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Phenotypic distinctions between the nodose and jugular TRPV1-positive vagal sensory neurons in the cynomolgus monkey.

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Abstract

Objective: Vagal capsaicin-sensitive afferent C-fibers play an important role in maintenance of visceral homeostasis and contribute to symptoms in visceral diseases. Based on their developmental origin two functionally distinct types of vagal C-fibers are recognized: those with neurons in the vagal nodose ganglia (derived from epibranchial placodes) and in the vagal jugular ganglia (from neural crest). Studies in non-primate species demonstrated that the vagal nodose and jugular C-fibers differ in activation profile, neurotrophic regulation and expression of neurotransmitters. We hypothesized that the expression of selected markers related to key phenotypic properties of vagal C-fibers in the cynomolgus monkey is similar to that reported in non-primate species.

Methods: We performed single cell RT-PCR on nodose and jugular putative C-fiber (TRPV1-positive) neurons isolated from the cynomolgus monkey.

Results: We found that the expression of purinergic P2X receptors that underlie selective responsiveness of nodose C-fiber terminals to ATP was conserved in that P2X₂ and P2X₃ subunits were expressed in nodose, but only P2X₃ subunit was expressed in jugular TRPV1-positive neurons. Also conserved was the preferential expression of neurotrophic receptor TrkB in the nodose and preprotachykinin-A (PPT-A) in the jugular TRPV1-positive neurons.

Conclusion: Several key distinctions in gene expression between nodose and jugular TRPV1-positive (C-fiber) neurons that have been noted in mice, rats, and guinea pigs, are conserved in the cynomolgus monkey. Our results support the translatability of distinct vagal C-fiber phenotypes to primates.

Keywords

vagus nerve; C-fibers; monkey; *Macaca fascicularis*; nodose ganglion; jugular ganglion

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AUTHORS' CONTRIBUTION

MK designed primers, performed data analysis, co-wrote the manuscript.

FR performed dissection, cell dissociation, single cell RT-PCR.

BJU initiated the study, formulated hypotheses, co-wrote the manuscript.

All authors have approved the final article.

Conflicts of Interest and Source of Funding

Conflict of interest: none declared.

Introduction

It is often underappreciated that vagal afferent innervation may comprise not only vagal nodose neurons, but also vagal jugular neurons. Unlike nodose neurons that developmentally originate from epibranchial placodes, jugular neurons originate from neural crest (similarly to dorsal root ganglia DRG neurons) [1]. In most species including humans, monkey, guinea pigs, cats, rabbits and dogs jugular neurons are located in a separate jugular ganglion proximal to nodose ganglion [2-8]. In mice, and rats, jugular neurons are located close to nodose and petrosal neurons often in a single variably-shaped structure termed jugular(-petrosal)-nodose ganglion complex, often simply referred to as nodose ganglion [6,9,10].

Studies in laboratory animals revealed that both nodose and jugular neurons project capsaicin-sensitive (TRPV1-positive) C-fibers into the airways, lungs and esophagus [11-13]. Importantly, there are significant phenotypic distinctions between the nodose and jugular C-fibers in the activation profile, expression of neurotrophic receptors, and neuropeptide content [11,12,14-17]. Growing evidence also indicates that nodose and jugular C-fibers connect to different pathways in CNS and initiate different reflex responses [10,13,18,19]). The results from guinea pigs, mice and rats suggest that these phenotypic distinctions between nodose and jugular C-fibers are conserved amongst common laboratory animals [6,10,16,18,20], but it is unknown whether they are conserved in primates.

The purpose of the present study was to determine if the expression of selected markers related to key phenotypic distinctions observed in nodose vs jugular C-fiber neurons in laboratory animals are conserved in the cynomolgus monkey (*Macaca fascicularis*). The markers that have been shown to differ between nodose vs. jugular C-fibers in mice and guinea pigs include ATP receptors (P2X₂ and P2X₃) [14], substance P expression (PPT-A) [5,11,12] and receptor expression for various neurotrophic factors (TrkA, TrkB and GFR α_3) [6,20]. We used single cell RT-PCR to evaluate these markers on vagal TRPV1-positive (presumed C-fiber) neurons. Our results indicate that several distinctions between nodose and jugular C-fibers are conserved in the cynomolgus monkey and may be therefore conserved in other primates including in humans.

