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Presynaptic ionotropic glutamate receptors modulate GABA release in the mouse dorsal motor nucleus of the vagus

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Abstract

Regulation of GABA release in the dorsal motor nucleus of the vagus (DMV) potentially influences vagal output to the viscera. The presence of functional ionotropic glutamate receptors (iGluRs) on GABAergic terminals that rapidly alter GABA release onto DMV motor neurons has been suggested previously, but the receptor subtypes contributing to the response is unknown. We examined the effect of selective activation and inhibition of iGluRs on tetrodotoxin-insensitive, miniature inhibitory postsynaptic currents (mIPSCs) in DMV neurons using patch-clamp recordings in brainstem slices from mice. Capsaicin, which activates transient receptor potential vanilloid type 1 (TRPV1) receptors and increases mIPSC frequency in the DMV via an iGluR-mediated, heterosynaptic mechanism, was also applied to assess GABA release subsequent to capsaicin-stimulated glutamate release. Application of glutamate, NMDA, or kainic acid (KA), but not AMPA, resulted in increased mIPSC frequency in most neurons. Inhibition of AMPA/KA receptors reduced mIPSC frequency, but selective antagonism of AMPA receptors did not alter GABA release, implicating the presence of presynaptic KA receptors on GABAergic terminals. Whereas NMDA application increased mIPSC frequency, blocking NMDA receptors was without effect, indicating that presynaptic NMDA receptors were present, but not activated by ambient glutamate levels in the slice. The effect of NMDA was prevented by AMPA/KA receptor blockade, suggesting indirect involvement of NMDA receptors. The stimulatory effect of capsaicin on GABA release was prevented when AMPA/KA or NMDA, but not AMPA receptors were blocked. Results of these studies indicate that presynaptic NMDAR and KA receptors regulate GABA release in the DMV, representing a heterosynaptic arrangement for rapidly modulating parasympathetic output, especially when synaptic excitation is elevated.

Keywords

capsaicin; kainate receptor; mIPSC; NMDA receptor; presynaptic; TRPV1

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INTRODUCTION

Neurons in the dorsal motor nucleus of the vagus (DMV) regulate parasympathetic output to most of the subdiaphragmatic viscera and therefore critically control feeding, digestion, hepatic glucose production, and insulin secretion, among other metabolic functions. DMV neurons tend to fire action potentials at fairly regular intervals, and this activity is modulated by synaptic input (Browning et al., 1999). In particular, GABAergic inhibitory inputs arising from the nucleus tractus solitarius and elsewhere in the brain prominently regulate moment-to-moment DMV neuron activity, whereas excitatory, glutamatergic synaptic inputs are thought to contribute phasically during periods of increased vagal afferent activity (Travagli et al., 2006, Browning and Travagli, 2011). Thus, the balance of glutamatergic and GABAergic synaptic inputs importantly regulates the activity of DMV neurons and consequently, vagal motor output to the viscera, and excitatory drive associated with specific vagal afferent signaling occurs in the context of ongoing synaptic inhibition.

In the DMV and elsewhere in the brain, glutamate acts as the principal excitatory neurotransmitter (Travagli et al., 1991, Davis et al., 2004, Babic et al., 2011), activating postsynaptic ionotropic glutamate receptors (iGluR) to generate excitatory postsynaptic currents (EPSCs). In addition, glutamate binds metabotropic glutamate receptors (mGluR) in the DMV to modulate responsiveness of GABAergic presynaptic terminals (Browning et al., 2006, Browning and Travagli, 2007, Babic et al., 2012, Babic and Travagli, 2014). In several brain regions, iGluRs on synaptic terminals (i.e., presynaptic receptors) have been identified functionally as autoreceptors to modulate glutamate release or heteroreceptors to alter GABA release (Berretta and Jones, 1996, Liu et al., 1999, Duguid and Smart, 2004). In the DMV, presynaptic N-methyl-D-aspartate (NMDA) receptors (preNMDAR) on glutamatergic terminals contribute to ongoing glutamate release tonically (Bach and Smith, 2012, Bach et al., 2015), and preliminary evidence has been presented to support the hypothesis that functional presynaptic iGluRs on terminals of inhibitory neurons may enhance GABA release (Derbenev et al., 2006). Activation of transient receptor potential vanilloid type 1 (TRPV1) receptors increases both glutamate and GABA release in the DMV; at least a component of GABA release modulation occurs subsequent to TRPV1-induced, glutamate-mediated heterosynaptic activation of iGluRs on GABAergic terminals (Derbenev et al., 2006, Derbenev and Smith, 2013). Since GABA plays a prominent role in regulating DMV motor neuron activity, rapid modulation of GABA release by presynaptic iGluR activation could potentially affect DMV neuron activity and, consequently, parasympathetic output to the viscera. The type(s) of terminally-located iGluR that serve to modulate GABA release in the DMV, however, is not known.

