Reflux disease and achalasia: Failure of the gatekeeper
Rohof, W.O.A.

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Chapter 4

Localisation of mGluR5, GABAB, GABAA and cannabinoid receptors on the vago-vagal reflex pathway responsible for transient lower esophageal sphincter relaxation in humans: An immunohistochemical study.

Wout Rohof, Eleonora Aronica, Hanneke Beaumont, Dirk Troost, Guy Boeckxstaens

ABSTRACT

Background and aims:
Transient lower esophageal sphincter relaxations (TLESRs) are the predominant mechanisms underlying gastroesophageal reflux. TLESRs are mediated by a vago-vagal reflex, which can be blocked by interaction with metabotropic Glutamate Receptor 5 (mGluR5), γ-aminobutyric acid type B (GABA_B), γ-aminobutyric acid type A (GABA_A) and cannabinoid (CB) receptors. However, the distribution of these receptors in the neural pathway underlying the triggering of TLESRs has not been evaluated in humans.

Methods:
Using immunohistochemistry we investigated the distribution of mGluR5, GABA_A, GABA_B, CB1 and CB2 receptors in the human nodose ganglion, the brain stem and the myenteric plexus of the esophagus.

Results:
MGluR5, GABA_B, CB1 and CB2 receptors are abundantly expressed in neurons of the myenteric plexus of the LES, nodose ganglion cell bodies and nerve fibres, the dorsal motor nucleus and nucleus of the solitary tract in the brain stem. GABA_A receptors are expressed in the same regions except in the nodose ganglion and myenteric plexus of the LES.

Conclusion:
Human mGluR5, GABA_A, GABA_B and CB_B receptors are abundantly expressed along the vago-vagal neural pathway and involved in the triggering of TLESRs. These findings are in line with the central side effects observed during treatment with reflux inhibitors such as GABA_A receptor agonists and mGluR5 antagonists, but also suggest that peripherally acting compounds may be effective.
INTRODUCTION

Gastroesophageal reflux disease (GERD) is a condition characterized by reflux of gastric contents causing troublesome symptoms, complications or both. In Western countries, GERD is one of the most common gastrointestinal diseases affecting approximately 10–20% of adults. Transient lower esophageal sphincter relaxations (TLESR) are the predominant mechanisms underlying gastro-esophageal reflux, both in normal subjects and in GERD patients.

TLESR are vago-vagally mediated motor patterns triggered by activation of vagal afferents in the cardia of the stomach by various stimuli, of which gastric distentions are the most important. The cell bodies of the vagal afferents are located in the nodose ganglion synapsing in the nucleus of the solitary tract (NTS) in the brain stem. Subsequently, neurons in the dorsal motor nucleus (DMV) of the vagus nerve are activated thereby initiating the specific motor pattern underlying TLESRs, i.e. relaxation of the lower esophageal sphincter (LES), esophageal shortening, and inhibition of the crural diaphragm. (Figure 1)
TLESRs and particularly the receptors involved in the underlying neural pathway have been identified as a novel therapeutic target. Animal data indicate that γ-aminobutyric acid type B (GABA\textsubscript{B}) receptors and metabotropic glutamate receptor 5 (mGluR5) are expressed along this pathway, in particular in the nodose ganglion and NTS of the ferret.\textsuperscript{7} More importantly, recent clinical studies have clearly demonstrated that GABA\textsubscript{B} receptor agonists baclofen and lesogaberan and mGluR5 antagonists inhibit TLESRs thereby reducing the number of reflux episodes and symptoms in healthy subjects and GERD patients.\textsuperscript{8-12} Activation of GABA\textsubscript{A} receptors, known to mediate fast postsynaptic inhibition, also leads to an inhibition of TLESRs in dogs, but the effect of GABA\textsubscript{A} agonists on the inhibition of TLESRs has not been studied in humans.\textsuperscript{13} Lastly, Δ\textsuperscript{9}-tetrahydrocannabinol, a receptor agonist of cannabinoid 1 and 2 receptor (CB1, CB2) reduced the number of TLESRs in humans and dogs.\textsuperscript{14}

Compounds targeting TLESRs, also referred to as reflux inhibitors, are currently evaluated as an add-on treatment for GERD patients that only partially respond to acid suppression with proton pump inhibitors. One of the main problems so far are central side effects such dizziness and nausea, most likely due to their interaction with receptors located in the central nervous system.\textsuperscript{10, 15, 16} However, the exact location of the receptors involved in TLESR neural pathway has not been evaluated in humans. Evidences from animal models, including receptor distribution, may differ significantly from the human situation; therefore an insight into the distribution of these receptors in human tissue is of utmost importance in optimising bench to bedside translation. Therefore, the aim of this study was to analyse the presence of the mGluR5, GABA\textsubscript{B}, GABA\textsubscript{A} and cannabinoid receptors in human nodose ganglion, DMV, NTS and the myenteric plexus of the lower esophageal sphincter.

