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Effects of chronic abdominal vagal stimulation of small-diameter neurons on brain metabolism and food intake.

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Abbreviations –

DVC – Dorsal vagal complex

VNS – Vagal nerve stimulation

PET – FDG – Positron emission tomography with 18Fluorodeoxyglucose

μ C – microcoulombs

HFAC – High frequency alternating current

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Abstract

Background

Abdominal bilateral vagal stimulation reduces food intake in animals. However, the classical square wave, mA range current generator is poorly effective to evoke action potentials on A δ and C neurons that represent the majority of vagal neurons at the abdominal level.

Objective/Hypothesis

(i) To ascertain the capability of very high-frequency stimulation schemes (pulsons) to trigger action potentials in abdominal vagal neurons in anaesthetized pigs. (ii) To compare these stimulation schemes with classical ones using PET imaging of brain metabolism and food intake behaviour in conscious pigs.

Methods

The current thresholds for pulsons (S2 & S3) and millisecond pulses (S1) required to trigger action potentials were calculated in 5 anaesthetized pigs using single fibre recording. Similar stimulation protocols were compared chronically to sham stimulation in 24 pigs. After two weeks of chronic stimulation, food intake and brain metabolism were investigated. The electrical characteristics and histology of the vagus nerve were also studied.

Results

S3 stimulation required a lower amount of charges to trigger an action potential. Chronically applied S2 & S3 activated the dorsal vagal complex and increased the metabolism of its afferent cortical structures. They also reduced energy intake together with a reduced ingestion of high fat and high sugar diets. All these effects were not observed for the S1 group. The vagal histology for the S1, S2 and S3 groups was not different from that of the sham.

Conclusions

These findings demonstrate that pulsons applied bilaterally on the abdominal vagus reduced food intake as a consequence of the activation of the brainstem and higher-order brain areas.

Keywords – Vagal stimulation, bariatric surgery, pulson, PET imaging, animal model.

Introduction

Obesity is an international public health issue that affects quality of life, increases the risk of illness, and raises health-care costs. Bariatric surgery remains the most effective treatment for obese patients (1). However, these procedures could be associated with major adverse-effects (2). There is an urgent need for alternative therapeutics.

Chronic vagal stimulation has the potential for weight control (3), but the animal and human data are ambiguous (4-6). This might relate to the different modalities of stimulation, including the location of the stimulating electrodes (7), the number of electrodes for bilateral or unilateral stimulation (8), the stimulation profile and current intensity (9, 10). In addition, body mass index (11) and diets (12) are confounding factors. Retrospectively, the most efficient stimulation strategy was close to that used for epilepsy, e.g., 500 millisecond pulses of 2.5 to 5 mA for 30 sec every 5 minutes (13), applied on both abdominal or thoracic vagal trunks. However, the optimal stimulation parameters need to be investigated (3).

At the abdominal level, most vagal neurons located either in the dorsal or ventral vagal trunks are small diameter myelinated and un-myelinated neurons, i.e., A δ or C type (14). Therefore, large current pulses are required for depolarizing the axon membrane and thus generating an action potential. To do so, more than 20 mA may be needed at this level (15). While they proved to be effective in an acute set-up, these currents are unrealistic in a chronic implant since they generate damages both to the electrode and to the tissues (16). Recently, Qing et al. (17) proposed to chop rectangular based waveforms into shorter rectangular waves lasting between 40 to 80 μ sec, called pulsons. While individual pulsons were unable to trigger an action potential, they appear collectively to be effective in

anaesthetized rats for stimulating C fibres at the cervical level. Furthermore, they achieved this with less charge injection than with a long lasting rectangular waveform. However, the efficacy of this method has never been tested in chronic conditions, and its capability to trigger action potentials in small diameter neurons at the abdominal or thoracic level remains to be proven.