We tested the hypothesis that activation of iGluRs on GABAergic synaptic terminals modulates GABA release in the mouse DMV. Whole-cell patch-clamp electrophysiological recordings from DMV neurons in brainstem slices were used to record GABAergic, miniature inhibitory synaptic current (mIPSC) responses to selective pharmacological activation and antagonism of NMDA, AMPA, or KA receptors, as well as to capsaicin, a TRPV1 agonist that induces glutamate receptor-mediated enhancement of GABA release.

EXPERIMENTAL PROCEDURES

Mice were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the University of Kentucky Animal Care and Use Committee (Animal Welfare Assurance Number A3336–01). All procedures were performed using coronal brain stem slices containing the dorsal vagal complex from young (4–8 wk) FVB mice (Jackson Laboratories, Bar Harbor, ME, United States). Mice were housed in a vivarium under a normal 14-h light/10-h dark cycle with food and water available ad libitum.

Brainstem slice preparation

Brainstem slices were prepared as described previously (Gao and Smith, 2010a, Gao and Smith, 2010b, Zsombok et al., 2011). Briefly, mice were anesthetized by isoflurane inhalation to effect (lack of tail-pinch response) and then decapitated while anesthetized. The brain was then rapidly removed and immediately immersed in ice-cold (0–4°C), oxygenated (95% O₂–5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 26 NaHCO₃, 1.4 NaH₂PO₄, 11 mM glucose, 1.3 CaCl₂, and 1.3 MgCl₂, pH = 7.2–7.4, with an osmolality of 290–305 mOsmol/kg H₂O. The brainstem was blocked rostral to the cerebellum and mounted on a metal stage using cyanoacrylate glue, and 300 µm coronal slices were cut using a Vibratome (Technical Products International, St. Louis, MO, United States). For consistency, slices from the caudal DVC near the level of the rostral area postrema (±600 µm rostrocaudally) were used. The slices were then transferred to a holding chamber containing warmed (32–34°C) ACSF for at least 1 h. The ACSF used for recordings was identical to that used in the dissection, except when drugs were added as described.

Patch-clamp recording

After an equilibration period of 1 h, whole-cell voltage-clamp recordings were obtained from DMV neurons under visual guidance on an upright, fixed-stage microscope equipped with infrared illumination and differential interference contrast (IR-DIC) optics (BX51WI, Olympus, Melville, NY, United States). Recording pipettes were pulled from borosilicate glass capillaries with 0.45 mm wall thickness (King Precision Glass, Claremont, CA, United States) and were filled with (in mM): 130 Cs-gluconate, 1 NaCl, 5 EGTA, 1 MgCl₂, 1 CaCl₂, 3 CsOH, 2 ATP; pH = 7.2–7.4. Open tip resistance was 2–5 MΩ, seal resistance was 1–5 GΩ, and series resistance was <25 MΩ (14.75±0.33 MΩ), uncompensated; recordings in which series resistance changed by >25% were excluded from analysis. Neural activity was recorded in voltage-clamp mode using an Axon 200B patch-clamp amplifier (Molecular Devices, Union City, CA, United States), low-pass filtered at 5 kHz and acquired at 20 kHz using a Digidata 1320A digitizer and pClamp 10.3 software (Molecular Devices). All recordings were performed in the presence of tetrodotoxin (TTX; 2 µM; Alomone Labs, Jerusalem, Israel) to block action potential-dependent neurotransmission. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded at a holding potential of 0 mV and had a fast (<1 ms) rise time and exponential decay. A value of twice the root mean squared noise level for a given recording was used as the detection limit for synaptic current amplitude. Synaptic currents were analyzed off-line on a PC-style computer with pCLAMP

programs (Molecular Devices) or Minianalysis 6.0.3 (Synaptosoft, Decatur, GA, United States).

Drug application

All experiments were performed in the presence of TTX (2 μ M). The K⁺ channel blocker, 4-aminopyridine (4-AP; 5 mM; Sigma-Aldrich, St. Louis, MO) was applied with the ACSF in some experiments. Agonists or antagonists of iGluRs, L-glutamate (50 μ M), *N*-methyl-D-aspartate (NMDA; 15 μ M), α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 3 μ M), kainic acid (KA; 1 μ M), the NMDA receptor antagonist, DL-5-aminophosphonovaleric acid (APV; 50 μ M), the non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μ M; all from Sigma-Aldrich), and the AMPA receptor antagonist, GYKI-54266 (50 μ M; Tocris Bioscience, Minneapolis, MN, United States) were bath applied for 5–15 min. The TRPV1 agonist, capsaicin (1 μ M; Tocris Bioscience) was dissolved in ethanol and diluted in ACSF (final concentration of ethanol <0.01 % by volume) and was bath applied in the presence of glutamate receptor antagonists to identify receptors mediating the iGluR-dependent effects of TRPV1 binding on GABA release in the DMV (Derbenev et al., 2006).