Material and methods

Tissues were harvested from cynomolgus monkeys (*Macaca fascicularis*, N=3) that were sacrificed at the end of unrelated studies not involving vagally-innervated tissues according to a protocol approved by Johns Hopkins Animal Care and Use Committee. Tissues from one animal were used for the dissection (Fig. 1) and whole ganglia RNA isolation. Single cell RT-PCR studies were performed on individual neurons dissociated from the monkey nodose and jugular ganglia (N=2 animals) using Super-Script III CellsDirect cDNA Synthesis System (Life Technologies) and HotStar Taq Polymerase Kit (Qiagen) for RT and PCR, respectively, as described previously [15,20]. The intron-spanning primers were designed by using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) based on the predicted *Macaca fascicularis* sequences (target, forward and reverse sequences 5' to 3', predicted product size in bp, sequence number. TRPV1,

CGCACCTTCTACTTCAACTTCTT, GTCCACAAACAGGGTCTTCA, 231, EF100779.1; P2X₃, GCCCAGAGAGTGAGGAGAAA, AAGAGGGGGAAACGGATG, 232, XM_005577867.1; P2X₂, ACCCCAAATTCCTTCTCC, AAGCTCTCCCCAGCCTTTT, 142, XM_005572674.1; PPT-A, GTGGCCTTGGCAGTCTTTT, GCTGAGGCTTGGGTCTCC, 175, XM_005550147.1; TrkB, CCCATCCCTGTCTCCTTCTC, GTTCTCCTTCCGCATTGT, 171, XM_005541397.1; TrkB, TTGGGATGTTGGTAATCTGGT, GTGGTGGTCTGAGGTTGGAG, 214, XM_015437336.1; GFR α_3 , TCCAGATGTCTACGAGCATACC, CTGGCTGTGAAAACGCATC, 207, XM_005557893.2). RNA was isolated using RNeasy Mini Kit (Qiagen) separately from one nodose and one jugular ganglion (N=1 animal), pooled and used as a positive PCR control. Water and bath samples were used as negative controls. The expression of markers in nodose vs. jugular neurons was compared by two tailed Fisher exact test.

Results

In the cynomolgus monkey the vagal nodose and jugular afferent ganglia were readily distinguishable with the jugular ganglion being appreciably smaller (approximately 25% of the size of the nodose ganglion) (Fig. 1). The jugular ganglion was located characteristically in jugular fossa of the skull while the nodose ganglion was located further distally. As shown in the dissection in figure 1 the arrangement of vagal ganglia and their relationships to adjacent structures including to vagal branches to laryngopharyngeal region, glossopharyngeal nerve and sympathetic trunk in the cynomolgus monkey was similar to that in humans as also partially described in the monkey before [2,4].

We obtained 27 nodose and 36 jugular vagal afferent neurons dissociated from 4 nodose and 4 jugular ganglia from 2 monkeys. We focused our single cell RT-PCR studies on the neurons that were positive for the capsaicin receptor TRPV1, as capsaicin-sensitivity largely defines C-fibers in the respiratory and upper gastrointestinal tract [6,11,12]. From our random selection of neurons 85% (23/27) of nodose and 61% (22/36) of jugular neurons expressed the TRPV1 mRNA.

In adult mice and guinea pig the purinergic receptor P2X₃ subunit is expressed in nearly all vagal sensory nodose and jugular neurons, whereas the purinergic P2X₂ subunit is expressed in nodose but not jugular neurons. Likewise, in the cynomolgus monkey we noted that the vast majority of TRPV1-positive nodose and jugular neurons expressed mRNA for P2X₃, whereas the P2X₂ mRNA expression was limited to the nodose neurons (Fig. 2, Tab. 1). This observation indicates that, as with mice and guinea pigs, many nodose C-fiber neurons in the cynomolgus monkey express the heteromeric P2X_{2/3} receptors, whereas the jugular neurons are more likely to express the homomeric P2X₃ receptors.