Our hypothesis was that the classical stimulation scheme using low intensity, long-lasting current pulses (1-5 mA, 500 to 1000 μ s) is unable to activate a large number of abdominal vagal neurons (15). This, in turn, might recruit only a limited number of neurons in the dorsal vagal complex (DVC). The extend of activated DVC neurons might be so small that it impossible to identify a DVC activation (versus background noise) because of the intrinsic limitations of our present imaging methods (18). Hence the reported absence of activation of the DVC in individuals with vagal stimulation irrespective of the brain imaging method used (15, 19-22). Conversely, using pulsons, large intensity currents could be applied to the nerve trunk, allowing effective triggering of a neuronal response through a larger amount of small diameter neurons being recruited.

Our primary objective was to demonstrate that, in anaesthetized animals, pulsons of large current intensity applied bilaterally on both abdominal vagal trunks are able to trigger action potentials in small diameter neurons (C and A δ types), while their activities were recorded at the cervical level using the single fibre method. Our secondary objective was to investigate the long-lasting consequences on brain metabolism of chronic vagal stimulation using pulsons. Our third objective was to evaluate the capability of this stimulation pattern to modify food intake and to correlate these changes with the alterations in brain metabolism measured by PET imaging. These objectives were carried out using the large white pig as a large animal model of normal human physiology (23). Furthermore, the size

of the animal is well suited for laparoscopic surgery and for brain imaging using high end clinical PET scanners (24).

In this study, we assessed, first in an acute set-up, the capability of three vagal stimulation schemes to trigger action potentials on slow adapting gastric and intestinal mechanosensitive vagal neurons (A δ and C types). One stimulation pattern reproduced the classical long lasting pulse (S1) while the others (S2 and S3) integrated pulsons. The same stimulation patterns were also compared, in a chronic experiment, for their capabilities to modify brain metabolism and food intake behaviour.

Methods

The experimental procedures were conducted in accordance with the current ethical standards of the European and French legislation (Agreement number A35-622 and Authorization number 01894, Ethical approval R-2012-CHM-03 and 00341.01).

Experimental paradigm

Acute experiment

The experiment consisted of recording evoked action potentials at the cervical level on the left vagal nerve after careful microdissection of the nerve bundle to obtain single neuron action potentials. Evoked action potentials were generated by applying current pulses on two cuff electrodes implanted on the anterior and posterior vagus nerve at the entrance of the diaphragm using a lateral thoracic approach. This terminal procedure was performed on 5 animals. Two to three neurons per animal were analysed for the entire range of stimulation, while 4 to 8 neurons were unable to be studied for all current/stimulation

scheme combinations, as a consequence of mechanical stress on the bundle or perineurium bleeding.

The stimulation pulses are presented in Figure 1 and consisted of a long lasting 1000 μsec pulse (S1), 14 constant amplitude pulsons forming a pattern lasting 1000 μsec (S2) and 14 pulsons of rising amplitude (S3) with a total duration identical to the two former patterns. Current increments in amplitude used in the last stimulation pattern were following a one-fourth sinusoidal series. The stimulating patterns were applied in a sequential manner, but the order was randomly selected. One minute without stimulation was allowed between each stimulation pattern.

Chronic experiment

The procedure was performed on 24 growing pigs (50% sex ratio and age-matched) distributed in 4 experimental groups consisting of sham, S1, S2 or S3 stimulation patterns (Figure 1). The experiment started with the application of one of the three stimulation patterns (Figure 1) or a sham stimulation immediately after the surgical procedure used to insert the stimulating electrodes (see supplementary material). The animals were placed for the entire experimental period in a robotic feed dispenser to evaluate their food intake. At the end of this period, the brain metabolism was mapped using PET-FDG imaging and the animal was euthanized afterwards. The vagal trunks were sampled for histological assessment of potential damage due to the stimulation.

