Acta Neurologica Scandinavica

Acta Neurol Scand DOI: 10.1111/ane.12462

ACTA NEUROLOGICA
SCANDINAVICA

Morphology of the human cervical vagus nerve: implications for vagus nerve stimulation treatment

Verlinden TJM, Rijkers K, Hoogland G, Herrler A. Morphology of the human cervical vagus nerve: implications for vagus nerve stimulation treatment.
Acta Neurol Scand: DOI: 10.1111/ane.12462.

Objectives – The vagus nerve has gained a role in the treatment of certain diseases by the use of vagus nerve stimulation (VNS). This study provides detailed morphological information regarding the human cervical vagus nerve at the level of electrode implant. *Results* – Eleven pairs of cervical vagus nerves and four pairs of intracranial vagus nerves were analysed by the use of computer software. It was found that the right cervical vagus nerve has an 1.5 times larger effective surface area on average than the left nerve [1,089,492 ± 98,337 vs 753,915 ± 102,490 μm^2, respectively, ($P < 0.05$)] and that there is broad spreading within the individual nerves. At the right side, the mean effective surface area at the cervical level (1,089,492 ± 98,337 μm^2) is larger than at the level inside the skull base (630,921 ± 105,422) ($P < 0.05$). This could imply that the vagus nerve receives anastomosing and 'hitchhiking' branches from areas other than the brainstem. Furthermore, abundant tyrosine hydroxylase (TH)- and dopamine ß-hydroxylase (DBH)-positive staining nerve fibres could be identified, indicating catecholaminergic neurotransmission. In two of the 22 cervical nerves, ganglion cells were found that also stained positive for TH and DBH. Stimulating the vagus nerve may therefore induce the release of dopamine and noradrenaline. A sympathetic activation could therefore be part of mechanism of action of VNS. Furthermore, it was shown that the right cervical vagus nerve contains on average two times more TH-positive nerve fibres than the left nerve ($P < 0.05$), a fact that could be of interest upon choosing stimulation side. We also suggest that the amount of epineurial tissue could be an important variable for determining individual effectiveness of VNS, because the absolute amount of epineurial tissue is widely spread between the individual nerves (ranging from 2,090,000 to 11,683,000 μm^2). *Conclusions* – We conclude by stating that one has to look at the vagus nerve as a morphological entity of the peripheral autonomic nervous system, a composite of different fibres and (anastomosing and hitchhiking) branches of different origin with different neurotransmitters, which can act both parasympathetic and sympathetic. Electrically stimulating the vagus nerve therefore is not the same as elevating the 'physiological parasympathetic tone', but may also implement catecholaminergic (sympathetic) effects.

T. J. M. Verlinden[1], K. Rijkers[2,3], G. Hoogland[2], A. Herrler[1]

[1]Department of Anatomy & Embryology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, the Netherlands; [2]Department of Neurosurgery, School for Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, the Netherlands; [3]Department of Neurosurgery, Zuyderland Hospital, Heerlen, the Netherlands

Key words: dopamine ß-hydroxylase; morphology; morphometric analysis; osmium tetroxide; peripheral autonomic nervous system; tyrosine hydroxylase; vagus nerve stimulation

T. J. M. Verlinden, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands
Tel.: 0031433881061
Fax: 0031433884134
e-mail: thomas.verlinden@maastrichtuniversity.nl

Accepted for publication June 23, 2015

Introduction

The (ortho)sympathetic and parasympathetic function of the autonomic nervous system (ANS) is essential to nearly all physiological processes. The vagus nerve is mainly known for its parasympathetic function. Apart from its physiological function, the vagus nerve has gained a

role in the treatment of certain diseases as well. The most important example of this role is the use of electrical stimulation of the vagus nerve (vagus nerve stimulation, VNS) for the treatment of epilepsy (1, 2). VNS is among others explored as treatment option for depression (3), cardiac disease states (4, 5) and inflammatory diseases such as rheumatoid arthritis (6, 7).

The exact mechanisms of VNS, however, remain elusive. Central effects are thought to be responsible for the anti-epileptic and antidepressant effects of VNS, while (mainly) peripheral and systemic effects are thought to be responsible for the immunomodulatory and cardiac effects (7). It has not been clarified yet whether the treatment effects can be explained by afferent/efferent, direct/indirect or ortho-/antidromic signalling. Furthermore, physiologic signalling towards intracranial structures can be afferent or efferent in origin depending on which division of the ANS is considered (i.e. parasympathetic or sympathetic). Dual peripheral and central effects are thereby likely too, through vago-vagal and vago-central (i.e. parasympathetic-sympathetic) reflexes (7). Stimulation intensity (output current), frequency, pulse width, signal on-time, signal off-time, surgical procedures and the choice of site are parameters that possibly influence the success rate of VNS therapy for the different pathologies. Classically, VNS is performed on the left side because of early observations that right-sided VNS caused a greater reduction in heart rate than left-sided VNS (7). Left-sided VNS became standard. The side effect profile of VNS investigated prospectively by Ben-Menachem et al. (1) and Handforth et al. (8), however, is positive and offers patients with refractory epilepsy prospects of good efficacy with only minor and often resolvable side effects (9).

Data on vagus nerve morphology are scarce (10–16). They include animal studies and qualitative studies on human vagus nerves. Quantitative data on human vagus nerve morphology at the level of electrode implant, however, are lacking. By giving a detailed morphological description of the human cervical vagus nerve using a computerized analysing system, we aimed to answer the following questions: What is the composition of human cervical vagus nerve? How many fascicles run through the nerve? What is its degree of myelination? Is the myelin equally distributed within the fascicles? What is the amount of epineurial (connective) tissue? How large is its effective surface area? Which percentage of this surface area is used by catecholaminergic neurotransmission? Do these variables differ between the right and left nerve? Do these variables differ between different nerves/individuals?