METHODS

Tissue samples
Brain and nodose ganglion tissues were obtained post-mortem from patients (5 male, median age 72 yrs, range 45-82) with prior informed consent for brain autopsy and for the use of human brain tissue for research. All autopsies were performed within 12 hours after death. Full thickness esophageal specimens (n=4) were obtained from surgical esophageal resections. Clinical data are presented in table 1. None of the patients had a reported history of any neurodegenerative diseases. Tissues were fixed in paraformaldehyde and embedded in paraffin. Cerebellum and cortex control tissue was obtained from the tissue bank of the department of neuropathology of the Academic Medical Centre.
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Table 1

**Immunohistochemistry and antibody characterisation**

Paraffin-embedded tissues were sectioned coronally with sections of 6 μm. Sections were mounted on pre-coated glass slides. Representative sections of all specimens were processed for haematoxylin and eosin and/or Nissl stains, to assure that specimens were of normal histology.

The sections were deparaffinised using xyrene, re-hydrated and incubated with 1% H₂O₂ diluted in methanol for 20 min. Slides were then washed with phosphate-buffered saline (PBS) (10 mM, pH 7.4) and heated in sodium citrate buffer (0.01 M, pH 6.0) for 10 minutes for antigen retrieval. After washing with PBS, the slides were incubated for 30 minutes in 10% normal goat serum followed by incubation with the primary antibodies overnight at 4 °C. Antibodies were visualized using the Powervision peroxidase system (Immunologic, Duiven, the Netherlands) and 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) for a brownish staining. Haematoxylin was used as a counterstain, and sections were subsequently dehydrated and mounted. As a negative control, slides were incubated without the primary antibody. Human cerebellum and cortex were used as positive control tissue when appropriate. The primary antibodies used in the study are summarized in Table 2. Studies in which the specificity of the antibodies was confirmed in humans are listed in the table.

**Evaluation of immunohistochemical staining**

Immunoreactivity of neurons, nerve fibres or ganglion cells was evaluated in the nodose ganglion, NTS, DMV and myenteric plexus. Localisation of the NTS and DMV was confirmed by an experienced neuropathologist (DT). The relative density of labelling was classified as absent (-),
rare (±), moderate (+) or extensive (++). All slides were scored independently by two observers and a consensus score was obtained. Additionally, immunopositive cells in nodose ganglion were counted (mean ± standard deviation [SD]). Sections were digitized using an Olympus microscope equipped with a DP-10 digital camera (Olympus, Japan). The images were imported into Adobe Photoshop for minor adjustments of brightness and sharpness.

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Table 2

RESULTS

Nodose ganglion

The presence of ganglion cells was confirmed using Nissl staining in all nodose ganglion sections (n=5). Immunostaining for mGluR5 and for GABA R1 and GABA R2 was scattered throughout the nodose ganglion and mainly expressed in ganglion cells and moderately in nerve fibres (Figure 2). No immunoreactivity for GABA α was observed whereas normal staining was observed in brain cortex, i.e. diffuse and strong neurophil immunoreactivity throughout the cortex. In contrast, immunoreactivity for GABA β2/3 was detected in ganglion cells, as shown in figures 2I and 2J. Extensive staining of both CB1 and CB2 was observed with a prominent immunoreactivity of ganglion cells and moderate staining of nerve fibres.

Medulla oblongata

Nuclei in the medulla oblongata were indentified using Nissl and haematoxylin and eosin (H&E) staining. A representative overview of a section stained for mGluR5 is shown (figure 3A). Both in the NTS and DMV, expression of mGluR5 was observed in nerve fibres and in neurons while the solitary tract remained unstained. Other stained regions comprised the olivary nuclei, but staining was less prominent compared to the cranial nerve nuclei like the nucleus of the hypoglossus nerve. A summary of staining data is given in table 3.
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Figure 2 | Immunostainings of the nodose ganglion. In panel A and B, mGluR5 immunoreactivity is detected in neuronal cell bodies and nerve fibres (A and B). GABA\_R1 (C and D) and GABA\_R2 (E and F) have a comparable staining pattern in ganglion cells and in nerve fibres. In contrast to GABA\_\alpha\_1 (G and H), GABA\_\beta\_2/3 receptor subunits (I and J) are present in the nodose ganglion. Strong and specific labelling of ganglion cells is observed for both cannabinoid receptors (CB1 (K and L) and CB2 (M and N)). Scale bar: A,C,G,I, K and M = 200 μm, B, D and J = 70 μm, F, L and N = 40 μm.

