NEW INSIGHTS INTO THE ROLE OF THE VAGUS NERVE IN HEALTH AND DISEASE: BASIC AND CLINICAL STUDIES

EDITED BY: Vitor Engracia Valenti, Luiz Carlos Marques Vanderlei, Moacir Fernandes Godoy, Davi J. A. Moraes and Brian Kliszczewicz

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NEW INSIGHTS INTO THE ROLE OF THE VAGUS NERVE IN HEALTH AND DISEASE: BASIC AND CLINICAL STUDIES

Topic Editors:
Vitor Engracia Valenti, São Paulo State University, Brazil
Luiz Carlos Marques Vanderlei, São Paulo State University, Brazil
Moacir Fernandes Godoy, Faculdade de Medicina de São José do Rio Preto, Brazil
Davi J. A. Moraes, University of São Paulo, Brazil
Brian Kliszczewicz, Kennesaw State University, United States

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EDITED AND REVIEWED BY
Vaughan G. Macefield,
Baker Heart and Diabetes Institute, Australia

*CORRESPONDENCE
Vitor E. Valenti
vitor.valenti@unesp.br

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Editorial: New insights into the role of the vagus nerve in health and disease: Basic and clinical studies

Vitor E. Valenti*

Autonomic Nervous System Center (CESNA), São Paulo State University (UNESP), Marília, SP, Brazil

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Editorial on the Research Topic
New insights into the role of the vagus nerve in health and disease: Basic and clinical studies

The vagus nerve plays an important role in the homeostatic control of several organ systems in health and disease (Karemaker, 2022). This Research Topic presents some of the latest work that explores different aspects of vagal control.

Kuchler et al. analyzed the role of the spleen in vagal activity and metabolism. The mentioned experimental study assessed the impact of vagotomy associated with splenectomy on white adipose tissue in obese rats. It is worth noting that obesity was induced *via* hypothalamic lesions, leading to lifelong obesity. The main finding of the study suggested that vagal-splenic mechanisms control the metabolism in health conditions.

Kobrzycka et al. conducted research on the hypothalamus. The researchers performed vagotomy in rats to understand the role of the vagus nerve on neurochemical alterations in the hypothalamus. The results indicated that, although mRNA expression was not changed, vagotomy affected the hypothalamic amino acids in the long term.

The study conducted by Yu et al. evaluated the interaction between transcutaneous auricular vagal nerve stimulation and cerebral hemodynamics in consciousness restoration. This open-label pilot clinical trial performed functional magnetic resonance imaging to analyze cerebral hemodynamics. The results indicated that this specific vagal stimulation technique activated the interoceptive and limbic systems, suggesting disorders of consciousness attenuation.

Due to the abovementioned limitation, Osińska et al. conducted a longitudinal case study to evaluate patients with disorders of consciousness submitted to vagal nerve stimulation. Electrophysiological measures included electroencephalography and heart rate variability. The authors reported that the 6-month intervention was able to improve the behavioral indices of consciousness and reinforced that the alpha wave electroencephalography power gradually increased.

Moreover, auricular vagal stimulation was analyzed during the COVID-19 pandemic. The abovementioned mentioned multicentric, randomized, controlled, double-blind study investigated the influence of auricular neuromodulation through auricular acupuncture using semipermanent needles on clinical outcomes in patients with COVID-19 (Rangon et al.). The outcome measures included the seven-category ordinal scale, time until clinical improvement, and transfer to the intensive care unit. Unfortunately, the authors did not observe a significant beneficial effect of this intervention on the outcomes of the COVID-19 patients.

Our Research Topic also explored psychiatric comorbidities and their association with autonomic reactivity. Ruschil et al. focused on heart rate variability, a non-invasive method that estimates vagal control of heart rhythm (Kloter et al., 2018). The authors examined neurological patients with medically unexplained sensory symptoms and analyzed the regulation of their HRV in different conditions, i.e., placebo application, cold-face test, and pain stimuli. The data pointed to altered vagal function in this specific condition.

The association between the vagus nerve, autonomic activity, and fetal development was investigated by Cerritelli et al. The authors conducted a simple review and highlighted the importance of non-invasive assessment of maternal and fetal autonomic function through heart rate variability. The authors reinforced that monitoring health status throughout the pregnancy, from its earliest stages, is necessary to identify risks and outcome projections.