Methods

Eleven pairs of cervical vagus nerves (right and left) were harvested by two anatomists from not previously dissected, fresh formalin-fixed (femoral infusion of ~10 l fixative, followed by 4 weeks of fixation in a formalin bath) specimens, ranging in age between 67 and 91 years. No previous surgical interventions of head and neck had been performed.

Vagus nerve sampling

The nerves were exposed using techniques that are similar to surgery for vagus nerve electrode placement (17, 18). A five-centimetre linear incision through the skin and platysma muscle was made one centimetre above the clavicle extending cranially and parallel to the anterior border of the sternocleidomastoid muscle. This muscle was retracted laterally to expose the carotid sheath. The omohyoid muscle was then dissected from the carotid sheath and retracted cranially. The carotid sheath was opened, and the vagus nerve was exposed between the common carotid artery and jugular vein. At this level, the vagus nerve was transected caudally and cranially, at a course of 3 cm, whereafter the nerve was sampled (Fig. 1).

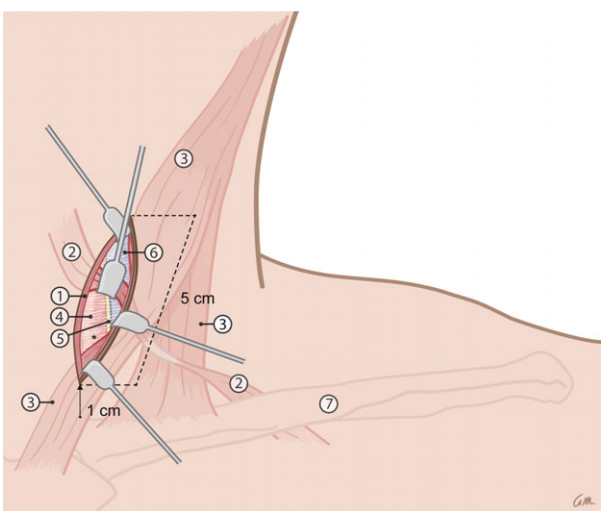

Figure 1. Harvesting of the vagus nerves using exposure techniques similar to the surgical approach applied during implantation of the electrodes of the VNS device. Legend: 1. platysma muscle, 2. omohyoid muscle, 3. sternocleidomastoid muscle, 4. common carotid artery, 5. vagus nerve, 6. internal jugular vein and 7. clavicle * carotid sheath.

From four additional specimens, vagus nerves (right and left) were collected inside the skull base (by means of opening the skull via a radial incision) between the brainstem and jugular foramen, proximal to the fusion of the vagus nerve with the accessory nerve.

Histological preparation

All cervical nerves were divided into three pieces, to provide the 'mean morphology' over the whole trajectory used for electrode placement. Every piece then was divided into two halves. The first halves were postfixated overnight with 1% osmium tetroxide (OsO_4) in phosphate-buffered saline (PBS) before they followed paraffin-embedding procedures. These paraffin blocks were cut into 4-μm transverse sections and mounted on slides. These slides were used for morphometric analysis. The other halves followed standard paraffin blocking and cutting (4-μm transverse sections) procedures and were used for immunohistochemistry staining with antityrosine hydroxylase (TH) (1:1000; Abcam AB112, Cambridge, UK) or antidopamine ß-hydroxylase (DBH) (1:150; Abcam, AB109112) for surface area measurement of catecholamine-containing fibres. Shrinkage of about 30% in tissue diameter before and during fixation and processing is thereby a recognized phenomenon (19).

Morphometric analysis (detailed description to be found in Data S1)

The slides were photographed with a Leica (type DMRD) photomicroscope in black and white using standardized settings. Surface areas of the specific stainings were measured with Leica Qwin v.3.5.1 analysis software at 10× magnification, including the following parameters: total surface area, effective surface area (nerve tissue exclusively within perineurium), connective tissue area, fascicle count and relative amount of myelination. Furthermore, the distribution of myelin within the fascicles was measured by dividing the effective surface area into three-thirds (inner, middle and outer). Two persons independently determined the grey value corresponding to positive staining. The average of their values was used as threshold.

Statistical analysis was performed with Graphpad Prism v5.0 software. Data were tested for normality with the use of Shapiro–Wilk normality test. Comparisons were made with the use of Student's t-tests. Data are presented as mean ± SEM. P values <0.05 were considered statistically significant.

Results

Eleven pairs of cervical vagus nerves were successfully harvested and studied. Transverse sections typically show several fascicles, connective tissue, fat and vessels. Figure 2 shows an example of a typical right and left human cervical vagus nerve of one specimen at the level of electrode implant. In 8 of the 11 specimens, the right nerve contained more fascicles than the left. On average, the right nerve contains 8 (±2) fascicles, compared to 5 (±1) within the left nerve ($P = 0.15$).

Surface area measurements

The mean effective surface area on the right side is significantly larger than on the left (1,089,492 ± 98,337 vs 753,915 ± 102,490 μm^2, respectively, $P < 0.05$). In 10 of the 11 specimens, the right nerve possesses a larger effective surface area.