Data analysis

Once in whole-cell configuration, cells were voltage-clamped near the resting membrane potential (determined by measuring the voltage at which $I=0$) for 10 min to allow equilibration of the intracellular milieu and recording electrode solutions. mIPSCs were recorded at 0 mV, using pipettes containing Cs-gluconate to block K⁺ currents and thereby reduce noise and improve voltage-clamp quality. At least 2 min of continuous synaptic activity prior to and during drug application was examined to identify drug effects of on amplitude and frequency of mIPSCs (minimum of 100 events). Effects were measured after 5 min application for agonists and 10 min for receptor antagonists. Where practical, drug effect washout (>75% restoration of effect after 15 min washout) was used to control for intrinsic variability in mIPSC parameters. In addition, correlation analysis of mIPSC frequency versus recording time was performed on each recording to assess stationarity (i.e., to determine if frequency changed independent of drug treatment). Recordings in which a correlation was observed ($R^2>0.05$) were excluded from further analysis. The intra-assay Kolmogorov-Smirnov test was used to determine significance of drug effects within a recording. Effects of drugs on mean mIPSC frequency and amplitude were determined using a paired, two-tailed Student's *t*-test. For all analyses, $p<0.05$ was considered significant. Values are reported as mean $\pm$ SEM.

RESULTS

Glutamate effects on mIPSCs

Previous experiments in rats and mice indicated that TRPV1 activation with capsaicin enhanced mIPSC frequency in the DMV, and that a significant proportion of this effect was due to TRPV1-mediated release of glutamate, which binds iGluRs located on the synaptic terminals of afferent GABAergic neurons (Derbenev et al., 2006, Derbenev and Smith, 2013). To establish that glutamate could act at presynaptic terminal receptors to increase

GABA release in the mouse DMV, we determined the effect on mIPSCs of L-glutamate (50 μ M) in nine DMV neurons, voltage-clamped at 0 mV to reduce direct effects of the agonist on the recorded neuronal membrane. In the presence of TTX (2 μ M), application of glutamate resulted in a rapid increase in mIPSC frequency in 7 of 9 recorded DMV neurons (2.77 ± 0.68 Hz control; 3.86 ± 0.69 Hz glutamate; $n=9$; $p<0.05$). The amplitude of mIPSCs was not changed (24.27 ± 1.97 pA vs. 23.24 ± 1.91 pA; $p>0.05$; Fig. 1). These findings were consistent with previous findings from rat indicating that glutamate accelerates GABA release in the DMV by acting at glutamate receptors located on GABA terminals (Derbenev et al., 2006).

Capsaicin effects on mIPSCs

To establish that TRPV1 activation could increase GABA release in the mouse DMV, the effect of the TRPV1 agonist, capsaicin (1 μ M) was examined on synaptic inhibition of the DMV neurons. Capsaicin application resulted in a rapid increase in mIPSC frequency 13 of 26 DMV neurons (1.62 ± 0.33 Hz control; 2.95 ± 0.53 Hz capsaicin; $n=13$; $p<0.05$; Fig 2). The frequency of mIPSCs returned to values near control levels (1.67 ± 0.39 Hz) after 15–20 min wash to normal ACSF (with TTX). There was no significant effect of capsaicin on mIPSC amplitude in responding neurons 24.13 ± 2.11 pA control; 28.29 ± 3.04 pA capsaicin; $p>0.05$; 23.47 ± 1.52 pA washout). There was no effect on mIPSC frequency ($p>0.05$) or amplitude ($p>0.05$) in the remaining nine cells.

A comparison of the background mIPSC frequency in the nine responding neurons with the nine non-responding cells indicated a significantly lower background frequency in neurons that responded to capsaicin (1.62 ± 0.33 Hz responding neurons; 3.11 ± 0.52 Hz non-responding neurons; $p<0.05$; Fig. 2C). To determine if the lack of effect was due to an intrinsic limit on mIPSC frequency, the K^+ channel blocker, 4-AP (5 mM) was applied in the presence of capsaicin in a subset of neurons ($n=8$). In each neuron, addition of 4-AP significantly increased mIPSC frequency in the presence of capsaicin (capsaicin alone: 2.39 ± 0.62 Hz; capsaicin+4-AP: 4.37 ± 0.88 Hz; $n=8$; $p<0.05$). In four of these neurons, capsaicin application was without effect on mIPSC frequency. Addition of 4-AP significantly increased mIPSC frequency in each of these four neurons ($p>0.05$). Similar to findings from rat, these data confirmed that TRPV1 application enhanced GABA release from synaptic terminals in the mouse DMV. Since a previous study indicated this effect could be significantly inhibited when iGluRs were blocked non-selectively (Derbenev et al., 2006), we investigated the effects of selectively activating individual subtypes of iGluR and determined the effects of capsaicin in the presence of selective glutamate receptor antagonists.

