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Endomorphin-1 Modulates Intrinsic Inhibition in the Dorsal Vagal Complex

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Glatzer NR, Derbenev AV, Banfield BW, Smith BN. Endomorphin-1 modulates intrinsic inhibition in the dorsal vagal complex. *J Neurophysiol* 98: 1591–1599, 2007. First published July 5, 2007; doi:10.1152/jn.00336.2007. Mu-opioid receptor (MOR) agonists profoundly influence digestive and other autonomic functions by modulating neurons in nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV). Whole cell recordings were made from NTS and DMV neurons in brain stem slices from rats and transgenic mice that expressed enhanced green fluorescent protein (EGFP) under the control of a GAD67 promoter (EGFP-GABA neurons) to identify opioid-mediated effects on GABAergic circuitry. Synaptic and membrane properties of EGFP-GABA neurons were assessed. The endogenous selective MOR agonist endomorphin-1 (EM-1) reduced spontaneous and evoked excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) in both rat and mouse DMV neurons. Electrical stimulation of the solitary tract evoked constant-latency EPSCs in ~50% of EGFP-GABA neurons, and the responses were reduced by EM-1 application. EM-1 reduced action potential firing, the frequency and amplitude of synaptic inputs in EGFP-GABA neurons and responses to direct glutamate stimulation. A subset of EGFP-GABA neurons colocalized mRFP1 after retrograde, transneuronal infection after gastric inoculation with PRV-614, indicating that they synapsed with gastric-projecting DMV neurons. Glutamate photolysis stimulation of intact NTS projections evoked IPSCs in DMV neurons, and EM-1 reduced the evoked response, most likely by activation of MOR on the soma of premotor GABA neurons in NTS. Naltrexone or H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), MOR antagonists, blocked the effects of EM-1. Our results show that GABA neurons in the NTS receive direct vagal afferent input and project to gastric-related DMV neurons. Furthermore, modulation by EM-1 of specific components of the vagal complex differentially suppresses excitatory and inhibitory synaptic input to the DMV by acting at different receptor locations.

INTRODUCTION

The dorsal vagal complex (DVC) forms a sensory-motor circuit that provides parasympathetic control of the thoracic and abdominal viscera through the vagus nerve. Viscerosensory information carried by vagal afferents is initially processed by neurons in the nucleus tractus solitarius (NTS). These neurons in turn make abundant glutamatergic and GABAergic synaptic connections with neurons in the dorsal motor nucleus of the vagus (DMV), which are the major source of preganglionic vagal motor control of the gastrointestinal tract and other subdiaphragmatic viscera. In particular, GABAergic connections between the NTS and DMV exert important control over vagal motor neuron activity and gastric function (Davis et al. 2004; Travagli et al. 1991, 2006).

Opioid peptides have inhibitory effects on synaptic activity in the DVC (Appleyard et al. 2005; Browning et al. 2002, 2004; Glatzer and Smith 2005; Rhim et al. 1993). Electrophysiological recordings in vivo and in vitro have shown that μ-opioid receptor (MOR) agonists reduce solitary tract input to the NTS in rats and mice (Appleyard et al. 2005; Glatzer and Smith 2005; Rhim et al. 1993; Sapru and Chitravanshi 2002). Endomorphins are endogenous neuropeptide agonists for the MOR and are prominent in the spinal cord and brain stem, including the NTS (Martin-Schild et al. 1999). Endomorphin-1 (EM-1) potently inhibited solitary tract and other synaptic input to NTS neurons in the rat (Glatzer and Smith 2005). MORs are found in abundance in the DVC, and their activation has been shown to affect gastric functions acting at the level of DMV motor neurons (Burks et al. 1985, 1987; Del Tacca et al. 1987; Ding et al. 1996).

In the DMV, previous studies analyzed effects of the MOR agonist met-enkephalin on synaptic input evoked after electrical stimulation of the NTS (Browning et al. 2002). Whereas evoked excitatory postsynaptic currents (EPSCs) were suppressed by activating MOR on glutamatergic terminals, there was no effect of met-enkephalin on inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation of the NTS under normal recording conditions (Browning et al. 2002). However, pretreatment with forskolin (Browning et al. 2004) or vagal deafferentation (Browning et al. 2006) induced a presynaptic inhibition of GABA release in response to MOR agonists, presumably caused by insertion of the receptors into the terminal membrane. However, effects in the DMV that are attributable to actions at the soma of afferent NTS neurons have not been determined.

Inhibitory neurons in the NTS potently regulate premotor output to the DMV in the vagal complex. Identification of this population of GABAergic NTS neurons in a slice preparation presents many technical challenges. To overcome this problem, we used the GIN (GFP-expressing inhibitory neurons) mouse line that contains a gene for enhanced green fluorescent protein (EGFP) under the control of a GAD67 promoter, which expresses EGFP at visibly detectable levels in known GABAergic neurons (Oliva et al. 2000, 2002) to target GABAergic NTS neurons in medullary slices prepared from these mice. We studied the effects of EM-1 on GABAergic synaptic transmission to the DMV and also on identified GABAergic NTS neurons. We tested the hypotheses that *1*) EM-1 modulates inhibitory synaptic input to rat and mouse DMV neurons; *2*) EM-1 modulation of NTS neurons decreases overall synaptic

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inhibition in the DMV; and *3*) EM-1 modulates GABAergic NTS to DMV connections within the slice.

METHODS

Gastric injection of PRV 152 and PRV 614

The gene encoding EGFP or monomeric red fluorescent protein (mRFP1, a generous gift of Dr. Roger Tsien) was inserted in the *gG* gene of the Bartha strain of the transneuronal viral tracer pseudorabies virus (Banfield et al. 2003; Smith et al. 2000). Male Sprague-Dawley rats 2–6 wk old (Harlan) or 3–9 wk old transgenic mice expressing EGFP under the control of the mouse GAD67 promoter [FVBTgN (GAD67GFP) 45704Swn; Jackson Laboratory; Oliva et al. 2000] were housed with a 12-h light/dark cycle, with food and water provided ad libitum. Breeding pairs of transgenic mice were purchased from Jackson Laboratory, and pups were raised in the animal facilities of Tulane University and the University of Kentucky.

Details of methods regarding use of viral constructs to label gastric-related brain stem neurons for electrophysiological and morphological analyses have been reported previously (Glatzer et al. 2003). Under sodium pentobarbital anesthesia (50 mg/kg, ip), animals received three to four injections (1 μl each) into the ventral stomach wall of PRV-152 (a generous gifts of Dr. Lynn Enquist) or PRV-614 over a 1-min interval using a 10-μl Hamilton syringe fitted with a 26-gauge needle. A fresh stock of virus was thawed for each injection. Animals were maintained in a biosafety level 2 facility for 60–72 h after injection.