Figure 3 shows a boxplot of the effective surface area of the individual nerves in relation to the mean and confidence interval of 95%. For both sides, four of the 11 nerves are not displayed within this 95% confidence interval, while Shapiro–Wilk normality test shows a normal

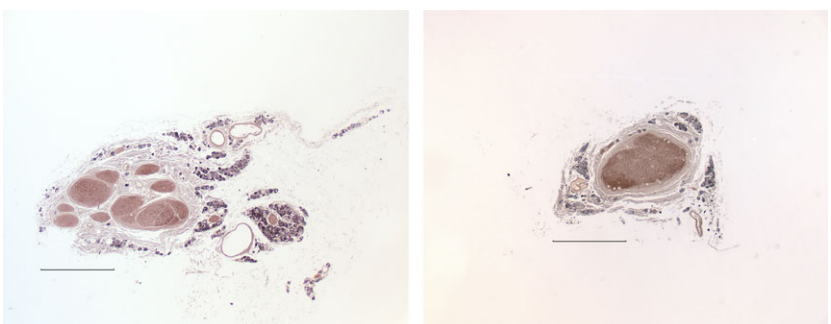

Figure 2. Example of a human cervical right (left picture) and left (right picture) vagus nerve within one individual (OsO_4 fixation, bar indicates 500 μm).

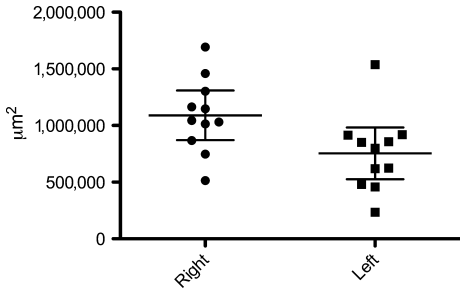

Figure 3. Mean effective surface area ±95% CI.

distribution for both the right ($P = 0.99$) and left ($P = 0.31$) nerve. To illustrate, Fig. 4 displays the largest and smallest left vagus nerve.

At the intracranial level ($n = 4$), no differences between the right (630,921 ± 105,422 µm^2) and left (855,808 ± 655,902 µm^2) nerve in terms of effective surface area were observed ($P = 0.54$).

On the right side, the mean effective intracranial surface area (630,921 ± 105,422 µm^2) is significantly less than the mean effective surface area at the cervical level of electrode implant (1,089,492 ± 98,337 µm^2) ($P < 0.05$), whereas on the left side, the mean effective surface areas do not differ between the intracranial and cervical level ($P = 0.69$). The typical intracranial vagus nerve hardly possessed connective tissue and fat in the epineurium and possessed multiple fascicles (Fig. 5).

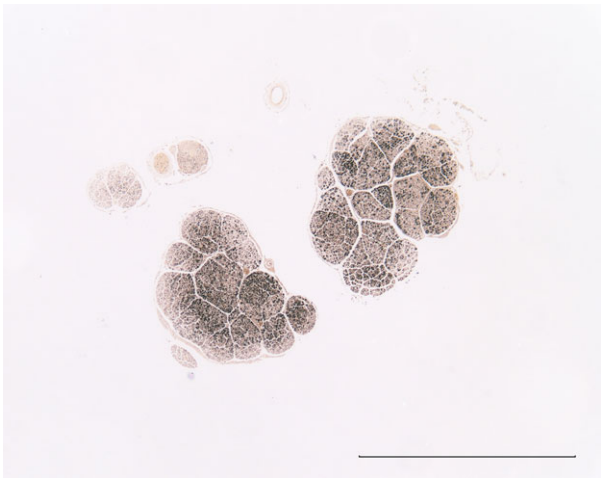

Figure 5. Example of a (left) human vagus nerve inside the skull base (OsO_4 fixation, bar indicates 500 µm).

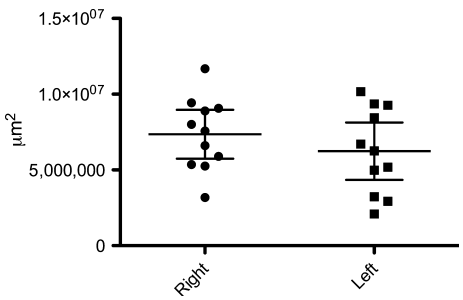

Figure 6. Mean amount of epineurial tissue ±95% CI.

Connective tissue

The mean total surface area of the cervical vagus nerve including epineurium (i.e. containing connective tissue, fat and vasa nervorum) did not differ between the right (8,448,000 ± 739,000 µm^2) and the left (6,992,000 ± 905,500 µm^2) cervical vagus nerve ($P = 0.23$).

The individual nerve's amount of epineurial tissue ranges from 2,090,000 to 11,683,000 µm^2. Figure 6 depicts these individual nerve's amount of epineurial tissue in relation to the mean and confidence interval of 95%. It is shown that five, respectively seven of the eleven nerves are not displayed within this 95% CI, while the Shapiro-Wilk normality test shows a normal distribution for both the right (P =0.98) and left (P =0.94) nerve.

Myelination

No difference in the amount of myelination relative to the effective surface area between the right

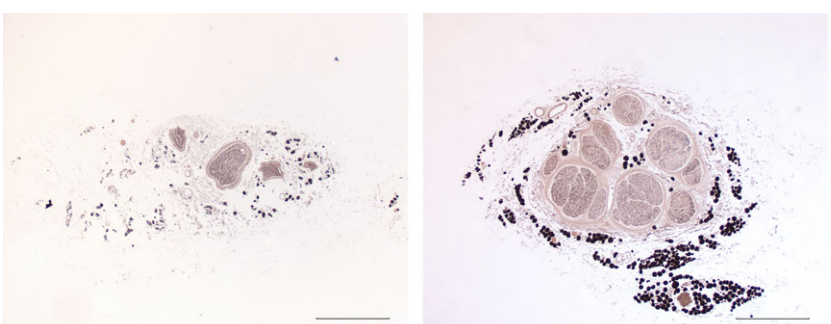

Figure 4. Example of two single left vagus nerves to illustrate the largest difference in surface area in-between nerves (OsO_4 fixation. Bar indicates 500 µm).

