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Glucose-dependent trafficking of 5-HT$_3$ receptors in rat gastrointestinal vagal afferent neurons

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Abstract

Background—Intestinal glucose induces gastric relaxation via vagally mediated sensory-motor reflexes. Glucose can alter the activity of gastrointestinal (GI) vagal afferent (sensory) neurons directly, via closure of ATP-sensitive potassium channels, as well as indirectly, via the release of 5-hydroxytryptamine (5-HT) from mucosal enteroendocrine cells. We hypothesized that glucose may also be able to modulate the ability of GI vagal afferent neurons to respond to the released 5-HT, via regulation of neuronal 5-HT$_3$ receptors.

Methods—Whole cell patch clamp recordings were made from acutely dissociated GI-projecting vagal afferent neurons exposed to equiosmolar Krebs' solution containing different concentrations of D-glucose (1.25–20mM) and the response to picospritz application of 5-HT assessed. The distribution of 5-HT$_3$ receptors in neurons exposed to different glucose concentrations was also assessed immunohistochemically.

Key Results—Increasing or decreasing extracellular D-glucose concentration increased or decreased, respectively, the 5-HT-induced inward current as well as the proportion of 5-HT$_3$ receptors associated with the neuronal membrane. These responses were blocked by the Golgi-disrupting agent Brefeldin-A (5μM) suggesting involvement of a protein trafficking pathway. Furthermore, L-glucose did not mimic the response of D-glucose implying that metabolic events downstream of neuronal glucose uptake are required in order to observe the modulation of 5-HT$_3$ receptor mediated responses.

Conclusions & Inferences—These results suggest that, in addition to inducing the release of 5-HT from enterochromaffin cells, glucose may also increase the ability of GI vagal sensory neurons to respond to the released 5-HT, providing a means by which the vagal afferent signal can be amplified or prolonged.

Keywords

vagus; glucose; 5-HT

Vagal neurocircuits are vitally important in the integration and co-ordination of homeostatic functions including the regulation of gastrointestinal (GI) reflexes. Blood glucose levels oscillate throughout the day and increase dramatically following food ingestion. Such acute changes in blood glucose levels, even within the physiological range, exert profound vagally-mediated effects upon gastric motility and emptying in order to stabilize these fluctuations and maintain homeostasis (1;2). Appropriate and effective homeostatic reflexes require the integration and co-ordination of a wide range of visceral sensations with

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neuronal, hormonal and metabolic signals. Vago-vagal sensory-motor reflexes are critically important in this regard since the majority of non-nociceptive information between the GI tract and the CNS is relayed via the vagus nerve.

Vagal sensory neurons, the cell bodies of which lie in the paired nodose ganglia, serve the classic afferent functions, relaying sensory information from the GI tract to the brainstem via a glutamatergic synapse at the level of the nucleus of the tractus solitarius (NTS; (3;4)). Neurons of the NTS assimilate this vast volume of sensory information with inputs from other brainstem and higher CNS centers and send this integrated signal to, amongst other areas, the adjacent dorsal motor nucleus of the vagus (DMV). The DMV contains the preganglionic parasympathetic motoneurons that provide the motor output back to the GI tract via the efferent vagus nerve (4).

Increases in both peripheral and central glucose levels inhibit gastric motility and tone (5–8), yet the precise mechanism responsible for these alterations still remains to be elucidated fully. A significant degree of glucose-mediated excitation of GI vagal sensory nerve fibers is due to an indirect, or paracrine, mechanism of action. Glucose induces the release of 5-hydroxytryptamine (5-HT) from enteroendocrine cells which, in turn, excites 5-HT_3 receptors present on GI vagal sensory nerve terminals (9–11). Certainly, agonists at 5-HT_3 receptors delay gastric emptying while antagonists accelerate gastric transit (10;12;13). Studies have also suggested, however, that the response of vagal afferent fibers to luminal glucose can be modulated by intravenous glucose, suggesting that glucose may also act directly upon vagal afferent neurons or fibers (14). More recent studies have demonstrated that some vagal afferent neurons are themselves glucose-sensitive, increasing their excitability in response to increased extracellular glucose levels via actions at ATP-sensitive potassium channels, in a manner similar to the canonical model of pancreatic β-cells (15).

We have demonstrated previously that glucose can modulate glutamate release from the central terminals of vagal afferent neurons via an alteration in the number of functional presynaptic 5-HT_3 receptors (16;17). This phenomenon does not appear to be restricted to central vagal terminals, however, since vagal afferent neurons are also capable of altering their neurotransmitter phenotype in response to physiological conditions (18–20). Since vagal afferent neurons also display functional 5-HT_3 receptors (21–24) and are capable of responding to circulating blood glucose levels, the present study aimed to determine whether extracellular glucose can modulate the response of GI vagal afferent neurones to 5-HT via alterations in 5-HT_3 receptor density and/or function.

MATERIALS AND METHODS

Ethical approval

All experiments were performed in accordance with the Pennsylvania State University's Institutional Animal Care and Use Committee, under an approved protocol.

Retrograde tracing

Experiments were performed in both male and female Sprague-Dawley rats. To label GI-projecting vagal afferent neurones, the fluorescent neuronal tracer 1,1'-dioctadecyl-3.3.3', 3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Grand Island NY) was applied to the GI tract as described previously (25). Briefly, after induction of anesthesia with isofluorane (2.5% with air, 600ml/min), the abdominal area of 14-day old rats (n=73) was cleaned before exposing the tomach and proximal intestine via a laparotomy. The stomach and proximal intestine were isolated from the surrounding viscera and crystals of DiI were placed onto the major curvature of either the fundus, the corpus or the antrum/pylorus areas or onto the antimesenteric border of the duodenum. The dye crystals were embedded in

place using a fast-hardening epoxy resin; the surgical area was washed with sterile saline before closing the abdomen. Rats were allowed to recover for 7–15 days before experimentation.

In another series of experiments, crystals of DiI were applied to the superior laryngeal nerve of 12 rats (28–35 days of age). Rats were anesthetized with a mixture of ketamine/xylazine/acepromazine in saline (80/1.6/5.0 $mg.kg^{-1}$, respectively); after a deep level of anesthesia was obtained (abolition of the foot-pinch withdrawal reflex) a ventral midline neck incision was made to expose the left cervical vagus nerve. The superior laryngeal nerve was isolated from surrounding tissue by a small piece of parafilm. Crystals of DiI were apposed to the superior laryngeal nerve and affixed in place with a fast-hardening epoxy resin. The surgical area was rinsed with sterile saline before the incision was closed. Rats were allowed to recover for 7–15 days prior to experimentation.

Dissociation of nodose ganglia

Rats were euthanized via anesthetic overdose (isoflurane, 5%) followed by administration of a bilateral pneumothorax. Nodose ganglia were removed under microscopic guidance and placed in chilled, oxygenated Hank's balanced salt solution (HBSS; Gibco, Life Grand Island, NY). The connective tissue surrounding the ganglia was removed before the ganglia were incubated in HBSS containing $10mg.ml^{-1}$ collagenase and $40mg.ml^{-1}$ dispase at 37°C for 45min. Ganglia were then placed in fresh HBSS and triturated through a pipette tip until tissue was no longer visible. Cells were centrifuged at 1000rpm for 5min, placed in fresh HBSS and centrifuged at 1600rpm for 8min. Neurons were resuspended in the plating medium (see below for composition), plated on poly-L-lysine coated coverslips and incubated in a humid incubator (5% CO_2) at 37°C. After 2 hours, cells were fed (see below for composition). Electrophysiological recordings and immunohistochemical studies were carried within 24 hours of dissociation.

