Novel attempts to optimize vagus nerve stimulation parameters on serotonin neuronal firing activity in the rat brain

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Background
Vagus nerve stimulation (VNS) is indicated for treatment-resistant epilepsy and depression. Electrophysiologic recordings in the rat brain have shown that VNS promptly increases the firing rate of NE neurons and subsequently that of 5-HT neurons. Thus far, it appears that the standard stimulation parameters currently used in depressed patients produce an optimal activation of 5-HT neurons.

Objective/Hypothesis
This study was therefore aimed at investigating additional alterations of stimulation parameters to optimize VNS efficacy to further increase 5-HT neuronal activity.

Methods
Rats were implanted with a VNS device and stimulated for 14 days using standard (0.25 mA/20 Hz/500 microseconds/30 seconds ON-5 minutes OFF, continuously) or various stimulation parameters: extension of the OFF period (30 seconds ON every 10 to 30 minutes), the OFF and ON periods, discontinuous stimulation (12 hours per day using standard parameters), and burst stimulation modes. Rat dorsal raphe 5-HT neurons were recorded under chloral hydrate anesthesia.

Results
Both 12-hour stimulation periods for 14 days, and the 30-second stimulation every 10 or 15 minutes significantly increased the firing activity of 5-HT neurons to the same extent as standard parameters while the 30-minute intervals were ineffective. Stimulations in a burst mode and the pseudo-one-pulse stimulations also significantly increased 5-HT neuronal activity.
Vagus nerve stimulation (VNS) has been approved several years ago as an adjunctive treatment for resistant depression. More recent lines of evidence support the efficacy of VNS. The most recent open label multicenter study on 74 patients with treatment-resistant depression showed a 53% response rate and a 33% remission rate after 1 year of treatment. Moreover, there are reports showing a progressive improvement of response and remission rates after the initiation of VNS. Inversely, depressive relapses have occurred after interruption of VNS.

VNS therapy consists of a surgical procedure that connects an electrical stimulator that looks like a pacemaker to the left vagus nerve. This stimulator can then be programmed to deliver electric pulses to the nerve according to a wide variety of stimulation parameters such as current intensity, pulse width, pulse frequency, and duration of the ON and OFF periods of stimulation.

The mechanisms of action of this treatment are still not completely understood, but a variety of approaches have shed light on altered brain functions. In particular, acute VNS produces an increase of the biomarker of short-term neuronal activation c-fos in several rat brain regions including the locus coeruleus (LC) but not the dorsal raphe nucleus (DRN). In contrast, an activation of the DRN is revealed by an enhancement of another gene product fosB, only after prolonged VNS treatment. These data were consistent with electrophysiologic studies showing that VNS increases after 1 hour the basal firing activity of LC norepinephrine (NE) neurons and secondarily that of DRN serotonin (5-HT) neurons only after 14 days, two key neurotransmitter systems in the pathophysiology of depression and in the mechanisms of action of antidepressants. The effects of VNS on the 5-HT system appear to be indirect and mediated through its robust effect in enhancing the firing rate and pattern of NE neurons because a lesion of LC NE neurons completely prevents the increase of 5-HT neuronal firing rate after chronic VNS. The additional increase in firing rates of 5-HT neurons after 3 months of VNS is consistent with the effect seen in clinical depression studies in which mean Hamilton Rating Scales for Depression scores tend to decrease progressively over time, suggesting a time-dependent improvement. Added to the fact that 5-HT transmission is an established target for the therapeutic effect of various antidepressant treatments, these previous studies suggested that 5-HT neuronal firing might be a predictor of the efficacy of VNS. This is also based on the premise that this increase in 5-HT neuronal firing leads to an enhanced 5-HT transmission in the rat hippocampus after 14 days of VNS.

It was already mentioned that VNS has proven to be efficient in about a third of treatment-resistant depressed patients so further studies are still necessary to further improve the success rate of this therapy. Clinical studies have already reported that the modification of stimulation parameters could increase the efficacy of VNS in refractory patients and on brain functions. Previous experiments have thus been conducted in our laboratory whereby variations of the standard parameters currently used in the clinic were studied in the rat brain. These experiments indicated that the optimal VNS parameters that activate 5-HT neurons are the ones routinely used in the clinic to treat resistant depression. These results were additionally perceived as an indicator of the use of the firing activity of 5-HT neurons as a target to possibly optimize parameters of VNS in patients.

The present in vivo electrophysiologic study thus investigated, in rats, the effects of more drastic alterations of the stimulation parameters on DRN 5-HT neuronal firing rate to potentially further optimize VNS effectiveness in treatment-resistant depression.

Materials and methods

Animals

The experiments were carried out on male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec, Canada) weighing a minimum of 275 g at the time of the implantation of the VNS device and housed individually under standard laboratory conditions (12:12 hours light-dark cycle with access to food and water ad libitum). Body temperature was kept at 37°C during surgery and electrophysiologic experiments. All experiments were performed in accordance with the Canadian Council on Animal Care, and the local animal care committee.