(26.8 ± 1.5%) and left (28.0 ± 1.7%) nerve could be observed ($P = 0.62$).

The myelin appeared to be distributed equally within the three-thirds of the fascicles. The surface areas of the outer thirds of the right and left nerve fascicles show 40.0 ± 1.5% and 37.9 ± 2.7% positive OSO_4 staining, respectively. The surface areas of the outer two-thirds of the right and left nerve fascicles show 66.5 ± 2.6% and 63.6 ± 2.6% positive OSO_4 staining, respectively.

Catecholamines

At the cervical level, all nerves stained positive for tyrosine hydroxylase, varying from 0.6% to 8.5% of the total effective surface area. The mean area of tyrosine hydroxylase (TH)-positive staining is significantly larger on the right side (43,393 ± 7,771 μm^2 i.e. 3.3%) compared to the left (20,748 ± 4,532 μm^2 i.e. 1.9%) ($P < 0.05$).

In two cases, ganglion cells in the right cranial cervical vagus nerve were detected that stained positive for TH (Fig. 7A). These nuclei stained positive for DBH as well (Fig. 7B). Furthermore, axons that stained positive for TH (Fig. 7C) also stained positive for DBH (Fig. 7D). In some cases, there was no positive DBH (Fig. 7F) staining at sites of TH-positive stained axons (Fig. 7E).

No positive staining for TH or DBH could be observed at the intracranial level.

Discussion

This is the first study that provides quantative data about human cervical vagus nerve morphol-

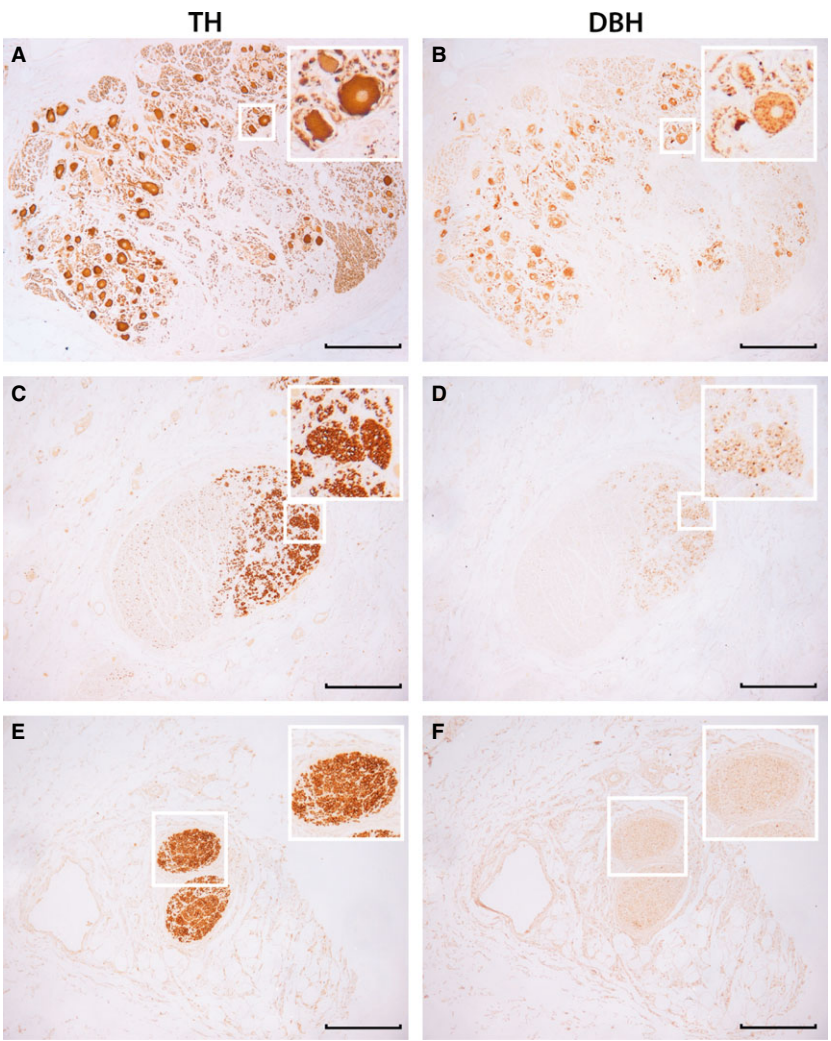

Figure 7. (A–F) Tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) staining (bar indicates 250 μm). (A) TH-positive ganglion cells in right cervical vagus nerve. (B) Ganglion cells that also stained positive for DBH. (C) TH-positive axons. (D) Axons that also stained positive for DBH. (E) TH-positive axons. (F) Axons that did not stain positive for DBH. Insets in the right upper corner show magnifications of the area surrounded with a white square.

ogy at the level of electrode implant for VNS. The following conclusions can be drawn from our results.

