Calibration of thresholds for functional engagement of vagal A, B and C fiber groups *in vivo*

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Vagal nerve stimulation is widely used therapeutically but the fiber groups activated are often unknown.

Aim: To establish a simple protocol to define stimulus thresholds for vagal A, B and C fibers. **Methods:** The intact left or right cervical vagus was stimulated with 0.1 ms pulses in spontaneously breathing anesthetized rats. Heart and respiratory rate responses to vagal stimulation were recorded. The vagus was subsequently cut distally, and mass action potentials to the same stimuli were recorded. **Results:** Stimulating at either 50 Hz for 2 s or 2 Hz for 10 s at experimentally determined strengths revealed A, B and C fiber thresholds that were related to respiratory and heart rate changes. **Conclusion:** Our simple protocol discriminates vagal A, B and C fiber thresholds *in vivo*.

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Vagal nerve stimulation (VNS) has been introduced or tested as a therapy for a range of disorders including epilepsy [1], cardiac arrhythmia [2], heart failure [3], depression [4], inflammatory bowel disease [5], rheumatoid arthritis [6] and obesity [7]. In the case of obesity, the vagal stimulation was designed to block the passage of afferent action potentials.

The mammalian vagus contains A, B and C fiber groups [8] whose diameters, conduction speeds, thresholds for activation and physiological roles differ from each other. A problem that arises with VNS is that it is difficult to predict which fiber groups are stimulated by a particular stimulus current. VNS is usually performed with bipolar electrodes implanted around the intact cervical vagus. Factors such as distances of electrodes from the nerve, electrode separation, fibrosis and unknown series or shunt resistances can all affect the efficacy with which a given stimulus current reaches the vagal nerve fibers to excite them. Variability of outcome, both in animal experiments and in human trials, may have contributed to differences in the fiber groups that are activated. However, standard functional measures of which fibers are being activated are not established.

In this work, we have developed a protocol to calibrate VNS functionally using physiological responses to detect when the different fiber groups (A, B and C) are being stimulated. The method is simple; all it requires is accurate moment-to-moment monitoring of respiration rate and heart rate. Additionally, it is important to have control over the stimulus frequency. While the protocol has been worked out for anesthetized rats, it is anticipated that the principles will apply across species and independently of anesthesia.

Materials & methods

Experiments were performed on male Sprague–Dawley rats (300–450 g), anesthetized with urethane (1.4 g/kg i.v.) after premedication with pentobarbital (30 mg/kg i.p.) and with instrumentation performed under 2% isoflurane in oxygen, applied by artificial ventilation. All experiments were approved by the Florey Animal Ethics Committee and the US Army Animal Care Use and Review Office.

Figure 1. The experimental arrangement to stimulate the vagus, record responses of the heart and lungs and to record mass action potentials. (A) A cuff stimulating electrode is placed around the intact vagus and secured in place with Kwik-Sil (WPI, Inc.). The open area is contained in a pool of mineral oil. Respiratory airflow and heart rate are recorded in the spontaneously breathing rat, and their responses to stimulation at a range of stimulus strengths are tested. **(B)** Later in the same experiment, the vagus nerve is cut distally, and its desheathed cut end placed over silver wire hook electrodes under mineral oil. It is crushed between the electrodes to record the MAP monophasically. MAPs in response to a full range of stimulus strengths are recorded. MAP: Mass action potential; NG: Nodose ganglion.