Distinctions have been noted in the expression of neurotrophic factor receptors in adult nodose vs. jugular neurons in mice, rats and guinea pigs. For example, nodose neurons are found to preferentially express TrkB, the receptor for BDNF and NT4 [6,15,20,21]. This holds true in the cynomolgus monkey with 96% of the nodose TRPV1-positive neurons expressing TrkB mRNA. In mice, rats, and guinea pigs TrkA is expressed in nearly all

jugular neurons, but much less so in nodose neurons. Surprisingly, the majority of both nodose and jugular TRPV1-positive neurons failed to express TrkA mRNA in the cynomolgus monkey. In mice and rats (but not guinea pig) the co-receptor for neurotrophic factor artemin, GFR α 3, is preferentially expressed by jugular but rarely in nodose neurons [6,15,20]. In the cynomolgus monkey GFR α 3 was expressed by approximately 40% of both nodose and jugular TRPV1-positive neurons.

Distinctions between nodose and jugular vagal neurons have also been noted in their neuropeptide content. We and others reported that the pre-protachykinin PPT-A mRNA and tachykinins are preferentially expressed in jugular neurons over nodose neurons [6,11,12,20]. We found that approximately 90% of jugular TRPV1-positive neurons expressed PPT-A whereas approximately 40% of nodose TRPV1-positive neurons expressed PPT-A in the cynomolgus monkey.

3. Discussion

The dissection of the vagus nerves in the cynomolgus monkey revealed the location of the nodose and jugular vagal sensory ganglia in this species (Fig. 1). We analyzed the expression of marker genes (P2X₂, P2X₃, TrkA, TrkB, PPT-A and GFR α ₃) as these markers serve to differentiate nodose from jugular C-fibers in the respiratory tract [13] and in the esophagus [12,14,20]. In general, the key distinctions in gene expression between nodose and jugular TRPV1-positive (C-fiber) neurons that have been noted in mice and guinea pigs were also found in the cynomolgus monkey. Our results therefore support the hypothesis that the distinctions in vagal nodose and jugular C-fiber phenotypes are largely conserved among different species including primates.

The expression of the purinergic P2X receptors provides an example of a well-conserved distinction between nodose and jugular C-fibers. Similar to findings in guinea pigs and mice, we found that the vast majority of nodose and jugular C-fiber neurons expressed the purinergic P2X₃ subunit (Fig. 2, Tab. 1). Also similar to mice and guinea pigs, P2X₂ subunit expression was selective for nodose neurons (Fig. 2, Tab. 1). Importantly, P2X₂ was always detected together with P2X₃ in the nodose neurons (Fig. 2). The P2X subunits form trimeric homomeric or heteromeric ionotropic receptors [22]. Stimulation of the homomeric P2X₃ receptors with ATP leads to transient rapidly-inactivating currents, whereas stimulation of P2X_{2/3} receptors leads to much larger and sustained currents [22]. In the guinea pig and mouse ATP and its analogues evoked large sustained P2X_{2/3}-like currents in nodose C-fiber neurons (expressing both P2X₂ and P2X₃ subunits) and induced strong action potential discharge in their nerve terminals in the lungs and esophagus [14,23]. In contrast, ATP and its analogues evoked only small transient homomeric P2X₃-like currents in jugular C-fiber neurons (expressing P2X₃ but not P2X₂) and failed to evoke action potential discharge in their nerve terminals [14]. Thus, the selective expression of P2X₂ in nodose C-fibers confers an important phenotypic distinction in that it mediates responsiveness of their nerve terminals to an important nociceptive mediator ATP often released in tissue injury and inflammation [22].

That the nodose neurons express P2X₃ was also demonstrated in humans [24]. A drug that inhibits both the homomeric and heteromeric receptors containing P2X₃ subunit has been demonstrated to effectively inhibit cough in subjects suffering from chronic idiopathic cough [25]. If this block is due to blocking the ATP-driven action potential discharge in vagal C-fiber terminals then the present non-human primate data together with the previous studies in mice and guinea pigs support the hypothesis that nodose C-fiber activation may underlie chronic idiopathic cough.