Surface area measurements

Although the total surface areas of the right and left cervical vagus nerves do not differ significantly, the effective surface area (nerve tissue exclusively within the perineurium) does. The effective surface area of the right cervical vagus nerve is 1.5× larger than the left nerve. This could have implications for the choice of side of stimulation when considering the possible amount of axons that will be stimulated. The (majority of the) right nerve fibres extend in the abdomen as the posterior trunk, running (among others) through the coeliac and superior mesenteric ganglia, thereby supplying more viscera (possibly including the spleen) than the anterior trunk. This could explain (among others, see further) the right–left difference in surface area at the cervical level. The effective surface area of four of the 11 nerves does not find themselves inside the ±95% CI. This large variation in surface area may affect VNS success rates. The variation in surface area may also be explained by the inferior cardiac branch, which branches off the vagus nerve at variable levels. It has even been reported to branch off the recurrent laryngeal nerve (11) (see Fig. 8). The possible costimulation of the cardiac branch upon VNS could therefore vary between individuals.

Our finding that the mean effective surface area at the right cervical level is significantly larger than at the level inside the right skull base, could implicate that the vagus nerve receives anastomosing and 'hitchhiking' branches from areas other than the brainstem. Other factors may contribute to this difference, such as the fusion of the accessory nerve with the vagus nerve and the difference in the amount of myelinisation. However, on the left side, these factors did not contribute to a significant difference and also the relative amount of myelinisation between right and left nerve appeared equal.

Myelination

High-degree myelinated fibres have the lowest amplitude–duration thresholds and are preferentially activated by low-level VNS to result in therapeutic effects (20, 21). It is thereby suggested that the myelinated axons are responsible for the anticonvulsive effect because destruction of C-fibres does not alter the effect of VNS upon

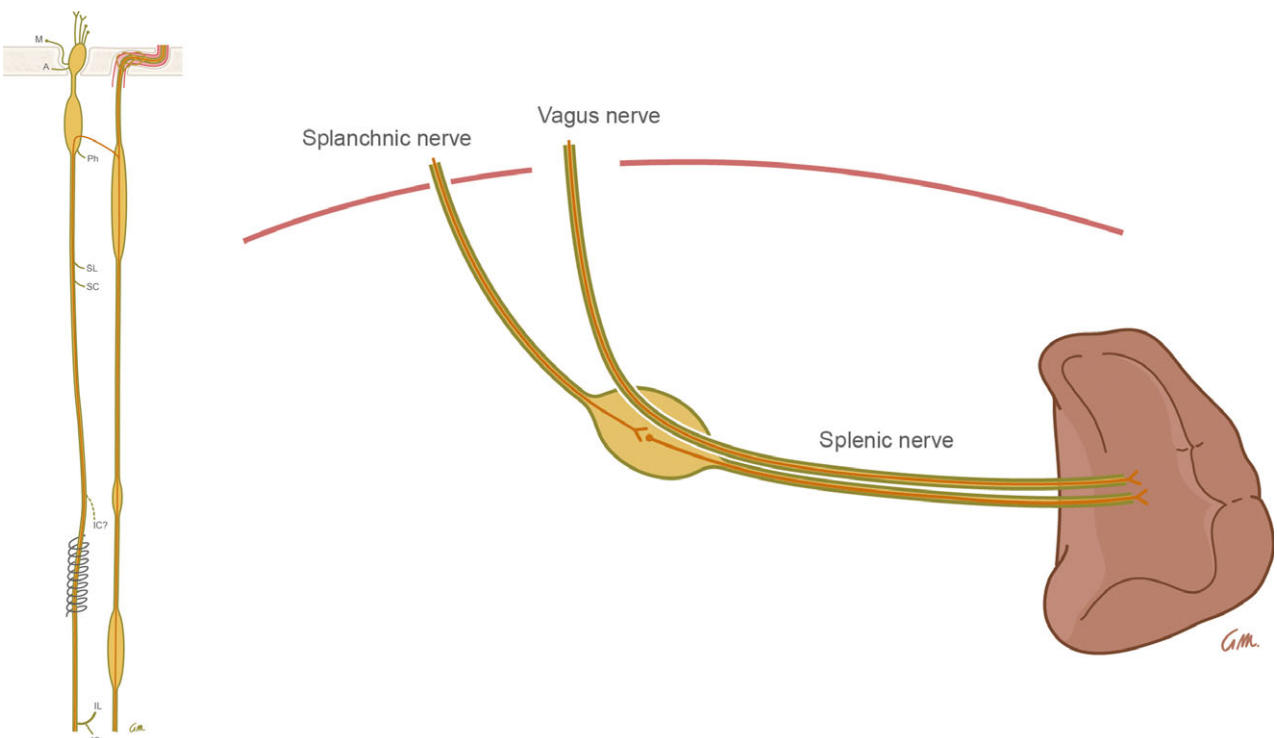

Figure 8. Left: Anastomising and 'hitchhiking' branches within the vagus nerve (left) from sympathetic chain (right). Right: hypothesis that the splenic nerve's catecholaminergic release originates directly from the vagus nerve upon VNS. M: meningeal branch, A: auricular branch, Ph: pharyngeal branch, SL: superior laryngeal branch, SC: superior cardiac branch, IC: inferior cardiac branch and IL: inferior laryngeal branch.

induced seizures in rats (22). Fibres located closer to the perineurium of a fascicle are exposed to a stronger electric field because they are closer to the electrode (23). We found that in humans, the cervical vagus nerve contains relatively equal amounts of myelination on both the right and left side and that the amount of myelination within the individual nerves lies within the 95% CI. Furthermore, this myelination is equally distributed within the nerve fascicles. Upon VNS, the electrode does not fully encircle the vagus nerve, but wraps approximately 270 degrees around it. This partial positioning of the electrode contributes to potential 'quiet spots' where higher stimulation may be required for the activation of the nerve fibres present (23). However, the large variation in epineurial connective tissue might influence the effectiveness of VNS to a greater extent (see further).