In this line, the association between the vagus nerve and bowel inflammation was investigated by Caravaca et al. The bowel disease model was based on an experimental indomethacin-induced acute intestinal inflammation in male Sprague Dawley rats. Vagal activation was performed *via* the left cervical vagus nerve, and the splenic nerve was also stimulated. Reduction of the TNF levels following vagal nerve stimulation evidenced that this intervention provided a benefic impact on small bowel inflammation.

Cardiac arrest, a very interesting issue, was also explored in our Research Topic. Kim et al. observed the effects of vagal nerve stimulation on cerebral mitochondrial dysfunction in an asphyxial cardiac arrest rat model. The respiration measurement in mitochondria was necessary to better understand this mechanism. The study evidenced that the stimulation of the cervical vagus nerve improved cerebral injury in male Sprague–Dawley rats.

Finally, a sophisticated investigation analyzed the spatial working memory in young adults who submitted to vagal stimulation (Sun et al.). The authors performed transcutaneous auricular vagus nerve stimulation to activate the vagus nerve and working memory tasks to better understand memory status. Among the main data, the authors highlighted that, although the vagus nerve increased the performance of offline spatial working memory tasks, there was no evidence of improvement of vagal stimulation on digit working memory tasks.

In summary, our Research Topic provides very interesting studies that add reliable data to better understand the role of the vagus nerve in health and disease in experimental and clinical conditions.

Author contributions

VV draft the editorial and gave final approval.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The Vagus Nerve and Spleen: Influence on White Adipose Mass and Histology of Obese and Non-obese Rats

Joice Cristina Kuchler[1,2], Bruna Schumaker Siqueira[2], Vanessa Marieli Ceglarek[3,4], Fernanda Vigilato Chasko[2], Isllany Carvalho Moura[2], Bruna Fatima Sczepanhak[2], Jean Franciesco Vettorazzi[5], Sandra Lucinei Balbo[2] and Sabrina Grassiolli[1,2]*

[1] Postgraduate Program in Applied Health Sciences, Western Paraná State University, Francisco Beltrão, Brazil, [2] Laboratory of Endocrine and Metabolic Physiology, Postgraduate Program in Biosciences and Health, Western Paraná State University, Cascavel, Brazil, [3] Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [4] Postgraduate Program in Biological Sciences, Physiology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [5] Educational Union of Cascavel, UNIVEL, Cascavel, Brazil

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Edited by:
Brian Kliszczewicz, Kennesaw State University, United States

Reviewed by:
Dervla O'Malley, University College Cork, Ireland
Matteo Cerri, University of Bologna, Italy

***Correspondence:**
Joice Cristina Kuchler
joicekuchler97@gmail.com

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The vagus nerve (VN) and spleen represent a complex interface between neural and immunological functions, affecting both energy metabolism and white adipose tissue (WAT) content. Here, we evaluated whether vagal and splenic axis participates in WAT mass regulation in obese and non-obese male Wistar rats. High doses of monosodium glutamate (M; 4 g/Kg) were administered during the neonatal period to induce hypothalamic lesion and obesity (M-Obese rats). Non-obese or Control (CTL) rats received equimolar saline. At 60 days of life, M-Obese and CTL rats were randomly distributed into experimental subgroups according to the following surgical procedures: sham, subdiaphragmatic vagotomy (SV), splenectomy (SPL), and SV + SPL (n = 11 rats/group). At 150 days of life and after 12 h of fasting, rats were euthanized, blood was collected, and the plasma levels of glucose, triglycerides, cholesterol, insulin, and interleukin 10 (IL10) were analyzed. The visceral and subcutaneous WAT depots were excised, weighed, and histologically evaluated for number and size of adipocytes as well as IL10 protein expression. M-Obese rats showed higher adiposity, hyperinsulinemia, hypertriglyceridemia, and insulin resistance when compared with CTL groups ($p < 0.05$). In CTL and M-Obese rats, SV reduced body weight gain and triglycerides levels, diminishing adipocyte size without changes in IL10 expression in WAT ($p < 0.05$). The SV procedure resulted in high IL10 plasma levels in CTL rats, but not in the M-Obese group. The splenectomy prevented the SV anti-adiposity effects, as well as blocked the elevation of IL10 levels in plasma of CTL rats. In contrast, neither SV nor SPL surgeries modified the plasma levels of IL10 and IL10 protein expression in WAT from M-Obese rats. In conclusion, vagotomy promotes body weight and adiposity reduction, elevating IL10 plasma levels in non-obese animals, in a spleen-dependent manner. Under hypothalamic obesity conditions, VN ablation also reduces body weight gain and adiposity, improving insulin sensitivity without changes in IL10 protein expression in WAT

or IL10 plasma levels, in a spleen-independent manner. Our findings indicate that the vagal-spleen axis influence the WAT mass in a health state, while this mechanism seems to be disturbed in hypothalamic obese animals.

