximal stomach.

In brain stem, cell bodies and primary dendrites of vagal efferents located within the dorsal motor nucleus of the vagus (DMN) were filled with CTB-AF555 rostrocaudally and bilaterally throughout obex (−2.0 to 2.0 mm, Fig. 4). Primary dendrites of filled DMN neurons were also clearly seen projecting medially caudal to obex along the ventral floor of the commissural subnucleus and at obex and rostral, dorsolaterally into the mnNTS (Fig. 4B, middle). On many occasions primary dendrites could be traced in serial sections into rostral regions of the mnNTS. A larger proportion of left DMN neurons were labeled by proximal stomach injection of CTB-AF555 compared with right DMN. Gastric vagal afferent terminal fields were filled with CTB-AF555 at and rostral of obex in the subnucleus gelatinosus (SG) and dorsomedial margins of the mnNTS (Fig. 4B, middle and right). Afferent terminal fields were diffuse compared with the punctate labeling seen in DMN dendrites, were restricted to a narrower rostral range (0.0–1.5 mm), and were separate from DMN dendrite fields except for a region immediately rostral to obex where they intermingled at the dorsomedial border of the mnNTS (0.5–1.0 mm, Fig. 4B, right). CTB-AF555-filled afferent fibers were also evident to a lesser extent in dorsolateral portions of the NTS over this anatomical range.

**mGluR5 Immunohistochemistry**

**Nodose ganglia.** In the nodose ganglia there was a widespread labeling of soma with mGluR5 immunolabel evident in cytoplasm. Of the total cells counted, 72 ± 4% of nodose ganglia neurons labeled for mGluR5. Of the retrogradely labeled gastric neurons, 79 ± 6% of nodose ganglia cells colabeled for mGluR5 (Fig. 5A). These data are directly comparable with those we obtained previously using a different retrograde tracer in ferrets (34).

**Medulla oblongata.** Within the brain stem mGluR5 immunolabel was seen caudal to obex in ventrolateral and dorsolateral regions of the NTS; label associated with fibers was also seen medial to these regions in the emerging mnNTS and in the area postrema (Fig. 5, B and C). At obex, dorsolateral regions of the NTS, likely representing the intermediate subnuclei (nI) of the NTS and dorsolateral mnNTS, contained high levels of mGluR5 label with intermediate levels seen in the medial portion of the mnNTS. mGluR5 label in these regions appeared to be associated with fibers rather than soma. mGluR5 label was largely absent from the SG (Fig. 5D). In regions rostral to obex the dorsolateral and medial portions of the mnNTS and nI remained immunopositive for mGluR5, and a thin band of intermediate level label was evident bordering the medial edge of the SG whereas ventrolateral regions of the NTS containing the subnucleus centralis (19) were mGluR5 immunonegative (Fig. 5E). At high power, gastric afferent terminal fields in the rostral SG were largely immunonegative for mGluR5, except for a small region that flanked the dorsomedial border of the mnNTS (Fig. 5E'). Throughout the rostrocaudal extent of the dorsal vagal complex DMN neurons and their dendrites were immunonegative for mGluR5 whereas solitary tract and hypoglossal nucleus (XII) showed either rare labeling of fibers or were immunonegative (Fig. 5D).

**DISCUSSION**

Our data provide the first evidence that mGluR5 antagonists reduce the mechanosensitivity of subtypes of primary vagal sensory endings. We have further demonstrated that the principal site of action of the mGluR5 antagonist MPEP in gastric vagal circuitry of the ferret is on afferent endings in the periphery where gastroesophageal tension and TM receptor mechanosensitivity was significantly reduced. In contrast, mGluR5 appear to play a minor role in the central vagal...
pathway linked to gastric distension, since there were minor effects of MPEP on NTS neurons excited by gastric distension and sparse mGluR5 immunolabeling in NTS subnuclei receiving input from retrogradely labeled gastric afferent terminals. Since MPEP potently inhibits triggering of transient LES relaxations in ferrets (12), these results suggest that development of peripherally restricted mGluR5 antagonists may provide a novel therapeutic for the management of patients with gastroesophageal reflux disease.

**mGluR5 Effects on Peripheral Gastroesophageal Afferents**

Results in ferret gastroesophageal vagal afferents extend our earlier findings with G protein-coupled receptor agonists to the...
inhibitory GABA<sub>B</sub> receptor (30) and inhibitory group II and group III mGluR (34) and show that an antagonist to the excitatory mGluR5 reduces mechanosensitivity of ferret tension and TM receptors. This provides the first direct evidence of mGluR5 involvement in excitatory modulation of the sensory receptive field of gastroesophageal afferents. MPEP effects on tension receptors were concentration dependent at lower levels of circular tension (0.5- to 3-g load) and had maximal effects on TM receptors in a similar range, confirming that pharmacological effects on mGluR5 occurred at physiologically relevant loads.

It is has been established that the mechanotransduction sites of vagal tension receptors in the stomach and esophagus correspond to intraganglionic laminar endings (IGLEs) (47, 48). IGLEs express the vesicular glutamate transporters VGLUT1 and VGLUT2 (10, 37, 38) and possess synaptic specializations like their central terminals (29). These facts point to a role for IGLEs in peripheral release of glutamate. Combined with our evidence of mGluR5 expression in ferret gastric afferents, peripherally directed vagal transport of mGluR5 (34), and inhibition of tension and TM receptor responses by mGluR5 antagonists, these data suggest that mechanical stimulation leads to endogenous glutamate release from gastroesophageal IGLEs, which in turn activates mGluR5 autoreceptors on the same endings. mGluR5 autoreceptors have been previously shown to facilitate synaptic neurotransmission in vitro and in vivo in rat brain (26, 42); mGluR5 autoreceptors on gastroesophageal IGLEs are candidates for serving such a facilitatory role in the periphery.