Electrophysiological Recordings

Cover glasses with plated dissociated nodose ganglion neurons were placed in a custom-made perfusion chamber (volume 500μl; Michigan Precision Instruments, Parma, MI) and maintained at 35°C by perfusion with warmed Krebs' solution at a rate of 2.5–3.0 $ml.min^{-1}$. Retrogradely labeled neurons (DiI-filled) were identified using a Nikon E600FN microscope (Tokyo, Japan) equipped with tetramethylrhodamine isothiocyanate (TRITC) epifluorescent filters. Once the identity of a DiI-filled neuron was confirmed, electrophysiological recordings were made under bright-field illumination.

Whole-cell recordings were made with patch pipettes of resistance 2–3MΩ when filled with potassium gluconate intracellular solution (see below for composition). Data were acquired using a single electrode voltage-clamp amplifier (Axopatch 1D; Molecular Devices, Sunnyvale, CA) at a rate of 10 kHz, filtered at 2 kHz, and digitized via a Digidata 1320 interface (Molecular Devices) before being stored and analyzed on a personal computer using pClamp9 software (Molecular Devices). Only those neurons with a series resistance of <15MΩ were considered acceptable.

Drugs and Krebs' solutions containing different equiosmolar concentrations of glucose were applied via perfusion through a series of manually operated valves for a period of time sufficient for the response to reach plateau. Neurons were allowed to recover fully between drug additions. A separate patch pipette was filled with 5-HT (100μM) and connected to a picospritzer (Parker Hannifin Corp. Cleveland, OH). Each neuron served as its own control and the response to picospritz application of 5-HT was assessed before, during and after perfusion with equiosmolar Krebs' solution containing different glucose concentrations

using ANOVA with repeated measures. Statistical significance was set at 5%. Results are expressed as mean + s.e.mean.

Immunohistochemistry

Dissociated nodose neurons were exposed to feeding medium with varying concentrations of glucose (1.25–20mM) for 30min before fixation in 4% paraformaldehyde for 15 min. Cells were washed for 5min in 50% ethanol in distilled water, then washed (3×5min) in phosphate buffered saline (PBS; see below for composition) containing 0.1% bovine serum albumin (BSA). Slides were then placed in rabbit anti-5-HT3B receptor antibody (1:500, Abcam ab39629; diluted in PBS containing BSA) and incubated overnight at room temperature. Slides were washed (3×5min) with PBS containing BSA before incubation in secondary antibody (AlexaFluor488-conjugated goat anti-rabbit IgG, 1:100; Invitrogen, Grand Island NY) for one hour at room temperature. Slides were washed (3×5min) in PBS containing BSA and mounted in Fluoromount-G (Southern Biotech., Birmingham AL).

Control experiments included (i) omission of the primary antibody, (ii) omission of the secondary antibody, and (iii) use of an inappropriate secondary antibody (AlexaFluor488-conjugated goat anti-donkey IgG 1:100, Invitrogen, Life Technologies, Grand Island NY). 5-HT3 receptor immunoreactivity was not observed under any of these conditions.

To determine whether glucose induced the trafficking of 5-HT_3 receptors, acutely dissociated neurons were exposed 5μM brefeldin-A, an inhibitor of protein transport, for 30min prior to exposure to different concentrations of glucose (2.5–20mM) containing brefeldin-A. Neurons were fixed subsequently and processed for 5-HT_3 receptor immunoreactivity as above. To determine whether cellular or metabolic events following glucose uptake into nodose neurons was responsible for trafficking of 5-HT_3 receptors, acutely dissociated neurons were also exposed to feeding medium containing 5mM D-glucose + 15mM L-glucose prior to fixation.

Confocal microscopy

Confocal photomicrographs of 5-HT_3 receptor immunoreactive cells were taken on an Olympus IX81 microscope and captured with Olympus Fluoview 1000 software. Images of individual cells were imported into Image J software (http://rsbweb.nih.gov/ij). The density of receptor on the membrane was quantified as described previously (26;27). Briefly, one confocal image (1μm thick) across the maximum diameter of the neuron was analyzed. The total number of pixels, defined by setting the threshold for immunoreactivity above the threshold for background staining, was determined within the area of a cell. The cell membrane was defined by outlining the inner and outer edges of the plasma membrane, within 0.5μM of each other. The number of pixels inside both lines was measured using the same parameters and the percentage of membrane-associated 5-HT_3 receptor density was calculated by subtraction. Data were analyzed by comparing the percentage of membrane-associated receptor density between different groups, using an ANOVA followed by the Student's *t*-test. Statistical significance was set at 5%. Results are expressed as mean ± s.e.mean. Linear regression analysis was used to determine the correlation between the glucose concentration and the percentage of membrane-associated 5-HT_3 receptor density.

Materials

Supplemented minimal essential medium (sMEM): Minimal essential medium (Gibco, Life Technologies, Grand Island NY) supplemented with 0.15g.100ml^{-1} $NaHCO_3$ and 29.2mg L-glutamine, pH 7.4

Plating medium: sMEM supplemented with fetal calf serum (10%), nerve growth factor (1%), penicillin-streptomycin (1%) and $CaCl_2$ (4mM)

Feeding medium: sMEM supplemented with fetal calf serum (10%), nerve growth factor (1%) and penicillin-streptomycin (1%)

Phosphate buffered saline (PBS; mM): 115 NaCl, 75 Na_2HPO_4, 7.5 KH_2PO_4

Paraformaldehyde fixative: 4% paraformaldehyde in PBS

Krebs' solution (mM): 126 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4 and 5 D-glucose maintained at pH 7.4 by bubbling with 95%O_2/5%CO_2

Intracellular pipette solution (mM): 128 K-gluconate, 10 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 NaATP and 0.25 NaGTP adjusted to pH 7.35 with KOH

Dispase was purchased from Roche Diagnostics (Indianapolis, IN); all other chemicals were purchased from Sigma Chemical Co (St. Louis, MO).

RESULTS

Picospritz application of 5-HT induces a 5-HT_3 receptor mediated current in GI vagal afferent neurons

In order to determine the type of receptor responsible for the inward current induced in response to picospritz application of 5-HT (100μM), GI vagal afferent neurons were voltage clamped at -50mV. Picospritz application of 5-HT induced an inward current of 1047±286pA (n=6). In the presence of the 5-HT_2 receptor selective antagonist, ketanserin (1μM), the response to re-application of 5-HT was unaffected (982±251pA, i.e., 96±3.3% of control amplitude, P>0.0%; data not shown). In contrast, in the presence of the 5-HT_3 receptor selective antagonist, ondansetron (1μM), the 5-HT-induced inward current was reduced to 179±77pA, i.e., to 16±3.6% of control amplitude (P<0.05; data not shown).

These results demonstrate that the inward current induced in GI vagal afferent neurons in response to picospritz application of 5-HT is mediated exclusively via activation of 5-HT_3 receptors.