Surgery

Using sterile surgical techniques, animals were operated under equithesine, 1 mL intraperitoneal (ip)/300 g rat (4.26% chloral hydrate and 0.96% sodium pentobarbital).
Supplemental doses of equithesine were given ip, 0.1 mL at a time, to maintain constant anesthesia and to prevent any nociceptive reaction to a tail pinch. A horizontal incision was made in the ventral aspect of the neck. The skin and muscles were meticulously separated and the left vagus nerve, which lies laterally to the carotid artery, was isolated. Bipolar leads were wrapped around the left carotid artery and the vagus nerve, allowing close contact between the electrodes with the vagus nerve. The leads were sutured in place to the underlying muscle. The leads were then tunneled subcutaneously toward an incision made in the back and were then connected to the stimulator. The stimulator was then placed in a dorsal pocket made under the back skin wiped with iodine and antibiotics and fluid replacement were given to ease recovery. Sham animals underwent the same surgical procedure with leads and a dummy 103-pulse stimulator was inserted as a control group. The lead impedance was checked to ensure a tight connection between the nerve and the coil, using the device diagnostic setting on the NeuroCybernetic Prosthesis (NCP) handheld-computer and programming wand from Cyberonics Inc (Houston, TX). After a 2-day recovery, the stimulator was turned on for 2 weeks in treated rats and programmed using either the standard parameters (0.25 mA/20 Hz/500 microseconds/30 seconds ON-5 minutes OFF, continuously) or different experimental settings described below:

- Extension of the OFF period (30 seconds ON from 10 to 30 minutes)
- Discontinuous stimulation (12 hours per day using standard parameters; from 7 AM to 7 PM and vice versa)
- Extension of the ON and OFF periods (180 seconds ON-180 minutes OFF)
- Double or quadruple pulses spaced by 5 milliseconds delivered every 2 seconds for 36 seconds every 4.5 minutes (equivalent, in number of pulses, to a frequency of 1 and 2 Hz, respectively, with standard parameters)
- Pseudo-one-pulse stimulation (POP): four pulses at 100 Hz delivered once every 4.5 minutes in a continuous manner for 14 days.
- Half-hour stimulation once a day for 4 days (1 mA/20 Hz/500 μseconds).

A system diagnostic (lead test) was performed again before the electrophysiologic experiments to check the electric connections of the whole system, as well as the integrity of the leads.

**Electrophysiologic experiments**

Experiments were performed with the VNS device in place but inactivated for the duration of the experiment to avoid electric interference with the recordings. Rats were anesthetized with chloral hydrate 400 mg/kg ip and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA).

**Recordings of dorsal raphe 5-HT neurons**

In vivo electrophysiologic extracellular unitary recordings were carried out using single glass micropipettes. The electrodes were filled with a 2 M NaCl solution, with an impedance range of 2 to 4 MΩ. A burr hole was drilled on the midline 1 mm anterior to lambda and dorsal raphe 5-HT neurons were encountered over a distance of 1 mm starting immediately below the ventral border of the Sylvius aqueduct.

Putative 5-HT DRN neurons were identified using the criteria of Aghajanian and Vandermaelen: a slow flow (0.5-2.5 Hz), a regular firing rate, and long-duration (0.8-1.2 milliseconds) asymmetric action potentials (Figure 1). These parameters are crucial in identifying 5-HT neurons because it is estimated that only a third of the neurons in the dorsal raphe area are serotonergic in nature. To determine the average firing rate for each group, at least five electrode descents were made per rat at 100 μm distances from the first descent. Each neuron was recorded for at least 2 minutes after stabilization. Neuronal firing rates were calculated by adding each action potential per 10-second periods (as obtained by the Spike2 program during recording) and divided by length of time recorded in seconds. All neuronal firing rates for single groups were added together and divided by the number of neurons recorded per group.

**Drugs and materials**

The leads, model 103-pulse stimulators, and dummy stimulators were provided by Cyberonics Inc.

**Statistics**

Electrophysiologic data were expressed as mean ± standard error of the mean (SEM) of firing rates. Nonparametric tests were used as firing rates were not normally distributed. Average firing rates for control and treated groups were compared using the Mann-Whitney rank sum test.

**Results**

**Effect of an extension of the OFF period of the stimulation on the firing of 5-HT neurons**

The duty cycle of the stimulation was modified by increasing the OFF period of stimulation from the standard 5 minutes to 30 minutes. The 30-second stimulation periods every 10 or 15 minutes significantly increased the firing activity of DRN 5-HT neurons (P = 0.015 and P < 0.001,
respectively; Figures 2 and 3) to the same extent as the standard parameters. The 30-second stimulation period every 30 minutes was not sufficient to enhance 5-HT neuronal firing ($P = 0.416$; Figure 3).

