Brain Stimulation 15 (2022) 1498–1507

Contents lists available at ScienceDirect

Brain Stimulation

journal homepage: http://www.journals.elsevier.com/brain-stimulation

Characterization of vagus nerve stimulation-induced pupillary responses in epileptic patients

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ARTICLE INFO

Article history:
Received 3 July 2022
Received in revised form
15 October 2022
Accepted 13 November 2022
Available online 17 November 2022

Keywords:
Biomarkers
VNS parameters
Dose dependency
Drug-resistant epilepsy
Pupillometry

ABSTRACT

Background: Modulation of the locus coeruleus (LC)-noradrenergic system is a key mechanism of vagus nerve stimulation (VNS). Activation of the LC produces pupil dilation, and the VNS-induced change in pupil diameter was demonstrated in animals as a possible dose-dependent biomarker for treatment titration.
Objective: This study aimed to characterize VNS-induced pupillary responses in epileptic patients.
Methods: Pupil diameter was recorded in ten epileptic patients upon four stimulation conditions: three graded levels of VNS intensity and a somatosensory control stimulation (cutaneous electrical stimulation over the left clavicle). For each block, the patients rated the intensity of stimulation on a numerical scale. We extracted the latency of the peak pupil dilation and the magnitude of the early (0–2.5 s) and late components (2.5–5 s) of the pupil dilation response (PDR).
Results: VNS elicited a peak dilation with longer latency compared to the control condition ($p = 0.043$). The magnitude of the early PDR was significantly correlated with the intensity of perception ($p = 0.046$), whereas the late PDR was not ($p = 0.19$). There was a significant main effect of the VNS level of intensity on the magnitude of the late PDR ($p = 0.01$) but not on the early PDR ($p = 0.2$). The relationship between late PDR magnitude and VNS intensity was best fit by a Gaussian model (inverted-U).
Conclusions: The late component of the PDR might reflect specific dose-dependent effects of VNS, as compared to control somatosensory stimulation. The inverted-U relationship of late PDR with VNS intensity might indicate the engagement of antagonist central mechanisms at high stimulation intensities.
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1. Introduction

Pupil size is regulated through a complex balance where lighting conditions and diverse neurotransmission systems related to brain function play an integrated role [1]. The activity of the locus coeruleus (LC), the main source of brain noradrenaline, correlates closely with pupil size, provided that luminance remains constant [2]. A phasic increase in LC neural firing produces a phasic pupil dilation [3]. These phasic responses are anti-correlated to the tonic level of LC activation, which is in turn reflected in the baseline pupil diameter [4]. Moreover, tonic stimulation of the LC may also correspond to a gradual increase in pupil diameter [5].

Vagus nerve stimulation (VNS) has been used for over 30 years as an adjunctive treatment for drug-resistant epilepsy, and VNS-induced activation of the LC-noradrenergic system is considered to be one of its key mechanisms of action [6–10]. The VNS modulation of LC activity relies on a disynaptic ascending pathway: from the nucleus of the solitary tract (NTS) – the main recipient of vagal afferents – excitatory inputs reach the LC via the nucleus paragigantocellularis [11]. The VNS-induced modulation of LC activity is graded with stimulation. In rats, VNS was shown to elicit a phasic dose-dependent peak in LC firing, which then persists tonically above the baseline levels throughout the stimulation period [12]. The LC firing increase proved to be correlated with VNS pulse width and current output. More recently, a study on mice described that VNS also induces pupil dilation with a graded relationship with VNS dose. Specifically, an indicator of the sustained pupil dilation

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https://doi.org/10.1016/j.brs.2022.11.002
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response (PDR) along the stimulation train was shown to increase as a function of the VNS total electric charge administered [13].

In humans, it has been shown that during VNS the average pupil diameter is larger compared to when VNS is turned off [14]. However, no stimulation-locked modifications of pupil size were reported. Regarding transcutaneous VNS (tVNS), recent studies showed a tVNS intensity-dependent dilation, and reported longer peak latencies compared to sham stimulation [15], or even occurring after the offset of 3.4 s trains [16]. This could suggest a relatively delayed activation of the LC-noradrenergic system by vagal pathways. Crucially, because both tVNS and VNS produce a relatively salient somatic sensation, the respective contribution of the direct effect of activating vagal afferents and indirect effects related to the arousal triggered by somatic input remains unclear.

If the effects of vagal stimulation on pupil diameter – and on epileptic seizures – are predominantly a consequence of the effect of VNS on the LC-noradrenergic system, the identification in patients of a graded pupillary response to VNS might lead to the development of a *dosing* biomarker. Such an indicator might help overcome the empirical selection of VNS parameters that is currently performed. Hence, the present study aimed to characterize the VNS-induced pupil dilation, addressing in particular its temporal dynamics and dose-dependency. Moreover, this study aimed to clarify the contribution of somatosensation in such responses, by applying a control electrical stimulation aimed to mimic VNS somatosensory perception.

2. Materials and methods

2.1. Patient selection

The patients eligible for this study were contacted and recruited from the Centre for Refractory Epilepsy follow-up database of Saint Luc University Hospital, Brussels, Belgium. The inclusion criteria were the following: (i) drug-resistant epilepsy; (ii) treated with VNS for at least three months; (iii) aged between 18 and 75 years; (iv) IQ > 55 (Wechsler scale) as evaluated at the latest neuropsychological assessment; (v) no recent history of use of: anticholinergic, alpha-adrenergic modulators, strong opioids (morphine), atypical antipsychotics, and psychedelic drugs, for their potential influence on acute pupil reactivity [17–20]. A VNS responder was defined as having ≥50% seizure reduction at the last follow-up visit. All patients signed the informed consent prior to any investigation. The Ethics Committee of Saint-Luc Hospital approved the study procedures (Reference No. 2018/07NOV/416).

2.2. Recordings and experimental setup

The pupil size recordings were performed for all patients in the same non-windowed room, providing a constant overhead LED lighting. The Eyelink 1000 Plus (SR Research, Canada) infrared camera was used to record the diameter of the left pupil with a 1000 Hz sampling rate. Patients were seated in front of a panel presenting a fixation cross. A chinrest was available to stabilize the gaze. The distance between the chinrest and the infrared camera (placed below the fixation panel) was ~35 cm. For the stimulation protocol, VNS was temporarily programmed to deliver trains with a total ON duration of 11 s, separated by OFF phases of 18 s. Pulse width (250–500 μs) and stimulation frequency (20-25-30 Hz) were left unchanged as per patient clinical routine programming. An anteroposterior ECG derivation enabled to retrieve the VNS electrical artefact. For the control cutaneous electrical stimulation, two Kendall H1245G electrodes (30 × 24mm) were placed on the left clavicle, with ~3 cm interelectrode distance. A customized script run on MATLAB 2018a was created to deliver trains of control stimulation through an external stimulator (DS7, Digitimer, United Kingdom). The parameters of the cutaneous electrical stimulation were programmed as comparable to the parameters of the VNS trains: 11 s duration, 500 μs pulse width, and 20-25-30 Hz frequency, depending on the subject's clinical parameters.