Slice preparation

Electrophysiological experiments were performed on brain stem slices from 3- to 6-wk-old adult male Sprague-Dawley rats (Harlan) or 3- to 9-wk-old transgenic mice [FVBTgN (GAD67GFP 45704Swn]. All the animals were treated according to the rules of the Tulane University or University of Kentucky Animal Care and Use Committee. The animals were deeply anesthetized with halothane (Sigma-RBI, St. Louis, MO) inhalation and decapitated. The brain was removed and blocked on an ice-cold stand, and the medulla was glued to a stage and mounted on a Vibratome. Slices (300–400 μm thick) were prepared in the coronal plane (i.e., transverse), cutting the brain stem from dorsal to ventral in 0–2°C artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 124 NaCl, 3 KCl, 1.3–2 $CaCl_2$, 1.3 $MgCl_2$, 1.4 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose, pH 7.2–7.4; osmolarity between 300 and 320 mOsm/kg. Slices were incubated for ≥1 h in 35°C oxygenated (95% O_2-5% CO_2) ACSF. After incubation, the slice was transferred to a superfusing recording chamber on a fixed stage under an upright microscope (Olympus, Melville, NY), where the slice was continually superfused by room temperature ACSF. Neurons expressing EGFP or mRFP1 were visualized in living tissue under epifluorescence by using fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) filter sets (Smith et al. 2000). Once the cell of interest was targeted, infrared/differential interference contrast (IR/DIC) optics were used to obtain recordings.

Electrophysiology

Voltage-clamp recordings made in whole cell mode with an Axopatch 200B or 700A amplifier (Axon Instruments, Union City, CA), digitized at 88 kHz (Neurocorder; Cygnus Instruments, Dorchester, UK), and low-pass filtered at 2–5 kHz were recorded onto videotape and a PC running Clampex 8.0 software (Axon Instruments). Patch pipettes (4–7 MΩ) were pulled from borosilicate glass (1.65 mm OD, 0.45 mm wall thickness; Garner Glass Company, Claremont, CA) and filled with (mM) 130 potassium-gluconate (or Cs-gluconate), 10 HEPES, 1 NaCl, 1 $CaCl_2$, 3 KOH (or CsOH), 5 EGTA, 2–4 Mg-ATP, and 0.1% biocytin. D-2-Amino-5-phosphono-pentanoic acid (AP-5; 50 μM; Sigma), picrotoxin (50 μM; Sigma), 6-cyano-7-nitroquinoxaline-2,3-dionedisodium (CNQX; 10 μM; Sigma), endomorphin-1 (Tyr-Pro-Trp-Phe-NH2; EM-1; 10 nM to 10 μM; Sigma), naltrexone HCl (20 μM; Sigma), and H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; 1–3 μM; Sigma) were bath applied with the ACSF. The criteria for detecting synaptic currents were fast 10–90% rise time (<1 ms) and exponential decays. Electrical stimulation was performed using a platinum-iridium concentric bipolar electrodes (125 μm diam, FHC, Bowdoinham, ME) placed in either the NTS or solitary tract (ST). For electrical stimulation of the NTS, the NTS was stimulated by paired current pulses (2–100 μA; 300–400 μs; A.M.P.I., Jerusalem, Israel) at pairing frequencies of 10–20 Hz at a cycle rate of 0.2 Hz. For electrical stimulation of the solitary tract, sets of two to five current pulses (2–25 μA; 300–400 μs) were applied at 40 Hz. Resting membrane potentials, assessed by removing the voltage clamp, were corrected for junction potential (approximately −8 mV). Action potential frequency was measured over a 2-min period of continuous recording. Frequency and amplitude changes for PSCs within a recording were examined over 2 min of continuous recording before, during, and after a drug application using a Kolmogrov-Smirnov test for frequency and an unpaired Student's *t*-test for amplitude, and across recordings with a paired Student's *t*-test. The concentration-response curve and IC_{50} were calculated as previously described (Glatzer and Smith 2005), by fitting a sigmoidal curve to the points of the concentration response plot using Prism 6.0 (Graphpad, San Diego, CA). All drug-induced changes were considered significant at $P < 0.05$. All values are shown as ±SE.

Chemical stimulation with caged glutamate photolysis

Similar to previous descriptions of glutamate photolysis in the vagal complex (Davis et al. 2003, 2004; Glatzer and Smith 2005; Williams et al. 2007), γ-(-carboxy-2-nitrobenzyl) ester, trifluoroacetic acid salt (i.e., CNB-caged glutamate, 250 μM; Molecular Probes, Eugene, OR) was added to recirculating ACSF and uncaged using brief pulses of UV light directed into the slice. Fluorescent light (UV filter, Chroma Technology, Rockingham, VT) was directed onto the slice through the ×40 objective used to obtain the recording, which was moved progressively further away from the recorded cell until a photolysis-mediated increase in synaptic events was found. Exposure time was electronically controlled using a shutter (Vincent Associates, Rochester, NY). Opening the shutter with no UV filter or with other filters in place (e.g., FITC) did not result in uncaging. Uncaging glutamate directly onto the recorded neuron (9-s interval, 20- to 30-ms exposure) resulted in a fast inward current (50–200 pA at a holding potential of −60 mV; $n = 4$), or a large depolarization ($n = 7$), which response was repeatable in single cells in >100 consecutive applications ($n = 4$). Photic stimuli were applied on the ST and at sites just outside the NTS for negative controls (i.e., no synaptic responses were observed). The effective diameter of the uncaging (~50 μm) was set by apertures in the light path and measured by moving the center of the illumination away from the cell and testing for a direct inward current after uncaging. The distance moved was analyzed posthoc by comparison with a scale slide.

Histology

After recording, brain slices were fixed in 4% paraformaldehyde in 0.15 M sodium phosphate buffer overnight at 4°C (pH = 7.4) and processed as previously described (Glatzer et al. 2003). After three rinses with 0.01 M PBS, whole mount slices were immersed in Avidin conjugated to AMCA or Texas Red (1:400; Vector Laboratories) in PBS containing 0.5% Triton X-100 and incubated overnight at 4°C to identify biocytin-filled neurons and confirm they were labeled with EGFP or mRFP1. Slices were rinsed 2–3 times with PBS and mounted on slides to air dry for 10 min. The slices were covered in Vectashield

(Vector Laboratories; Burlingame, CA). Cells labeled with biocytin during a recording and/or with EGFP or mRFP1 or in transgenic mice were identified with a Leica DMLB microscope, and images were captured with a Spot RT CCD camera (Diagnostic Instruments) using filters for the Avidin-conjugated fluorescent dyes and EGFP or mRFP1. Neurons were required to be positively identified and documented by one or both of these methods to be considered gastric-related DMV neurons or EGFP-GABA neurons.