Presynaptic NMDA receptors

To determine whether GABA release was modulated by activation of NMDA receptors located on presynaptic terminals (preNMDAR), the effect of NMDA (15 μ M) application on mIPSCs in DMV neurons was identified. In each of 6 neurons, the frequency of mIPSCs was significantly increased in the presence of NMDA (Fig. 3A–C). Mean mIPSC frequency was increased from 3.06 ± 1.73 to 4.40 ± 2.04 Hz after NMDA application ($n=6$; $p<0.05$; 44%

increase). The amplitude of mIPSCs was increased slightly (16%) in 3 of the 6 neurons (17.41 ± 0.49 pA control; 20.14 ± 1.28 pA NMDA; $n=6$; $p<0.05$).

Ambient glutamate levels in the slice preparation are sufficient to bind preNMDAR on glutamatergic afferent terminals and modulate the tonic release of glutamate in the DMV (Bach and Smith, 2012, Bach et al., 2015). To determine if preNMDARs on GABAergic terminals were similarly activated by ambient glutamate in the slice, the effects on mIPSCs of APV (50 μ M), a selective NMDA receptor antagonist, were identified in DMV neurons (Fig. 3D–E). There was no significant change in mIPSC frequency (control, 3.64 ± 1.71 Hz; APV, 3.28 ± 1.18 Hz; $n=10$; $p>0.05$) or amplitude (20.36 ± 1.76 pA control; 18.53 ± 1.29 pA APV; $n=10$; $p>0.05$) in the presence of APV, although the K-S test indicated that APV induced a reduction in mIPSC frequency in a minority of cells ($n=3$; $p<0.05$). We reasoned that the lack of effect of NMDAR antagonism might be due to Mg^{2+} -dependent blockade of NMDAR at the resting membrane potential of the terminal. To test this, we repeated the APV experiment in ACSF that was nominally Mg^{2+} -free (no Mg^{2+} added to the ACSF) in order to relieve the voltage-dependence of NMDA receptor activation. The effect of APV in Mg^{2+} -free ACSF was similar to that in normal ACSF (with TTX), with mIPSC frequency being unaffected overall ($p>0.05$) and significantly reduced in only two of five neurons.

Activation of NMDA receptors located on glutamatergic terminals can enhance glutamate release in the DMV (Bach and Smith, 2012). Since NMDAR-mediated glutamate release might have indirect effects on mIPSC frequency, and because of the inconsistent effects between NMDA and APV, we hypothesized that the NMDA effect was due to heterosynaptic activation of glutamate receptors on GABAergic terminals. In the presence of CNQX, which blocks AMPA/KA receptors, application of NMDA had no overall effect on mIPSC frequency (3.83 ± 1.37 Hz in CNQX; 4.27 ± 1.34 Hz in CNQX + NMDA; $n=9$; $p>0.05$). However, NMDA significantly increased mIPSC frequency in three of nine neurons, suggesting direct effects in a subset of cells. Results of agonist application indicated that activation of preNMDARs located on synaptic terminals could enhance GABA release in the DMV. Consistent with a previous report, however, preNMDARs were not active tonically in the slice preparation (Bach and Smith, 2012), and their effect on GABA release appeared to require AMPA/KA receptor function in most neurons.

To determine if TRPV1 receptor-stimulated glutamate release could bind preNMDAR on GABAergic terminals, we determined the effect of heterosynaptic enhancement of GABA release by capsaicin. In the presence of APV, capsaicin application failed to induce a change in mIPSC frequency (3.28 ± 1.18 in capsaicin; 3.34 ± 1.56 Hz in capsaicin+APV; $n=10$; $p>0.05$) or amplitude (18.53 ± 1.29 pA in APV; 19.10 ± 1.37 pA in capsaicin+APV; $n=10$; $p>0.05$; Fig. 3D–E). Together, these data suggested that activation of preNMDAR on synaptic terminals can modify GABA release and that capsaicin-mediated increase in mIPSC frequency typically requires activity of preNMDAR.

Non-NMDA receptors

Application of the AMPA/KA receptor antagonist, CNQX (10 μ M) blocks all spontaneous EPSCs in the DMV (Derbenev et al., 2004, Gao and Smith, 2010a) and was applied in nine recordings to determine if presynaptic, non-NMDA receptors altered mIPSCs or the