The experimental design is detailed in Fig. 1. Each patient underwent four stimulation conditions: three different intensities of VNS (hereafter, VNS *level*), and one control stimulation. Stimulation trains (1 train = 1 trial) were delivered in blocks of 6, and repeated for 3 series in a pseudorandomized order (i.e. the high stimulation was not used as first block), equalling 3 blocks and 18 trials per condition overall. Patients were asked to keep their gaze on the fixation cross for the whole 3-min duration of the block. At the end of each block, they were asked to rate the intensity of perception on a numerical scale from 0 to 100 as described above.

2.3. Data analysis

2.3.1. Pre-processing and subject ratings

Data processing was performed on MATLAB 2018b, using custom-made functions and the open-source Letswave 6 toolbox (http://letswave.org). Continuous pupil size measurements, with amplitude logged in arbitrary units, were segmented in 29 s windows from −8 to +21 s with respect to the first pulse of each train. A custom-made algorithm was implemented in MATLAB to reject blinking artifacts. Blinks were detected as sharp peaks of the first derivative of the signal. Linear interpolation was performed in the -/+ 250 ms interval around the blink. Epochs requiring interpolation for more than 50% of their total length were rejected. The pupil size arbitrary units were expressed as percent of change relative to a baseline interval of 3 s preceding the trial onset, which allowed to account for possible fluctuations of pre-stimulus baseline pupil diameter [21].

The perception ratings given for each block were z-score transformed per subject. This was done to obtain an estimate of the intra-subject variability of ratings. Based on this transform, trials were classified into three categories of relative perception of the stimuli: light (lower third of percentile z-score distribution), medium (middle third), and strong (upper third).

2.3.2. Feature extraction

Waveforms expressing pupil size as a function of time were averaged per block. We extracted the following three main stimulus-locked features:

i) peak dilation latency: the time lapse between the stimulus train onset and the peak pupil dilation throughout the 11 s of stimulation;
ii) early PDR: the peak amplitude reached within the first 2.5 s of stimulation onset;
iii) late PDR: the mean value of the pupil size between +2.5 and + 5 s of stimulation

The application of a 2.5 s delay after the beginning of the stimulation for the latter feature extraction was motivated by the previous study of Mridha et al. on VNS-evoked pupillary dilation in mice, which found significant effects of VNS on pupil size starting from 2.5 s after stimulation onset [13].

2.3.3. Statistical analysis

Statistical analyses were performed using R (version 4.1.2) and MATLAB 2018a. Each block was considered as a single observation, and the features extracted from each block as the dependent variables. A linear mixed model with interaction was used to analyze the relationships between PDR (dependent variables) and the

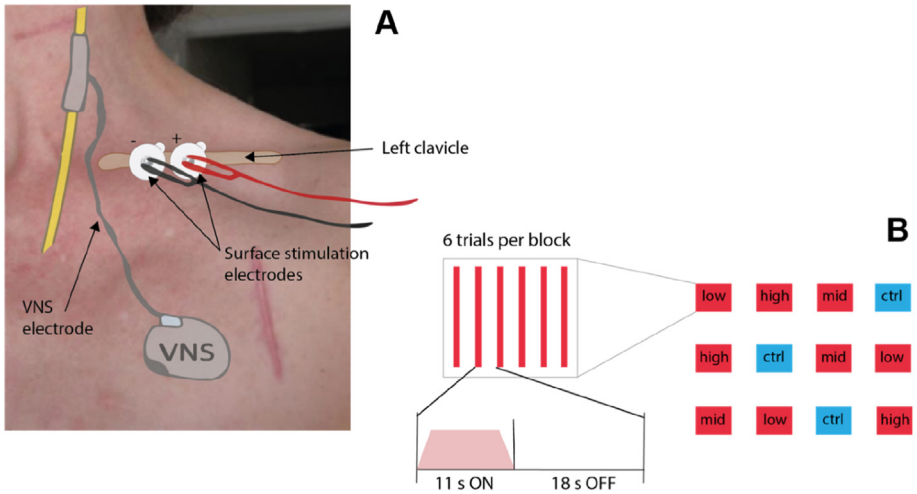


Fig. 1. Experimental design. Pupil size was recorded upon 4 stimulation conditions: 3 VNS levels, i.e., VNS at current clinical output (mid), clinical+0.25 mA (high), and clinical−0.50 mA (low), plus one control stimulation. **(A)** The control stimulation consisted of a cutaneous electrical stimulation administered over the left clavicle, programmed with the same train duration and frequency as VNS and a 500 μs pulse width. The intensity (mA) selection for the control stimulation was preceded by a staircase procedure on single pulses to determine the perception threshold. Once established the threshold, pilot trains of 11 s length were delivered to the patient, starting at intensities equalling 5-fold the threshold. The intensity was then adjusted under the guidance of the subjects (decreasing to 4-fold or 3-fold if needed), aiming at eliciting a perception comparable to the routinely applied VNS intensity (i.e., mid-VNS), which was used as a reference (value of 50 in a 0 to 100 numerical scale rating). **(B)** In the experimental protocol, trains of 11 s length were delivered in blocks of 6, and repeated for 3 series with pseudorandomized order (i.e., 18 trials per condition per subject).

perception rating (independent variable), with the stimulation type (VNS vs. control) as interaction factor. This analysis was performed in a category of perception with a similar number of VNS and control observations to have a homogeneous distribution of data along the axis of the independent variable. Inclusion of the random effect for subject was also performed to tease out the influence of the inter-subject variability. One-way repeated-measures ANOVA was used to compare the peak dilation latency across the four stimulation conditions. Post-hoc t-tests were performed, applying a Bonferroni correction to limit the false significant detections in multiple comparisons. PDR values were correlated with VNS therapy duration. Mann-Whitney *U* test were computed for a subgroup analysis comparing patients with short (<12 month) vs. long (>12 months) duration of VNS implant. Mean (± standard deviation) values are reported unless stated otherwise.

With respect to VNS trials only, a mixed-design ANOVA was used to compare the perception ratings, early and late PDR (as dependent variables) across the three VNS levels (within-factor). Since a range of different intensities (from 0.5 mA to 2 mA, in steps of 0.25 mA) were tested between subjects, the VNS absolute intensity (in mA) was taken into account in the model as a between-factor. The absence of interactions between the within and between factors would prove that the same intensity of VNS would elicit similar responses even if administered at a different level. In addition, three equations (linear, sigmoid, and Gaussian) were tested to fit the distribution of the dependent variables as a function of the VNS absolute intensity. Visual inspection, R_2, and Root-Mean-Squared Error (RMSE) of the models were assessed as a measure of the suitability of the fit.