Connective tissue

The nerves were exposed using techniques that are similar to surgery for vagus nerve electrode placement (17, 18). Attention was paid to isolate the nerve from its surroundings, while not harming the nerve proper. As already depicted in Fig. 6, the absolute amount of epineurial tissue (i.e. connective tissue (including perineurium), fat and vasa nervosum) varied widely between the individual nerves, ranging from 2,090,000 to 11,683,000 μm^2. The amount of 'epineurial tissue' could isolate the nerve tissue proper from the electrical impulses generated by the electrical stimulation upon VNS treatment. Helmers et al. (23) calculated in their mathematical working model of VNS (with 1.5-mA current and 500-μs pulse width) that the presence of 1,300,000 μm^2 fibrous tissue around the nerve led to a decrease of 46.5% in A and B fibre activation. The conductivity of the extraneural medium is of prime importance to fibre recruitment. An insulating 'extraneural' medium generally requires more current to reach the stimulus threshold (24). We suggest that the amount of epineurial tissue could be an important variable for determining individual effectiveness of VNS. These findings support the general belief that careful surgical exposure of the nerve prior to electrode application is extremely important.

Catecholaminergic neurotransmission

Tyrosine hydroxylase-positive nerve fibres were detected within the cervical vagus nerve. This confirms the early finding of H. Gray and H.V. Carter in 1858 (25) who found communicating branches between the vagus nerve and sympathetic trunk. To date, no quantative data about TH-positive nerve fibres in human nerves exist, especially concerning the level of electrode implant upon VNS. The right cervical vagus nerve contains on average two times more TH-positive nerve fibres than the left cervical nerve. The presence of DBH-positive staining in the matching axons confirms the presence of not only dopamine but also noradrenaline (NA). No TH- or DBH-positive nerve fibres could be identified inside the skull base, confirming the early findings (25) that the origin/destination of these positive fibres must be the sympathetic chain. Above-mentioned findings have important implications for the contribution of the VNS treatment principle hypotheses.

Central mechanism-of-action hypothesis

Stimulating the vagus nerves may implicate the release of dopamine and noradrenaline centrally as well as peripherally. Dopamine and noradrenaline might be released centrally via the sympathetic chain, thereby reaching all central areas that are innervated by the sympathetic nervous system. This implies that VNS may have a catecholaminergic (sympathetic) effect that could contribute to the mechanism of action. This hypothesis is supported by the finding that VNS-induced anti-epileptic effects are associated with increased hippocampal NA levels (26). Furthermore, the locus coeruleus, the principal brain noradrenergic nucleus, mediates at least some of the seizure-attenuating effects of VNS (27). The sympathetic component might also receive interest because of its role in regulating cerebral perfusion, because VNS has shown to induce cerebral (thalamic) blood flow changes in experimental settings (3). All together, an increased release of noradrenaline in widespread cerebral regions upon VNS is thought to be one mechanism of action (2). Furthermore, VNS is thought to have anti-inflammatory effects that are suspected to result from increased corticosteroid release by VNS-induced HPA axis activation (28). Lesions of the noradrenergic projections to the hypothalamus in rats impair the increase in plasma corticosterone induced by intraperitoneally injected IL-1 (29). Further imaging studies are necessary to characterize the activity of 'sympathetic and dopaminergic brain regions' upon VNS treatment. As in many experimental studies, however, the electronic device upon 'VNS' is (necessarily) implanted around the vagus nerve and common

carotid artery (including sympathetic fibres therefore) together, the positive results from these studies might additionally be explained by the sympathetic component that was stimulated by chance.

Peripheral mechanism-of-action hypothesis

(Peripheral) anti-inflammatory effects of VNS appear to rely on an intact catecholamine-containing splenic nerve (30, 31). Our findings support this hypothesis by stating that the splenic nerve's catecholaminergic release possibly originates directly from the vagus nerve itself, and not from a synapse in the coeliac ganglion, as has been proposed earlier (30). Catecholamine-containing nerve fibres in the anterior nerve of Latarjet, for instance, have been described previously (32). A possible direct catecholaminergic, sympathetic effect upon VNS treatment could be the mechanism of action (see Fig. 8) because ß- and α-receptors are present in different types of immunocompetent cells, and because of the direct contact between TH-positive nerve terminals and lymphocytes in the spleen (33). Despite several studies on spleen innervation (34–38), the question whether the human spleen receives (catecholaminergic) innervation from the vagus nerve has not been answered yet. The origin of catecholamines within the subdiaphragmatic vagus nerves remains controversial. Ahlman et al. (39) and Liedberg et al. (40) demonstrated that the abdominal vagus nerve receives catecholamines from the superior cervical ganglion, which may be supported by our findings, as well as from the stellate ganglion and possibly also from the intrathoracic sympathetic ganglia. Muryobayashi et al. (41) found that the origin of catecholamines in the abdominal vagus is restricted to the superior cervical ganglion. However, these studies were conducted on cats and dogs. As we found that the cervical vagus nerve on the right side contains more TH-positive nerve fibres, and the right (posterior) vagus nerve extends in the abdomen through the coeliac trunk and possibly the splenic plexus, experimental studies studying the anti-inflammatory effect of VNS could investigate the difference in effects upon right vs left nerve stimulation. In theory, stimulation of the direct sympathetic supply to the spleen should induce even stronger anti-inflammatory effects.

In two of our cases, ganglion cells were found in the human cervical vagus nerve at the level of electrode implant. These nuclei stained positive for TH as well as DBH, implicating a catecholaminergic origin. The presence of these nuclei here implies that the destination of at least part of the catecholaminergic fibres are second-order neurons that travel peripherally down the vagus nerve.