Keywords: vagotomy, splenectomy, autonomic nervous system, adipocyte, hypothalamic obesity

INTRODUCTION

White adipose tissue (WAT) exerts a central role in energy homeostasis, a function related to the endocrine activities of adipocytes (Ghaben and Scherer, 2019). Adipocytes present a narrow association between metabolism (lipogenesis and lipolysis), cell size (larger and small cells), and adipokine secretion (pro or anti-inflammatory substances) (Gustafson and Smith, 2015). Thus, in obesity conditions, the pronounced WAT expansion is primarily characterized by increased lipogenesis, adipocyte hypertrophy, and increases in pro-inflammatory proteins, such as tumor necrosis factor-alpha (TNFα) and interleukin 1 beta (IL1β), with simultaneous reduction in anti-inflammatory substances, such as interleukin 10 (IL10) and adiponectin (Van Meijel et al., 2019).

The imbalance between cellular and secretory functions of WAT is a key point for the development of metabolic abnormalities, such as insulin resistance, hyperglycemia, dyslipidemia, and hypertension, characterizing the metabolic syndrome (MS) (Klöting and Blüher, 2014; Chu et al., 2018). However, the origin of these processes is unknown. In this sense, the interplay of neuronal and immunological aspects seems to have an important impact in metabolic diseases, including those associated with WAT expansion (Seoane-Collazo et al., 2015). Two central arms in the immune and metabolic interface are the bi-directional influence of the vagus nerve (VN) and spleen on WAT function (Martin et al., 2015; Pavlov and Tracey, 2017; Ai et al., 2018).

Autonomic nervous system (ANS) imbalance is commonly observed in obesity, with VN hyperactivity involved in hyperinsulinemia, insulin resistance, glucose intolerance, and excessive WAT mass expansion (Cohen et al., 2013; Balbo et al., 2016). The WAT vagal innervation is a matter of discussion for several research groups (Kreier et al., 2002; Giordano et al., 2006). In this regard, some argue a lack of significant vagal innervation in WAT, while others report the presence of a parasympathetic input in WAT (Giordano et al., 2006; Holland et al., 2019). Independent of these discussion points, it is clear that VN ablation (vagotomy) induces WAT mass reduction, a response observed in obese human (Miyato et al., 2012) and rodent obesity models (Andrews et al., 1985; Balbo et al., 2016). The impact of VN ablation in adiposity probably involves neuro-immune system interplay with repercussions on metabolic state. In this sense, the reduction in brain-melanocortin signaling promotes fat mass gain, by activating the lipogenic program in adipocytes and the proliferation of endothelial cells in WAT depots, a response dependent of the efferent hepatic VN branch (Holland et al., 2019). Moreover, VN modulates WAT content by controlling the sympathetic peripheral tonus via central nervous system (CNS). Subdiaphragmatic vagotomy impairs the brown adipose tissue (BAT)-mediated diet-induced thermic response (Andrews et al., 1985), while acute VN stimulation increases norepinephrine concentrations and transmission in the rat brain (Follesa et al., 2007).

A well-known vagal-immune interaction is the anti-inflammatory vagal reflex (Pavlov and Tracey, 2017) for which the spleen is required (Rosas-Ballina et al., 2015). The immunological activities of the spleen are modulated by the ANS and the best recognized anti-inflammatory pathway in this organ is the sympathetic activity via the splanchnic nerve (Martelli et al., 2019). However, more recent data suggest that parasympathetic vagal activity is also able to alter immune splenic responses, being functionally relevant for the sympathetic tone control of the spleen (Rosas-Ballina et al., 2015). Despite the VN does not directly innervate splenic cells, the vagal preganglionic fibers synapse with postganglionic sympathetic neurons in celiac ganglion, subsequently traveling through splenic nerves (Berthoud and Neuhuber, 2019). As such, the sympathetic nervous system (SNS) and the VN synergically act through the splenic nerve, to inhibit the release of TNFα by macrophages in the spleen (Pavlov and Tracey, 2017).