3. Results

Ten patients were included in the study (7 F, 3 M; 5 responders, 5 non-responders). The age range of the participants was 31–70 years old (median: 45 years, interquartile range: 15 years) with no significant outliers in the distribution. VNS therapy duration ranged from 4 to 133 months. No significant correlations could be found between PDR values and VNS duration. Also, no significant differences between short-duration and long-duration subgroups were detected after Mann-Whitney *U* test. All patients were under concomitant antiseizure medication treatment (range: 1–4), most commonly lamotrigine (7/10 patients), levetiracetam, and brivaracetam (4/10), and carbamazepine (3/10). The clinical characteristics of the study cohort are detailed in Table 1. The group-level averaged waveforms (across the 10 included patients, averaged per condition) of the stimulus-locked pupil size variation are displayed in Fig. 2.

3.1. Variance of perception ratings and pupillary responses in VNS and control stimulation

The perception ratings were significantly different across the four conditions ($F = 68.15$, $p < 0.0001$), with both control and high-VNS eliciting stronger perception compared to low-VNS and mid-VNS (post-hoc, $p < 0.0001$). No significant differences between the perception elicited by control and high-VNS were found (Fig. 3A).

Control stimulation elicited a peak pupil dilation with a significantly shorter latency compared to all VNS levels (control: 1.92 ± 1.56 s, low-VNS: 2.87 ± 2.06 s, mid-VNS: 2.82 ± 1.96 s, high-VNS: 3.36 ± 1.72 s, repeated-measures ANOVA $F = 4.14$, $p = 0.043$; Fig. 3B). The magnitude of both early and late PDR differed across the four conditions ($F = 10.6$, $p < 0.0001$ and $F = 4.23$, $p < 0.007$, respectively). In particular, the early PDR was significantly greater for control stimulation compared to low- and mid-VNS ($p < 0.0001$ and $p < 0.001$, respectively), mirroring the differences in perception ratings found across conditions. No significant grading of the early PDR was found across VNS levels (high-VNS vs. mid-VNS, $p = 0.393$; mid-VNS vs. low-VNS, $p = 1$; high-VNS vs. low-VNS, $p = 0.051$) (Fig. 3C). The largest late PDR response was found in the high-VNS level (high-VNS: 3.43% ± 3.61, mid-VNS: 1.88% ± 2.9, low-VNS: 0.77% ± 2.37, control: 2.69% ± 3.15), with a significant difference between high-VNS and low-VNS ($p = 0.0057$, Fig. 3D).

3.2. Linear mixed model analysis in perception-matched VNS and control stimulation

Linear mixed models were performed using the "strong perception" category only (from +0.6 to +2 of z-scored perception

Table 1
Clinical records and demographics of the study population. In the rightmost column, the number of multiplications of the perceptual threshold (PT) used for the control stimulation is reported as (xn) after the PT value. LEV, levetiracetam. LTG, lamotrigine CBZ, carbamazepine. BRV, brivaracetam. PER, perampanel. LCM, lacosamide. TPM, topiramate. PRG, pregabalin.

PAT n°	Age	Duration of VNS (months)	VNS Normal parameters	IQ (Wech-sler scale)	Epilepsy type	Clinical response to VNS (%sz. reduction)	Antiseizure medical treatment	Perceptual threshold (PT) and control stimulation (CTRL)
PAT 1	46	7	1.25 mA; 30Hz; 500usec; 30sec On 3min Off	>80	Focal epilepsy, unknown etiology	R (−75%)	LEV, LTG, PRG	PT = 0.33 mA (x3) CTRL = 1.00 mA
PAT 2	49	133	1 mA; 20 Hz; 250usec; 30sec ON 5min Off	70	Focal epilepsy, unknown etiology	R (−100%)	LEV, LCM	PT = 0.40 mA (x5) CTRL = 2.00 mA
PAT 3	70	100	1.75 mA; 20 Hz; 250usec; 14sec On 1,1min Off	73	Focal epilepsy, unknown etiology	R (−75%)	LTG, CBZ, PER, benzodiazepines	PT = 0.34 mA (x3) CTRL = 1.00 mA
PAT 4	47	10	1.50 mA; 20Hz; 250usec; 30sec On 5min Off	78	Focal epilepsy, unknown etiology	R (−80%)	LEV, LCM	PT = 0.33 mA (x3) CTRL = 1.00 mA
PAT 5	43	11	1.75 mA; 20 Hz; 250usec; 30sec On 5 min Off	91	Focal epilepsy, unknown etiology	NR (−33%)	LTG	PT = 0.37 mA (x5) CTRL = 1.85 mA
PAT 6	57	4	1.50 mA; 30Hz; 250usec; 30sec On 5min off	94	Focal epilepsy, structural etiology	NR (−30%)	LTG, BRV, TPM	PT = 0.4 mA (x5) CTRL = 2.00 mA
PAT 7	44	47	1.75 mA; 20Hz; 500usec; 30sec On 5min Off	>100	Focal epilepsy, unknown etiology	NR (−33%)	LTG, BRV, CBZ, PER	PT = 0.68 mA (x2) CTRL = 1.36 mA
PAT 8	34	8	1.5 mA; 30Hz; 250usec; 30sec On 5 min Off	>75	Focal epilepsy, unknown etiology	NR (−20%)	BRV, CBZ, PER	PT = 0.37 mA (x3) CTRL = 1.12 mA
PAT 9	31	48	1 mA; 20Hz; 250usec; 30sec On 5 min Off	>80	Generalized epilepsy, idiopathic	R (−100%)	LEV, LTG	PT = 0.34 mA (x2) CTRL = 0.68 mA
PAT 10	36	97	2.00 mA; 25Hz; 250usec; 30sec On 1.1min Off	>80	Focal epilepsy, genetic etiology	NR (−47%)	BRV, LTG	PT = 0.50 mA (x2) CTRL = 1.00 mA

rating), as this category was found to contain an approximately equal number of control and VNS observations (23 and 20,