We selected purposely different current amplitudes for S1 and for S2/S3 with the aim to inject the same amount of electrical charges per unit of time irrespective of the stimulation pattern. As a consequence, the S1 current was set to the maximal current used during unilateral vagal stimulation at the cervical level for epilepsy therapy, i.e., 5 mA supplying 5 μC . This value was also proven to be effective when applied bilaterally at the juxta-

diaphragmatic level to alter food intake in pigs (6, 13). For the S2 stimulation pattern, the current required to inject the same 5 μC charge was 15 mA. Therefore, this current was selected for the S2 and S3 stimulation patterns. Consequently, S3 stimulation pattern injected 3.52 μC , i.e., approximately one third less charges than the S2 or S1 stimulation patterns.

Animals

All experiments were performed on young Large-White pigs weighing 32 ± 4 kg at the time of surgery. The animals were housed in individual cages, one week before the beginning of the experiment to allow adaptation to their new environment, and subjected to standard 12 : 12 h light \square dark cycle. During this period, the animals used in the acute experiment were fed daily between 08.00 h and 09.00 h with 1 kg of granulated porcine meal (3.63 kcal/g).. The animals used for the chronic experiment were placed in special cages that include a robotic feeder, during the same time period and until the completion of the experiment 15 days after the surgery. Within this device, they had simultaneous access to three types of test feeds during 30 minutes at 9:00, 12:30 and 17:00 (see food intake and analysis below). Water was provided ad libitum.

Stimulating electrodes and device

Acute experiment

The animals were anaesthetized and surgically prepared for a dissection of the cervical vagus as previously described (25). A surgical access to the mediastinal area allowed the insertion of the cuff electrodes around both vagal trunks. They comprised two pairs of Pt-Ir 10% half-circular contacts (4 in total), short-circuited together to form a bipolar configuration. The overall dimension of the cuff was 25 ± 0.1 mm. The stimulation pattern

was created using a D/A card (National instrument, USA) running a custom made software written using Labview. The voltage output of the D/A card was connected to a battery-operated buffer amplifier that created a mirror current output while allowing electrical insulation with an optical coupling between the computer and the animal.

Chronic experiment

Two sets of cuff electrodes were fixed on the dorsal and ventral trunks using laparoscopy as described in the supplementary material. The electrode leads exited in the interscapular space and were connected to an external portable stimulator placed between the shoulders and held in position by a harness. The stimulators used were custom built either at the INRA (S1 pattern) or Axonic (S2 and S3 patterns) laboratories.

Recordings and data analysis

Vagal recording and analysis

Electrical activity from single vagal afferent neurons was recorded by classical neurophysiological methods adapted to the pig (25) and described in the supplementary material. Evoked potentials were recorded on characterized gastric or duodenal afferent neurons. Neurons with increased firing frequency during light distension of either the stomach or the proximal duodenum were selected by microdissection of the vagal bundle. To achieve bowel distensions, a mid-line laparotomy was performed prior to nerve dissection to insert an inflatable balloon in the stomach and another one in the proximal duodenum. The balloons were connected to a home-made inflator/deflator capable of inflating the gastric or the duodenal balloon to a set pressure (20 mmHg) within 2 to 3 seconds.

Evoked potentials were analysed using dedicated software that allowed the identification of the occurrence or the absence of an action potential in three dimensions: time of occurrence during the sweep, sweep number and amplitude of the action potential. The conduction speed was calculated knowing the time of occurrence of the action potential and the distance between the stimulating and recording electrodes.

Impedance measurements

The impedance of the stimulating electrodes was recorded periodically using the method described elsewhere (26) and detailed in the supplemental material.

PET imaging

After 15 days of chronic stimulation or sham procedure, a PET imaging with ¹⁸Fluorodeoxyglucose was performed in anaesthetized animals as already described (27). The 3D images were spatially normalized according to a porcine template (28) using PMod (Pmod Switzerland). They were analysed afterward with statistical parametric mapping (SPM8, Wellcome Trust Centre for Neuroimaging, London, UK).

Food intake measurement and analysis

The pigs received all their food from a robotic feeder comprising three troughs placed side by side. The animals had simultaneous access to the control (balanced), high lipids and high glucose test feeds at 9:00, 12:30 and 17:00, 30 minutes each, to assess their food preferences and food intake patterns. Details of the robotic feeder and data analysis were given in the supplementary material section.