response to capsaicin. Overall, neither frequency (4.30 ± 0.81 Hz, control; 3.62 ± 0.89 Hz, CNQX; $n=9$; $p>0.05$) nor amplitude (23.95 ± 1.84 pA, control; 23.28 ± 1.99 pA, CNQX; $n=9$; $p>0.05$; Fig. 4) of mIPSCs were significantly altered after application of the mixed AMPA/KA antagonist. The intra-assay K-S test, however, indicated a significant effect of CNQX on mIPSC frequency in 5 of 9 neurons (4.98 ± 0.90 Hz control; 3.29 ± 0.90 Hz CNQX; $n=5$; $p<0.05$; Fig. 4). Further, application of capsaicin in the presence of CNQX failed to modulate mIPSC frequency (3.01 ± 1.00 Hz; $p>0.05$) or amplitude (22.06 ± 2.32 pA; $p>0.05$; Fig. 4), suggesting that non-NMDA iGluRs on presynaptic terminals contributed to the TRPV1-stimulated, glutamate-mediated increase in GABA release. These data suggested that tonically-activated, CNQX-sensitive receptors participated in the modulation of GABA release by glutamate in most neurons.

Presynaptic AMPA receptors

In some neural systems, AMPA receptors located on presynaptic terminals (i.e., preAMPA) potentially modulate GABA release (Satake et al., 2000, Lee et al., 2002, Engelman and MacDermott, 2004, Shyphshyna and Veselovsky, 2015). To determine if activation of preAMPA altered mIPSCs in the DMV, AMPA ($3 \mu\text{M}$) was bath-applied in seven recordings (Fig. 5A,B). Application of AMPA failed to increase mIPSC frequency in any of seven DMV neurons (2.22 ± 0.63 Hz control; 1.65 ± 0.44 Hz AMPA; $n=7$; $p>0.05$), and there was no change in mIPSC amplitude (24.24 ± 2.48 pA control; 26.81 ± 2.35 pA AMPA; $n=7$; $p>0.05$). These data suggested that preAMPA do not participate in the glutamate-mediated modulation of GABA release.

To further assess the possibility that preAMPA activity modulates GABA release in the DMV, mIPSCs were assessed in the presence of the selective, non-competitive AMPA receptor antagonist, GYKI-52466 ($50 \mu\text{M}$; Fig. 5C–E). Application of GYKI-52466 resulted in no significant change in mIPSC frequency (2.84 ± 0.7 Hz control; 2.64 ± 0.54 Hz GYKI-52466; $n=11$; $p>0.05$) and a small ($\sim 10\%$) change in mIPSC amplitude (21.18 ± 1.98 pA control; 18.96 ± 1.48 pA GYKI-52466; $n=11$; $p=0.04$). In the presence of GYKI-52466, capsaicin application resulted in increased mIPSC frequency in four of eight neurons ($P<0.05$; K-S test), similar to effects in the absence of antagonist (Fig. 5C–E). These results indicated minimal, if any, involvement of preAMPA in modulating GABA release in the DMV.

Presynaptic kainate receptors

Since AMPA was without effect in most neurons, and CNQX, but not GYKI-52466, suppressed the capsaicin-evoked increase in mIPSC frequency, we reasoned that KA receptors were present on GABAergic terminals (i.e., preKAR) and were involved in the capsaicin-induced response. The effect of KA on mIPSCs was therefore determined in six DMV neurons. In the presence of KA, mIPSC frequency was significantly increased in 5 of 6 recorded DMV neurons ($p<0.05$; K-S test; Fig. 6). Overall, KA induced a significant increase in the frequency of mIPSCs (3.49 ± 1.18 Hz control; 5.66 ± 1.92 Hz KA; $n=6$; $p<0.05$). No significant change in mIPSC amplitude was detected (23.18 ± 3.47 pA control; 24.96 ± 2.89 pA KA; $n=6$; $p>0.05$; Fig. 6A,B,E). Further, when AMPA and NMDA receptors were blocked in the presence of GYKI-52466 and APV, application of KA resulted in

significantly increased mIPSC frequency in four additional neurons (2.10 ± 1.35 Hz to 3.56 ± 1.48 Hz; $n=4$; $p<0.05$; Fig. 6C–E). These data indicated that GABA release can be modulated by activation of KARs on GABAergic terminals.

DISCUSSION

This study identified iGluRs that increase GABA release onto DMV neurons when activated. All recordings were performed in the presence of TTX, which blocks action potentials. Thus, drug effects on mIPSC frequency, especially in the absence of effects on amplitude, were interpreted to be due to actions at receptors located on or near synaptic terminals. Presynaptic modulation of GABA or glutamate release mediated by mGluRs in the DMV and NTS have been described (Jin et al., 2004, Browning and Travagli, 2007, Babic et al., 2012, Babic and Travagli, 2014), but functional iGluR activity at synaptic terminals contacting DMV neurons has been suggested only indirectly. A previous report suggested the presence of iGluRs on GABA terminals in the DMV, but did not identify which receptor types were involved in the response (Derbenev et al., 2006). Further, TRPV1 activation increases GABA release in the DMV, at least in part by acting via a heterosynaptic mechanism involving glutamatergic activation of iGluRs located on GABAergic terminals, with presynaptic mGluRs contributing after a period of time (Derbenev et al., 2006). Here, we found that activation of both NMDAR and KAR, but not AMPAR, increased mIPSC frequency in most DMV neurons. Agonist and antagonist effects were observed in the presence of TTX (i.e., they were action potential independent). The mIPSC frequency, but not amplitude, was affected, indicating effects were most likely at the level of the synaptic terminal. Together, the results are consistent with the presence of both preNMDA and preKA receptor subtypes on synaptic terminals that influence GABA release in the DMV.