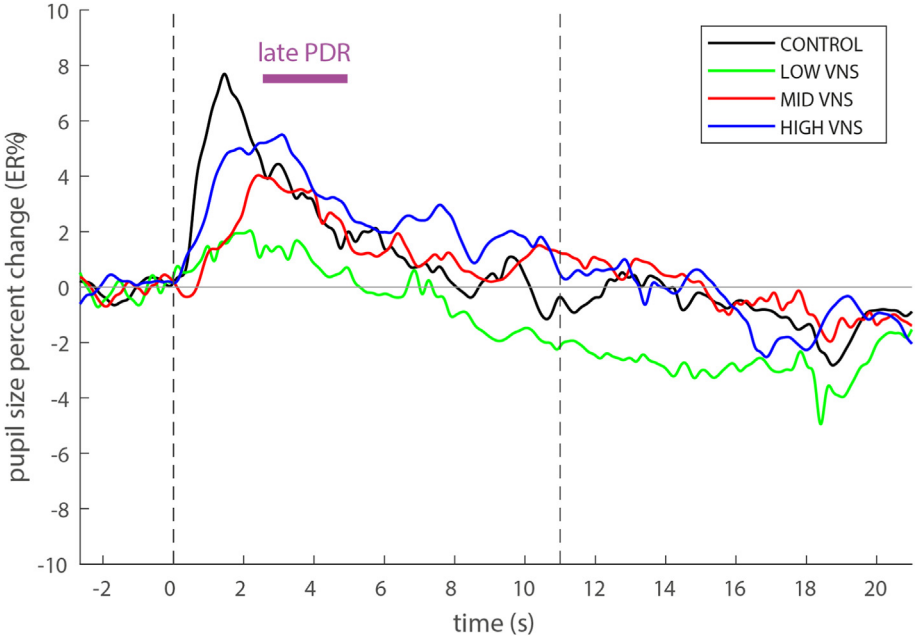


Fig. 2. Group-level averaged waveforms. The grand average of all trials pooled per condition, after averaging per patient (n of subjects = 10). Different peak latencies between VNS (later) and control (earlier) can be seen. The purple horizontal bar represents the +2.5 s to +5 s interval, whose mean magnitude was used to extract the late pupil dilation response (PDR). The late PDR was found to be graded with VNS intensity (see Fig. 5). The early PDR (not highlighted in the figure for aesthetical reasons) corresponded to the magnitude of the peak value found in the interval between 0 and + 2.5 s. The vertical dashed bars indicate the onset (0 s) and the offset of the stimulation (11 s). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

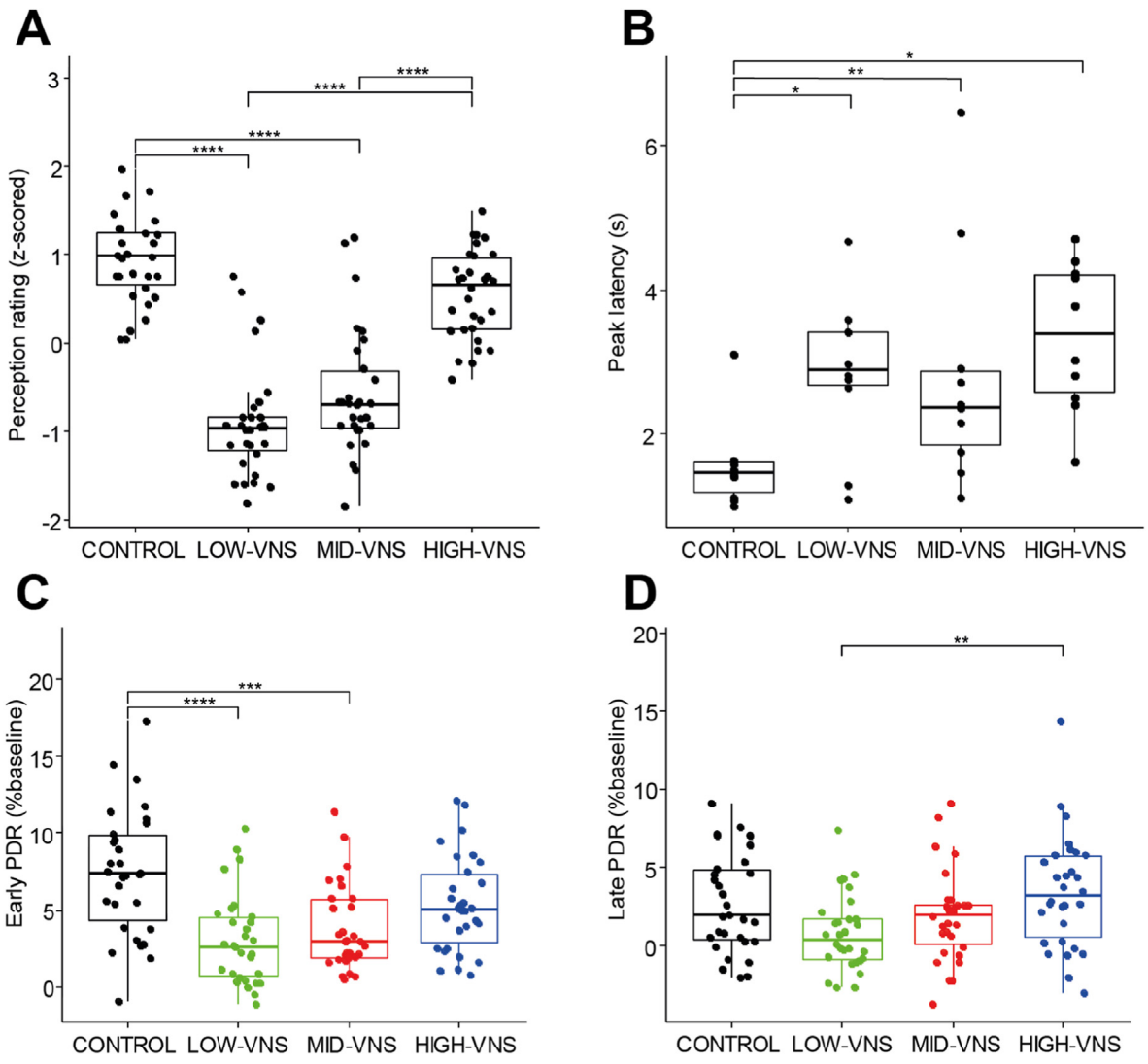


Fig. 3. Block-level analysis across the four conditions of stimulation. In these boxplot graphs, the median is represented as a horizontal line, the interquartile range (IQR) as the box's boundaries, and the whiskers indicate the 1.5*IQR limit. Each dot represents a single block. **(A)** The differences in the perception rating are significant between control and high-VNS compared to low- and mid-VNS. **(B)** Control stimulation elicited a peak pupil dilation with a significantly shorter latency compared to all VNS levels. **(C)** Early PDR was significantly higher for control stimulation, particularly compared to low- and mid-VNS mirroring the respective differences in perception ratings. **(D)** The greatest mean PDR was found at high-VNS level, with a significant difference between high-VNS and low-VNS. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

respectively). As shown in Fig. 4, when considering the effect of perception only, the early PDR was significantly influenced by perception ($t = 2.06$, $p < 0.05$). By contrast, the late PDR did not show a significant influence of perception ($t = 1.51$, $p = 0.14$). When taking into account the interaction effect, perception was found to affect more the control stimulation than VNS, both for the early PDR (slope difference VNS - control $= -4.25$, $t = -1.04$, Fig. 4A), and for the late PDR (slope difference VNS - control $= -2.54$, $t = -0.68$). The main hypothesis was reinforced with the inclusion of random effects for subject: taking into account subject-based variability, the influence of perception on the early PDR increased further ($t = 2.69$), while its influence on the late PDR decreased ($t = 1.44$).