The basic properties of GI vagal afferent neurones responsive to 5-HT differ from non-responsive neurons

The basic membrane properties of GI vagal afferent neurons were recorded as described previously (25). Basic membrane properties did not differ between GI regions (fundus, corpus, antrum/pylorus, duodenum); the results were therefore combined. The input resistance of neurons responsive to 5-HT (289±32MΩ; n=33) did not differ from non-responsive neurons (298±27MΩ; n=34). The duration of the action potential at threshold was significantly shorter for GI vagal afferent neurons responsive to 5-HT as compared to non-responsive neurons (3.8±0.28ms vs 4.8±0.32ms, respectively, P<0.05). Furthermore, while the action potential afterhyperpolarization amplitude did not differ between GI responsive vs non-responsive neurons (18.1±1.06mV vs 17.9±1.15mV, respectively, P<0.05) the duration of the afterhyperpolarization was significantly shorter in responsive neurons (16.6±1.90ms vs 28.3±3.94ms, P<0.05).

Extracellular glucose concentration modulates the amplitude of the 5-HT-induced current in GI vagal afferent neurons

Previous studies have demonstrated that subpopulations of gastric-projecting vagal afferent neurons are glucose-excited, i.e., increasing extracellular glucose concentration induces a membrane depolarization, whereas decreasing extracellular glucose concentration induces a membrane hyperpolarization (15). In the present study, increasing extracellular glucose concentration from 5 to either 10m or 20mM induced an inward current of 43±6pA or 58±21pA, respectively, in 7 of 31 neurons (22%; n=3,4 respectively). Conversely, decreasing extracellular glucose concentration from 5 to 2.5mM induced an outward current of 20±5pA in 4 of 22 neurons (18%).

In the presence of control concentrations of extracellular glucose (5mM), picospritz application of 5-HT (100μM) induced an inward current of 286±50.1pA amplitude in GI vagal afferent neurons voltage clamped at −50mV (n=33). In 28 of these neurons (85%), the magnitude of the 5-HT-induced inward current was modulated by the extracellular glucose concentration; the 5-HT-induced current in the remaining 5 (15%) of neurons was unaffected. Perfusion with equiosmolar Krebs' solution containing 1.25mM or 2.5mM D-glucose decreased the 5-HT-induced inward current to 58±9.6% and 54±5.3% of the control response, respectively ($P<0.05$; Figure 1A). The change in amplitude of the 5-HT-induced current reached a steady-state within 5–8 minutes and was stable thereafter. In contrast, increasing the extracellular glucose concentration to 10 or 20mM increased the magnitude of the 5-HT-induced inward current to 133±8.9% and 187±16.7% of control amplitude, respectively ($P<0.05$ for both; Figure 1B).

Extracellular glucose concentration modulates the density of 5-HT_3 receptors on the neuronal membrane of GI vagal afferent neurons

Immunohistochemical studies were performed to assess the ability of the extracellular glucose concentration to modulate the proportion of 5-HT_3 receptors associated with the membrane of GI vagal afferent neurons. The proportion of membrane-associated 5-HT_3 receptors, expressed as a percentage of total 5-HT_3 receptor density, was assessed following 30min exposure to concentrations of extracellular glucose ranging from 1.25–0mM (Figure 1C). At 1.25mM extracellular glucose, only 19.8±1.58% of the total 5-HT_3 receptor density was membrane-associated (n=24) but this proportion increased linearly to 36.6±2.62% at 20mM glucose (n=28; $r^2=0.97$; Figure 1D). The linear nature of this relationship suggests that even physiological changes in glucose level (4–8mM) may alter the proportion of membrane-associated 5-HT_3 receptors on GI vagal afferent neurons, although this needs to be examined in more detail. While the immunohistochemical data do not exclude the possibility that increasing extracellular glucose concentration induces degradation of intracellular 5-HT_3 receptors (thus increasing the relative proportion of membrane-associated receptors), taken together with the electrophysiological data, these data suggest that extracellular glucose concentration rapidly and reversibly modulates both the response to activation of 5-HT_3 receptors in GI-projecting vagal afferent neurons and the proportion of 5-HT_3 receptors present on the neuronal membrane.

Extracellular glucose does not modulate either the response to activation of 5-HT_3 receptors or the density of membrane-associated 5-HT_3 receptors in superior laryngeal nerve-labeled vagal afferent neurons

To examine whether the glucose-dependent modulation of the 5-HT-induced inward current was restricted to vagal afferent neurons innervating the GI tract, recordings were made from vagal afferent neurons labeled following application of DiI to the superior laryngeal nerve (SLN), i.e., neurons presumed to innervate the larynx. In the presence of Krebs' solution containing 5mM glucose, picospritz application of 5-HT induced an inward current of

amplitude 890±485pA (n=5). In the presence of equisomolar Krebs' solution containing 2.5mM or 20mM glucose, however, the amplitude of the 5-HT-induced inward current was unaffected at 98±2.5% and 101±4.2% of control amplitude, respectively (Figure 2A).

Immunohistochemical studies were also performed to assess whether the extracellular glucose concentration modulated the proportion of 5-HT_3 receptors associated with the membrane of SLN-labeled vagal afferent neurons (Figure 2B). In the presence of 1.25mM glucose, 25±0.7% of the total 5-HT_3 receptor density was membrane associated (n=21); this proportion was not changed by increasing the extracellular glucose concentration up to 20mM (25±1.3%; n=14; P>0.05; Figure 2C).

These results suggest that the glucose-dependent modulation of 5-HT_3 mediated inward currents is not a ubiquitous property of vagal afferent neurons, but may be restricted to neurons innervating the GI tract.

Extracellular glucose modulates the ability of ondansetron to antagonize the 5-HT-induced inward current

To confirm that the extracellular glucose modulates 5-HT-induced inward current in GI vagal afferent neurons by modulating the number of available receptors, the ability of the 5-HT_3 receptor selective antagonist, ondansetron (0.1μM) to inhibit the response to 5-HT was tested at different extracellular glucose concentrations. In the presence of 5mM glucose, ondandsetron inhibited the 5-HT-induced inward current by 49±10.9%, decreasing the current amplitude from 667±179pA to 348±116pA (n=7; P<0.05; Figure 3A). In the presence of 2.5mM glucose, however, the same concentration of ondansetron inhibited the 5-HT response to a greater extent, decreasing the inward current from 704± 314pA to 122± 68pA, i.e., a 81± 10.9% decrease (n=5; P<0.05 vs control; P<0.05 vs inhibition in the presence of 5mM glucose). In contrast, in the presence of 10 or 20mM glucose, ondansetron was less effective and inhibited the 5-HT-induced inward current by only 45±10.9 and 37±10.7%, respectively, from 740±261pA to 497±289pA and from 342±82pA to 209±67pA, respectively (P<0.05; Figure 3B). These results demonstrate that the ability of the receptor selective antagonist, ondansetron, to inhibit the 5-HT-mediated inward current was dependent upon the extracellular glucose concentration. Since a single concentration of the antagonist would be expected to occupy the same number of 5-HT_3 receptors, the decreased ability of ondansetron to block the response to 5-HT as glucose concentration increases suggests that elevating glucose levels may increase the number of active 5-HT_3 receptors on the membrane of GI vagal afferent neurons.