Experimental studies have demonstrated beneficial effects of VNS for the treatment of various cardiac disease states such as arrhythmias (4). These cardiac effects appear to rely on both parasympathetic and sympathetic activation. For instance, the anti-arrhythmic effect of VNS in undiseased hearts is abolished by treatment with propranolol (42). On the other hand, several investigators have shown a reduction in the anti-arrhythmic effect of VNS following treatment with atropine, supporting the role of muscarinic receptor activation in these studies (42, 43). Furthermore, Hopkins et al. (44) stated that the intrinsic cardiac nervous system can become directly involved in cardiac pathology. At the level of the intrinsic cardiac nervous system, complex functional interconnectivity exists. These act as a stabilizing feature to prevent various cardiac disease states. Components within this neuroaxis may interact abnormally to alter myocyte function (45). These findings have lead to the concept of VNS-induced restoration of the loss of autonomic balance, in order to provide treatment effects such as electrical stability (5, 6). Furthermore, Levy demonstrated that in the presence of a substantial background of sympathetic activity, the same level of vagal activity exerted a more prominent effect upon different cardiac effector tissues than in the absence of substantial sympathetic background activity (46). Concurrent stimulation of both sympathetic and parasympathetic cardiac nerves increases myocardial contractility without increasing heart rate (5). The presence of catecholaminergic fibres within the vagus nerve at the level of electrode implant could therefore be of special interest when stimulating the nerve in patients with cardiac disease. This fact could be of particular interest upon choosing the side of stimulation. As the right vagus nerve at the level of electrode implant contains more catecholaminergic fibres, it would therefore be interesting to study whether the effects of human VNS on the right nerve possibly exert a greater beneficial effect.

Conclusion

Our finding that the human cervical vagus nerve differs at the level of electrode implant between both sides in various ways has interesting implications for the choice of stimulation side for VNS. Future studies should test this in various conditions instead of merely try to standardize the procedure. In support of the general

belief, careful surgical exposure of the vagus nerve prior to electrode application seems extremely important due to various amounts of epineurial tissue. Furthermore, we conclude by stating that one has to look at the vagus nerve as a morphological entity of the peripheral autonomic nervous system. 'The parasympathetic vagus nerve' does not exist. Rather, we have to speak of the morphological vagus nerve, a composite of different fibres and branches with different neurotransmitters, which can act parasympathetic, sympathetic, somatoefferent or viscero-afferent in function. This 'morphological' view at nerves is further illustrated by the difference in effective surface area and amount of fascicles at different levels within in the nerve, indicating various anastomising and 'hitchhiking' branches within morphological nerve entities of different origin. Electrically stimulating the vagus nerve therefore is not the same as a pure elevation of the 'physiologic vagal parasympathetic tone', but also implements catecholaminergic (sympathetic) effects. We recommend surgeons and scientists taking this in consideration when investigating the autonomic nervous system and related areas.

Acknowledgements

We would like to thank Paul van Dijk and Els Terwindt for their technical assistance and Greet Mommen for her illustrations.

Conflict of interest

K. Rijkers received speaker's fee from Cyberonics, Inc.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Data S1. Morphometric analysis.

References

1. Ben-Menachem E, Manon-Espaillat R, Ristanovic R et al. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 1994;**35**:616–26.
2. Beekwilder JP, Beems T. Overview of the clinical applications of vagus nerve stimulation. J Clin Neurophysiol 2010;**27**:130–8.
3. Nemeroff CB, Mayberg HS, Krahl SE et al. VNS therapy in treatment-resistant depression: clinical evidence and putative neurobiological mechanisms. Neuropsychopharmacology 2006;**31**:1345–55.
4. Brack KE, Winter J, Ng GA. Mechanisms underlying the autonomic modulation of ventricular fibrillation initiation–tentative prophylactic properties of vagus nerve stimulation on malignant arrhythmias in heart failure. Heart Fail Rev 2013;**18**:389–408.
5. Kobayashi M, Massiello A, Karimov JH, Van Wagoner DR, Fukamachi K. Cardiac autonomic nerve stimulation in the treatment of heart failure. Ann Thorac Surg 2013;**96**:339–45.
6. Koopman FA, Stoof SP, Straub RH, Van Maanen MA, Vervoordeldonk MJ, Tak PP. Restoring the balance of the autonomic nervous system as an innovative approach to the treatment of rheumatoid arthritis. Mol Med 2011;**17**:937–48.
7. Bonaz B, Picq C, Sinniger V, Mayol JF, Clarencon D. Vagus nerve stimulation: from epilepsy to the cholinergic anti-inflammatory pathway. Neurogastroenterol Motil 2013;**25**:208–21.
8. Handforth A, DeGiorgio CM, Schachter SC et al. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 1998;**51**:48–55.
9. Ben-Menachem E. Vagus nerve stimulation, side effects, and long-term safety. J Clin Neurophysiol 2001;**18**:415–8.
10. Pereyra PM, Zhang W, Schmidt M, Becker LE. Development of myelinated and unmyelinated fibers of human vagus nerve during the first year of life. J Neurol Sci 1992;**110**:107–13.
11. Standring S. Gray's anatomy. Churchill Livingstone: Elsevier Health Sciences, 2008.
12. Hoffman HH, Schnitzlein HN. The numbers of nerve fibers in the vagus nerve of man. Anat Rec 1961;**139**:429–35.
13. Kawagishi K, Fukushima N, Yokouchi K, Sumitomo N, Kakegawa A, Moriizumi T. Tyrosine hydroxylase-immunoreactive fibers in the human vagus nerve. J Clin Neurosci 2008;**15**:1023–6.
14. Onkka P, Maskoun W, Rhee KS et al. Sympathetic nerve fibers and ganglia in canine cervical vagus nerves: localization and quantitation. Heart Rhythm 2013;**10**:585–91.
15. Asala SA, Bower AJ. An electron microscope study of vagus nerve composition in the ferret. Anat Embryol (Berl) 1986;**175**:247–53.
16. Ruffoli R, Giorgi FS, Pizzanelli C, Murri L, Paparelli A, Fornai F. The chemical neuroanatomy of vagus nerve stimulation. J Chem Neuroanat 2011;**42**:288–96.
17. Reid SA. Surgical technique for implantation of the neurocybernetic prosthesis. Epilepsia 1990;**31**(Suppl 2):S38–9.
18. Patil A, Chand A, Andrews R. Single incision for implanting a vagal nerve stimulator system (VNSS): technical note. Surg Neurol 2001;**55**:103–5.
19. Stickland NC. A detailed analysis of the effects of various fixatives on animal tissue with particular reference to muscle tissue. Stain Technol 1975;**50**:255–64.
20. Groves DA, Brown VJ. Vagal nerve stimulation: a review of its applications and potential mechanisms that mediate its clinical effects. Neurosci Biobehav Rev 2005;**29**:493–500.
21. Mollet L, Grimonprez A, Raedt R et al. Intensity-dependent modulatory effects of vagus nerve stimulation on cortical excitability. Acta Neurol Scand 2013;**128**:391–6.
22. Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus

nerve stimulation-induced seizure suppression in rats. Epilepsia 2001;**42**:586–9.
23. Helmers SL, Begnaud J, Cowley A et al. Application of a computational model of vagus nerve stimulation. Acta Neurol Scand 2012;**126**:336–43.
24. Frieswijk TA, Smit JP, Rutten WL, Boom HB. Force-current relationships in intraneural stimulation: role of extraneural medium and motor fibre clustering. Med Biol Eng Comput 1998;**36**:422–30.
25. Gray H, Carter HV. Anatomy descriptive and surgical. West strand, London: John W. Parker and Son, 1858;498.
26. Raedt R, Clinckers R, Mollet L et al. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. J Neurochem 2011;**117**:461–9.
27. Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. Epilepsia 1998;**39**:709–14.
28. Dantzer R, Konsman JP, Bluthe RM, Kelley KW. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton Neurosci 2000;**85**:60–5.
29. Chuluyan HE, Saphier D, Rohn WM, Dunn AJ. Noradrenergic innervation of the hypothalamus participates in adrenocortical responses to interleukin-1. Neuroendocrinology 1992;**56**:106–11.
30. Rosas-Ballina M, Ochani M, Parrish WR et al. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. Proc Natl Acad Sci USA 2008;**105**:11008–13.
31. Tracey KJ. Understanding immunity requires more than immunology. Nat Immunol 2010;**11**:561–4.
32. Lundberg J, Ahlman H, Dahlstrom A, Kewenter J. Catecholamine-containing nerve fibres in the human abdominal vagus. Gastroenterology 1976;**70**:472–4.
33. Hori T, Katafuchi T, Take S, Shimizu N, Niijima A. The autonomic nervous system as a communication channel between the brain and the immune system. NeuroImmunoModulation 1995;**2**:203–15.
34. Reilly FD. Innervation and vascular pharmacodynamics of the mammalian spleen. Experientia 1985;**41**:187–92.
35. Bellinger DL, Lorton D, Hamill RW, Felten SY, Felten DL. Acetylcholinesterase staining and choline acetyltransferase activity in the young adult rat spleen: lack of evidence for cholinergic innervation. Brain Behav Immun 1993;**7**:191–204.
36. Heusermann U, Stutte HJ. Electron microscopic studies of the innervation of the human spleen. Cell Tissue Res 1977;**184**:225–36.
37. Kudoh G, Hoshi K, Murakami T. Fluorescence microscopic and enzyme histochemical studies of the innervation of the human spleen. Arch Histol Jpn 1979;**42**:169–80.
38. Nance DM, Burns J. Innervation of the spleen in the rat: evidence for absence of afferent innervation. Brain Behav Immun 1989;**3**:281–90.
39. Ahlman BH, Larson GM, Bombeck CT, Nyhus LM. Evidence for innervation of the small intestine from the cervical sympathetic ganglia. J Surg Res 1978;**24**:142–9.
40. Liedberg G, Nielsen KC, Owman C, Sjoberg NO. Adrenergic contribution to the abdominal vagus nerves in the cat. Scand J Gastroenterol 1973;**8**:177–80.
41. Muryobayashi T, Mori J, Fujiwara M, Shimamoto K. Fluorescence histochemical demonstration of adrenergic nerve fibers in the vagus nerve of cats and dogs. Jpn J Pharmacol 1968;**18**:285–93.
42. Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation 2004;**109**:120–4.
43. Wang X, Gerdes AM. Chronic pressure overload cardiac hypertrophy and failure in guinea pigs: III. Intercalated disc remodeling. J Mol Cell Cardiol 1999;**31**:333–43.
44. Hopkins DA, Macdonald SE, Murphy DA, Armour JA. Pathology of intrinsic cardiac neurons from ischemic human hearts. Anat Rec 2000;**259**:424–36.
45. Armour JA. Potential clinical relevance of the 'little brain' on the mammalian heart. Exp Physiol 2008;**93**:165–76.
46. Levy MN. Cardiac sympathetic-parasympathetic interactions. Fed Proc 1984;**43**:2598–602.