Beside the known neuro-immune responses involving the spleen, this organ also affects energy homeostasis (John and King, 1914; Ai et al., 2018). Splenectomy changes WAT content, glucose, and lipids homeostasis, and insulin sensitivity in obese rodents (Leite et al., 2015). Obesity provokes fat accumulation and induces higher inflammatory responses in the spleen (Turbitt et al., 2019). Leptin, the primary WAT adipokine, increases SNS flux to the spleen when centrally administered (Tanida et al., 2019). Interestingly, the spleen is also an important source of the anti-inflammatory cytokine IL10, and several splenic metabolic functions could be a consequence of changes in IL10 secretion or action. For example, infusion of adipose tissue-derived stem cells (ADSCs) reduces hyperglycemia and insulin resistance in diabetic rats, a response dependent on spleen-derived IL10 expression (Zhang et al., 2017). Obesity is hypothesized to suppress the synthesis of IL10, resulting in chronic inflammation in WAT (Gotoh et al., 2012a). In obese humans, IL10 expression in WAT was inversely associated with insulin resistance (Mclaughlin et al., 2014). Interesting, adipocytes are able to modulate immune responses in the spleen, including IL10 production (Vielma et al., 2013; Toda et al., 2020). Moreover, WAT depots also represent an abundant source of IL10 in the context of viral infections (Garcia-Valtanen et al., 2020). IL10 knockout mice develop systemic inflammation and alterations in mitochondrial lipid metabolism (de-Lima-Júnior et al., 2019). Finally, VN stimulation increases IL10 endogenous production, a response mediated by splanchnic nerves in the spleen (Komegaea et al., 2018). Together, these data suggest a bi-directional vagal-splenic interaction impacting WAT mass through mechanisms still unknown.

Elevated doses of neonatal glutamate monosodium (M) administered to newborn rats induce hypothalamic lesions, primarily affecting the arcuate nucleus (ARC), resulting in massive adiposity associated with insulin resistance, glucose intolerance, and dyslipidemia, reproducing central elements of MS (Balbo et al., 2007; Grassiolli et al., 2007). Vagal hyperactivity, spleen abnormalities, and altered IL10 plasmatic levels have already been confirmed in M-Obese rats (Caetano et al., 2017; Guareschi et al., 2019). Based on these findings, M-Obese rats can be considered a substantial obesity model for investigating possible vagal–spleen interactions via IL10 actions and their repercussions on WAT histology and function. Thus, in the present work, we evaluated the effect of vagotomy associated with splenectomy on WAT content and histology in hypothalamic M-Obese and non-obese male Wistar rats, assessing whether changes in IL10 plasma levels or IL10 protein expression in WAT are involved in vagal-splenic responses.

MATERIALS AND METHODS

Animals

Pregnant Wistar rats (n = 10) were obtained from the central animal facility of the Western Paraná State University (Unioeste) and transferred to the sectorial animal facility of the Laboratory of Endocrine and Metabolic Physiology (LAFEM). Animals were allocated into individual cages and received water and rodent chow (Biobase; SC; BR) *ad libitum* until the birth of offspring. At birth, the offspring size was adjusted to 6–8 male pups per dam, which were maintained under controlled luminosity cycles (12 h light–dark) and temperature (23 ± 2°C) during the lactation phase. All experimental procedures were approved by the local Ethics Committee on Animal Use (CEUA) on March 16, 2017, according to the Brazilian guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Hypothalamic Obesity

On the second day after birth, the offspring were divided into two groups. One group (n = 44) received a daily subcutaneous injection of monosodium glutamate (MSG) in a dose of 4 g/Kg of body weight (bw) during five consecutive days according to a previously established protocol (Olney, 1969). Elevated MSG doses to neonates provoke hypothalamic lesions, inducing lifelong obesity (Timper and Brüning, 2017; Torrezan et al., 2019). This group was denominated M-Obese. Non-obese or Control rats (CTL; n = 44) received equimolar saline solution. After weaning (21 days of life), M-Obese and CTL animals were randomly distributed into cages (three rats/cage) and at 60 days of life subdivided according to the surgical procedures described below.