Interestingly, inhibition of gastroesophageal vagal afferent mechanosensitivity by MPEP was restricted to tension sensitivity with no change in responses to mucosal stroking. This finding contrasts with our earlier findings using GABA<sub>B</sub> receptor and group II and III mGluR agonists, which also inhibited mucosal receptor responses in ferrets (30, 34). This difference suggests that mGluR5 are either absent from mucosal receptors in ferrets or not endogenously activated because they are inaccessible to endogenous glutamate released from IGLEs. This modality-restricted action of MPEP may also be relevant in the context of reflux disease, in which tension-sensitive gastric vagal afferents are considered the primary trigger for transient LES relaxation and thus gastroesophageal reflux (9, 18). Pharmacotherapy targeted at peripheral mGluR5 in reflux disease patients may therefore offer advantage over strategies targeting other receptors within this vagal pathway because of a reduced likelihood of off-target effects related to reducing mucosal receptor input. This study also provides evidence that mGluR5 are constitutively active in ferret tension-sensitive afferents, because MPEP concentration dependently reduced basal activity in TM receptors and showed a

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**Fig. 4.** Retrogradely traced gastric vagal inputs to the ferret dorsal vagal complex. A: representative diagrams of the ferret dorsal vagal complex (0.5 mm caudal to 1.0 mm rostral of obex) adapted from Boissonade et al. (4). B–D: representative images of CTB-AF555 through the rostrocaudal extent of the ferret dorsal vagal complex traced from proximal stomach. B: 0.5 mm caudal to obex; gastric traced dorsal motor nucleus of the vagus (DMN) perikarya and dendrites extending medially toward commissural subnucleus of NTS (ncom). B': higher power view of boxed area in B showing traced gastric DMN neurons and processes. C: 0.0 mm obex; traced gastric DMN perikarya with dendrites extending dorsomedially into medial subnucleus of the NTS (mNNTS); afferent terminal fields first visible in subnucleus gelatinosus (SG). C': higher power view of boxed area in C showing punctuate labeling of DMN dendrite fields. D: 1.0 mm rostral of obex; gastric traced DMN perikarya with dendrites visible in mNNTS; DMN dendrites and gastric afferent terminal fields intermingling at the mNNTS-SG border. D': higher power view of boxed area in D showing diffuse gastric afferent terminal filling in the SG by CTB-AF555. cc, Central canal. Scale bars (A) = 1 mm, (B–D) = 500 µm, (B'–D') = 100 µm.
similar trend in tension receptors. We conclude that mGluR5 autoreceptors on mechanosensitive gastrointestinal vagal afferent endings are preferential targets for endogenous glutamate under both basal and stimulated conditions.

Although ours is the first report of the actions of MPEP on vagal afferents, there is existing indirect evidence for a role of peripheral mGluR5 in nociceptive transmission in somatic afferents. Following intraplantar inflammation in rat hindpaw, in vivo and in vitro evidence show that MPEP is antihyperalgesic and acts solely at a peripheral site of action (41, 45). This has led to speculation that mGluR antagonists might provide a novel management tool for treatment of chronic inflammatory pain. We propose that, equally, pharmacotherapy with peripherally directed mGluR5 antagonists may be effective in diseases that may involve disordered afferent signaling from the gut, such as gastroesophageal reflux disease.

**mGluR5 on Gastric NTS Neurons**

Electrophysiological studies showed that single neurons excited or inhibited by gastric distension could be identified in the ferret NTS as in our laboratory’s earlier report (35). Recording site markings and stereotaxic coordinates showed that single neurons recorded were within the lateral mNTS near obex, although we cannot exclude the possibility that some extracellular recordings were from DMN dendrites penetrating the mNTS. MPEP acted rapidly and potently to inhibit a minority of NTS neurons excited by gastric distension. These neurons were located in both dorsal and ventral margins of the mNTS, indicating a broad anatomical distribution and confirming efficient penetration of MPEP following icv administration. However, the majority of neurons tested with MPEP over a similar dorsoventral range showed no effect or were modestly excited or inhibited.
afferent types. In contrast, mGluR5 immunolabeling was strong and notably in dorsolateral regions of the NTS. In combination with our electrophysiological data, these results suggest either a predominant peripheral site of action of the group I mGluR antagonist MPEP on gastric vagalafferent endings, with a minor role of central mGluR5 on higher order NTS interneurons linked to gastric mechanosensory input. This action of MPEP in the periphery is likely to be the major contributing mechanism to the efficacy of mGluR5 antagonists reported on transient LES relaxation, and we propose that peripherally restricted, well-targeted mGluR5 antagonists may be an effective strategy in disease management.

GRANTS
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REFERENCES


ferrets, regions that integrate and relay complex input from the upper esophagus, larynx, pharynx, trachea, soft palate, and tongue in other species (46). This may in part explain the reduced swallowing seen in conscious ferret studies with mGluR5 antagonists (12), although this has yet to be directly tested.

In conclusion, these studies have completed a comprehensive characterization of mGluR5 in ferret gastric vagal pathways. Results here point to a predominant peripheral site of action of the group I mGluR antagonist MPEP on gastric vagal afferent endings, with a minor role of central mGluR5 on higher order NTS interneurons linked to gastric mechanosensory input. This action of MPEP in the periphery is likely to be the major contributing mechanism to the efficacy of mGluR5 antagonists reported on transient LES relaxation, and we propose that peripherally restricted, well-targeted mGluR5 antagonists may be an effective strategy in disease management.
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