Animals were given a tracheostomy, through which they were ventilated with isoflurane in oxygen throughout instrumentation. A femoral artery and vein were cannulated. Either the right (eight rats) or left (one rat) vagus nerve was dissected in the neck and cleared for as long a distance caudally as possible, but left intact. A cuff electrode pair (stainless steel braided wire in plastic; separation 2–3 mm; a kind gift from Dr Michael Kilgard [9]) was placed around the intact vagus, just caudal to the nodose ganglion and the origin of the superior laryngeal nerve. It was separated from the underlying tissue by a sliver of black plastic sheet, and after carefully wicking away any free fluid, secured in place with Kwik-Sil biocompatible adhesive (World Precision Instruments, FL, USA). The edges of the wound were stitched to a brass wire ring, forming a hollow containing the vagus nerve in the neck that was then filled with mineral oil (Figure 1). After surgery was completed, isoflurane was gradually withdrawn and isoflurane anesthesia was replaced by urethane over 20–30 min. Artificial ventilation was then withdrawn, allowing the animal to breathe oxygen-enriched air spontaneously.

Blood pressure was measured from the femoral artery cannula with a pressure transducer. Data were recorded using a CED Power 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). Instantaneous heart rate was derived from the blood pressure trace and respiratory flow was measured from the air pressure in the side tube of a T-piece placed in series with the tracheostomy (Figure 1). Instantaneous respiratory rate was calculated from that signal.

After completing the study of physiological responses to a full range of stimulus strengths, the vagus nerve was then cut as low as possible in the neck. Under mineral oil, the cut end was desheathed, placed over a pair of silver wire hook electrodes (2–3 mm separation) and crushed between them so as to record monophasic mass action potentials (MAPs). The temperature of the pool was approximately 35°C. The distance between the stimulating and recording electrodes was 15–21 mm. All stimuli were 0.1 ms constant voltage square pulses delivered from an isolated stimulator (Type 2433, Digitimer Ltd, Herts, UK) at 1 or 2 Hz, with the caudal cuff electrode as the cathode.

Figure 2. Discrimination of A, B and C fiber responses.

MAPs in response to stimulation at four-times A fiber threshold (4T, green trace) activated only A fibers. At 20T, A and B fiber groups were activated (blue trace) and at 200T all three fiber types were activated (red trace).

MAP: Mass action potential.

Figure 3. Segments of chart record showing respiratory and cardiovascular responses to 50 Hz stimulation at stimulus strengths from 1.4- to 4-times A fiber threshold.

Note the graded decrease in respiratory rate with no change in heart rate (or blood pressure). Corresponding MAPs are shown at the bottom, using the same stimulus strengths (1.4, 2, 3, 4T).

MAP: Mass action potential.

Results

Figure 1 shows the two phases of the experimental setup. Physiological responses were measured using setup A; MAP recordings were made in the second part of every experiment after cutting the vagus nerve distally (setup B). Figure 2 shows an example of the A, B and C fiber components of the mammalian vagal MAP, as originally described by Heinbecker [8] and Gasser and Grundfest [10,11]. For standardization, all stimulus strengths are quoted as multiples of the threshold of the lowest threshold fibers (A fibers) in the MAP of that animal, designated T. In these experiments, T varied between 0.05 and 0.1 V. The lowest threshold B fibers were recruited at between 2.5 and 5T, while for C fibers, this was between 25 and 67T. Stimulating the left vagus nerve (one rat) produced results indistinguishable from stimulating the right vagus (eight rats). They have therefore been considered together.

The protocol that we have developed to distinguish fiber groups (Figures 3–5) has two components. To investigate the actions of myelinated (A and B) fibers, the standard test was a 2 s stimulus burst at 50 Hz. Starting from low levels, the stimulus was gradually increased until there was a change in respiratory pattern. Figure 3 shows this in a series of records taken from one animal. In this animal, a strength of 1.4T was needed to cause a brief slowing of respiration (in other animals this was between 1.0 and 3T). Increasing the stimulus caused complete cessation of breathing during the stimulus. As can be seen from the corresponding MAPs from those stimulus strengths, this response can be attributed to A fibers. This is a reflex response to stimulating vagal afferent fibers because it survived cutting the vagus distally (not shown). It is attributable to activation of the large, myelinated pulmonary stretch afferent fibers [12] that activate pathways of the Hering–Breuer reflex, which inhibits inspiration and delays the next breath.

Figure 4. Recruitment of cardioinhibitory fibers by stimuli activating B fibers.