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Figure 3 | Images A to G show representative stainings of the medulla oblongata at the level of the upper medulla. Immunoreactivity for mGluR5 can be observed in A, B and C with different magnification, revealing mGluR5 immunoreactive neurons in the DMV. GABA\textsubscript{B}R1 immunoreactivity on neurons and nerve fibres in the DMV is shown in image D, and reactivity for GABA\textsubscript{B}R2 is shown in the DMV (E) and at a larger magnification (F). A typical plasma membrane and dendrite staining is observed for GABA\textsubscript{A\textalpha{}} subunits in the NTS (G) with a larger magnification in H. Comparably, extensive staining of axons and dendrites is observed for CB1 (I) and CB2 (J) in DMV. Scale bars: A 400 μm BG 150 μm CDEIJ 50 μm FH 20 μm. Abbreviations: DMV: dorsal motor nucleus of the vagus, NTS: nucleus of the solitary tract, Sol: Solitary tract, VL: Ventrolateral solitary nucleus, DFr: dorsal fringe of the dorsal nucleus of the vagus, MFr: medial fringe of the dorsal nucleus of the vagus, In: Intercalated nucleus of the vagus.
In control cerebellum, GABA_α1 and GABA_β2/3 co-staining was observed along with a cellular pattern similar to that previously described. Positive stainings of Purkinje cell and molecular layers were also observed. In the medulla oblongata, GABA_α receptors were localized in the DMV and NTS mostly on the cell bodies, as shown in figures 3D and 3E. Moderate staining was observed in the solitary tract, whilst a profound staining in the olivary nucleus and in the dorsal olivary nucleus was observed.

A typical staining pattern of the plasma membrane and dendrites was observed in GABA_α1 and CB1 and CB2 stainings, as shown in figures 3G-3J. Cannabinoid receptors were observed in the DMV and NTS and other nuclei in the brain stem, comparable to GABA_α receptor distribution, whereas staining in the solitary tract could not be detected.

**Myenteric plexus**

Four LES resection specimens were studied in which the myenteric plexus could easily be identified between the two muscle layers. The ganglion cells in the myenteric plexus clearly expressed mGluR5, GABA_α receptor subunits and both cannabinoid receptors (Figure 4). In contrast, no staining for GABA_α1 was observed in any of the esophageal resection specimens. Additionally, immunolabeling of nerve fibres was observed for GABA_β2/3 (I and J) and CB1 (L).
Figure 4 | The presence of mGluR5 (A and B), GABA_Aβ2/3 (C and D), GABA_A (E and F), GABA_Aβ2/3 (I and J), CB1 (K and L) and CB2 (M, N and O) receptors was observed in ganglion cells in the myenteric plexus. Additionally, immunolabeling of nerve fibres was observed in GABA_Aβ2/3 (I and J) and CB1 (L). No immunoreactivity for GABA_Aα1 (G and H) was observed. Scale bars A, C, E, G, J, K, M, N 150μm, B, D, F, H, I, L and O 50 μm.

DISCUSSION

This is the first human study that analyzed the distribution of mGluR5, GABA and cannabinoid receptors along the vago-vagal neural pathway involved in the triggering of TLESRs. Similar to previous animal studies, all receptors were abundantly expressed in the brain stem, and all but GABA_Aα1 were present in the nodose ganglion and myenteric plexus of the LES. These findings are
in line with the central side effects reported during treatment with reflux inhibitors such as GABA\_A receptor agonists and mGlur5 antagonists, and underscore that peripheral acting compounds may still be effective as reflux inhibitors.