3.3. Analysis of VNS-related trials: effects of VNS level and VNS intensity, and dose-dependency models

By means of mixed-ANOVA analysis, the VNS level was found to have a significant main effect on the late PDR magnitude ($F = 4.85$, $p = 0.01$). In particular, post-hoc t-tests revealed a significant difference between low- and high-VNS ($p < 0.05$, after Bonferroni correction). By contrast, no significant effect of VNS level was found on the early PDR ($F = 1.67$, $p = 0.20$), as displayed in Fig. 5. No interaction between VNS level and intensity was detected for the late PDR (interaction VNS level: intensity, $F = 0.265$, $p = 0.85$) nor for the early PDR ($F = 1.34$, $p = 0.27$). Hence, the assumption that one same intensity administered as different levels elicits comparable pupil responses was satisfied. This allowed us to pool VNS intensities between-subjects and to perform dose-dependency fittings (Fig. 5).

Fig. 6 displays the distribution of the observations as a function of the VNS intensity. The results from the different models examined for fitting the stimulus-response relationship are summarized in Table 2. The magnitude of the late PDR showed an inverted-U profile, with a Gaussian model as best fitting ($R_2 = 0.888$, RMSE $= 0.52$) and maximal responses at 1.5 mA. The early PDR as well as the perception ratings showed best results for sigmoid and linear fittings, respectively (early PDR: sigmoid fit $R_2 = 0.905$, RMSE $= 0.52$; perception: linear fit $R_2 = 0.678$, RMSE $= 0.38$).

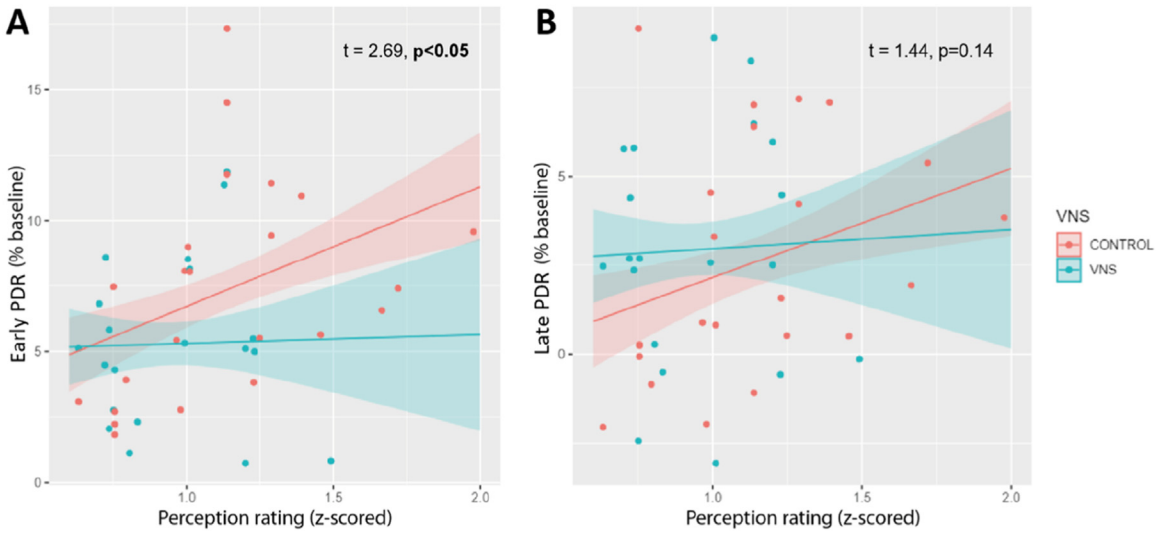


Fig. 4. Linear mixed model to explain the influence of perception on VNS and control-induced responses at block level. Although a similar trend may be noticed, early PDR is significantly depending on perception values **(A)**, while late PDR is not **(B)**. The t and p values reported in the figures take into account the inclusion in the model of the random effect for subject. When considering the interaction effect (i.e. slope difference) in both **A** and **B**, VNS-induced responses are less depending on perception than control, when considering the interaction: for late PDR, difference in slopes VNS − control: −2.539, t = −0,68); for early PDR, difference in slopes VNS − control: −4.251, t = −1,04).

4. Discussion

The main research questions addressed by the present work are: i) does cervical VNS induce stimulation-locked pupil responses in humans? ii) what are the temporal dynamics of these responses? iii) are such responses graded with VNS intensity? iv) are there features specific to VNS, i.e., what is the influence of stimulus-evoked somatic sensations? This proof-of-concept study demonstrates that a VNS-induced pupil dilation, graded with VNS intensity, can be recorded in human subjects. The VNS-induced dilation, compared to cutaneous control stimulation, was shown to have a more sustained, delayed profile, and to be less dependent on the intensity of the perception.

4.1. Specificity of VNS-induced pupil dilation: possible modulation of underlying LC activity

Due to the fixed location of the implanted VNS electrode, the possibilities of delivering a "sham" stimulation using the same generator are limited. Previous studies attempted to deliver a VNS with a 1 Hz frequency as a control stimulation [22]. However, such stimulation would entail a very different somato-sensation compared to standard 20–30 Hz VNS. In our experiment, both VNS and a control non-noxious electrical stimulation (having the same train duration and pulse frequency as VNS) elicited a stimulation-locked pupil dilation. Hence, our study failed to identify a pupillary reaction "evoked" by VNS only. However, the different relationship between PDR magnitudes and perception ratings exhibited by control and VNS condition may still signify that VNS-induced effect is more pronounced on the late component of the PDR, while the early component may be critically influenced by the stimulus-related arousal. Nonetheless, our results suggest that the VNS-related effect on pupil dilation is less dependent on the perceived sensation of the stimulus. In particular, we found that the feature of the pupil dilation that may be more specifically related to stimulation of vagal afferents is the later component of the pupil response (>2.5 s), referred here as the late PDR. In contrast, the early component of pupil dilation (<2.5 s) did not prove a significant dependency on VNS intensity, and, instead, showed a strong positive relationship with perception intensity (Fig. 4). This dependence on perception was most pronounced in the control condition, corresponding to the condition where salience of the stimulus is expected to be the main determinant of the pupil response. The mixed-ANOVA analysis further proved that, when taking into account the different intensities tested across patients, the VNS *dose* (i.e., the VNS level) showed an effect on the magnitude of the late PDR but no effect on the magnitude of the early PDR.