At 16- and 18.4-times A fiber threshold, heart rate is unaffected. There is a substantial decrease when slower-conducting B fiber vagal efferents (arrowed) are recruited. MAP: Mass action potential.

Figure 5. Recruitment of C fibers is revealed by the effects of stimulation at 2 Hz.

At a stimulus strength of 40T, recruiting A+B fibers maximally, 50 Hz stimulation causes a profound bradycardia (panel 1, which is the same as Figure 4 panel 5, on a compressed timescale). Stimulation at the same strength, but at 2 Hz, has almost no effect (panel 2). Further increasing the stimulus strength to recruit more C fibers (see corresponding MAPs at bottom) causes a strong bradycardia at 2 Hz stimulation. MAP: Mass action potential.

The second fixed point that was encountered as stimulus strength was progressively increased was the threshold for bradycardia, which occurred at 15–20T. Figure 4 shows an example taken from another animal. The effect has a rapid onset (within 2–3 heartbeats of the start of the stimulus), and is generally strong and obvious. It works whether the right or the left vagus is stimulated, and is attributable to stimulating preganglionic parasympathetic efferent nerves to the heart. It did not survive distal section of the vagus (data not shown). The nerves responsible are B fibers, but are among the slower-conducting, higher threshold members of that group.

The third fixed point in the discrimination uses responses to stimulating unmyelinated axons (C fibers). Stimuli that activate C fibers, also always activate A and B fibers. Effects of C fiber actions can be demonstrated selectively, however, by using low-frequency stimulation. In this protocol, we used a 10 s train, stimulating at 2 Hz. Figure 5 demonstrates this using the same animal as in Figure 4. The first panel in Figure 5 shows the same response as the last panel in Figure 4 (on a different timescale). The 50 Hz stimulation at 40T, maximally activating B fibers, caused a profound bradycardia. At 2 Hz, however, stimulation at 40T does almost nothing. But when the stimulus strength was increased to 60–80T, to recruit substantial numbers of C fibers, 2 Hz stimulation caused a strong bradycardia (accompanied by hypotension and usually tachypnea). The bradycardia is slower in onset than that due to B fiber activation. It was a reflex response to stimulating unmyelinated afferent fibers (it survived distal section of the vagus – not shown), most likely those afferents from the heart and lungs that mediate the Bezold–Jarisch reflex [12].

Summary of protocol

The method requires accurate, moment-to-moment recordings of heart rate and respiratory rate:

- Stimulate either cervical vagus with 50 Hz pulses for 2 s periods. Increase stimulus strength until respiration slows or stops for the 2-s period, but heart rate does not change. This is due to activation of the largest A fibers (pulmonary stretch afferents).
- Increase stimulus strength until there is an abrupt (within 2-3 beats of stimulus onset) fall in heart rate. This measures the threshold of the cardioinhibitory vagal B fibers. With increasing stimulus strength, this response saturates when all B fibers are activated.
- Reduce stimulus frequency to 2 Hz, and increase pulse train duration to 10 s. Increasing stimulus strength recruits a slow-onset bradycardia, whose magnitude reflects the recruitment of vagal C fibers.

Discussion

The method described here uses established techniques and principles to provide a physiological calibration of the thresholds of different vagal fiber groups by monitoring changes in heart and respiratory rates. These can be monitored noninvasively, for example, by a thoracic strain gauge and an electrocardiogram. The sequence of fixed threshold points at increasing stimulus strengths should not be affected by pulse shape [13], so it can be applied equally with different pulse forms such as charge balanced bipolar pulses. But the pulse frequencies in the protocol are important. They should be used in a dedicated calibration run, not as an ongoing monitor during therapeutic VNS with different frequencies or durations.