Histological preparation and measurements

For histological analysis, 6-cm segments were collected from the ventral and dorsal vagus at the level of the cuffs and prepared as already described (6). The number of the nerve bundles and the nerve area were calculated manually.

Statistics

Brain PET first-level analysis was performed as a series of paired t-tests ($S1 > S0$, $S2 > S0$ and $S3 > S0$) with a provision for type II error cancellation. These analyses were thresholded at $p < 0.001$ (false discovery rate - FDR). A conjunction analysis with a 3*6 full factorial analysis of variance with the study as an independent factor, and the effect (stimulation) as a dependent factor, was performed to identify the areas activated in all stimulation patterns versus sham. Other continuous dependent variables were analysed with ANOVA using Stata software.

Results

Effect of the stimulating patterns on current threshold in anaesthetized animals

Based on their conduction speed, seven neurons were identified as A δ type while the remaining were C type. Seven of them had their receptor field located in the duodenum while the remaining 8 had their receptor field located in the stomach. The mechanical half adaptation time equalled 4.8 ± 0.08 sec for the duodenal projecting neurons, and 3.2 ± 0.04 sec for the gastric ones. The firing threshold of the gastric neurons was higher than the duodenal ones: 20 ± 2.8 vs 13 ± 3.1 mmHg, respectively.

The most efficient stimulating pattern to elicit an action potential was the rising burst pattern (S3) without differences between gastric versus duodenal neurons (Table 1, Figure

2). Indeed, the quantity of charges required to trigger action potentials was 18.7 ± 0.50 , 6.8 ± 0.23 and $4.8 \pm 0.16 \mu\text{C}$ for S1, S2 and S3, respectively ($p < 0.001$ from each other).

Effects of the stimulating patterns on brain activity and food intake behaviour

We did not identify any clinically relevant deleterious side-effects during the experimental period irrespective of the stimulation pattern group.

Large differences in brain metabolism were observed between stimulation patterns compared to sham stimulation (Figure 3). S1 stimulation was unable to activate the DVC, unlike the S2 and S3 stimulation patterns. These two stimulation patterns were equally effective to increase the metabolism of the posterolateral region of the medulla including the solitary tract and the parabrachial area. However, due to the PET's spatial resolution, it was impossible to identify the exact nucleus involved. All primary or secondary afferent structures of the DVC were also activated with S2 or S3 stimulations (thalamus, insular cortex, superior colliculus, cingular cortex; Table 3 and 4). Both S2 and S3 stimulation patterns had an impact on the prefrontal cortex, which was unaltered with S1 stimulation. Unlike S2 or S3 stimulations, S1 was able to activate the ventral segmental area (Table 2). Conjunction analysis allowed us to identify the area(s) that were activated by the three stimulation patterns. Surprisingly, only the medial hypothalamic area was found to be activated in all stimulation conditions compared to the sham group ($P_{\text{FWE}} = 0.001$, Figure 4).

Food intake was significantly modified 14 and 15 days after the onset of the stimulation. The S2 and S3 stimulation patterns only reduced the overall daily intake ($p < 0.05$) compared to sham, without any significant difference between S2 and S3. This reduction

was due to (1) a change in diet preferences and (2) a reduction in the duration of the eating bouts within a single meal: (1) The high-fat diet and high sugar diet intake were both significantly reduced, while the ingestion of the balanced diet was unchanged compared to sham group (Figure 5). (2) The duration of eating bouts was also significantly reduced within the 30 minutes of diet access for S2 and S3 groups compared to sham (101 ± 8.2 ; 94 ± 18.5 ; 61 ± 3.3 and 62 ± 5.1 sec for Sham, S1, S2 and S3, $p = 0.919$; 0.036 and 0.044 for Sham vs S1; Sham vs S2 and Sham vs S3 respectively). However, this reduction was not significantly different depending on the diet. Furthermore, the ingestion speed and the daily number of eating bouts were unchanged in S1, S2 and S3 groups compared to sham (Figure 6). Finally, most of these changes in food intake pattern were observed during the last meal of the day and they were not spread over the three-daily food distribution.