The observed responses may be interpreted as the sum of two physiologically independent components: the *early PDR* might reflect a phasic, rapid, saliency-driven activation of the LC, while the *late PDR* could depend on a possibly tonic, more sustained LC activation related to vagus nerve afferent input, graded by VNS intensity rather than by the perception of the stimulus. Animal studies showed that phasic stimulations of the LC induce abrupt variations in the pupil size, while a sustained tonic stimulation of LC may induce a pupil response with a gradual, delayed build-up [5]. Our results suggest that VNS might modulate pupillary function (and the underlying LC activity) similarly to the latter pattern. This view is supported by the study of Mridha et al. in mice, in which they applied stimulation trains of 10 s length and found an effect of VNS intensity building up in the later phases of the stimulation period (+2.5 to +7.5 s) [13]. In line with this finding, only the late PDR in our study was significantly affected by VNS intensity. It remains unclear why, dissimilarly to what was reported by Mridha et al., we could not observe a long-lasting dilation until the end of VNS trains. A gradual release of attention over the course of each stimulation train, leading to a reversion of LC activity towards baseline levels, might be a possible explanation. The impossibility to fully fixate the gaze and head of the subject might also have introduced a "noise" in the second part of the recording. However, it cannot be excluded that VNS induces a pupil dilation with a more transient duration as compared to other species such as mouse. Nonetheless, the late component of the PDR remains the component significantly modulated by VNS intensity, whereas the early PDR (<2.5 s from stimulation onset) did not. Further studies are warranted to better explore the temporality of PDR across different time intervals after +5s from stimulus onset, as well as in longer trains (such as the routinely programmed 30 s ON duration). An

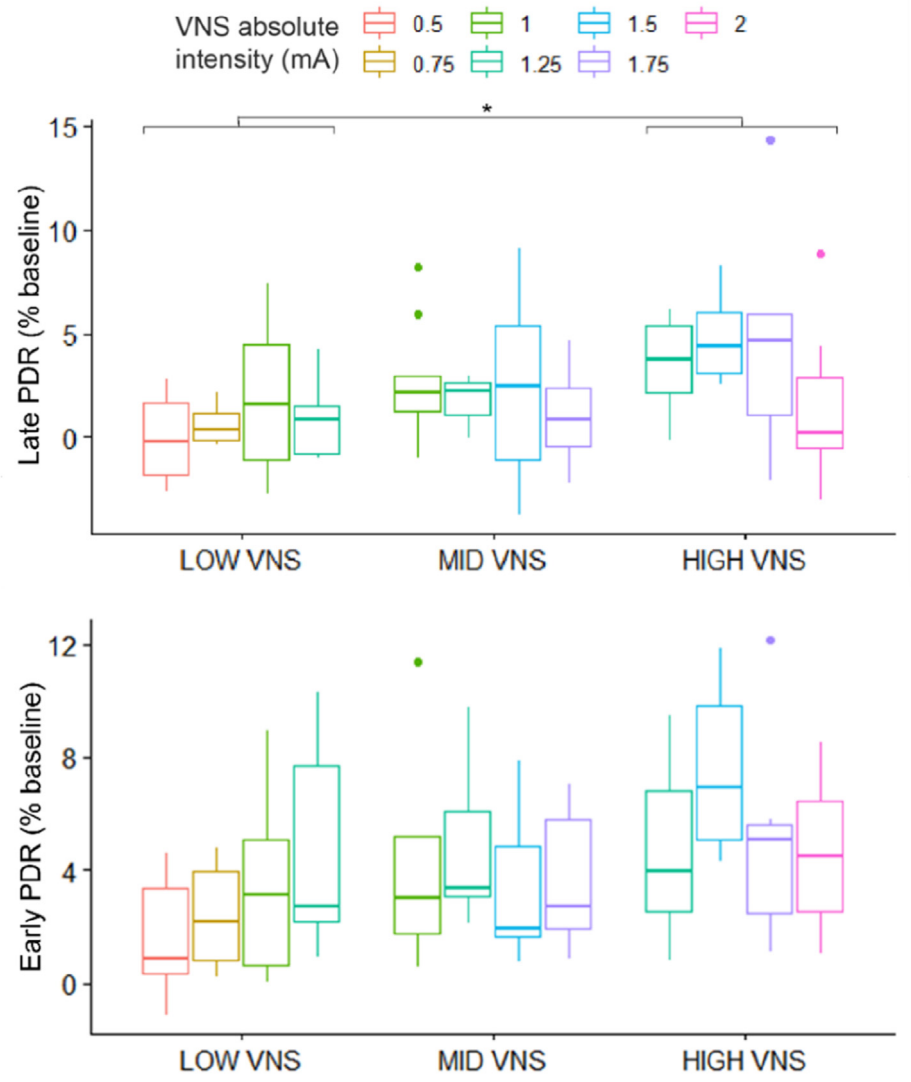


Fig. 5. Effect of VNS dose on mean PDR, mixed-ANOVA. There is a significant effect of VNS level on the variance of mean PDR values ($F(2,78) = 4.846$, $p = 0.01$) when considering the between-subject testing of different VNS absolute intensities. In particular, post-hoc *t*-test revealed a significant difference between low- and high-VNS ($p < 0.05$, after Bonferroni correction). By contrast, no significant effect of VNS level was detected for the early PDR ($F(2,78) = 1.67$, $p = 0.20$). No interaction was detected between VNS intensity and VNS level ($F(3.78) = 0.265$, $p = 0.85$), suggesting that even if administered under different conditions in the experiment, the same VNS absolute intensities elicit a comparable PDR. Outliers are displayed as dots outside of the boxplots.

additional finding that remains to be fully explored and elucidated is a potential pupil constriction offset response, visible for the low-VNS condition (Fig. 2). Such a response might mirror a similar response observed by Hulsey et al., and elicited by low-intensity VNS (0.2–0.8 mA) in the firing of the LC at the offset of short trains of VNS, although in the rat model [12]. It cannot be excluded that such phenomenon might also reflect an underlying unmasking of a vagal-mediated parasympathetic effect at peripheral level.

4.2. Dose-dependency modeling: the VNS-related late PDR showed an inverted-U profile

In the present study, we could explore how pupillary responses and the evoked perception varied as a function of eight graded VNS intensities. In the cohort of subjects investigated, 1.5 mA yielded the maximal pupil dilation. In contrast, a drop in the PDR values was observed at higher intensities, particularly at 2.00 mA. By fitting linear and non-linear models, we observed that the stimulus-response relationship of PDR is consistent with a Gaussian equation. We could therefore argue that the VNS-related modulation of the LC-noradrenergic system (due to its established close correlation with pupil size [23–25]) shows an inverted-U profile, in which moderate intensities induce a stronger modulatory effect compared to low and to excessively high intensities. By contrast, the early PDR and perception ratings showed as best fittings a sigmoid and linear profile respectively (Table 2).