Surgery Protocols

At 60 days of life, M-Obese (n = 22) and CTL (n = 22) groups were submitted to subdiaphragmatic vagotomy (SV) and/or splenectomy (SPL) (Balbo et al., 2007; Gotoh et al., 2012b). Briefly, after 12 h of fasting, animals were anesthetized with isoflurane (1%) and maintained in spontaneous ventilation with oxygen (1 ml/min). Then, the abdominal cavity was opened throughout an incision (± 2 cm) performed immediately below the sternum. Liver was moved for visualization of the anterior and posterior VN ramus in the esophagus wall. The VN ramus were placed away from the esophageal wall, tied, and posteriorly sectioned. For splenic surgery, the abdominal incision was done as described above, the blood vessel connected to the spleen was tied, and the organ excised and weighed. A group of animals had both SV and SPL surgeries performed in the same procedure, while another group was submitted to a sham surgery. At the end, eight experimental groups were originated (n = 11 rats), as illustrated in **Figure 1**. After the surgical procedures, all rats were transferred to individual cages, receiving water and rodent chow *ad libitum* for 1 week, to guarantee postoperative recovery. After this period, animals were regrouped (three rats/cage) according to the surgical protocol until 150 days of life.

Biometric and Plasma Parameters

From 70 to 150 days of life, rats were weighed for body weight gain (g) calculation. At 150 days of life, the naso-anal length (NAL; cm) was evaluated, and after 12 h of fasting, rats were euthanized, total blood was collected in heparinized tubes, and plasma used for dosage of glucose, triglycerides, and total cholesterol by enzymatic kits (Bioloquid, Laborclin, Pinhais, Brazil). Plasma insulin was measured by radioimmunoassay. Glucose and triglycerides values in fasting were used for TyG Index calculation using the formula: log [triglycerides (mg/dl)*glucose (mg/dl)/2] (Guerrero-Romero et al., 2010). Plasma samples were also used for IL10 dosage by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Novex, Bender MedSystems GmbH, Vienna, Austria). Immediately after euthanasia, abdominal cavity was opened, the stomach was excised, emptied, cleaned, and the net weight registered. Stomach's weight was used as a parameter of SV efficacy (Campfield et al., 1983). In non-splenectomized rats, the spleen was also excised, cleaned and the net weight registered. The final body weight and NAL were used to obtain the Lee index [$\sqrt[3]{}$body weight (g)/NAL (cm)]. The Lee index is a biomarker of adiposity in obese rodents, including those with hypothalamic obesity as previously proposed (Bernardis and Patterson, 1968).

White Adipose Tissue Histological Analysis

After euthanasia, the mesenteric (WAT-M) and inguinal (WAT-I) WAT depots were excised, weighed, and a fragment was immediately transferred to Alfac, a histological fixation solution constituted by a mixture of alcohol (80%), formol (10%), and glacial acetic acid (5%), during 24 h. After this period, the WAT tissue samples were transferred to an alcoholic (70%) solution for histological procedures. For this, WAT depots were diaphanized in xylol, dehydrated in alcoholic solution and embedded in paraplast (McCormickTM; Leica Microsystems Pty Ltd., Sydney, Australia), being finally submitted to the microtomy procedures. Semi-serial cuts (5 μm) were performed and stained with hematoxylin and eosin (H&E). Tissues from five to six rats per