Impedance and histological consequences of the stimulating patterns on abdominal vagal nerve structure

There was no significant difference between dorsal and ventral nerve bundles, which were pooled later. R_f (in parallel resistor with the capacitive load) was increased in the S2 and S3 groups compared to sham group. R_s (in series resistor) was also significantly larger in the S2 group (Figure 7).

There was a large quantity of connective tissue around the electrode cuffs that doubled the size of the vagus irrespective of the experimental group. The number of neuron bundles within the nerve was not significantly different between the groups: 39 ± 2.9 , 37 ± 1.7 , 36 ± 2.5 and 38 ± 2.7 for sham, S1, S2 and S3, respectively (Figure 8). The area of the nerve was altered for sham versus all the other groups, but it was not possible to identify a significant difference between sham and each of the stimulation groups using post hoc

multiple comparisons test: 2.0 ± 0.15 , 2.8 ± 0.38 , 2.8 ± 0.37 and $2.6 \pm 0.20 \mu\text{m}^2 \cdot 1\text{E}^5$ for sham, S1, S2 and S3, respectively.

Discussion

We demonstrated that pulson-type stimulation applied to the abdominal vagus has the capability to evoke action potentials on small-diameter C and A δ neurons, which represent the majority of abdominal vagal neurons. Similarly, it had the capability to increase the metabolism of the DVC and other brain areas that are primary or secondary afferent projections from the DVC. Finally, pulson stimulation was able to alter food intake, within two weeks, unlike the classical millisecond stimulation pattern that takes several weeks to be effective (6, 13). Since this stimulation pattern required approximately one-third of the charge needed for a classical long lasting pulse to evoke an action potential, it has the potential to be used chronically without altering the integrity of the nerve. Indeed, we could not find a difference in the histological remodelling of the nerve between stimulation groups.

Several limitations should be acknowledged. First, we did not evaluate different amplitudes for the pulsons stimulation (S2 and S3 groups) during our chronic experiment. This was the consequence of the difficulty to handle a large animal model, including brain imaging sequences and behavioural recordings, over several weeks. Using growing pigs instead of miniature pigs made it difficult to extend the experiment over several months, which would have been the optimal experimental design. However, at the time of study, we were not able to have an implantable stimulator capable of supplying S2 or S3 stimulations over several months, and an external stimulator would have been unethical for such an extended period of time. Second, we did not compare the same current amplitude in S1 and in S2/S3

groups. However, this was done on purpose because currents up to 15 mA for the S1 pattern were unrealistic due to safety and nerve damage concerns (15, 19).

While our initial end-point was to elicit brain metabolism changes with the pulson stimulation that was different from those observed with the classical pulse-type stimulation, we were able to identify altered food intake as early as 14 to 15 days after the onset of very high frequency stimulation. This delay is sharply less than the 5 weeks one demonstrated in prior experiments using S1 stimulation either in growing lean or morbid obese minipigs (6, 13). To our knowledge, this is the first demonstration of such early (e.g occurring 2 weeks only after the onset of the stimulation instead 5 weeks) behavioural effect of chronic vagal stimulation (3, 5, 10, 29), and it is a strong argument in favour of the efficacy of pulsons stimulation. Furthermore, the comprehensive use of a robotic feeder together with the development of algorithms for precise evaluation of food intake behavior allowed to identify the origin of the overall reduction in food intake. It relates both on a decreased duration of eating bouts and a reduced preference for high fat and high sugar diets.