Extracellular glucose modulates the 5-HT-induced inward current in GI vagal afferent neurones via trafficking of 5-HT_3 receptors

To determine whether increasing extracellular glucose concentration potentiates the responses of vagal afferent neurons to 5-HT by trafficking of 5-HT_3 receptors to the cell membrane, the ability of glucose to modulate the 5-HT-induced inward current was assessed before and after exposure to the Golgi-disrupting agent, brefeldin-A, which we have demonstrated previously inhibits receptor trafficking within vagal neurocircuits (28). In GI-vagal afferent neurons, picospritz application of 5-HT (100μM) in the presence of 5mM extracellular glucose, induced an inward current of 397±109pA (n=5). In the presence of 20mM glucose, the 5-HT-induced inward current was increased by 56±27% to 508±40pA (P<0.05). Following re-perfusion with 5mM glucose and recovery of the 5-HT-induced inward current amplitude to control levels (389±115pA, i.e., 94±8.5% of control amplitude, P>0.05), neurons were perfused for 5 minutes with brefeldin-A (5μM) which itself did not alter the amplitude of the 5-HT-induced inward current (385±117pA, i.e., 97±2.9% of control amplitude; P>0.05). In the continued presence of brefeldin-A, however, re-

application of 20mM glucose no longer increased the amplitude of the 5-HT-induced inward current (338±125pA, i.e., 81±7.0% of amplitude in the presence of 5mM glucose + brefeldin-A; P<0.05; Figure 4A).

Immunohistochemical studies were also performed to assess the ability of brefeldin-A to disrupt the actions of extracellular glucose concentration to modulate the proportion of membrane-associated 5-HT_3 receptors (Figure 4B). In the continued presence of brefeldin-A (5μM), 30±0.7% of total 5-HT_3 receptor density was associated with the neuronal membrane following exposure to 5mM glucose (n=27). Decreasing the concentration of glucose to 1.25mM did not increase receptor internalization, i.e., the proportion of membrane-associated receptor was unchanged at 28±0.9% (n=26; P>0.05). Similarly, the externalization of receptors associated with increasing the extracellular glucose concentration to 20mM was blocked by brefeldin-A (28±0.6%, n=41, P>0.05; Figure 4C).

These data suggest that the actions of extracellular glucose to modulate the response to activation of 5-HT_3 receptors, as well as the density of 5-HT_3 receptors present on the neuronal membrane of GI vagal afferent neurons, is due to trafficking of receptors to and from the membrane.

D-glucose, but not L-glucose, modulates the 5-HT-induced inward current in GI vagal afferent neurons

We have demonstrated previously (16;17) that D-glucose, but not L-glucose, increases glutamate release from the central terminals of vagal afferents via modulation of 5-HT_3 receptors (17). In order to determine whether the observed effect of extracellular glucose on 5-HT-mediated inward currents in nodose ganglion neurons was similarly dependent upon D-glucose, recordings were made from GI vagal afferent neurons voltage clamped at −50mV. In the presence of 5mM D-glucose, picospritz application of 5-HT induced an inward current of amplitude 712±326pA. Following perfusion with equiosmolar Krebs' solution containing 20mM D-glucose, the magnitude of the 5-HT-induced inward current was increased by 43±9.2% to 981±474pA (n=5; P<0.05). After re-exposure to 5mM D-glucose and reversal of the increased 5-HT current (to 693±289pA, i.e., 104±4.8% of control amplitude; P>0.05), neurons were perfused with Krebs' solution containing 5mM D-glucose + 15mM L-glucose. The combination of D- and L-glucose had no effect on the amplitude of the 5-HT-induced inward current (738±335pA, i.e., 100±5.1%, P>0.05; Figure 5A).

Immunohistochemical studies were also carried out to investigate whether the ability of the extracellular glucose concentration to traffic 5-HT_3 receptors to and from the membrane of GI vagal afferent neurons was dependent upon D-glucose. In the presence of 5mM D-glucose, 26±1.19% of 5-HT_3 receptor density was membrane-associated (n=64), increasing to 37±2.6% in the presence of 20mM D-glucose (n=38; P<0.05). In the presence of 5mM D-glucose + 15mM L-glucose, however, the proportion of membrane-associated 5-HT_3 receptor density was unchanged at 26±1.2% (n=31; P>0.05; Figure 5B).

These data suggest that the glucose-dependent modulation of receptor density and response to activation of 5-HT_3 receptors in GI vagal afferent neurons is dependent upon D- but not L-glucose, implying the involvement of metabolic events downstream to neuronal glucose uptake.

DISCUSSION

Results of the present study demonstrate that, in GI vagal afferent neurons, the extracellular concentration of glucose modulates: 1) the proportion of 5-HT_3 receptors associated with the neuronal membrane, 2) the magnitude of the 5-HT_3 receptor-mediated inward current, and

3) the ability of the 5-HT_3-receptor selective antagonist, ondansetron, to antagonize the 5-HT-induced inward current. The present study also demonstrates that: 4) the extracellular glucose concentration does not modulate either the 5-HT-induced inward current or the proportion of membrane-associated receptors in vagal afferent neurons innervating the SLN, suggesting that these responses may be selective for neurons innervating the GI tract, 5) these effects are blocked by the Golgi-disrupting agent Brefeldin-A, suggesting that glucose induces trafficking of pre-made receptors rather than *de novo* receptor synthesis, and 6) that L-glucose does not mimic the actions of D-glucose, suggesting that a downstream metabolic pathway subsequent to neuronal glucose uptake is involved.

Since the initial studies demonstrating that glucose within the lumen of the intestine increases the firing rate of vagal afferent fibers (14;29;30), several studies have indicated that glucose acts, at least in part, via the release of 5-HT from mucosal enterochromaffin cell. In turn, this activates 5-HT_3 receptors present on vagal afferent terminals within the GI tract, allowing the excitatory signal to be relayed to the brainstem (9–11;31). Electrophysiological studies on isolated afferent neurons have often been used as a convenient model for events at the peripheral terminal, based on the assumption that biophysical events are identical at both locations. While it remains unclear as to the exact extent to which this assumption holds true, previous studies have demonstrated that the response of GI vagal afferent neurons to neurohormones such as glucagon-like peptide 1 (GLP-1) do, indeed, mimic those of the afferent terminals (32). Intraluminal 5-HT itself increases the discharge rate of nodose ganglion neurons (11) and, further, studies in which dye was injected into the internal occipital artery have shown relatively uniform dye penetration into the nodose and jugular ganglia, suggesting that circulating factors have free access to vagal afferent neurons (33). Vagal afferent neurons themselves display a wide variety of receptors for neurotransmitters and neuromodulators hence, presumably, are capable of responding directly to diverse circulating factors. In this regard, following ingestion of a meal, the levels of platelet-free 5-HT rise from approximately 25nM to 88nM; (34), Since vagal afferent neurons from several species, including the rat, display both 5-HT_2 and 5-HT_3 receptors, one may reasonable assume that vagal afferent neurons may be additionally excited by the increase in circulating 5-HT levels following ingestion of a meal (21–24).

In addition, therefore, to inducing the release of 5-HT from enterochromaffin cells and activating 5-HT_3 receptors on vagal afferent terminals within the GI tract, glucose may also increase the ability of vagal sensory neurons and nerve fibers to respond to the released 5-HT, providing a means by which the vagal afferent signal can be amplified and/or prolonged in a temporally distinct manner. Trafficking of 5-HT_3 receptors via regulated exocytosis has been shown previously to occur in expression systems (35;36) but, to our knowledge, this is the first functional and physiological demonstration of 5-HT_3 receptor trafficking within peripheral neurons.