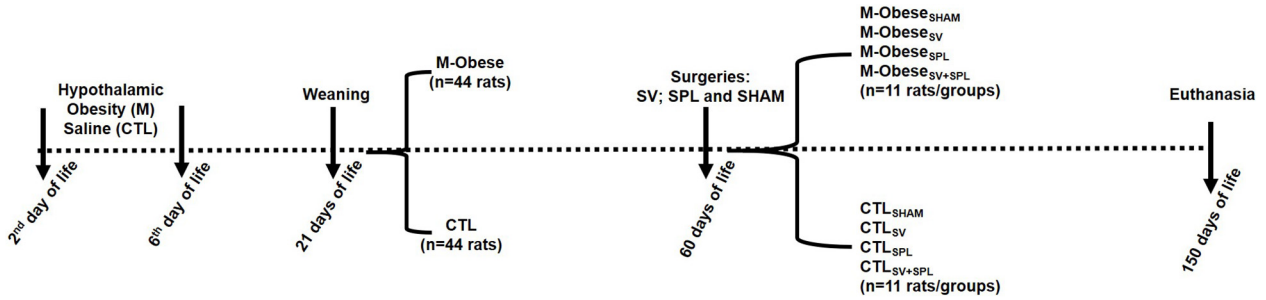

FIGURE 1 | Experimental design. CTL animals were divided into CTL_{SHAM}, Sham surgery control; CTL_{SPL}, splenectomized control; CTL_{SV}, subdiaphragmatic vagotomy control; CTL_{SV+SPL}, subdiaphragmatic vagotomy + splenectomized control; $M\text{-}Obese_{SHAM}$, simulated surgery MSG; $M\text{-}Obese_{SPL}$, Splenectomized MSG; $M\text{-}Obese_{SV}$, subdiaphragmatic vagotomy MSG; $M\text{-}Obese_{SV+SPL}$, subdiaphragmatic vagotomy + splenectomized MSG *N* = number of animals. The dashed line indicates the events over time (from birth to euthanasia), with vertical arrows evidencing specific points.

group were used to assemble the slides for histology (three slides per rat, containing at leastthree slices each). Images of the slides were captured using a photomicroscope (Olympus BX60, Tokyo, Japan) at a magnification of 40x. Adipocytes, size (μm^2), and number (number/field) were measured using an image analysis system (Image J 1.39f, NIH–Bethesda, MD, United States). A total of 50 adipocytes were analyzed per section.

White Adipose Tissue Western Blotting

Fragments of WAT-M and WAT-I depots were homogenized in 200 μl of lysis buffer (10 mM EDTA, 100 mM Tris base, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 1% triton X-100, and 1 μg/ml aprotinin). Protein concentration was measured using Bradford reagents (SIGMA, B6916). Of the protein samples, 30 μg was homogenized and boiled (5 min at 100°C) in Laemmli buffer. Proteins were then separated by electrophoresis in a 15% polyacrylamide gel. The transfer to nitrocellulose membranes was performed in Trans Blot transfer for 2 h at 110 V, with Tris/glycine buffer. Membranes were blocked in a Tris-buffered saline [10 mM tris base, 150 mM NaCl and 0.25% (vol./vol.) of tween 20] containing 5% (wt./vol.) of non-fat milk for 1 h at room temperature. After blocking, membranes were incubated overnight at 4°C with primary antibodies against IL10 (sc-8438–Santa Cruz, Dallas, Texas, United States) and Tubulin (sc-5286–Santa Cruz, Dallas, Texas, United States). Detection of specific protein bands was performed by incubating membranes with appropriate secondary antibodies (sc-2005–Santa Cruz, Dallas, Texas, United States) and bands detection was performed by measuring chemiluminescence (LOCCUS). Band's intensity was quantified by optical densitometry using the software LabImageID. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States), unless otherwise stated.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). CTL and M-Obese groups were compared using Student's *t*-test ($p < 0.05$). The main outcome measurements were analyzed by two-way ANOVA, followed by Tukey's *post hoc* test ($p < 0.05$). *Glass's Delta*, for effect size (ES) evaluation, was also calculated and physiological relevance was interpreted as small ($d = 0.2$), medium ($d = 0.5$), or large ($d = 0.8$).

RESULTS

White Adipose Tissue Visceral and Subcutaneous Hypertrophy, Dyslipidemia, and Insulin Resistance in M-Obese Rats

As shown in **Table 1**, hypothalamic lesions induced by MSG neonatal treatment had a large effect on biometric and biochemical parameters in adult life, as evidenced by the *ES*-values from M-Obese rats. Thus, at 150 days of life, M-Obese rats had lower body weight (29%; *ES* = −3.83), NAL (16%; *ES* = −10.30), and spleen weight (26%; *ES* = −1.29) compared

TABLE 1 | Adiposity, biometric, and metabolic profile of M-Obese rats.