PET data demonstrated that all stimulation patterns activated the insular cortex as already demonstrated with unilateral cervical VNS in humans and animals (22, 29). Furthermore, we also observed an activation of the cingular cortex irrespective of the stimulation pattern, which is compatible with DVC projection (20, 22). Finally, the activation of the prefrontal cortex could reflect its connectivity with the thalamo-insular pathway, which is altered during VNS-dependent alterations in synaptic plasticity (30). Undoubtedly, the activation of the medulla in the vicinity of the DVC was an important observation that was found with the S2 and S3 stimulation patterns only. To our best knowledge, we are not aware of animal or human imaging data demonstrating activation of the DVC during cervical or abdominal VNS (31), while neuroanatomical studies provided extensive evidence that the

sensory vagus projects to the NTS or, more liberally, to the DVC (32). A recent study was able to observe a widespread activation of the NTS by cymbal conchae stimulation in human. However, the parameters used for brainstem analysis were insufficiently conservative (33). In contrast, we used highly conservative parameters (18) and consequently the number and size of the activated areas are robust. Most likely, the best demonstration of the adequacy of our analysis workflow was the absence of activation of the medulla in S1 group compared to S2 or S3 groups versus S0 group. Since the activation of the DVC occurred concurrently with a significant change in food intake behaviour, it is tempting to speculate a causal relationship.

While using a conservative brain analysis workflow, the hypothalamus was not found to have an increased metabolism in all three stimulated groups compared to S0 group. Conjunction analysis, because of its statistical power, identified the hypothalamus as a key activated area common for all the VNS groups. This is not surprising since unilateral stimulation of the cervical vagus has been shown to increase the activity within the hypothalamus, measured using radioactive water PET imaging (21). While it is tempting to speculate that hypothalamic activation might reflect the involvement of the homeostatic network controlling food intake (34), such extrapolation must take into account the actual limited PET spatial resolution and the intricacy of the hypothalamic blood supply.

Both acute and chronic results demonstrated that pulsons stimulations evoked action potentials and that they were responsible for activation of the DVC, then the insula and prefrontal cortex, via the thalamo-insular pathway. It is likely that an identical cascade is also involved with the S1 stimulation pattern, but to a lesser extent as a consequence of the small number of axons actually stimulated (15). Since the number of axons involved in the generation of action potentials during S1 stimulation represents only a small fraction of the

nerve, the activation of the primary brain integrator, i.e., the DVC, may not be strong enough to be detected with PET imaging (35). Such phenomena have already been noticed during vagal stimulation performed at the cervical vagus level (20).

The most effective stimulation pattern to trigger action potentials had a frequency of approximately 13 kHz. High frequency alternating current (HFAC) of approximately 5 kHz is known to inhibit action potentials (36) and has been used on the vagus nerve to improve weight control in obese individuals (37). While the therapeutic success of the latter is still to be proven (3), the differences between the two strategies encompassed (i) the duty cycle 25/50 vs 50/50, (ii) the pulse frequency 13 vs 5 kHz, (iii) the current amplitude 15 mA vs 6 mA, positive pulses follow by passive discharge for our strategy vs pure alternative pulses for the VBLOC solution.

We were not able to study the impact of the electrode placement itself on the nerve integrity since the cuffs were implanted in all experimental groups. However, the macroscopic observation of the electrode area during necropsy confirmed the presence of important connective tissue growth as already described in pigs (38). While there were no significant changes in the number of neurons bundles or in the nerve area, we observed a significant increase in resistance in parallel and in series for S2 and S3 compared to sham group. These could be early signs of a more profound alteration in the nerve structure. Indeed, the 2 week stimulation protocol might be too short for the reorganization of the microstructure of the nerve. In contrast, despite the large currents injected in the S2 and S3 groups, one could have expected a large reorganization of the nerve (19), a situation that was not observed in our experiment.

Conclusions

The present findings demonstrate that very high-frequency pulsons applied bilaterally at the abdominal vagus were capable to activate brainstem and, subsequently, several higher order areas connected to the DVC. These changes in brain metabolism are associated with reduced daily food intake. All the former changes were likely the consequence of the induction of action potentials in small diameter neurons, which are extremely difficult to activate using the classical millisecond-lasting low intensity electrical pulses. The absence of deleterious effects observed after several days of chronic stimulation suggests that pulson stimulation might be worthwhile to investigate in the long term.