A previous study identified preNMDARs on glutamatergic terminals contacting DMV neurons (Bach and Smith, 2012), activation of which increases glutamate release. These receptors were tonically activated by ambient glutamate levels in the slice, since the NMDA antagonist, APV, suppressed glutamate release. Consistent with our previous findings, the present results indicate that ambient glutamate levels in the slice are insufficient to activate preNMDAR that modulate GABA release tonically, since APV was without effect on mIPSCs. The presence of preNMDAR, however, was revealed by increased mIPSC frequency subsequent to NMDA application. Likewise, KA application increased mIPSC frequency, consistent with the presence of preKAR. Unlike preNMDAR, preKAR appeared to be tonically active because application of CNQX, which blocks both AMPA and KA receptors, decreased mIPSC frequency. Further, KA application increased mIPSC frequency when NMDA and AMPA receptors were blocked. The failure of NMDA to increase GABA release in the presence of CNQX in most cells, along with the small NMDA-induced increase in mIPSC amplitude, however, suggested that participation of preNMDAR on GABA terminals may be limited to a subset of terminals or neurons. Instead, preNMDAR located on glutamate terminals might mediate their effect by enhancing glutamate release and thus heterosynaptically activate preKARs on GABA terminals. Although preNMDAR appear to participate in the glutamate receptor-mediated GABA release, our data are consistent with the hypothesis that activation of preKAR located on GABAergic terminals

enhances GABA release in the DMV. Conversely, neither AMPA nor the selective AMPA antagonist GYKI-52466 modified mIPSC frequency significantly, indicating that these receptors do not normally play a major role in modulating GABA release in most DMV neurons. The identification of presynaptic KA receptors on GABAergic terminals represents a novel mechanism regulating inhibitory neurotransmission in the DMV.

Although blockade of NMDAR with APV did not affect mIPSC frequency, it did inhibit the effect of TRPV1-evoked glutamate receptor activation on GABA release. This is consistent with previous findings indicating that the TRPV1 agonist, capsaicin increases the release of glutamate, which binds preNMDAR and enhances GABA release in DMV neurons (Derbenev et al., 2006, Derbenev and Smith, 2013). Similarly, blockade of AMPA/KA receptors with CNQX, but not selective AMPAR blockade with GYKI-52466, suppressed the capsaicin-induced increase in mIPSC frequency. These data suggest that glutamate released via TRPV1 activation is sufficient to bind KAR on GABA neuron terminals. Presynaptic NMDARs also participate in the response. In the case of preKARs, it is reasonable to conclude that the receptors are activated tonically by ambient glutamate levels in the slice preparation and thereby contribute to GABA release characteristics.

In rats, capsaicin potently enhances glutamate and GABA release in nearly all DMV neurons. Glutamate receptor blockade significantly reduces, but does not eliminate, the TRPV1-mediated increase in mIPSC frequency, suggesting effects of TRPV1 receptor activation at the level of both glutamate and GABAergic terminals (Derbenev et al., 2006). The present study in FVB mice found that GABA release was enhanced by capsaicin in about 50% of neurons, suggesting that TRPV1 regulation of GABA release may be somewhat less prominent in this mouse strain than in rats. Interestingly, the effect of capsaicin was less likely to be observed in neurons with higher baseline mIPSC frequency than in neurons exhibiting lower frequency of mIPSCs. Although this could be attributed to a “ceiling” effect due to TRPV1 or presynaptic glutamate receptor saturation, addition of 4-AP increased mIPSC frequency, even when capsaicin did not. The capsaicin concentration used here (1 μ M) was relatively high, and more consistent effects would be expected if TRPV1 were expressed robustly and ubiquitously in GABA or glutamate terminals, suggesting that TRPV1 may affect GABA release in only about half the DMV neuron population. Synaptic glutamate release onto DMV neurons, extrapolated from mEPSC frequency, is greater in mice than in rats (Zsombok et al., 2011, Bach et al., 2015), which could contribute to relatively smaller effects of TRPV1-stimulated increase in iGluR-mediated GABA release. Blockade of preKAR (82%) or preNMDAR (100%) prevented capsaicin effects on mIPSC frequency in most cases, consistent with the notion that TRPV1-mediated modulation of GABA release was mainly due to heterosynaptic activation of preKAR located on GABAergic terminals, and also involved preNMDAR; both receptors appeared necessary to observe the effect. If the TRPV1-mediated enhancement of GABA release in mice occurs mainly via a heterosynaptic, glutamate-dependent mechanism (versus direct TRPV1 actions on GABA terminals), this could also contribute to the lower percentage of neurons in which mIPSC modulation was observed in mice.

