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Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms

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ABSTRACT

Objectives: G protein-coupled receptors (GPCRs) act as transmembrane molecular sensors of neurotransmitters, hormones, nutrients, and metabolites. Because unmyelinated vagal afferents richly innervate the gastrointestinal mucosa, gut-derived molecules may directly modulate the activity of vagal afferents through GPCRs. However, the types of GPCRs expressed in vagal afferents are largely unknown. Here, we determined the expression profile of all GPCRs expressed in vagal afferents of the mouse, with a special emphasis on those innervating the gastrointestinal tract.

Methods: Using a combination of high-throughput quantitative PCR, RNA sequencing, and *in situ* hybridization, we systematically quantified GPCRs expressed in vagal unmyelinated $Na_V1.8$-expressing afferents.

Results: GPCRs for gut hormones that were the most enriched in $Na_V1.8$-expressing vagal unmyelinated afferents included NTSR1, NPY2R, CCK1R, and to a lesser extent, GLP1R, but not GHSR and GIPR. Interestingly, both GLP1R and NPY2R were coexpressed with CCK1R. In contrast, NTSR1 was coexpressed with GPR65, a marker preferentially enriched in intestinal mucosal afferents. Only few microbiome-derived metabolite sensors such as GPR35 and, to a lesser extent, GPR119 and CaSR were identified in the $Na_V1.8$-expressing vagal afferents. GPCRs involved in lipid sensing and inflammation (e.g. CB1R, CYSLTR2, PTGER4), and neurotransmitters signaling (CHRM4, DRD2, CRHR2) were also highly enriched in $Na_V1.8$-expressing neurons. Finally, we identified 21 orphan GPCRs with unknown functions in vagal afferents.

Conclusion: Overall, this study provides a comprehensive description of GPCR-dependent sensing mechanisms in vagal afferents, including novel coexpression patterns, and conceivably coaction of key receptors for gut-derived molecules involved in gut-brain communication.

Keywords G protein-coupled receptors; Vagal afferent nerves; Gut-brain axis; Gut hormones; GLP1R; NTSR1

1. INTRODUCTION

The gastrointestinal (GI) mucosa has long been known to be richly innervated by unmyelinated axons [1,2]. These axons typically wander within the lamina propria, often approaching its epithelium without penetrating the basal lamina. While many axons present in the GI mucosa are of enteric origin [3], anterograde tracing studies revealed that vagal afferents also abundantly innervate the GI mucosa [4–8]. Some vagal unmyelinated afferents supplying the GI tract are polymodal [9], and electrophysiological studies have demonstrated that mucosal vagal afferents respond to a wide range of molecules absorbed across the epithelium or locally released, in addition to the distension of the alimentary canal, as well as noxious, thermic and osmotic stimuli [10–17]. Thus, it is not surprising that a variety of sensations and autonomic reflexes can be elicited by the mechanical or chemical stimulation of the GI mucosa [18–21].

Many of the molecules that modulate the activity of vagal afferents, including neurotransmitters, gut peptides, lipids, and metabolites, are G protein-coupled receptors (GPCRs) ligands [22]. However, due to a lack of understanding of GPCRs distribution in vagal afferents, it is unknown whether the aforementioned molecules directly act on these neurons. For instance, a prevalent view suggests that vagal GI mucosal afferents directly respond to peptides released from enteroendocrine cells including, cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) [23–27]. In support of this view, the receptors for CCK and GLP-1 are abundantly expressed in vagal afferents [7,28–30], and

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CCK and GLP-1 acutely enhance the activity of vagal afferents [10,31]. Moreover, the anorectic effects of peripheral CCK and GLP-1 are attenuated in deafferented animals [32–35]. Despite these findings, several other studies have suggested that gut-derived CCK and GLP-1 act through potentiating vagal responses to distending loads and/or directly on the brain [36–40]. A recent study further challenged the common view that CCK and GLP-1 can be directly detected by mucosal afferents by demonstrating that neurons expressing receptors for these gut hormones do not innervate the mucosa [7]. Lastly, considering that at least 10 different peptides are known to be secreted by different types of enteroendocrine cells [41], it is unknown which gut peptides, other than CCK and GLP-1, can be detected by vagal afferents.

Here, we determine the expression profile of all GPCRs in the vagal afferents of the mouse. A special emphasis was given to vagal afferents expressing $Na_v1.8$, a voltage-gated sodium channel involved in the generation of action potentials in vagal unmyelinated afferents [16,42,43]. $Na_v1.8$-expressing vagal afferents richly innervate the entire GI mucosa [5], making these neurons ideally positioned to detect nutrients and molecules locally released in the GI tract. To better understand how the vast assortment of GPCRs contribute to vagal sensing mechanisms, we fully characterized GPCRs expression in $Na_v1.8$-expressing vagal afferents using a combination of quantitative PCR (qPCR), RNA sequencing, and double *in situ* hybridization studies.

2. MATERIALS AND METHODS

2.1. Mice

Male C57BL/6JRj and C57BL/6J wild-type mice were purchased from Janvier Breeding Centre (France) and the ARC at UT Southwestern Medical Center (Dallas), respectively. These mice were used for qPCR assays, chromogenic Brown, Red, and Duplex *in situ* hybridization.