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C-H.M. D.G. and C.P. planned the experiments, conducted the studies, analysed the data and wrote the manuscript. E.B designed one of the stimulators and the interface used for acute studies. J-L.D. designed the stimulating electrodes and the stimulator for chronic experiments. C.H. was responsible of the integration of the work in the grant. C-H.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors thank the staff of the animal facilities of Pegase unit for animal care: Mickael Genissel, Julien Georges, Alain Chauvin, Francis Le Gouevéc and Vincent Piedvache. The authors thank Gwenaëlle Randuineau (ADNC, INRA, 35590 Saint-Gilles) for the histological analyses. The authors also thank the members of Aniscan imaging centre for data collection.

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Table 1 - Charge injection threshold (in $\mu\text{Coulombs}$) for triggering an action potential in a single neuron depending on the pattern of the stimulating pulses.

| Neuron | Conduction speed (m.s^{-1}) | Receptive field | Pulse pattern (S1) | Constant burst pattern (S2) | Rising burst pattern (S3) |
|---------------|--|------------------------|---------------------------|------------------------------------|----------------------------------|
| 1 | 2.3 | Stomach | 20.4 | 8.6 | 5.9 |
| 2 | 2.3 | Stomach | 20.4 | 8.0 | 5.5 |
| 3 | 2.4 | Stomach | 18.1 | 6.2 | 4.3 |
| 4 | 6.8 | Stomach | 16.0 | 5.6 | 3.9 |
| 5 | 3.5 | Stomach | 16.2 | 5.5 | 4.1 |
| 6 | 4.8 | Stomach | 17.5 | 6.7 | 4.8 |
| 7 | 3.9 | Stomach | 16.6 | 6.2 | 4.3 |
| 8 | 4.0 | Stomach | 18.2 | 6.4 | 4.7 |
| 9 | 2.3 | Duodenum | 18.9 | 7.2 | 5.0 |
| 10 | 2.9 | Duodenum | 16.2 | 5.7 | 4.0 |
| 11 | 2.9 | Duodenum | 18.1 | 6.8 | 4.9 |
| 12 | 3.0 | Duodenum | 21.2 | 7.8 | 5.6 |
| 13 | 3.1 | Duodenum | 20.6 | 6.9 | 4.9 |
| 14 | 3.2 | Duodenum | 20.8 | 6.9 | 5.1 |
| 15 | 3.5 | Duodenum | 21.3 | 7.8 | 5.8 |

Table 2 - Statistical parameter mapping analysis of activation patterns (local maxima) in S1 stimulated animals compared to sham group.

| Coordinates of local maximum (x, y, z in mm) | Tentative anatomical localization | T Value (voxel level) | P_{FWE} corrected |
|---|--|------------------------------|----------------------------------|
| -6, 9, 15 | Dorsal posterior cingular cortex | 4.98 | 0.003 |
| -2, 0, -8 | Ventral tegmental area | 4.70 | 0.003 |
| -24, 6, 10 | Insular cortex | 4.27 | 0.004 |
| 2, 11, -4 | Anterior hypothalamic area | 4.02 | 0.004 |

Table 3 - Statistical parameter mapping analysis of activation patterns (local maxima) in S2 stimulated animals compared to sham group.

| Coordinates of local maximum (x, y, z in mm) | Tentative anatomical localization | T Value (voxel level) | P_{FWE} corrected |
|---|--|------------------------------|----------------------------------|
| -4, 20, -8 | Rostral medulla | 8.22 | 0.002 |
| -2, 0, 14 | Ventral anterior cingular cortex | 7.45 | 0.003 |
| 11, -9, 1 | Superior colliculus | 5.21 | 0.004 |
| 7, 19, -1 | Putamen | 4.66 | 0.004 |
| -4, 6, -3 | Ventral anterior thalamic nucleus | 3.94 | 0.005 |
| -10, 36, -1 | Anterior prefrontal cortex | 3.85 | 0.005 |