RESULTS

Recordings in DMV neurons

Whole cell patch-clamp recordings were made from 52 neurons in the rat DMV and 7 DMV neurons from transgenic mice. Qualitative or quantitative differences were not seen between infected and uninfected DMV neurons, so the data were pooled unless otherwise specified. Using a potassium gluconate intracellular solution (K-gluconate), average resting membrane potential was -57 ± 3 mV, and input resistance averaged 466 ± 96 MΩ ($n = 16$, including 5 PRV-152–labeled neurons). Consistent with previous findings (Browning et al. 2002), EM-1 caused an outward current in 7/16 neurons, including 4/5 PRV-152–labeled DMV neurons. The average amplitude of the outward current was 13.6 ± 3.6 pA at a holding potential of -65 mV ($n = 7$). Neurons that responded to EM-1 application with an outward current also showed a decrease in input resistance that averaged -54 ± 20 MΩ ($n = 7$; $P < 0.05$; paired *t*-test). No significant change to input resistance was observed in response to EM-1 application using intracellular solution with Cs^+ as the primary cation (425 ± 73 MΩ in control ACSF, 455 ± 92 MΩ in EM-1 1 μM; $n = 21$; $P > 0.05$).

Effects of EM-1 on excitatory synaptic inputs to DMV neurons

Electrical stimulation of the NTS using a concentric bipolar stimulating electrode resulted in excitatory and inhibitory synaptic inputs to DMV neurons. Pharmacological blockade of the inhibitory inputs (picrotoxin, 50 μM) resulted in expression of only excitatory glutamatergic inputs ($V_m = -65$ mV); all EPSCs were blocked by *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists. Analogous to other MOR agonists (Browning et al. 2004), application of EM-1 (10 nM to 10 μM) resulted in a concentration-dependent reduction in the amplitude of the evoked EPSC ($IC_{50} = 136$ nM). The reduction was blocked by the opioid receptor antagonist naltrexone HCl (25 μM) and was reversible after 15–20 min of wash in normal ACSF. Bath application of EM-1 (1 μM) reduced the average NTS-evoked EPSC amplitude from -38.0 ± 6.0 to -20.0 ± 4.2 pA ($n = 7$; $P < 0.05$; paired *t*-test). The paired-pulse ratio (PPR) of two consecutive stimuli was measured to see if EM-1 may have caused a change in the presynaptic glutamate release. The PPR significantly increased in the presence of EM-1 from 0.70 ± 0.09 to 1.00 ± 0.09 ($n = 7$; $P < 0.05$; paired *t*-test). Application of EM-1 (1 μM) reduced mean spontaneous EPSC frequency from 4.2 ± 1.3 to 1.3 ± 0.4 Hz ($P < 0.05$); EPSC amplitude was not significantly affected (15.7 ± 4.4 pA, control; 12.6 ± 2.4 pA in EM-1; $P > 0.05$; $n = 6$). To determine whether the reduction in EPSC frequency by EM-1 could occur independently of Na^+-dependent synaptic release (i.e., action potentials in afferent intact neurons), the effects of EM-1 on miniature EPSCs (mEPSCs) were measured in the presence of TTX (2 μM) in five neurons (including 2 EGFP-labeled neurons). The mean frequency of mEPSCs was 2.1 ± 0.5 Hz, and amplitude was -8.5 ± 0.8 pA in control ACSF. The mEPSC frequency was significantly reduced to 1.2 ± 0.5 Hz in EM-1 (1 μM; $n = 5$), whereas amplitude was unaltered (-7.8 ± 0.7 pA; $n = 10$; $P > 0.05$, paired *t*-test). The reduction in mEPSC frequency was blocked by CTOP or CTAP, MOR-selective antagonists (1–3 μM; $96 \pm 2\%$ of control frequency; $n = 5$; $P > 0.05$).

Effects of EM-1 on inhibitory synaptic inputs to DMV neurons

We tested the effects of EM-1 on spontaneous IPSCs (sIPSCs) in gastric-related and unlabeled DMV neurons. Spontaneous IPSCs were recorded at -10 to 0 mV, and fast glutamate currents were blocked using CNQX (10 μM) and AP-5 (50 μM). In the rat DMV, the average frequency of sIPSCs was 1.3 ± 0.2 Hz, and average amplitude was 25.5 ± 6.5 pA ($n = 9$). After application of EM-1 (1 μM), sIPSC frequency and amplitude were significantly reduced to 0.8 ± 0.2 Hz and 22.9 ± 6.0 pA, respectively (Fig. 1; $n = 9$ including 6 PRV-labeled neurons; $P < 0.05$; Student's paired *t*-test). In the presence of TTX, the average mIPSC frequency was 0.9 ± 0.3 Hz, and the average amplitude was 27.6 ± 2.0 pA. After application of EM-1 (1 μM), mIPSC frequency was 0.8 ± 0.3 Hz, and average amplitude was 25.6 ± 2.6 pA, with no significant change to either measurement (Fig. 1; $n = 6$, including 2 PRV-152–labeled neurons; paired *t*-test; $P > 0.05$). EM-1 reduced sIPSC frequency in rat DMV neurons, but the effects were not seen when action potentials were blocked with TTX.

Spontaneous IPSCs were also recorded in unlabeled DMV neurons in slice preparations from GAD67-EGFP mice to verify that MOR activation affected inhibitory synaptic input in a manner similar to the rat. Average frequency of sIPSCs was 3.9 ± 1.8 Hz, and average amplitude was 32.3 ± 2.7 pA ($n = 5$) in this set of cells. After application of EM-1 (1 μM), sIPSC frequency and amplitude were significantly reduced to 1.8 ± 0.7 Hz and 25.7 ± 4.3 pA, respectively ($n = 5$; Kolmogrov-Smirnov; $P < 0.05$). The effects of EM-1 were prevented by CTAP ($n = 4$; $P > 0.05$). The effects of EM-1 on sIPSCs in DMV neurons in the GAD67-EGFP mouse were similar to the effects of EM-1 on DMV neurons in the rat.

Effects of EM-1 on IPSCs evoked after electrical stimulation of the NTS

Electrical stimulation of the NTS evoked constant latency GABAergic IPSCs in DMV neurons (Davis et al. 2004; Travagli et al. 1991). The average amplitude of the IPSCs was 73.3 ± 12.9 pA ($V_m = 0$ mV; $n = 7$). EM-1 (1 μM) caused a reduction in the amplitude of electrically evoked GABAergic IPSCs, reducing the average IPSC amplitude to 28.4 ± 6.9 pA ($n = 6$, including 2/2 PRV-152–labeled DMV neurons; Fig. 2). The effect of EM-1 was blocked by application of CTAP ($n = 4$; Fig. 2). The PPR did not change in the presence of EM-1 (1 μM; 0.90 ± 0.06 in control, 0.97 ± 0.08 in EM-1; $n = 6$; paired *t*-test; $P > 0.05$). That the PPR was not altered by EM-1