Studies in both animals and humans have shown that 5-HT_3 receptor selective agonists induce relaxation of the proximal stomach (12;37) whereas 5-HT_3 receptor selective antagonists accelerate gastric transit (10;38). Indeed, the 5-HT_3 receptor selective antagonist ondansetron is effective in treatment of several GI motility disorders (39–41). Significantly, studies have also demonstrated that a larger dose of a 5-HT_3 antagonist is required to induce the same increase in gastric motility and intestinal transit in diabetic animal models (38;42) suggesting that hyperglycemia may affect the response to 5-HT, possibly via an increase in the number of 5-HT_3 receptors present on vagal neurocircuits.

We have demonstrated previously that glucose modulates excitatory synaptic transmission from the central terminals of vagal afferent fibers (16) due to alteration in the number of

presynaptic 5-HT_3 receptors (17). In the present study, we extend these earlier findings by demonstrating that glucose also regulates the trafficking of 5-HT_3 receptors on GI-projecting vagal afferent neurons and, by consequence, modulates the responsiveness of these neurons to 5-HT. The results of the present study suggest that circulating glucose levels modulate vagally-mediated GI responses via alteration in the number of 5-HT_3 receptors associated with the neuronal membrane of GI vagal afferent neurons. The effect of ondansetron to inhibit the 5-HT induced inward current is more apparent at glucose levels below 5mM suggesting that higher concentrations of ondansetron may be required to inhibit the 5-HT-induced response in the diabetic state (see also 38). Although it remains to be determined, it is possible that this glucose-dependent trafficking of 5-HT_3 receptors may be a generalized phenomenon within vagal neurocircuits and, as such, may also occur on the peripheral nerve terminals within the intestine.

An elevation in extracellular glucose levels induced a rapid, and reversible, increase in the number of functional 5-HT_3 receptors associated with the membrane of GI-projecting vagal sensory neurons, hence increased the magnitude of the response to 5-HT. This suggests that even a relatively small change in receptor distribution is able to produce a marked difference in the response to 5-HT. This phenomenon appears to be restricted to vagal afferent neurons innervating the GI tract, however, since glucose altered neither the response to 5-HT nor the proportion of 5-HT_3 receptors associated with the neuronal membrane of SLN-labeled neurons (i.e., neurons presumed to innervate the larynx). As such, these data suggest that the vagal afferent neurons involved in vagally-mediated gastrointestinal reflexes such as oropharyngeal and esophageal peristalsis as well as relaxation of the lower esophageal sphincter (43) are not susceptible to modulation by glucose.

The exact mechanism by which glucose regulates the density of membrane-associated 5-HT_3 receptors on GI vagal afferent neurons remains to be elucidated. The the relatively rapid speed with which glucose can increase the density of membrane-associated 5-HT_3 receptors (within minutes) argues against *de novo* synthesis of receptors but, rather, implies that glucose traffics receptors to and from the membrane surface, as observed previously (36). We have demonstrated previously that G-protein coupled (28;44–46) as well as ionotropic (17) receptors on the nerve terminals of central vagal neurocircuits are exceptionally susceptible to trafficking to and from the neuronal membrane and, thus, are able to modulate the excitability and responsiveness of vagally-mediated responses in accordance within physiological conditions in an on-demand fashion. While several reports have described changes in neurotransmitter receptor distribution patterns within GI vagal sensory neurons in response to alterations in feeding conditions (18–20), the present study is the first to report that such receptor trafficking may occur rapidly enough to alter ongoing physiological responses on a minute-to-minute basis. Cumulatively, these studies all suggest that vagally-mediated homeostatic reflexes are exceptionally ‘plastic’ and open to modulation by a variety of circulating factors.

Glucose is also able to alter the excitability of GI vagal afferent neurons directly, via closure of an ATP-sensitive potassium channel, in a manner similar to the canonical model described in pancreatic β-cells (15). In the present study, however, increasing extracellular glucose concentration was rarely observed to induce an inward current in GI vagal afferent neurons, possibly due to methodological differences in electrophysiological recordings. In the present study, which used conventional whole-cell patch clamp recording techniques, after entering whole-cell mode, an equilibration period of at least 5min was allowed prior to examining the neuronal responses. Since ATP was routinely included within the patch pipette solution, it is reasonable to assume that ATP-sensitive potassium channels were already closed prior to alteration in extracellular glucose concentration, rendering it difficult, if not impossible, to observed further glucose-dependent channel closure. In the present

study, however, the actions of extracellular glucose to modulate 5-HT_3 receptor distribution and 5-HT-mediated responses were dependent upon D-glucose, since L-glucose was without effect (see also (17). These results suggest that metabolic events downstream of neuronal glucose uptake are required in order to observe the modulation of 5-HT_3 receptor mediated responses. The nature of these downstream metabolic events are still open to investigation but previous studies have demonstrated that 5-HT_3 receptors can be trafficked to and from the membrane of receptor expression systems by protein kinase C (PKC) (35;36). Furthermore, both acute and chronic hyperglycemia activate and upregulate PKC activity (47;48), suggesting that this second messenger system may be a likely candidate in the trafficking of 5-HT_3 receptors within GI vagal afferent neurons.

CONCLUSIONS

The present study suggests that circulating glucose levels may affect GI functions via actions at multiple levels, both peripheral and central, confirming and extending the emerging concept that vagally-mediated neurocircuits regulating autonomic functions are highly integrative and capable of a remarkable degree of plasticity and modulation (49). By regulating the number of functional 5-HT_3 receptors present on the cell membrane of GI vagal afferent neurons, glucose is able to modulate its own ‘perception’, hence amplify or prolong vagal afferent signaling. The rapid time-scale of the observed electrophysiological effects suggest that glucose may alter ongoing physiological responses of vagal afferent neurons on a minute-to-minute basis, although further studies to investigate these effects within physiological glucose ranges and timescales are required. Whether such receptor trafficking actions of glucose are restricted 5-HT_3 receptors, or whether this is a more generalized phenomenon and can occur with other receptors involved in GI homeostatic reflexes, remains to be elucidated.

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TB, AET, SR and KNB performed the research and analyzed the data