A TLESR is characterised by relaxation of the LES and crural diaphragm occurring in the absence of swallowing, triggered by the activation of mechanosensitive receptors located on vagal afferents within the stomach muscle layer, or as recently hypothesised, by activation of chemo- or tension receptors within the gastric mucosa\(^1\). These vagal afferents have their cell body in the nodose ganglion and terminate in the NTS. Tracing studies using pseudorabies virus, known to cross neural synapses, elegantly showed that the signal is relayed to the NTS and nuclei in the dorsal vagal complex in the brainstem.\(^2\) Finally, after integration of the sensory information, DMV neurons activate inhibitory neurons in the myenteric plexus of the LES leading to relaxation. According to recent evidence, the crural diaphragm is also directly innervated from these regions in the DMV, which explains the co-ordination of the motor pattern.\(^3\,4\) To study the distribution of receptors along the neural pathway involved in the triggering of TLESRs, human nodose ganglia, brain stems and lower esophageal sphincters were analysed for the presence of mGluR5, GABA\_A, GABA\_B, and cannabinoid receptors.

Vagal afferents utilize glutamate as their principal neurotransmitter to communicate with NTS neurons by activation of both ionotropic and metabotropic glutamate receptors.\(^5\,6\) Metabotropic Glutamate 5 receptors located on vagal afferents are involved in visceral perception, as demonstrated by the finding that mGluR5 antagonists reduce mechanosensitivity to gastric distention.\(^7\,8\) In multiple species such as dogs, ferrets and rodents, mGluR5 has been demonstrated in the nodose ganglion, DMV and NTS using polymerase chain reactions (PCR) and immunohistochemical stainings.\(^7\,9\) In humans, the presence of mGluR5 has been previously described in the nodose ganglion using PCR.\(^7\) In our study, we confirmed these findings using immunohistochemistry illustrating labelling for mGluR5 of the nodose ganglion, NTS and DMV. However, retrograde labelling of gastric projecting neurons in the brain stem and nodose ganglion in ferrets revealed that the expression of mGluR5 receptors was confined to the afferent soma in the nodose ganglion, whilst gastric projecting neurons in the DMV and NTS did not express mGluR5 receptors.\(^10\) In contrast to this observation, microinjection of L-glutamate in the DMV in ferrets abolishes LES pressure suggesting that glutamate excites neurons targeting the LES, possibly via mGluR5 receptors.\(^11\) Clearly, these approaches cannot be used in humans making it impossible to determine if the LES is innervated by mGluR5 expressing neurons. In addition, we demonstrated the presence of mGluR5 in the myenteric plexus neurons for the first time. Previously, the presence of mGluR5 receptors has been demonstrated in rats and guinea pigs in the myenteric plexus of ileum and colon, but not in the esophagus.\(^12\)
For an appropriately functioning GABA$_B$ receptor, both GABA$_B$R1 and GABA$_B$R2 receptor subtypes are needed. GABA$_B$ receptors produce slow prolonged inhibitory signals, and therefore agonism of GABA$_B$ receptors on the vago-vagal reflex arch results in a reduction of the number of TLESRs. The presence of GABA$_B$ receptors in the nodose ganglion was previously demonstrated in rats and ferrets, as well as their presence in the brain stem of rodents and humans. Our study confirms the presence of both receptor subtypes in human nodose ganglion, NTS and DMV. In addition, we demonstrate the presence of GABA$_B$ receptors in the myenteric plexus, comparable to a recent study by Torashima et al. These authors demonstrated that both GABA$_B$ receptor subtypes are present in the myenteric plexus and form heterodimers. This suggests that the myenteric plexus is a possible site of action of GABA$_B$ agonists, thereby contributing to the reduction in TLESRs.

In contrast to mGluR5 receptors, GABA$_B$ receptors are highly expressed in LES-projecting neurons in the DMV. In line with this, injection of baclofen into the DMV inhibits the firing rate of vagal motor neurons that are responsive to gastric distension. Interestingly, the DMV is composed of heterogeneous neuronal subpopulations in terms of both membrane as well as pharmacological properties. These subpopulations can further be distinguished based on their peripheral targets, at least in rats. Notably, activation of GABAB-receptors in one neuronal subpopulation projecting to the stomach leads to an increase in vagal excitatory cholinergic drive mediating an increase in rhythmic contractions, whereas activation of another neuronal subpopulation leads a decrease in tonic vagal drive to NANC inhibitory neurons resulting in an increase in gastric pressure. To what extent this also applies to human remains unclear and cannot be concluded from our data.