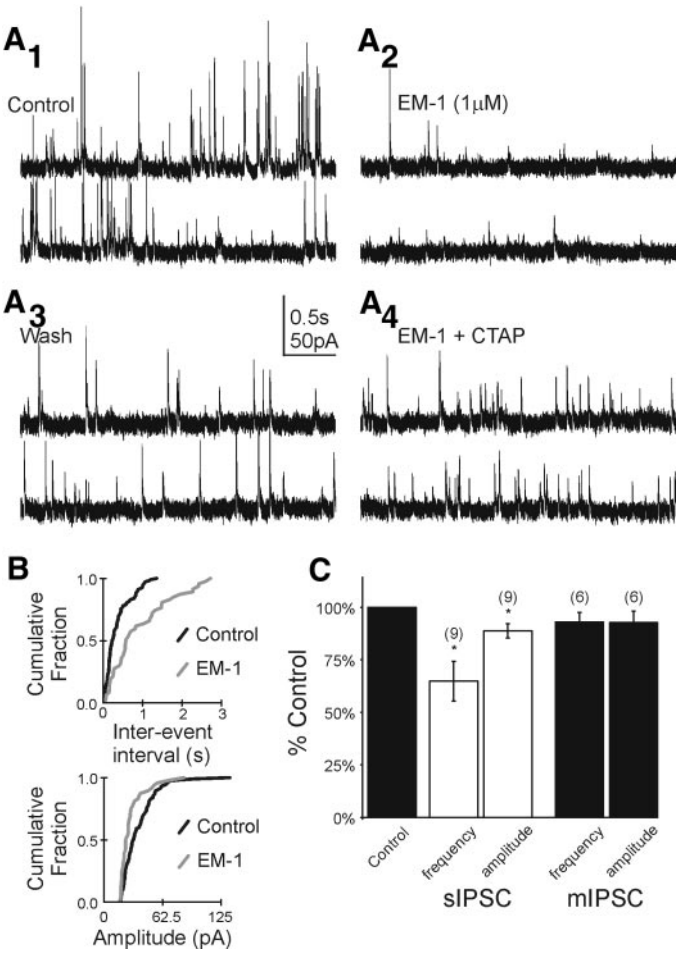

FIG. 1. Endomorphin-1 (EM-1) suppressed spontaneous inhibitory postsynaptic currents (IPSCs) in the dorsal motor nucleus of the vagus (DMV). *A*: sample traces from a DMV neuron voltage clamped at 0 mV in the presence of AP-5 (50 μM) and CNQX (10 μM). Cs^+ was present in the recording pipette. *A1*: control. *A2*: in EM-1 (1 μM). *A3*: wash. *A4*: 2nd application of EM-1 in the presence of H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP; 3 μM). Effects of EM-1 were prevented by pretreatment with CTAP. *B*: cumulative fractions of interevent interval (*top*) and amplitude (*bottom*) for the sample neuron over a 2-min continuous recording before (control) and during EM-1 application. *C*: effects of EM-1 (1 μM) on spontaneous IPSC (sIPSC) and miniature IPSC (mIPSC) frequency and amplitude. Numbers of replicates in parentheses. *Significant change vs. control ($P < 0.05$).

suggested that the reduction in amplitude of the first response was not caused by the effects of EM-1 on receptors located on terminals contacting DMV cells under normal conditions, as previously shown (Browning et al. 2004). However, unlike that study, the amplitude decreased significantly in our hands, and sIPSCs but not mIPSCs were reduced in frequency. We therefore reasoned that the suppression of IPSCs was caused by inhibition of intact GABA neurons by EM-1.

Recordings from GABAergic NTS neurons in transgenic mice

EM-1 reduced electrically evoked IPSCs, but did not seem to involve MOR activity at GABAergic terminals in the DMV under normal conditions. We therefore tested the hypothesis that identified GABA neurons in the NTS were directly inhibited by EM-1. Recordings were made from 26 EGFP-labeled NTS neurons from a transgenic mouse line that expresses EGFP under the control of a GAD67 promoter (Oliva et al. 2000).

Neurons within the NTS that expressed EGFP were widespread throughout the rostral-caudal and dorso-lateral NTS (Fig. 3). Although the neurons were distributed throughout the NTS, there was a relatively dense cluster found in the interstitial nucleus in the dorsolateral NTS (Paxinos et al. 1999; Stornetta and Guyenet 1999). Ten of 14 GABAergic NTS neurons fired spontaneous action potentials at >0.5 Hz, and only one neuron did not fire action potentials at rest, although it did fire in response to current injection (Fig. 4). Membrane properties of GABAergic NTS neurons are shown in Table 1.

GABAergic NTS neurons innervated gastric-projecting DMV neurons

Injection of the retrograde viral tracer PRV-614 (see METHODS) in the wall of the stomach of GAD67-EGFP mice resulted in transsynaptic labeling of parasympathetic preganglionic motor neurons in DMV and gastric-related putative premotor neurons in the NTS (Fig. 3), similar to other PRV constructs (Glatzer et al. 2003; Glatzer and Smith 2005; Rinaman et al. 1999). PRV-614 expresses mRFP1 (Campbell et al. 2002) and can be used for multicolor labeling with other fluorophores such as EGFP (Banfield et al. 2003). The number of NTS neurons labeled by PRV-614 was quantified in a previous study in rats and shown to be between 10 and 20% of NTS neurons at 60–72 h after infection (Glatzer et al. 2003). Our study showed a subset of the GABAergic neurons were also labeled with PRV-614 (Fig. 3, *C–E*). Some of these neurons, as well as EGFP-GABA neurons that were not retrogradely labeled transneuronally from the stomach, were targeted for recording (Figs. 3 and 4).

Effects of EM-1 on solitary tract input to GABAergic NTS neurons

The synapse of the central primary afferents from the vagus and glossopharyngeal nerve has characteristic properties that

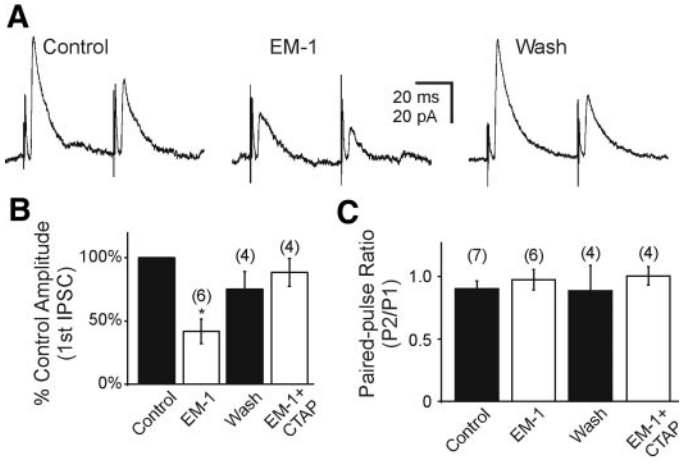

FIG. 2. EM-1 suppressed IPSCs evoked after electrical stimulation of the nucleus tractus solitarius (NTS). *A*: sample average traces from a DMV neuron showed inhibition of evoked IPSCs during application of EM-1. Stimulus artifact was partially reduced for clarity. Ten to 15 traces are averaged for each trace. *B*: average decrease in amplitude of the 1st pulse compared with control. *C*: ratio of *pulse 2* to *pulse 1* in averages of traces where both stimulations resulted in an IPSC. Number of replicates averaged in parentheses.