Presynaptic iGluRs located on GABA neuron terminals have also been observed elsewhere in the brain, where glutamate released from synaptic terminals has been proposed to “spill

over” and activate glutamate heteroreceptors located on GABAergic terminals to increase or decrease GABA release (Berretta and Jones, 1996, Glitsch and Marty, 1999, Liu et al., 1999, Sjostrom et al., 2003, Duguid and Smart, 2004, Mathew and Hablitz, 2011). Presynaptic iGluRs that act as autoreceptors to increase glutamate release (Bach and Smith, 2012), or heteroreceptors that enhance GABA release, demonstrated here, likely represent a relatively widespread mechanism of rapid synaptic release modulation, especially during periods of increased glutamatergic synaptic activity. The glutamate-induced enhancement of GABA release via a presynaptic mechanism in the DMV observed here might be expected to stabilize DMV neuron activity and help control membrane potential after robust glutamatergic synaptic activation. Motoneurons in the DMV that project to gastrointestinal viscera tend to fire action potentials in a regular fashion (Browning et al., 1999), which allows them to increase or decrease in firing rate subsequent to subtle changes in synaptic input. Release of GABA from synaptic terminals in the DMV exerts a potent and tonic inhibitory influence on DMV motoneuron activity (Travagli et al., 2006, Gao and Smith, 2010a, Gao and Smith, 2010b, Browning and Travagli, 2011, Boychuk et al., 2015). During periods of increased afferent vagal activation, DMV neurons are depolarized phasically by increased synaptic glutamate release, allowing them to respond transiently to specific afferent drive (Travagli, 2007, Browning and Travagli, 2011). Assuming synaptically-released glutamate activates preKAR on GABAergic terminals in addition to postsynaptic receptors on the DMV neuron, the heterosynaptic enhancement of GABA release would tend to suppress the influence of sustained glutamatergic drive by increasing GABA release in a feedforward inhibitory fashion, which would serve functionally to improve the fidelity of temporally-discrete, phasic excitatory inputs, such as those occurring during meal ingestion and digestion.

Conclusions

Ionotropic glutamate receptors located on GABAergic terminals represent a heterosynaptic regulatory arrangement for modulating GABA release when synaptic excitation is elevated in the DMV. Here, we identified functional preKAR and preNMDAR that participate in increasing synaptic GABA release in the DMV, with preKAR located on GABAergic terminals contributing most directly to the effect. The circumstances under which these receptors are recruited into circuit modulation are not known, but since they mediate effects of TRPV1 receptor activation on GABA release, it is reasonable to postulate that conditions leading to the release of the endocannabinoid, anandamide or other ‘endovanilloid’ substances, which result in TRPV1-mediated modulation of GABA release in the DMV and elsewhere (Boychuk et al., 2013, Derbenev and Smith, 2013), might recruit this heterosynaptic mechanism of GABA release modulation during periods of excessive excitatory synaptic drive.

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Highlights

- GABA release is enhanced by activation of ionotropic glutamate receptors (iGluRs) located on GABAergic terminals in the DMV
- Both NMDA and kainate receptors, but not AMPA receptors, contribute to the heterosynaptic enhancement of GABA release
- TRPV1 receptor activation enhances GABA release mainly via kainate receptors located on GABAergic terminals
- iGluR activation heterosynaptically regulates GABA release in the DMV under conditions of elevated synaptic excitation.

Figure 1. Modulation of inhibitory synaptic input to DMV neurons by glutamate

A. In the presence of tetrodotoxin (TTX; 2 μ M), glutamate (50 μ M) induced an increase in miniature inhibitory postsynaptic current (mIPSC) frequency. Continuous traces showing recording of mIPSCs before and during glutamate application are shown. Neuronal membrane was voltage-clamped at 0 mV. **B.** Cumulative fraction plots of mIPSC interevent interval and amplitude prior to and during glutamate application in the DMV neuron shown in **A**, indicating increased mIPSC frequency with no change in amplitude. **C.** Plots showing mean frequency (left) and amplitude (right) of mIPSCs from DMV neurons ($n=9$) prior to and after glutamate application. Asterisk indicates significant effect of glutamate ($p<0.05$).