A limitation of the present study is that it has been performed on anesthetized animals. In other species (cats and rabbits) also under anesthesia, similar physiological responses that were graded with stimulus strength were observed [R McAllen, Unpublished Observations]. Although it remains to be tested directly how applicable the method is in the absence of anesthesia and whether similar thresholding can be applied to humans, there is no reason to doubt that the same fiber groups would be recruited in the same sequence with increasing stimulus strength [14–16].

The respiratory slowing that we observed in response to stimulating A fiber afferents is deduced to be due to activating the slow-adapting pulmonary stretch afferents that mediate the Hering–Breuer reflex [12]. These are large diameter myelinated fibers with a fast conduction velocity and a low electrical threshold; they are present in all mammals so far tested; and have been recorded directly from the human vagus [17]. Moreover, experiments in conscious human volunteers have shown the presence of a Hering–Breuer reflex that is dependent on innervation of the lungs [18]. Although the reflex in humans has a higher threshold than that seen typically in experimental animals [19], we expect that 50 Hz stimulation of this whole afferent population should comfortably exceed that threshold. The Hering–Breuer reflex has been observed to habituate during continuous [20], or repetitive afferent stimulation over minutes [21]. This effect is reversible after cessation of the stimulus [20]. The 2-s stimulus trains used here are brief and sufficiently intermittent to avoid habituation of this type.

There seems no reason to doubt that stimulating the parasympathetic cardioinhibitory fibers (B fibers) at 50 Hz would slow the heart in all mammals, including humans, and that this direct efferent effect should occur in the conscious state as well as under anesthesia. The currents used for VNS for epilepsy are designed to activate A fibers, but if they also activate B fibers during intraoperative lead testing at 20 Hz, bradycardia is observed [22]. In an earlier report, stimulation of the left vagus at 25 Hz for 5 s in humans caused the heart to stop beating [16]. Thus thresholds for B fiber activation should be possible to determine in human and in animal models.

In the present study, we recorded bradycardia by stimulation of C fibers at 2 Hz. The observation that C fiber afferents cause strong reflex effects at much lower stimulus frequencies (typically 1–2 Hz) than A fiber afferents (typically 25–50 Hz) is not new [12,23]. Recently, synaptic mechanisms that underlie the effectiveness of low frequency fiber stimulation have been identified. Both A fiber and C fiber vagal afferent terminals impinging on secondary neurons in the nucleus of the solitary tract cause a synchronous excitatory synaptic event (and usually a postsynaptic action potential) in their respective second order neurons. But C fiber afferents then release further transmitter (glutamate), asynchronously giving rise to further action potentials over the next second or two, rather than the single spike evoked by A fiber afferents [24]. The TRPV1 receptor on C fiber afferents underlies this further release of transmitter [24].

Conclusion

The physiological outcomes that have been used to identify thresholds for activating vagal A, B and C fibers are common to all mammals so far as we are aware, including humans. Because respiratory and heart rates can be recorded noninvasively in humans and in nonhuman mammals, the methods described here have applicability to set stimulus strengths for neuromodulation using VNS.

Future perspective

We hope that this simple method, and perhaps other similar protocols, will be adopted widely in an effort to remove the present uncertainty about which vagal fiber groups are responsible for which therapeutic actions. We look forward to a better understanding of the diversity of actions of the various vagal afferent and efferent fiber groups, and thence to better-targeted therapeutic VNS.

Summary points

- Vagal nerve stimulation has been introduced as a therapy for a range of diseases.
- For adequate therapy, it is necessary to know whether threshold stimulus strengths to engage appropriate nerve fiber groups have been reached.
- Here we use physiological responses to determine when thresholds for A, B and C fibers are achieved.
- Engagement of A fibers was indicated by respiratory slowing without heart rate change with 50 Hz stimuli for 2 s at low strength; B fiber engagement was indicated by bradycardia that starts at 2.5- to 5-times this stimulus strength; and C fiber engagement was indicated by slow onset bradycardia using 2 Hz for 10 s at over 40 times A fiber threshold.
- This simple protocol discriminates vagal A, B and C fiber thresholds *in vivo*.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimentation investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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