Preprotachykinin-A (PPT-A) mRNA encodes peptides of tachykinin family including substance P. In all small laboratory animals studied (mice, rats, guinea pigs, and rabbits) tachykinins are preferentially expressed in jugular over nodose neurons [5,6]. In the guinea pig PPT-A mRNA and substance P are expressed in ~80% of jugular C-fiber neurons compared to only 20-50% of nodose C-fiber neurons [11,12].

This was similar in the monkey in which PPT-A was expressed in ~90% of jugular and ~40% of nodose TRPV1-positive neurons. Consistent with this observation, substance P was detected by immunostaining in approximately 30% of nodose neurons and in nearly all jugular neurons in the macaca fascicularis monkey [3].

In the guinea pig TrkB is preferentially expressed in nodose C-fiber neurons (80% vs. 50% jugular)[15]. This was similar in the adult monkey (100% vs. 70%, $P<0.05$). GFR α_3 was found in both nodose and jugular C-fiber neurons in the guinea pig (70% and 90%, respectively) [15]. This was similar in the monkey albeit in smaller proportions of nodose and jugular TRPV1-positive neurons (50% and 40%, respectively). Interestingly, this differentiated the guinea pig and monkey from the mouse in which GFR α_3 was selectively expressed in a proportion of jugular neurons[6,20]. Thus, neurotrophins and neurotrophic factors such as BDNF and artemin may play an important neuromodulatory role in visceral organs of adult primates.

One difference between the monkey and the guinea pig and mouse observed in this study was in the TrkA expression. In the guinea pig and mouse TrkA is preferentially expressed in jugular neurons and only rarely expressed in nodose neurons [15]. In the monkey the TrkA expression in the nodose TRPV1-positive neurons (30%) was similar to that in the guinea pig. However, in the jugular C-fiber neurons TrkA was expressed in notably lower proportion in the monkey (~20%) than in the guinea pig and mouse (~90%). These data would argue that NGF may have less influence on vagal sensory neurons in adult primates as compared to small laboratory animals like guinea pigs and mice.

It should be kept in mind that our previous studies in the guinea pig and mice were generally carried out on the organ-specific nodose and jugular neurons that were retrogradely labeled from the airways, lungs or the esophagus. In contrast, in the present study we evaluated nodose and jugular neurons in which the innervation target organs were unknown. This may account for some quantitative differences between observations in the monkey and the guinea pig and mouse. Nonetheless, the preserved main expression pattern of most markers argues for conserved phenotypic distinctions between nodose and jugular C-fibers in mammals.

In guinea pigs, activation of nodose vs. jugular C-fibers can lead to opposing respiratory reflexes that are often differentially perturbed in respiratory diseases[19]. Distinct central terminations of vagal C-fiber subtypes suggest that they have similarly distinct roles in regulation of other organs [10,18]. Understanding of translatability of the knowledge of vagal C-fiber subtypes to humans is expected to focus the development of treatment strategies that target vagal C-fibers to reduce the symptoms of those suffering from visceral inflammatory diseases.

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Figure 1. Vagal jugular and nodose afferent ganglia and adjacent structures (right side) in the cynomolgus monkey.

Figure 2.

The expression of mRNA of selected phenotypic markers detected by single cell RT-PCR in nodose and jugular TRPV1-positive neurons in the cynomolgus monkey. Representative gels. Individual neurons are numbered. +, positive control, -, negative control.

Table 1.

The expression of selected phenotypic markers in nodose and jugular TRPV1-positive neurons in the cynomolgus monkey.

	Nodose	Jugular
P2X ₃	87% (20/23)	68% (15/22)
P2X ₂	52% (12/23)	0% (0/22) **
PPT-A	43% (10/23)	86% (19/22) **
TrkA	30% (7/23)	23% (5/22)
TrkB	96% (22/23)	68% (15/22) *
GFR α_3	48% (11/23)	41% (9/22)

*
P<0.05

**
P<0.01, Fisher exact test (two tailed).