Figure 2. Effects of the TRPV1 receptor agonist, capsaicin on mIPSCs

A. Traces showing the effect of bath-applied capsaicin (1 μ M) on mIPSCs in DMV neurons. Left, mIPSCs recorded in control ACSF containing TTX (2 μ M). Middle, the same neuron in the presence of capsaicin. Right, 15 min after washout of capsaicin. Traces in each panel are continuous. **B.** Cumulative probability plots of mIPSC frequency and amplitude under the three conditions (control, capsaicin, washout) from the recording in **A**. **C.** Plots showing mean the effect of capsaicin on mIPSC frequency and amplitude in 26 DMV neurons. Capsaicin induced an increase in the frequency of mIPSCs in each of 13 DMV neurons with lower baseline mIPSC frequency ($p < 0.05$), whereas DMV neurons with relatively higher baseline mIPSC frequency were unaffected by capsaicin ($n = 13$; $p > 0.05$). Asterisk indicates a significant difference from mIPSC frequency in cells that responded to capsaicin ($p < 0.05$).

Figure 3.

NMDA receptor activation increased mIPSC frequency in DMV neurons. **A.** Sample traces showing mIPSCs in control ACSF (containing TTX) and in the additional presence of NMDA (15 μ M). Continuous activity is shown for each condition. **B.** Cumulative fraction plot indicating an increase in mIPSC frequency in the presence of NMDA in the neuron shown in **A** ($p < 0.05$; K-S test). **C.** Plots of NMDA effect on mean mIPSC frequency (left) and amplitude (right). Effects are shown in ACSF with TTX ($n = 6$) and in the additional presence of CNQX ($n = 9$). Asterisks indicate significant effect of NMDA in ACSF; no significant change in frequency of amplitude was detected in the presence of CNQX. **D.**

Sample traces from a different neuron in control ACSF, the NMDA receptor antagonist, APV (50 μ M), and with addition of capsaicin (CAP; 1 μ M). **E.** Cumulative probability plot indicating no significant effect of APV or CAP in the presence of APV on mIPSC frequency in this neuron. **F.** Plots indicating no significant effect of APV or APV+CAP on mean mIPSC frequency (left) and amplitude (right) in 11 DMV neurons.

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Figure 4.

Blockade of non-NMDA receptors decreased mIPSC frequency and prevented effects of capsaicin. **A.** Sample traces showing mIPSCs in control ACSF (with TTX), CNQX (10 μ M), and after addition of capsaicin in the presence of CNQX. **B.** CNQX reduced mIPSC frequency in this neuron ($p < 0.05$; K-S test), and addition of capsaicin had no additional effect ($p > 0.05$; K-S test). **C.** Graphs of mean mIPSC frequency and amplitude for nine DMV neurons in control ACSF, CNQX, and CNQX+capsaicin (CNQX+CAP). Asterisk indicates significant effect of CNQX. There was no significant effect of capsaicin when applied in the presence of CNQX in any neuron.

Figure 5.

AMPA receptor modulation was without effect on mIPSCs or the effect of capsaicin on mIPSC frequency. **A.** Sample traces showing the effect of AMPA (3 μ M) on mIPSCs. **B.** Cumulative probability plots for the recording in **A** indicate no significant effect of AMPA on mIPSC frequency ($p > 0.05$) or amplitude ($p > 0.05$). **C.** Sample traces showing effect of the selective AMPA receptor antagonist, GYKI-52466 (GYKI; middle traces) and capsaicin in the presence of GYKI (GYKI+CAP; right traces). **D.** Cumulative probability plots indicating no significant effect of AMPA receptor blockade, but a significant increase in mIPSC frequency with the addition of capsaicin ($p < 0.05$). **E.** Graphs indicating mean mIPSC frequency and amplitude in the presence of AMPA ($n = 7$) or GYKI and GYKI+CAP

(n=11). There was no significant effect of AMPA (n=7; $p>0.05$) or GYKI (n=11; $p>0.05$) versus control ACSF. Addition of capsaicin in the presence of GYKI increased mIPSC frequency (n=11; $p<0.05$); a small but significant effect of GYKI on mIPSC amplitude was detected ($p<0.05$).

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Figure 6.

Kainate (KA) receptor activation increased mIPSC frequency in DMV neurons. **A.** Sample traces showing the effect of KA (1 μ M) application on mIPSCs. Continuous recording is shown for each panel. **B.** Cumulative probability plots indicate that KA induced a significant increase in mIPSC frequency in this neuron ($p < 0.05$). **C.** Sample traces showing mIPSCs in the presence of GYKI+APV (left), which selectively block NMDA and AMPA receptors, respectively, and the same neuron after the addition of KA (right). **D.** Cumulative probability plot showing a significant effect of KA on mIPSC frequency in the presence of GYKI+APV in the neuron shown in **C** ($p < 0.05$; K-S test). **E.** Graphs of mean mIPSC frequency and amplitude indicate a significant effect of KA on mIPSC frequency in control

ACSF containing TTX ($p < 0.05$; $n = 6$). A significant effect of KA on mIPSC frequency was also observed when applied in the presence of GYKI+APV ($p < 0.05$; $n = 4$). There was no effect on mIPSC amplitude under either condition.

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