TB and KNB designed the research study

TB and KNB wrote the paper

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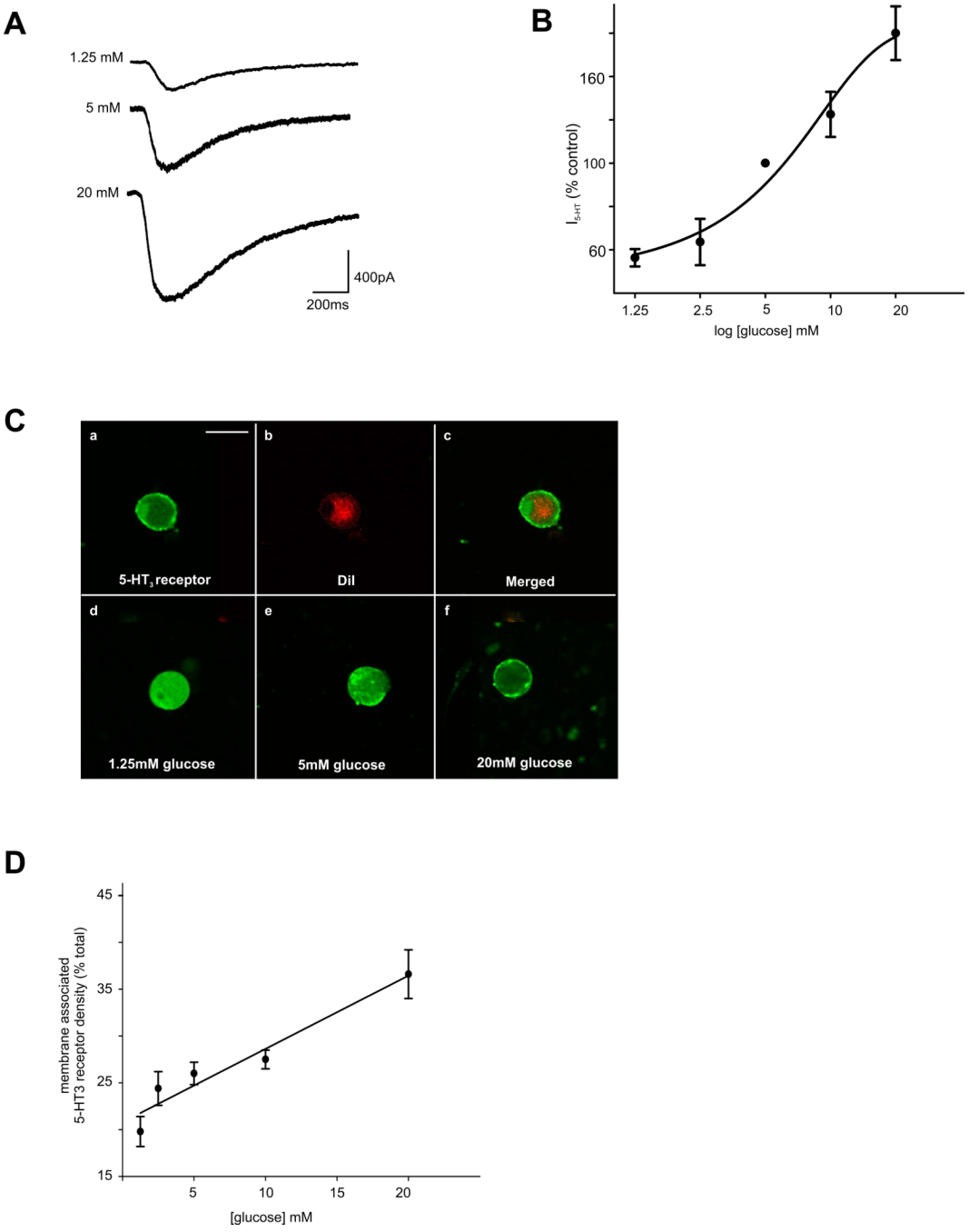

Figure 1. Extracellular glucose concentration modulates the response to 5-HT and the density of membrane-associated 5-HT$_3$ receptors in gastrointestinal-projecting vagal afferent neurons

A. Electrophysiological traces illustrating the 5-HT-induced inward current in a single GI-projecting vagal afferent neuron voltage clamped at -50mV. Superfusion with equiosmolar Krebs' solution containing different concentrations of glucose modulated the amplitude of the 5-HT-induced inward current; decreasing the glucose concentration to 1.25mM decreased the magnitude of the inward current whereas increasing the glucose concentration to 20mM increased the magnitude of the inward current.

B. Concentration-response curve illustrating the modulation of the 5-HT-induced inward current in GI vagal afferent neurons by glucose. Neurons were voltage clamped at −50mV prior to picrospritz application of 5-HT; each neuron was exposed to equiosmolar Krebs' solution containing at least 3 different concentrations of glucose. The 5-HT-induced inward current was normalized to the amplitude of the current induced in the presence of 5mM glucose. N=6–41 for each point.

C. Photomicrographs of representative confocal images (1μm thick) of GI-projecting vagal afferent neurons (5-HT_3 receptors - green; DiI - red). (a) an acutely dissociated vagal afferent neuron illustrating 5-HT_3 receptor distribution (20mM glucose); (b) the same neuron as in (a) depicting DiI-labeling from the GI-tract; (c) merged image. 5-HT_3 receptor distribution in acutely dissociated GI-projecting vagal afferent neurons following exposure to 1.25mM (d), 5mM (e) or 20mM (f) glucose prior to fixation. Note that as the extracellular glucose concentration was elevated, membrane-associate 5-HT_3 receptor-density increased. Calibration bar = 20μm.

D. Concentration-response curve illustrating the modulation of 5-HT_3 receptor density in GI vagal afferent neurons induced by extracellular glucose concentration. 5-HT_3 receptor density associated with the neuronal membrane was expressed as a proportion of that neurons' total 5-HT_3 receptor density. Note that an increase in glucose level was associated with a linear increase in 5-HT_3 receptor density associated with the neuronal membrane ($r^2 = 0.97$).

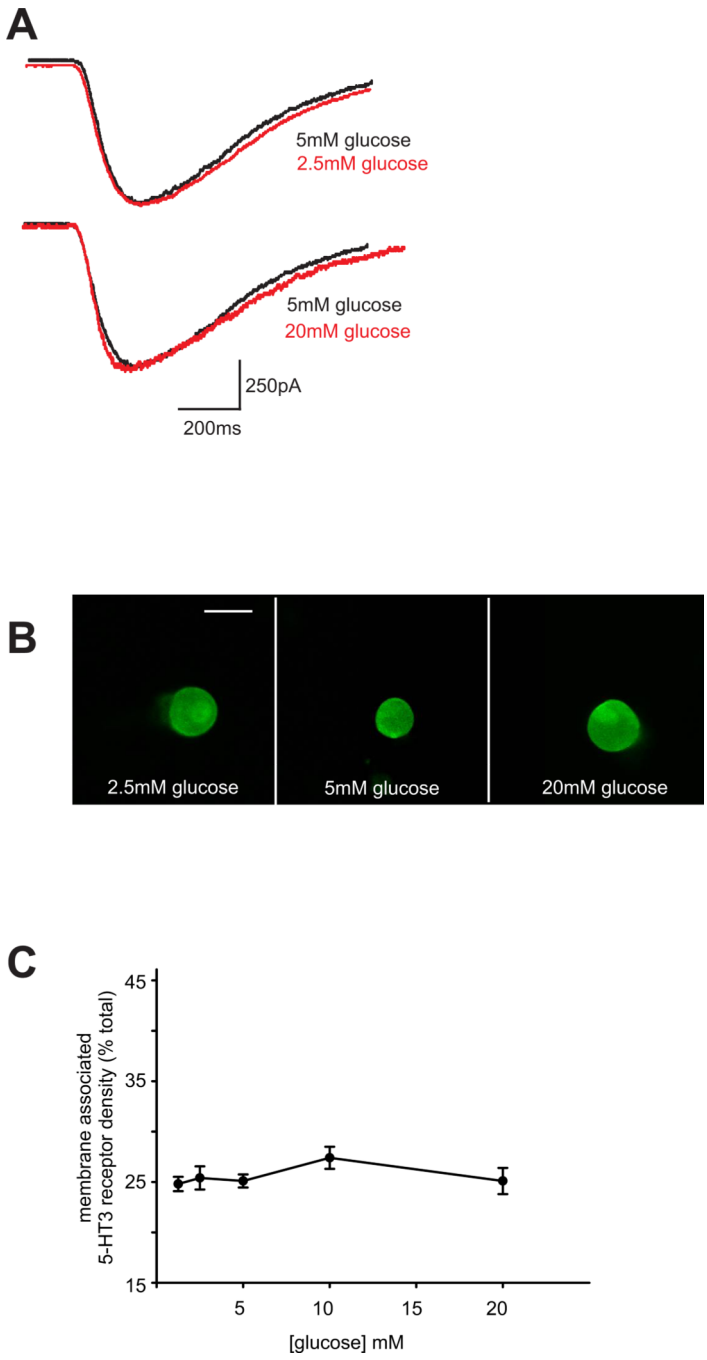

Figure 2. Extracellular glucose concentration does not modulate the response to 5-HT or the density of membrane-associated 5-HT$_3$ receptors in SLN-labeled vagal afferent neurons

A. Electrophysiological traces illustrating the 5-HT-induced inward current in a single a vagal afferent neuron labeled following application of DiI to the SLN. The neuron was voltage clamped at -50mV prior to picospritz application of 5-HT. Superfusion with equiosmolar Krebs' solution containing either 1.25 or 20mM glucose failed to modulate the amplitude of the 5-HT-induced inward current.