Table 4 - Statistical parameter mapping analysis of activation patterns (local maxima) in S3 stimulated animals compared to sham group.

| Coordinates of local maximum (x, y, z in mm) | Tentative anatomical localization | T Value (voxel level) | P_{FWE} corrected |
|---|--|------------------------------|----------------------------------|
| -24, 8, 12 | Insular cortex | 9.62 | 0.002 |
| -2, 6, 18 | Dorsal posterior cingular cortex | 7.73 | 0.002 |
| -11, -13, 7 | Superior colliculus | 7.01 | 0.002 |
| -5, -18, -8 | Rostral medulla | 4.45 | 0.004 |
| -5, 8, 2 | Ventral anterior thalamic nucleus | 4.32 | 0.005 |
| -5, 33, 14 | Dorsal prefrontal cortex | 4.3 | 0.005 |

Figure 1 - Stimulation patterns used during acute (line C only) and chronic experiments (lines A, B and C). For example, for stimulation pattern S2 applied chronically, the 1 msec pulse is chopped into 14 pulsons of 25 μ sec duration each. The 1 ms composite waves occurred at 30 Hz for 30 seconds to create a train of pulses that were applied to the vagus every 5 minutes.

Figure 2 - Single fibre action potentials triggered by the three different patterns of the stimulating pulses. Note the spread of the onset of the action potentials for S2 and S3 patterns. The vertical arrow represents the stimulation onset. The neuron presented was neuron 7 of Table 1

Figure 3 - Results from voxel-based statistical parametric mapping analysis showing the differences in glucose metabolism between stimulated animals versus the sham-stimulated animals. $p < 0.005$ cluster level FDR corrected. To improve reading, panel labelling refers to stimulation pattern only while the contrast was actually $S1 > S0$, $S2 > S0$ and $S3 > S0$.

Figure 4 - Results from conjunction analysis showing the area(s) that have identical metabolism for all stimulation groups versus sham group. Threshold level was set to 0.05 at cluster level - cluster size = 100 voxels.

Figure 5 - Food intake pattern recorded 14 and 15 days after the onset of stimulation. Top left panel - total number of calories ingested irrespective of the nature of the diet. The quantities of food ingested during the three daily meals were summed. Bottom left panel - number of calories ingested during the 17:00 meal. The number of calories corresponded to the sum of the three different diets ingested. Top right and bottom right panel - energy ingested during the last daily meal as the consequence of high lipids and high glucose diet preference. * indicates a significant difference compared to sham ($p < 0.05$).

Figure 6 - Characteristics of the microstructure of the meals recorded 14 and 15 days after the onset of stimulation. Data from the three troughs/diets and from the three daily meals were concatenated to increase statistical power. Ingestion speed was calculated during eating bouts and the periods of no ingestion during the meal were not taken into account for the measurement. Eating bouts were defined on the basis of the ingestion speed evaluated every 30 seconds. Note that daily ingestion is reduced for S2 and S3 groups only due to a reduced duration of the eating bouts. On the contrary, the ingestion speed within

eating bouts was unchanged by stimulation. * indicates a significant difference compared to sham ($p < 0.05$).

Figure 7 - Changes in impedance 15 days after implantation and chronic stimulation for S1, S2 and S3 groups. Transient pulse responses were analysed according to a Randles equivalent circuit. Dorsal and ventral electrodes data were pooled for the analysis. * denotes a significant difference from sham group.

Figure 8 - Cross section of the vagus nerve immediately at the exit of the stimulating cuff. Note the slight alteration in the repartition of the nerve bundles for S1, S2 and S3 groups compared to sham group. Improvements in the stimulating cuff design together with the minimally invasive surgical procedure reduced the impact on the connective tissue development around the nerve bundle.