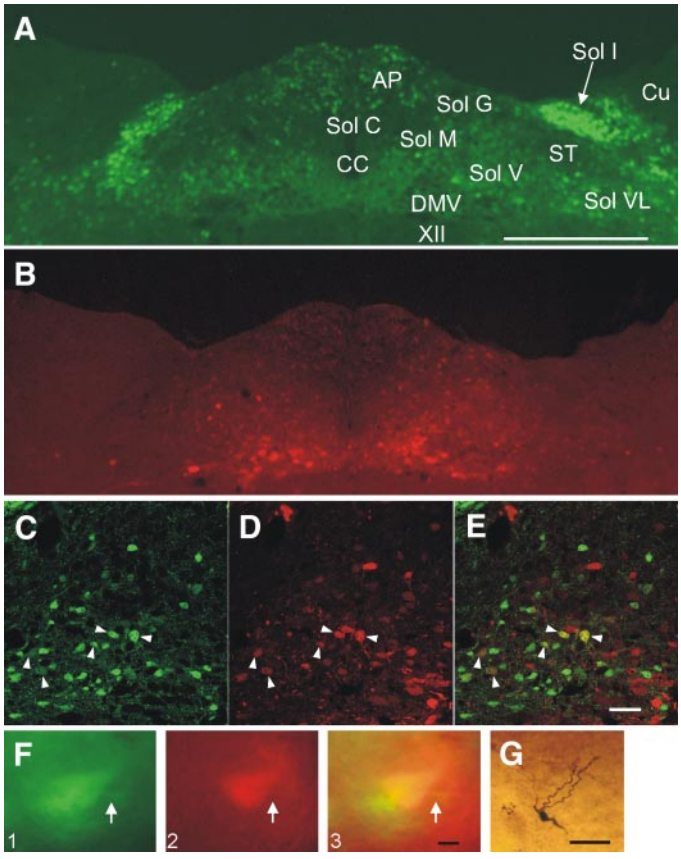

FIG. 3. Distribution and recording of GAD67-enhanced green fluorescent protein (EGFP) and PRV-614–labeled neurons in the dorsal vagal complex (DVC). *A*: GABAergic neurons were scattered throughout the DVC, including many subnuclei of the NTS and a small number in the DMV. Scale bar equals 500 μm. *B*: PRV-614 infected motor neurons in the DMV and neurons in the NTS. *C–E*: confocal images of the NTS; optical sections had a thickness of 1 μm. A small subset of the EGFP-labeled neurons in the medial NTS were double-labeled with mRFP1. Scale bar = 50 μm. *F*: GABAergic neuron that was also retrogradely labeled with PRV-614. *1*: on-line image of a cell targeted for recording showing EGFP. *2*: PRV-614. and *3*: overlay of the two images. Arrow points to the recording pipette in each image. Scale bar = 5 μm. *G*: post hoc identification of the recorded neuron in *F* after labeling with biocytin during whole cell recording. Scale bar = 50 μm. AP, area postrema; CC, central canal; Cu, nucleus cuneatus; DMV, dorsal motor nucleus of the vagus; Sol C, commissural NTS; Sol G, gelantinous NTS; Sol I, interstitial NTS; Sol M, medial NTS; Sol V, ventral NTS; Sol VL, ventrolateral NTS; ST, solitary tract; XII, hypoglossal nucleus.

distinguish it from other inputs received in the NTS. Notably, there is a frequency-dependent depression of the input and an almost total blockade by CNQX near resting membrane potential (Doyle and Andresen 2001; Miles 1986; Smith et al. 1998). Previous studies in rats indicated that MOR activation reduced ST-evoked input to a phenotypically unidentified subset of NTS neurons (Rhim et al. 1993; Glatzer and Smith 2005). Of 14 EGFP-GABA NTS neurons tested, 10 received EPSCs that followed at constant latency (i.e., <400-μs variability in response latency over 10 stimuli) after electrical stimulation of the solitary tract (Figs. 4 and 5). At a V_m of −65 mV, the average amplitude of the ST-evoked EPSC was –52.5 ± 10.9 pA and was blocked completely by CNQX (10 μM; Fig. 4). EM-1 (1 μM) reduced the average EPSC amplitude in to –27.9 ± 9.4 pA ($n = 5$; $P < 0.05$). In the presence of CTAP (1–3 μM), a MOR antagonist, the effect of EM-1 on ST input to EGFP-GABA NTS neurons was blocked (102 ± 5% of

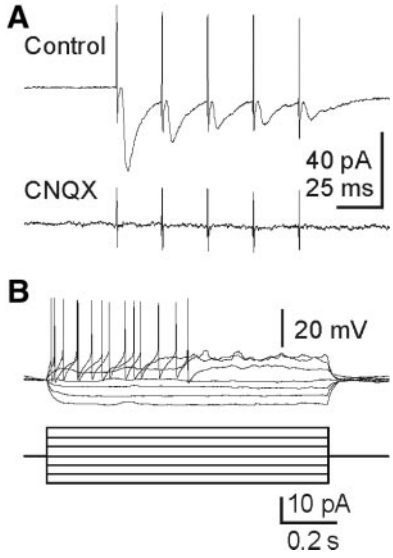

FIG. 4. Electrophysiological responses of GABAergic NTS neurons identified in a mouse model expressing EGFP under a GAD67 promoter. *A*: most of the EGFP-GABA neurons received constant latency excitatory postsynaptic currents (EPSCs) after electrical stimulation of the solitary tract (ST). An example trace from a GABAergic neuron is shown. Evoked EPSCs were blocked by CNQX (10 μM) in the same neuron. Traces shown are averages of 10–15 trials. *B*: EGFP-GABA NTS neurons displayed membrane properties similar to other NTS neurons, including action potentials in response to injected current. A single trace is shown in response to each current injection.

control ESPC amplitude, $n = 5$; Fig. 5*B*). The PPR increased in the presence of EM-1 (58 ± 6% in control; 108 ± 20% in EM-1; $P < 0.05$; 1 μM, $n = 5$; Fig. 5*C*). These data indicated direct ST input to identified GABA neurons was commonly observed. This input was inhibited by EM-1, and MORs were likely present on ST terminals contacting GABA neurons.