Next, we evaluated the distribution of CB1 receptors, as blockade of these receptors has been shown to inhibit TLESRs in humans. CB1 receptors have a comparable distribution and function as GABA$_B$ receptors. Comparable to rats and ferrets, CB1 receptors were present in the human nodose ganglion, DMV and NTS. Interestingly, in animals ganglionectomy or vagotomy did not lead to an alteration receptor expression of DMV or NTS neurons. As vagal motor neurons degenerate after vagotomy, these findings support an intrinsic interneuronal localization of immunoreactive terminals in the brainstem. In line with this, no staining of CB1 receptors was observed in dorsal motor nucleus neurons projecting to the LES and gastric fundus identified using retrograde labelling studies in ferrets. Although we cannot directly extrapolate animal data to humans, these data suggest that it is questionable whether these neurons in the DMV and NTS in humans project to the LES.

In contrast to previous immunohistochemical evidences, a more recent study by van Sickle showed that CB2 receptors are expressed in rat and ferret brainstem.
humans, expression in the brain was most prominent in the DMV, although van Sickle et al report that the levels of CB2 were much lower compared to the spleen. Due to the high levels of CB2 receptors in the spleen, agonism of CB2 receptors leads to immunosuppression. Therefore, this receptor is probably not a suitable candidate for the reduction of TLESRs in GERD.

GABA\textsubscript{A} induces fast synaptic ionotropic inhibition upon activation, and the receptor consists of various subunits. In contrast to GABA\textsubscript{A} receptors, no GABA\textsubscript{A}\textsubscript{\textalpha} were present in the myenteric plexus and nodose ganglion, which is comparable to the absence of these receptors in the periphery in dogs. The lack of effect of peripherally acting GABA\textsubscript{A} antagonists on TLESR rates in dogs substantiates the absence of peripheral GABA\textsubscript{A} receptors on the vago-vagal reflex arch.

To date, clinical studies have indeed demonstrated the favourable effect of GABA\textsubscript{B} agonism and mGluR5 antagonism on the number of TLESRs, reflux episodes and symptoms in humans compared to placebo. However, currently there are no reflux inhibitors available for clinical use except baclofen, which also has sustainable side effects. The same holds true for mGluR5 antagonists inducing central side effects such as dizziness and vertigo in 16% to 12% of patients. These data are in line with the profound distribution of both GABA and mGluR5 in the brain stem, cortex and cerebellum. Therefore, centrally acting agents are anticipated to have sustainable side effects, emphasizing the need for development of peripherally acting agents. In view of these central side effects, pharmaceutical companies have developed peripherally acting compounds. As shown in the present study, these receptors are abundantly present in the periphery. Furthermore, functional evidence has established a peripheral site of action of baclofen on GABAB receptors and of mGluR5 antagonists on mGluR5 receptors in gastric vagal afferents, suggesting that reflux inhibitors targeting these receptors may have the potential to block TLESRs.

Two strategies specifically targeting peripheral receptors can be considered: First, polarized compounds that do not pass the blood barrier and thus do not enter the central nervous system can be used. It should be emphasized though that the blood brain barrier at the level of the DMV and NTS is leaky and thus polarized compounds could also – at least partly – have an effect on receptors in this region. Second, targeting specific subpopulations of receptors or transporters could also affect the TLESR reducing properties or side effect profile of a compound. For instance, lesogaberan, a GABA\textsubscript{B} agonist reducing the number of TLESRs and GERD symptoms, is taken up by the GABA transporter (GAT), and is therefore sequestrated by CNS neurons leading to reduced intracerebral levels. Baclofen however does not bind to GAT most likely explaining the finding that lesogaberan has fewer CNS side effects than baclofen. Still, Lesogaberan effectively reduces the number of TLESRs leading to a significantly better symptom relief than placebo.
Our study shows the expression of several receptors along the entire neural pathway in human tissue. Studies in laboratory animals are usually hard to translate to the human situation, and therefore conflicting results in animal and clinical studies often arise.\textsuperscript{43} however the limitation of our study is that the target organ of the neurons cannot be determined. To this end, anterograde and retrograde labelling is required which unfortunately is not feasible in humans due to ethical concerns.

In conclusion, this study shows that mGluR5, GABA\textsubscript{A,B}, and CB\textsubscript{1,2} receptors, which are the targets of known reflux inhibitors, are present throughout the vago-vagal reflex pathway involved in the triggering of TLESRs. These findings are in line with the central side effects observed during treatment with GABA\textsubscript{A} receptor agonists and mGluR5 antagonists, but also suggest that peripherally acting compounds may be effective as reflux inhibitors.
REFERENCE LIST