$Na_v1.8$-Cre-DTA mice were generated and genotyped as previously described by us [44]. Briefly, we crossed $Na_v1.8$ knock-in Cre-recombinase mice (gift from Dr. John Wood, University College London) on a pure C57Bl/6J background with ROSA26-eGFP-DTA mice (stock #006331; The Jackson Laboratory, USA) to generate litters with a 1:1 ratio of littermate controls ($Na_v1.8$-Cre) and ablated mice ($Na_v1.8$-Cre-DTA). Young male ablated and control mice were used for RNA-Seq studies.

$Na_v1.8$-Cre-ChR2-YFP male mice were also used for the purpose of ISH combined with immunohistochemistry. Mice carrying one $Na_v1.8$-Cre and one ChR2-YFP alleles were generated and genotyped exactly as previously described by us [45]. In these mice, YFP clearly delineates the membrane of $Na_v1.8$ neurons, hence facilitating double labeling analysis.

All mice used were young males (6–10 weeks old) housed in a barrier facility with temperature controlled environment (~23 °C). Mice were fed *ad libitum* with standard chow. The Danish Animal Experiments Inspectorate or Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas approved all animal procedures.

2.2. qPCR array of nodose ganglia

Wild-type mice (C57/Bl6JRj, Janvier) were euthanized using cervical dislocation and the nodose ganglia from 2 mice were dissected into RNAlater at room temperature and kept at 4°C overnight prior of RNA extraction using the NucleoSpin® RNA XS (MACHEREY-NAGEL) and RT-PCR was performed using SuperScript III Reverse Transcriptase (Invitrogen). Custom-designed RT2 Profiler PCR Arrays (Qiagen) was used to analyze 377 GPCRs and 3 Receptor-Activity Modifying Proteins (RAMPS) on a LightCycler480 (Roche). Relative expression was calculated using the formula:

$$Relative\ expression = 2^{-(CqTarget - CqRef)} \times C$$

where CqTarget = the quantification cycle (Cq) for the target gene; CqRef = the geometrical mean of the quantification cycle for the reference genes (Ywhas, Actb and Gapdh); C is an arbitrary constant dependent on CqRef used to shift the relative expression so Cq values of 35 on average are equal to 1, here C = 24221. Undetectable targets were assigned a Cq value of 40. In total 8 mice was used to generate the 4 samples analyzed. Information of genes on the qPCR array is given in Table S1.

2.3. RNA-sequencing of nodose ganglia

RNA sequencing was performed on the nodose ganglia of ablated and control mice (n = 3/group). The two nodose ganglia (left and right) from one mouse were pooled and RNA was extracted as described above for qPCR. Library construction (Truseq, Illumina), sequencing (Hiseq 100 PE, Illumina) and read quality control filtering was performed at BGI Tech solutions (Hong Kong). The sequencing data was processed using the "Tuxedo tools" (Tophat2, Cufflinks2, cummeRbund) as outlined in [46] using the mm10 reference annotation only. Briefly, normalized expression quantification and differential expression tests for Ensembl genes were obtained using Cuffdiff2 with "–frag-bias-correct" [47] and "–multi-read-correct" enabled. The output from Cuffdiff2 [46] was analyzed with the R package cummeRbund (version 2.12.1).

2.4. *In situ* hybridization

2.4.1. RNAScope chromogenic "brown" assay

Wild-type mice received an overdose of chloral hydrate (500 mg/kg, i.p.) and their nodose ganglia were rapidly dissected and frozen on dry ice. Ganglia were cut using a cryostat at 14 μm and stored at −80 °C. Following the manufacturer's protocol (Advanced Cell Diagnostic), ganglia were hybridized with double-Z oligo probes for the genes listed in Table S2. Signal detection was achieved using DAB and tissue was counterstained with Nuclear Fast Red (Sigma).

2.4.2. RNAScope chromogenic "red" assay combined with immunohistochemistry for YFP

$Na_v1.8$-Cre-ChR2-YFP mice were anesthetized with chloral hydrate (500 mg/kg, i.p.) and transcardiacally perfused with 10% formalin (Sigma). Fixed nodose ganglia were kept in 10% formalin for 48 h at 4 °C before being transferred into 20% sucrose in PBS for an additional 24 h. Ganglia were next frozen and cut at 14 μm onto SuperFrost Plus slides (1:5 series). Tissue was baked at 60 °C for 30 min, washed in 1X PBS, treated in H_2O_2 for 10 min. Pretreatment consisted of hot 1X Target Retrieval solution for 1–2min. Next, slides were rinsed in distilled water and dehydrated in fresh 100% ethanol. Tissue was incubated with Pretreatment 3 at 40 °C for 15mins. After rinsing the slides in distilled water, hybridization was performed using the standard ACD procedure and reagents from the RNAscope® 2.5 HD Detection Kit (RED). The probes listed in Table S2 were applied at 40 °C for 2hrs. Amplification steps were done using the following the manufacturer's instructions. Lastly, signal detection was achieved using a mix of Fast RED-B and Fast RED-A in a ratio of 1:60 at room temperature for about 10 min. Slides were washed in distilled water. The native tdTomato fluorescence was greatly diminished following *in situ* hybridization. Nodose ganglia were next labeled using a GFP