Effects of EM-1 on inhibitory synaptic transmission to GABAergic NTS neurons

Spontaneous IPSCs recorded in EGFP-labeled NTS neurons were blocked by application of picrotoxin ($n = 3$). Spontaneous IPSCs were recorded either using Cs-gluconate electrode solution at a depolarized holding potential (−10 to 0 mV) or using KCl-containing electrode solution in the presence of AP5/CNQX, which blocked all EPSCs (Derbenev et al. 2004; Glatzer et al. 2003; Smith et al. 1998), and revealed reversed IPSCs. EM-1 reversibly reduced the frequency and amplitude of sIPSCs in NTS neurons. Average frequency of sIPSCs was 2.0 ± 0.5 Hz, and average amplitude was 26.0 ± 3.8 pA. After application of EM-1 (1 μM), sIPSC frequency was reduced to

TABLE 1. *Electrophysiological Properties of GAD-67 EGFP-labeled NTS neurons*

Properties	Value
RMP, mV	−53 ± 2.2 (18)
AP frequency, Hz	2.4 ± 0.7 (14)
Threshold, mV	−33 ± 1.7 (14)
Input resistance, MΩ	1180 ± 144(16)
Capacitance, pF	12.5 ± 0.4 (16)
AHP, mV	−57 ± 1.8 (14)

Number of replicates is shown in parentheses. RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization.

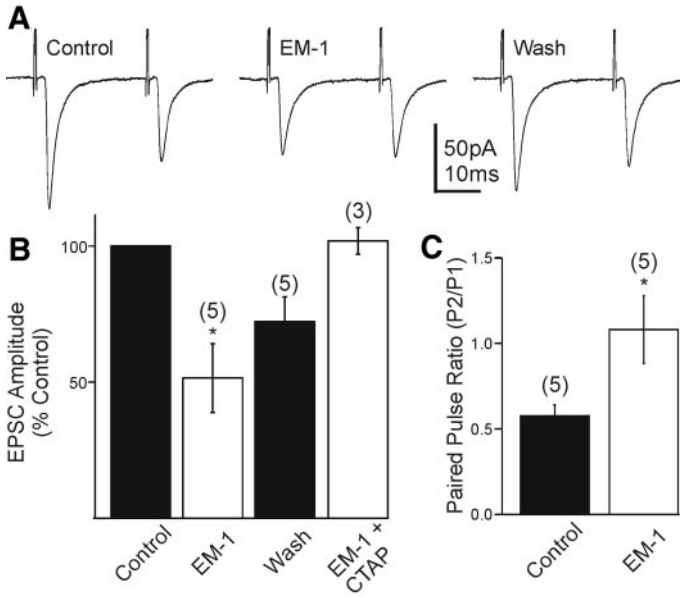

FIG. 5. EM-1 reduced ST-evoked, constant-latency EPSCs in EGFP-GABA NTS neurons. *A*: traces showing average responses to pairs of ST stimulations before, during EM-1 application (1 μM), and after application of EM-1 (V_m = −65 mV). Pairs of EPSCs evoked 25 ms apart (40 Hz) resulted in paired-pulse depression of the 2nd response. *B*: percent change in evoked EPSC amplitude from NTS neurons in the presence of EM-1, after washout, and in the presence of mu-opioid receptor (MOR) antagonist CTAP (1–3 μM). *C*: EM-1 (1 μM) reduced amplitude of the 1st pulse to a greater extent than the 2nd compared with control (V_m = −65 mV). *Significant change vs. control ($P < 0.05$). Numbers of replicates in parentheses.

0.7 ± 0.2 Hz and amplitude to 16.8 ± 4.7 pA ($n = 6$; $P < 0.05$, Student *t*-test). The effects of EM-1 were blocked by CTAP ($n = 4$) and TTX (2 μM; $n = 4$; $P > 0.05$; paired *t*-test), indicating an effect on MORs that reduced action potential firing in GABAergic neurons afferent to the recorded neurons. In addition to decreasing ST input and hyperpolarizing GABA neurons, inhibitory input to GABA neurons was also suppressed.

Effects of EM-1 on membrane potential of GABAergic NTS neurons

A hyperpolarization from resting membrane potential (−8.2 ± 2.3 mV; range, −4 to −18 mV) was seen in six of eight GABAergic NTS neurons in response to 1 μM EM-1, recorded using a K-gluconate electrode solution. No hyperpolarization was seen in any of five GABAergic NTS neurons recorded with Cs^+ in the recording pipette. Spontaneous action potential firing was reduced from an average of 2.4 ± 0.7 Hz in control to 0.2 ± 0.1 Hz in the presence of EM-1 (Fig. 6; $n = 5$; $P < 0.05$; *t*-test), including in GABAergic NTS neurons that were also labeled with PRV-614 after gastric inoculation ($n = 2$). In addition to modulating endogenous action potentials, the effect of EM-1 on glutamate-evoked action potential firing was examined. Caged glutamate was photoactivated at the site of the recorded neuron to cause a glutamate-evoked depolarization of the cell ($n = 7$). EM-1 (1 μM) suppressed the activity evoked by uncaging glutamate directly on the recorded NTS neuron ($n = 5$ GABA neurons and 2 unidentified neurons; Fig. 6). EM-1 therefore hyperpolarized the membrane potential of GABA neurons, including those related to motor output to the stomach, and reduced responsiveness to glutamate-induced depolarization.

Effects of EM-1 on glutamate photolysis stimulation of the NTS to DMV inhibitory circuit

Previous studies have focused on activation of MOR located on afferent terminals in the DMV, using mainly electrical stimulation of the NTS to measure evoked IPSCs (Browning et al. 2004, 2006), which can activate both somata and cut axons. Electrical stimulation and the physical disruption caused by introduction of the electrode may actually disrupt neuronal activity and result in preferrential activation of axons versus somata (Callaway and Katz 1993; Davis et al. 2004). To identify the effects of EM-1 on input from intact neurons within the slice, we used caged glutamate, which is activated by UV light, to depolarize intact NTS neurons while recording in the DMV (Callaway and Katz 1993; Davis et al. 2003, 2004; Glatzer and Smith 2005). Neurons were recorded at a holding potential of 0 mV so that evoked EPSC amplitude was minimal