**Effect of discontinuous stimulation on the firing of 5-HT neurons**

The 12-hour stimulation per day was applied during the light or dark phase of the rat diurnal cycle using the standard stimulation parameters. Both 12-hour stimulation periods significantly increased 5-HT neurons firing rate ($P = 0.001$ during the light period and $P = 0.01$ during the dark period) as effectively as the continuous stimulation (24 hours; Figure 4).

**Effect of stimulations in a burst mode on the firing of 5-HT neurons**

The mode of stimulation was modified by using twin or quadruple pulses at 200 Hz delivered every 2 seconds for 36 seconds every 4.5 minutes that correspond to the number of pulses using frequencies of 1 or 2 Hz, respectively, with standard stimulation parameters. Both stimulation settings significantly increased the firing activity of DRN 5-HT neurons to the same extent as standard parameters ($P < 0.001$ with doublets and $P < 0.01$ with quadruple pulses; Figure 5).

**Effect of the use of POP stimulations on the firing of 5-HT neurons**

The stimulation parameters were modified to deliver four pulses at 100 Hz once every 4.5 minutes, 24 hours per day for 14 days. This mode of stimulation significantly increased DRN 5-HT neuron firing rate as high as the standard parameters ($P < 0.001$; Figures 2 and 6).

**Effect of the extension of the ON period with prolongation of the OFF period on the firing of 5-HT neurons**

The duty cycle was modified by extending the period of stimulation from 30 to 180 seconds and the OFF period from 5 minutes to 3 hours while keeping other standard parameters constant. Such stimulations significantly decreased 5-HT neuronal firing rate compared with sham rats ($P = 0.024$; Figures 2 and 7).

**Effect of the extension of the ON period with daily delivery on the firing of 5-HT neurons**

Rats were stimulated once daily for 30 minutes for 4 days to reproduce the stimulation parameters used by Krahl and colleagues in 2004 before performing the forced swim test experiments. This mode of stimulation did not affect the firing activity of DRN 5-HT neurons ($P = 0.67$; Figure 8).
Discussion

The results of the current study support the notion that 5-HT neuronal activation is not proportional to the intensity of stimulation of the vagus nerve. Indeed, several modalities delivering less stimulations than the standard one are sufficient to achieve the same effectiveness in increasing 5-HT neuronal firing activity, whereas some
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modalities delivering more stimulations attenuate their discharge rate.

The duty cycle parameter determining the ON and OFF period of the stimulations was already shown to be safe and effective with a 30-second stimulation every 5 minutes for epilepsy and depression as well.\(^{19-21}\) The efficacy of such a duty cycle was also confirmed in a preclinical study conducted in our laboratory whereby decreasing the OFF period of stimulation induced the loss of VNS effect on increasing 5-HT neuronal firing activity.\(^{15}\) Furthermore, an ON period longer than the OFF period was even shown to induce degenerative nerve damage.\(^{22}\) Thus, in the present experiments, longer intervals were tested between the stimulation periods (although keeping others standard parameters constant, i.e., intensity of 0.25 mA/frequency of 20 Hz/pulse width of 500 microseconds) for 14 days. It was found that 30-second stimulations every 10 or 15 minutes, instead of the usual 5 minutes, were also effective to
increase 5-HT neurons firing rate to the same extent, but the enhancing action was lost if the stimulation occurred every 30 minutes (Figure 3). Even though the firing of 5-HT neurons was not further increased, these results demonstrate that less stimulations are sufficient to achieve the same VNS efficacy on 5-HT neurons. The latter findings could be relevant for patients using VNS because adverse events can be encountered due to the delivery of stimulations. Indeed, the main side effects seen in long-term treatment with VNS are infections, voice alteration, throat pain, hoarseness, dyspnea, cough, and paresthesia. Even if these symptoms are generally of mild-to-moderate intensity, and tend to decrease over time, they may still be cumbersome for the patients. As these adverse events are mostly stimulus related, it would be helpful for the patients treated with VNS to minimize the intensity and/or duration of the stimulation periods after a response is achieved. For instance, if the 30-second stimulation period delivered every 15 minutes maintained the effectiveness of standard VNS parameters in depression, this means that the period of experiencing the side effects would occur three times less often. The second issue is in regard to the battery life of the stimulator that lasts approximately 6 to 8 years using the standard parameters after which an additional surgery is required for the battery to be replaced. Decreasing the time of stimulation would then increase the battery life and minimize the risks related to the surgery and anesthesia, as well as the cost of the treatment.