Mridha et al. reported a stimulus-response relationship best fit by a log-logistic plateauing model [13]. Critically, it should be noted that in this study, the experimental protocol consisted of intensities reaching a maximum of 0.9 mA (for pulse widths in regular ranges up to 500 μs). Compared to the observations of Morrison et al. described above, these intensities would correspond to *moderate* intensities yielding a maximal modulatory effect. It may then be hypothesized that with their design, Mridha et al. could not observe the descending slope of the stimulus-response curve that might have occurred at higher intensities.

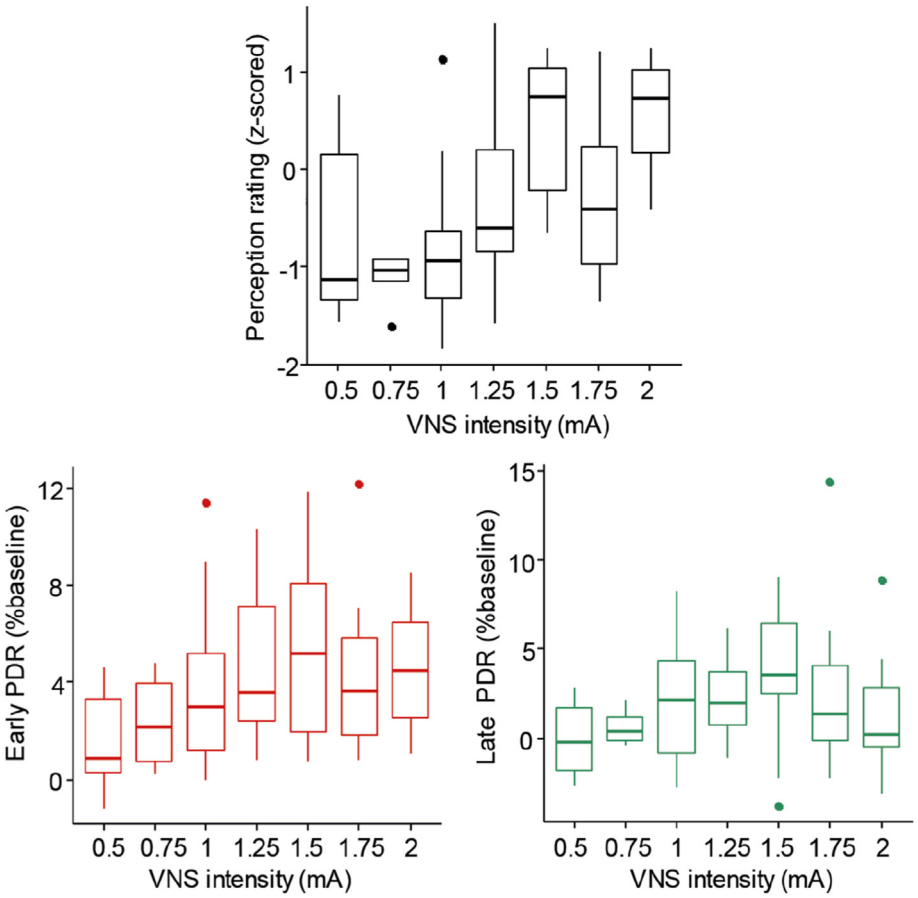


Fig. 6. Stimulus-response relationship between VNS intensity and perception ratings, early and late PDR. In this figure, boxplots illustrating the distribution of the dependent variables are plotted as a function of the VNS absolute intensity. The late PDR (right panel) shows maximal values at 1.5 mA, with a decrease in magnitude at 1.75 mA and a further decrease at 2 mA. At 2 mA, a drop in magnitude is also observed for the early PDR, albeit slighter (left panel), while no drop is observed for the perception ratings (upper panel). Outliers are displayed as dots outside of the boxplots.

Although it is known that a sigmoid relationship explains best the stimulus-response model of vagal fiber recruitment [26–28], a series of studies have consistently described an inverted-U model for the dose-dependency of central VNS-induced effects. Early research in epileptic patients demonstrated that moderate intensities of VNS enhanced memory performance better than low or excessively high intensities [29]. More recently, Morrison and colleagues proved that the VNS effects on cortical plasticity (which, like the antiepileptic, are mediated by LC modulation and noradrenergic transmission [30]) show an inverted-U dose-dependency, where moderate intensities (0.8 mA) elicit a greater rearrangement of cortical maps in rats compared to sham, 0.4 mA and 1.6 mA stimulations [31–33]. In human subjects, one study exploring the stimulation-locked fMRI (functional Magnetic Resonance Imaging) acute changes induced by VNS-induced, found peak activations in the thalamus at intermediate levels of intensity, whereas excessive increases in intensity led to decreased patterns of BOLD fMRI activation in the same regions [34].

In conclusion, since LC activation correlates linearly with VNS antiepileptic effects [8], we may therefore hypothesize that the antiepileptic effects might also be greater at moderate vs. low and high VNS intensities. This hypothesis suits the findings from Mollet et al., who explored in a rat model the VNS-induced reduction of cortical excitability as a measure of the antiseizure effects of VNS. In this work, it was found that low intensities of VNS (0.25–0.50 mA) elicit the strongest increase of motor seizure threshold, with no further increase – and even a slight decrease in effects – observed at higher intensities [35]. Crucially, future studies are justified to evaluate the inverted-U-shaped relationship with VNS dose of the antiepileptic effects.

Table 2
Curve fitting analysis for the stimulus-response relationship. The three dependent variables (perception rating, early peak dilation, and PDR) were modeled as a function of VNS absolute intensity. Equations were computed using mean values for each intensity. The perception rating showed the best fit for a linear model, the early PDR for a sigmoid model, while the PDR relationship with intensity was best fit by a Gaussian model, suggesting an inverted-U dose-dependency. PDR, pupil dilation response. RMSE, Root-mean-square error.