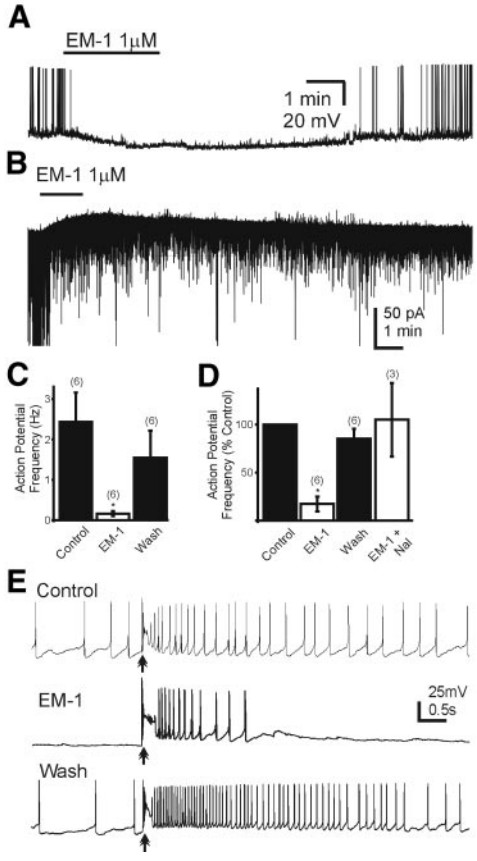

FIG. 6. EM-1 inhibited the membrane potential and firing properties of GABAergic NTS neurons. *A*: EM-1 (1 μM) reduced action potential firing and holding current in a sample neuron recorded in current clamp with no injected current. *B*: EM-1 induced an outward current and reduction in spontaneous EPSCs in voltage clamp (V_m = −65 mV). *C*: graph of action potential frequency in control, EM-1, and after washout. *D*: graph of action potential frequency as a percentage of control in the presence of EM-1, after washout, and in EM-1 applied with opioid receptor antagonist naltrexone (25 μM). *Significant change from control ($P < 0.05$). *E*: glutamate photolysis directly on a recorded GABAergic NTS neuron resulted in a barrage of action potentials. *Top*: control. *Middle*: EM-1 (1 μM). *Bottom*: wash. Arrows indicated uncaging. Potassium gluconate was used for electrode solution in all traces.

and IPSC amplitude was outward and relatively large. Focal, short-duration exposures to UV light directed through the microscope objective into the NTS (<50 μm diam; 15–30 ms) resulted in a glutamate uncaging that increased synaptic activity in DMV neurons ($n = 8$; Fig. 7). Moving the spot >50 μm from the area that generated a response resulted in loss of the stimulated response, suggesting an effective uncaging diameter of <100 μm. Stimuli directed onto the ST or to areas adjacent to the NTS were ineffective, suggesting that responses to NTS stimulation were caused by activation of neurons within the NTS that connected to DMV neurons. Application of TTX (2 μM) blocked the evoked IPSCs ($n = 3$), indicating that they were caused by actions potentials generated in intact, afferent neurons, as described in several previous studies (Callaway and Katz 1993; Dalva and Katz 1994; Davis et al. 2003, 2004; Katz and Dalva 1994). At a holding potential of 0 mV, uncaging glutamate in various areas of the NTS resulted in an increase in the number of IPSCs recorded during a variable period (100–500 ms) after the stimulation (Fig. 7). The average number of events recorded in the period before the stimulation was subtracted from the number after stimulation to measure the changes in the increase between the control condition and during EM-1 application. EM-1 significantly reduced the glutamate-stimulated increase in IPSC frequency in four of five DMV neurons (including each of 3 PRV-152–labeled neurons; paired t-test, $P < 0.05$). Inhibitory NTS input to DMV neurons was inhibited by EM-1 and appeared to involve suppression of action potentials in afferent GABA neurons rather than binding to MORs on GABAergic terminals.

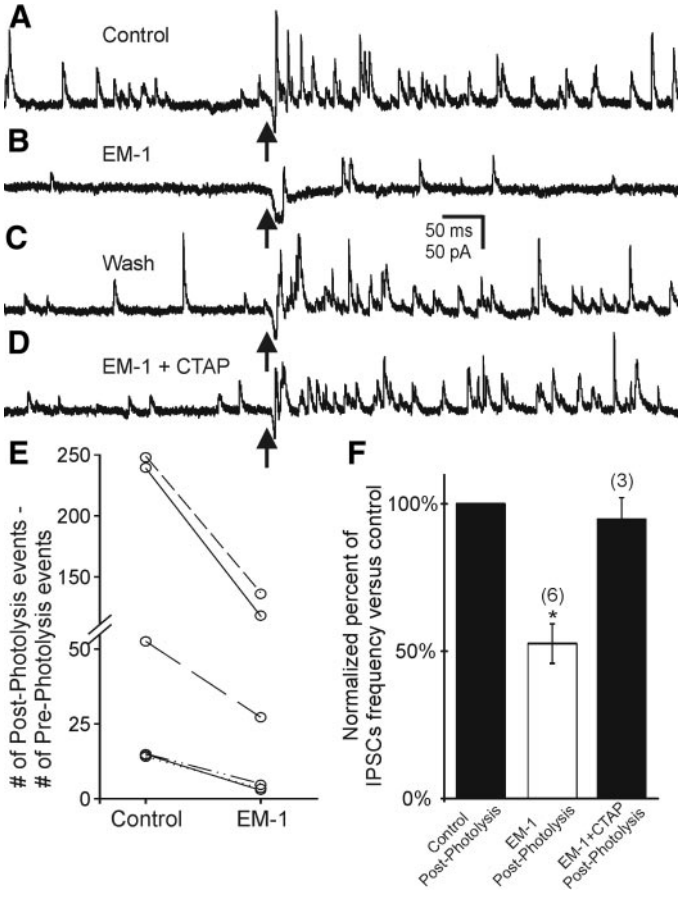

FIG. 7. EM-1 suppressed inhibitory currents recorded in the DMV evoked by photolysis of caged glutamate within NTS. *A*: glutamate uncaging in the NTS (arrow) resulted in evoked IPSCs (holding potential = 0 mV) in a rat DMV neuron. Synaptic responses to glutamate uncaging (arrow) are shown in control artificial cerebrospinal fluid (ACSF). *B*: responses to glutamate uncaging (arrow) are shown in the presence of EM-1 (1 μM). *C*: responses to uncaging (arrow) after 20-min wash to control ACSF. *D*: responses to glutamate uncaging are not reduced by EM-1 in the presence of CTAP (1 μM). *E*: average frequency of IPSCs during 100-ms periods before and after glutamate uncaging ($n = 6$). Uncaging occurred at *time 0* (arrow). *F*: normalized effects of EM-1 (1 μM; $n = 6$) and MOR antagonist CTAP (1 μM; $n = 3$) on pre- and postphotolysis IPSC frequency (500 ms) and on the evoked currents. *Significant effect of the agonist vs. preagonist response ($P < 0.05$).

DISCUSSION

Immunohistochemistry for endomorphins has shown they are present at sensory afferent terminals and in second-order sensory neurons within the NTS (Martin-Schild et al. 1999; Pierce and Wessendorf 2000). Robust staining for EM-1 in perikarya in the dorsomedial NTS suggests a role in gastric sensory regulation (Martin-Schild et al. 1999), because this is the area primarily contacted by gastric sensory afferents (Shapiro and Miselis 1985). MOR agonists, including EM-1, have consistently been shown to inhibit glutamatergic synaptic transmission in the NTS and DMV by acting at glutamatergic synaptic terminals (Appleyard et al. 2005; Browning et al. 2002; Glatzer and Smith 2005; Rhim et al. 1993). These results support the hypothesis that MOR activation in the DMV reduces excitatory glutamatergic inputs to DMV neurons and specifically to the gastric-projecting DMV motor neurons. The magnitude and consistency of the MOR inhibition of electrical and spontaneous EPSCs in this study were similar to that observed by Browning et al. (2002, 2004). This study also showed that MORs are functional on GABAergic NTS neurons and that they inhibit both local GABAergic transmission and GABAergic projections to the DMV. The glutamate photolysis experiments show that EM-1 reduces the IPSCs caused by inhibition of "premotor" GABAergic NTS neurons, suppressing intact inhibitory connections in the slice. Previous studies concluded that the effect of MOR agonists on electrically evoked IPSCs in the DMV could occur subsequent to activating receptors on presynaptic GABAergic terminals, but only after translocation of the receptors into the membrane (Browning et al. 2004, 2006). Our results further show that receptors on the soma-dendritic compartment of GABA neurons in the NTS can normally mediate an MOR-induced reduction in inhibitory drive to the DMV. EM-1 thus exerts a strong inhibitory effect on both glutamatergic and GABAergic synaptic input to NTS and DMV neurons, albeit by different means. This implies a high degree of specificity for opioideregic modulation of different neural components of the DVC.