B. Photomicrographs of representative confocal images (1μm thick) of acutely-dissociated SLN-labled vagal afferent neurons illustrating 5-HT$_3$ receptor

distribution (green). Neurons were exposed to 1.25, 5 or 20mM glucose prior to fixation. Note that there was no alteration in 5-HT_3 receptor distribution in response to change in extracellular glucose concentration. Calibration bar = 20μm.

C. Concentration-response curve illustrating the modulation of 5-HT_3 receptor density in SLN-labeled vagal afferent neurons in response to extracellular glucose concentration. 5-HT_3 receptor density associated with the neuronal membrane was expressed as a proportion of that neurons' total 5-HT_3 receptor density. Note that extracellular glucose concentration had no effect upon the proportion of membrane-associated 5-HT_3 receptor density.

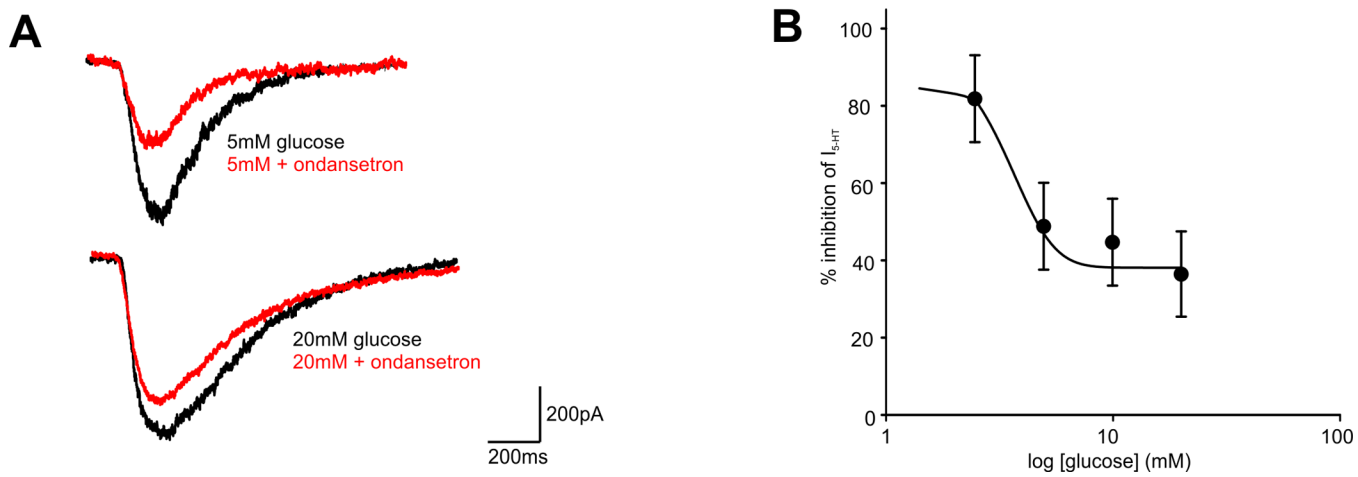

Figure 3. The ability of the 5-HT$_3$ receptor selective antagonist, ondansetron, to inhibit the 5-HT-induced inward current in GI vagal afferent neurons is dependent upon extracellular glucose concentration

A. Electrophysiological traces illustrating the effects of the 5-HT$_3$ receptor selective antagonist, ondansetron (0.1μM) to inhibit the 5-HT induced inward current in a GI-projecting vagal afferent neuron. The neuron was voltage clamped at -50mV prior to picospritz application of 5-HT. Ondansetron decreased the amplitude of the 5-HT-induced inward current. Superfusion of that same neuron with equiosmolar Krebs' solution containing 20mM glucose increased the amplitude of the 5-HT-induced current; at this higher concentration of glucose, ondansetron was less effective at inhibiting the 5-HT-induced inward current.

B. Concentration-response curve illustrating the actions of extracellular glucose concentration to modulate the ability of ondansetron (0.1μM) to inhibit the 5-HT-induced inward current in GI vagal afferent neurons. Note that as the extracellular glucose concentration increased, ondansetron became less effective at inhibiting the 5-HT-induced inward current, suggesting that as extracellular glucose levels increased, the number of membrane-associated 5-HT$_3$ receptors also increased.

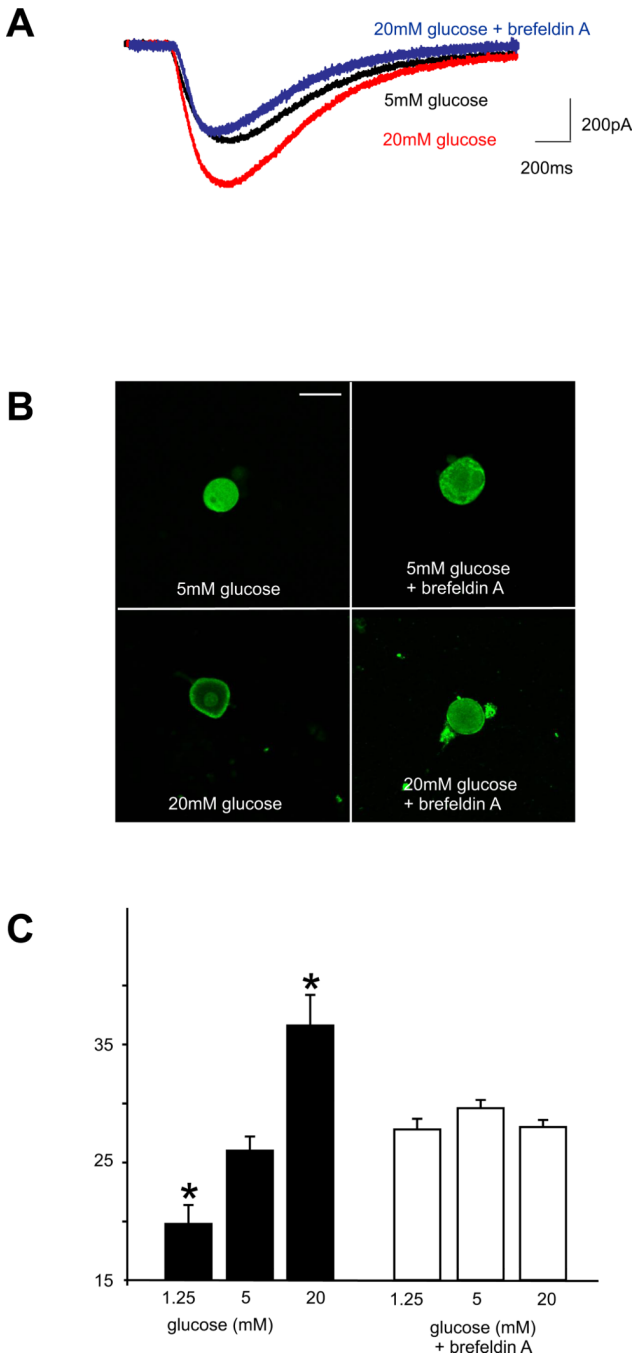

Figure 4. Inhibition of protein trafficking prevents the glucose-induced modulation in response to 5-HT and the density of membrane-associated 5-HT$_3$ receptors in GI-projecting vagal afferent neurons