	Linear	Sigmoid	Gaussian
Perception rating	**$R_2 = 0.678$** **RMSE = 0.38**	$R_2 = 0.596$ RMSE = 0.40	NA (reverse fit)
Early PDR	$R_2 = 0.707$ RMSE = 0.82	**$R_2 = 0.905$** **RMSE = 0.52**	$R_2 = 0.860$ RMSE = 0.65
Late PDR	$R_2 = 0.411$ RMSE = 1.06	$R_2 = 0.772$ RMSE = 0.74	**$R_2 = 0.888$** **RMSE = 0.52**

4.3. Limitations and further perspectives

The assumptions behind the LC activation remain speculative, as no recording of LC activity could be provided in this study. Our hypothesis places the LC as the main driver of the VNS-related pupil dilation: the inputs received from the NTS cause the LC to inhibit the Edinger-Westphal nucleus (via α2 receptors), while activating

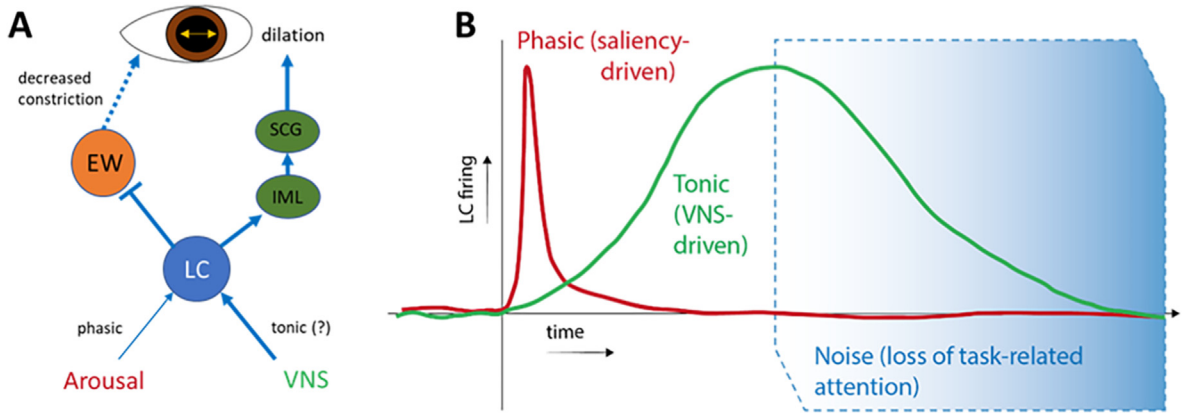


Fig. 7. Physiological hypotheses of VNS-induced modulation of pupil size. (A) Pathways leading to the locus coeruleus (LC)-dependent modulation of pupil size. VNS modulates LC activity via an indirect relay mediated from the nucleus of the solitary tract (not depicted in the figure). The LC send inhibitory inputs to the nucleus of Edinger-Westphal (which will in turn lead to decreased pupil constriction). On the other hand, the LC is responsible for an excitatory pathway leading to the activation of dilator muscle of the pupil, via the intermedio-lateral column (IML) and the superior cervical ganglion (SCG). **(B)** The observed results may be interpreted through a physiological model where early peak dilation reflects a phasic, early, saliency-driven activation of the LC (red line), while the later PDR expresses a rather tonic LC activation induced by VNS (green line). In a real-life human setup for pupil size recording, we expect a likely influence of noise increasing with time from the trial onset (blue shade), due to a physiological loss of task-related attention, required for gaze fixation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the dilator muscle of the pupil via the intermedio-lateral column [1] (Fig. 7A). In addition, we postulate that, unlike the arousal (phasic) response, the VNS-induced modulation might occur in a more tonic way, justifying the appearance of intensity-related effects on the late PDR component (as previously described in Section **4.1**, Fig. 7B). Future research should ascertain whether the inverted-U profile seen for the late PDR depends on a reduced LC activation at high intensities (e.g. through fMRI), or on the concomitant engagement of an inhibitory pathway through other structures. Also, in addition to the noradrenergic modulation, pupil size modulation induced by VNS was correlated with the cholinergic nuclei of the basal forebrain [36]: the latter may also have played a role in the observed responses.

We also acknowledge that the overlap between the possible VNS-related late PDR and the phasic arousal-related early PDR implies that the magnitude of the late PDR evoked by VNS is not solely determined by vagus nerve afferent input. A clear disentanglement of these two components in a human setup remains challenging. To this extent, this study provides new insights on those characteristics of the PDR which might be more associated with vagal modulation rather than with simple somatosensory effect. In addition, we cannot exclude that part of the differences in the latency of the pupil dilation following VNS and control stimulation may be related to the ramp-up (~2 s) of the VNS stimulation, which is intrinsically programmed for the LivaNova pulse generators, and not present in the control condition.

In order to propose the PDR as a dosing biomarker, a higher number of VNS intensities should be tested for each subject. Future studies should evaluate whether fewer trials per intensity might lead to consistent PDR magnitudes. This could enable to reduce the recording duration and maintain its feasibility in clinical routine. On a pharmacological level, this study was conducted on a clinical population with heterogeneous concomitant medical treatment, in particular regarding antiseizure medicines. It is in accordance with the purpose of developing a real-life biomarker acquisition for potential clinical use in epileptic patients. There is no specific effect reported in the literature of antiseizure medicines, including benzodiazepines, on pupil reactivity. Although the effect of the certain medications that acutely influence pupil size and reactivity was mitigated by the exclusion criteria, we cannot exclude that the remaining variety of treatment might have affected the results at individual level. Future studies aiming to use PDR as a response and/or predictive classifier (i.e., comparing its levels between subjects) should carefully consider potential baseline brain function modulation by factors such as fatigue and concomitant medications. Also, the limited sample size, as well as a potential selection bias for well-responding patients, led to the inclusion of 2/10 (20%) of seizure-free patients, a value that is beyond the generally reported rate of seizure freedom. Future works should ensure a larger population with seizure response rates better reflecting the reality of VNS clinical efficacy.

Further avenues to fully exploit the PDR as a dosing or response biomarker may be foreseen in two directions. Firstly, by testing whether PDR values can distinguish between responders and non-responders to therapy. One might hypothesize that, in the light of previous evidence showing higher acute modulation of LC function in responders [37], these patients might show higher maximal values of PDR, or different intensities leading to maximal PDR, when compared to non-responders. Secondly, exploring how VNS parameters that influence the summation of charge delivery over time (such as VNS frequency) might affect the observed stimulus-response relationship, as the observed pupil dilation is not evoked by single VNS pulses but emerges as a reflection of the overall stimulation throughout a train.

Funding sources

This work was supported by the F.R.S.-FNRS (Fonds National de la Recherche Scientifique, Belgium): SV is a Research Fellow "Aspirant" [Grant no: 40000947]; GL is a Research Associate. RET is funded by the WELBIO program, Wavre, Belgium. This work was also supported by the Fonds de Recherche Clinique, Saint-Luc University Hospital, Brussels, Belgium.

CRediT authorship contribution statement

Simone Vespa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Lars Stumpp:** Data curation, Formal analysis, Investigation, Software, Writing – review & editing. **Giulia Liberati:** Conceptualization, Methodology, Writing – review & editing. **Jean Delbeke:** Conceptualization, Methodology, Supervision, Validation, Writing

– review & editing. **Antoine Nonclercq:** Conceptualization, Methodology, Supervision, Writing – review & editing. **André Mouraux:** Conceptualization, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing. **Riëm El Tahry:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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