The 12-hour daily stimulation periods using standard parameters for 14 days, applied either during the light or dark phase of the rat diurnal cycle, significantly increased the firing activity of 5-HT neurons (Figure 4). In addition to enhancing the battery life of the stimulator, and potentially decreasing the side effect burden, the stimulation of the vagus nerve for only 12 hours during the sleep-wake cycle may also alter favorably the sleep architecture of depressed patients. Indeed, sleep disturbances are part of the diagnostic criteria in the DSM-IV for major depressive disorder with more than 80% of depressed inpatients and 40-60% of outpatients reporting troubles in initiating and maintaining sleep, as well as feeling fatigued on awakening. In addition to impaired sleep efficiency, alterations of sleep architecture in depression are characterized by increased rapid eye movement (REM) sleep and decreased slow-wave sleep (SWS) stage 3 and 4. These last stages, considered as deep sleep, reflect the sleep intensity and quality required by the human body to recover. A recent report studying the effect of VNS on sleep EEG in depression showed an improvement in sleep architecture with sleep EEG rhythms restored to near normal after VNS treatment. However, even if stage 2 sleep tends to increase after VNS treatment, no effect was observed on stages 3 and 4. It may then be speculated that the side effects of VNS during the night could be subconsciously detected, thereby limiting the extension of the deep sleep phases. It would then be interesting to explore if the 12-hour stimulation period only during the day would improve further the sleep architecture of depressed patients. Even if such sleep studies turned out negative, then the 12-hour stimulation period only during the night would prevent the discomforts occurring during the time of stimulation thereby improving the quality of life of the patients during the day and prolonging battery life of the stimulator.

More drastic alterations of stimulation parameters were then studied to potentially increase the firing activity of 5-HT neurons. About 30-40% of NE neurons in the LC discharge in a burst fashion with an average of two single spikes occurring closely together. In rats that received long-term VNS, over 80% of NE neurons displayed this mode of firing and the number of action potentials per burst increased to four single spikes. Thus, in another series of experiments, double or quadruple pulses at 200 Hz were delivered every 2 seconds for 30 seconds every 5 minutes to mimic the burst firing activity of NE neurons and eventually more robustly influence 5-HT neuronal firing (Figure 5). Such stimulations, that are the equivalent of a global stimulation of 1 or 2 Hz in terms of total number of pulses, were as effective as the standard frequency of 20 Hz, thus showing that the pattern of stimulation could also influence the impact of VNS on 5-HT neuronal firing. These results corroborate the possibility that less stimulations could be sufficient to obtain VNS efficacy. This was also confirmed when using the 3-minute stimulation periods every 3 hours that significantly decreased the firing rate of 5-HT neurons (Figure 7). Indeed in this condition, even if the OFF period of the duty cycle was relatively long, the prolonged duration of the ON period delivering six times the number of pulses compared with the standard one actually dampened the firing rate of 5-HT neurons. The only other condition that significantly decreased the firing rate of 5-HT neurons was the 144-Hz stimulations, which delivered about seven times the usual number of stimulations.

In the next experimental series, POP stimulations were delivered once every 4.5 minutes in a continuous manner for 14 days and tested in their capacity to increase 5-HT neuronal firing. The rationale for such stimulation was that numerous studies have shown that four pulses delivered at 100 Hz can produce neurotransmitter release that can only be achieved with much longer stimulation periods with pulses delivered at much lower frequencies. This is attributable to the duration of stimulation in this situation to be too short for the development of autoinhibition of the neurons through the activation of their autoreceptors. Once again, this mode of stimulation of the vagus nerve significantly increased 5-HT neuronal firing to the extent, but not further, of standard parameters (Figure 6). It would thus be interesting to assess how such stimulations are perceived by patients receiving VNS. Indeed, given the fact that POP stimulations occur within a very brief period (30 milliseconds) and only once about every 5 minutes, they have the potential to abolish the discomforts related to the stimulus periods.
Finally, Krahl and colleagues showed that 30 minutes of stimulation of the vagus nerve at 1 mA once a day for 4 consecutive days reduced the immobility time in rat the forced-swim test (FST), which is an indicator of an antidepressant-like effect. This mode of stimulation had, however, no effect on 5-HT neuronal firing rate (Figure 8). This could be explained by the fact that the FST is divided into two specific behavioral measures: the swimming behavior that is predominantly increased by antidepressants increasing serotonergic transmission, and the climbing behavior that is related to an enhancement of catecholaminergic transmission. Thus, the antidepressant-like effect observed by Krahl and colleagues may have been a pure noradrenergic action, consistently with such a brief stimulation period affecting only the firing rate of NE neurons.

Taken together, the present results showed that alternative stimulation parameters can be as effective as the standard ones in increasing the firing activity of DRN 5-HT neurons. Even if the current experiments did not reveal a further augmentation of the firing rate of 5-HT neurons, it would still be interesting to test some of these new parameters in patients with depression and also epilepsy in order to potentially minimize and/or even prevent side effects in VNS-responsive patients.

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