A. Electrophysiological traces illustrating the effects of the Golgi-disrupting agent, brefeldin-A (5μM) to block the glucose-induced alteration in 5-HT-mediated inward current in a GI-projecting vagal afferent neuron. The neuron was voltage clamped at -50mM prior to picospritz application of 5-HT (black trace). The magnitude of the 5-HT-induced inward current was increased following superfusion with equiosmolar Krebs' solution containing 20mM glucose (red

trace). Following wash-out and recovery, however, superfusion with brefeldin-A inhibited the ability of 20mM glucose to increase the response to 5-HT (blue trace), suggesting involvement of a protein trafficking pathway.

B. Photomicrographs of representative confocal images (1μm thick) of acutely-dissociated GI-projecting vagal afferent neurons illustrating 5-HT_3 receptor distribution (green). Neurons were exposed to either 5 or 20mM glucose in the absence or presence of the Golgi-disrupting agent brefeldin-A (5μM) prior to fixation. Note that exposure to brefeldin-A inhibited the ability of 20mM glucose to increase the proportion of 5-HT_3 receptors associated with the neuronal membrane, suggesting involvement of a protein-trafficking pathway. Calibration bar = 20μm.

C. Graphical representation of the effects of brefeldin-A (5μM) to disrupt to ability of extracellular glucose to modulate the proportion of 5-HT_3 receptors associated with the membrane of GI vagal afferent neurons. In control conditions, varying the extracellular glucose concentration to 1.25 or 20mM, respectively, altered the proportion of 5-HT_3 receptors associated with the neuronal membrane (black bars). In the presence of brefeldin-A, however, altering extracellular glucose concentration had no effect upon membrane-associated 5-HT_3 receptor density. *$P<0.05$ vs control (5mM) glucose.

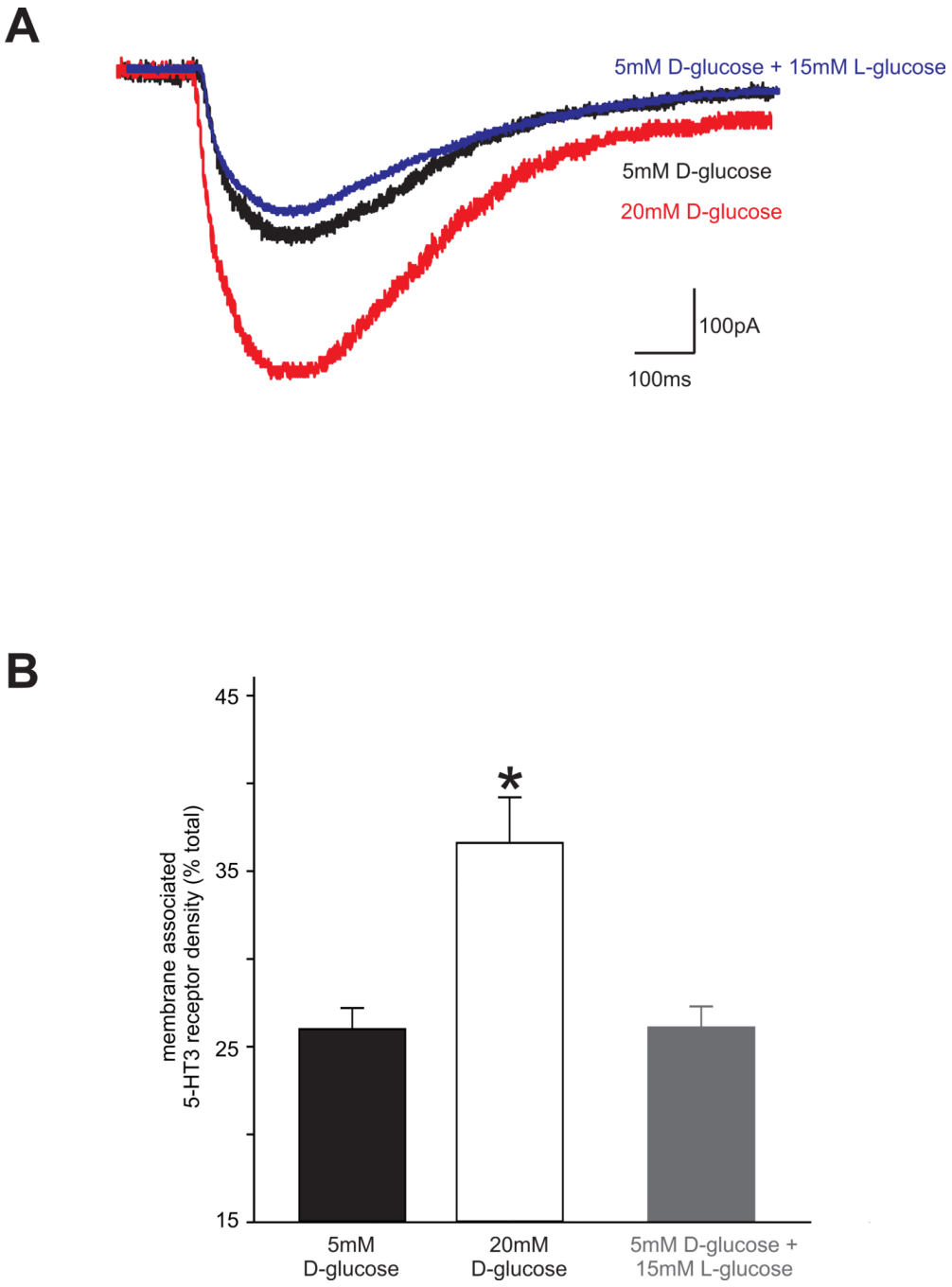

Figure 5. D- glucose, but not L-glucose, modulates the response to 5-HT and the density of membrane-associated 5-HT$_3$ receptors in GI-projecting vagal afferent neurons

A. Electrophysiological traces illustrating the effects of D- and L-glucose on the response to 5-HT in a GI-projectin vagal afferent neuron. The neuron was voltage-clamped at −50mV prior to picospritz application of 5-HT (black trace). Superfusion with equiosmolar Krebs' solution containing 20mM D-glucose increased the magnitude of the 5-HT-induced inward current (red trace) whereas superfusion with 5mM D-glucose + 15mM L-glucose had no effect (blue trace).

B. Graphical representation of the effects of D- and L-glucose on the proportion of 5-HT_3 receptors associated with the membrane of GI-projecting vagal afferent neurons. Neurons were exposed to 5 or 20mM D-glucose or 5mM D-glucose + 15mM L-glucose prior to fixation. Elevating the extracellular D-glucose concentration increased the proportion of 5-HT_3 receptors associated with the neuronal membrane whereas elevating the extracellular L-glucose concentration had no effect. These results suggest that the effects of glucose are dependent upon glucose metabolism, rather than uptake, within the vagal afferent neurons. *p,0.05 vs control (5mM) glucose.