Modulation of inhibition in the DVC by EM-1

Synaptic responses in the DMV to stimulation of the NTS reflected the complexity of the excitatory and inhibitory projections. Electrical stimulation produces a constant latency fast EPSC or IPSC and sometimes results in a transient increase in PSC frequency (Davis et al. 2004; Travagli et al. 1991). We used a model wherein a reduction in the presynaptic probability of release in response to electrical stimulation of the NTS in the

presence of EM-1 would result in a greater reduction in the first PSC of the pair, whereas a change in postsynaptic response would result in equal reduction of both PSCs (Kline et al. 2002; Regehr and Stevens 2001). A change in evoked EPSC PPR was observed after EM-1 application, and mEPSCs were reduced in frequency. These results support the hypothesis that functional MOR are normally located in glutamatergic terminals in the DMV.

Electrically evoked responses occur subsequent to activation of neural elements originating in or passing through the NTS to contact the DMV neurons. Glutamate microstimulation of the NTS produces both excitatory and/or inhibitory responses in DMV neurons caused by depolarization and increased action potentials in intact NTS neurons projecting to the DMV (Davis et al. 2004) and not fibers of passage. The inhibitory response evoked by caged glutamate stimulation was reduced dramatically by application of EM-1. Previous reports indicated preterminal MORs require adenyl cyclase activity to be functional (Browning et al. 2004). Our data, which specifically identified responses originating in activity of afferent GABA neurons, indicate that MOR-mediated inhibition of intact GABA neurons (i.e., on the somatodendritic portion of NTS GABA neurons) suppresses inhibitory input to the DMV that arises in the NTS. In support of this, identified GABAergic NTS neurons were hyperpolarized by EM-1, and the response to glutamate-induced activation of GABAergic NTS neurons was suppressed. There was also an EM-1–induced reduction in input resistance in the DMV, consistent with a reduction in both sEPSC and sIPSC amplitude. Consistent with the findings of Browning et al. (2002, 2004), we found no evidence for preterminally located MORs under normal conditions. Thus neither the electrically evoked IPSC PPR nor the mIPSC frequency, measures of putative preterminal receptor activation, was altered by EM-1. Inhibition of action potentials in GABA cells afferent to the DMV could reflect functionally different MOR effects on inhibitory circuits in the DVC, depending on the location of the opioidergic input (NTS vs. DMV) and the functional state of the system (e.g., elevated cAMP).

GABAergic NTS cells are premotor and/or second order sensory neurons

We tested the hypothesis that MOR activation inhibited GABAergic NTS neurons using a transgenic mouse line (i.e., GIN mice) in which GABAergic neurons express EGFP. The transgene has been shown to label GABAergic neurons, although the subtypes of GABAergic neurons expressing GFP varies through different brain regions (Heinke et al. 2004; Oliva et al. 2000). The distribution of the EGFP-labeled neurons in the NTS appeared qualitatively similar to that of immunohistochemical staining for glutamic acid decarboxylase (GAD) (Blessing et al. 1984) and GABA (Izzo et al. 1992) performed in other studies. Use of this transgenic mouse allowed a straightforward method for identifying and targeting these neurons for recording a priori. The finding that most GABA neurons receive primary viscerosensory input, as shown by constant-latency EPSCs generated after stimulation of the TS, is suggestive of their position as second-order sensory neurons. As such, they could act as either a direct relays to the DMV or as interneurons, helping to coordinate afferent signals from various viscera at the level of the first central viscerosensory synapse. In addition, the NTS neurons double-labeled with both mRFP1 (through viral label from the stomach) and EGFP (through transgene expression) were considered to be GABAergic gastric-related premotor NTS neurons. Our findings regarding the membrane properties of GABAergic NTS neurons are consistent with previous conclusions from immunohistochemically identified cells (Kawai and Senba 1999).

Implications of EM-1 effects on GABAergic NTS neurons

Generalized administration of MOR agonists such as morphine can cause a pronounced reduction in all synaptic activity in the DVC. In contrast, spatially specific endogenous release of opioid agonists would presumably bind MORs to reduce specific aspects of synaptic input. If released in the DMV, opioids like met-enkephalin would normally cause an inhibition of the preganglionic neurons, through membrane hyperpolarization and/or inhibition of glutamatergic input to gastric-projecting DMV neurons (Browning et al. 2002). However, GABA release would not be reduced by MOR activation at terminals in the DMV under basal conditions because the MORs on GABAergic terminals are not active except under specific circumstances (Browning et al. 2002, 2004, 2006). Conversely, a *disinhibition* of DMV activity would occur if EM-1 were selectively released within the NTS because of the site of activity on glutamatergic synaptic terminals and GABAergic cell bodies and dendrites in the NTS. Application of MOR agonists to the NTS results in increased parasympathetic output (Kotz et al. 1997), which tends to be orexigenic, and this could be caused by inhibition of a tonically active inhibitory GABAergic projection to gastric-projecting DMV neurons. Thus activation of MORs in the DVC can result in variable responses in preganglionic motor neurons, which depends on the location of the release within the DVC in addition to the functional state of the system. Changes in preganglionic function can alter the excitatory/inhibitory balance in the enteric nervous system, causing up- or downregulation of digestive and other autonomic functions.

The results of this study indicate that EM-1 and other MOR agonists released in the NTS can have a net excitatory effect on vagal motor neuron output by suppressing excitatory solitary tract and other glutamatergic input to GABAergic NTS neurons and by hyperpolarizing them. MORs are present on glutamatergic terminals of NTS neurons in DMV and on glutamatergic terminals contacting NTS cells, and they are present on the somatodendritic region of NTS GABA neurons, including neurons projecting to the DMV. Activation of MORs in the NTS tends to decrease inhibition of DMV neurons, including gastric-projecting DMV neurons associated with digestion, whereas activation of MORs in DMV tends to decrease excitation of DMV neurons under normal conditions. These results support the hypothesis that spatially specific opioid peptide connections and cellular activity levels combine to produce precise regulation of gastric-related DMV neuron activity.

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