Parameters	CTL	M-Obese	*p*-value	ES
Body weight (g)	433 ± 9	306 ± 5	<0.0001	−3.83
NAL (cm)	23.26 ± 0.11	19.36 ± 0.28	<0.0001	−10.30
Lee Index	0.32 ± 0.02	0.34 ± 0.02	0.0008	2.59
WAT-I (g/100 g)	0.27 ± 0.03	0.55 ± 0.05	0.0007	3.12
WAT-M (g/100 g)	0.99 ± 0.060	2.26 ± 0.25	0.0004	7.89
Stomach (g/100 g)	0.48 ± 0.01	0.49 ± 0.02	0.6064	0.55
Spleen (g/100 g)	0.14 ± 0.01	0.10 ± 0.01	0.0049	−1.29
Insulinemia (ng/ml)	0.09 ± 0.02	0.18 ± 0.02	0.0206	1.60
Glucose (mg/dl)	93 ± 8	75 ± 6	0.1124	−0.78
Triglycerides (mg/dl)	121.1 ± 16	217.1 ± 35	0.0337	2.44
TyG	2.00 ± 0.03	2.13 ± 0.04	0.0157	2.01
Cholesterol (mg/dl)	113 ± 3.8	146 ± 16.4	0.0722	3.25
IL-10 (pg/ml)	39.31 ± 4.57	50.13 ± 3.94	0.1122	0.68

Data are expressed as mean ± SEM, n = 8–11 rats per group.
NAL, naso-anal length; TyG, triglycerides and glucose index; IL10, interleukin 10; g, grams; cm, centimeters; mg, milligrams; dl, deciliters; ng, nanograms; ml, milliliters; CTL, control; M-Obese, MSG; ES, Effect size; WAT-I, inguinal white adipose tissue; WAT-M, mesenteric white adipose tissue; Student's t-test (p < 0.05).

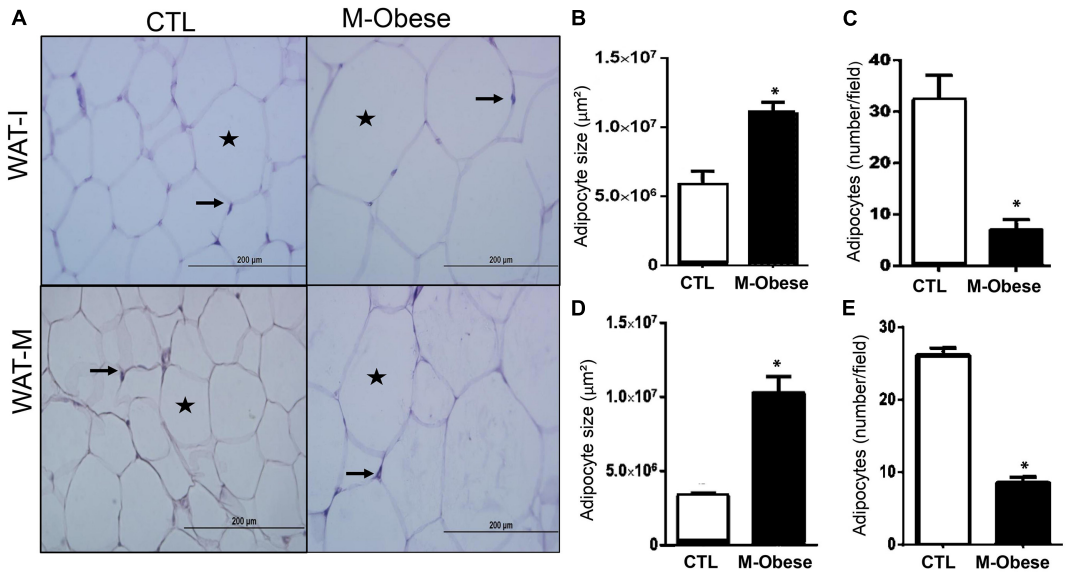

FIGURE 2 | M-Obese rats show intense adipocyte hypertrophy in WAT. **(A)** Representative photomicrographs of WAT-I and WAT-M, respectively, stained with H&E, under light microscopy, 40-fold magnification; **(B,D)** illustrate the average adipocyte size; **(C,E)** number of adipocytes per field in WAT-I and WAT-M, respectively. Adipocyte nuclei are indicated by arrows, and the deposition of fat in the cytosol is marked by the star. CTL, control; M-Obese, MSG; WAT-I, inguinal white adipose tissue; WAT-M, mesenteric white adipose tissue. Data are mean ± SEM (n = 6 rats/group). Asterisk (*) represent statistical differences between the groups. Student's t-test ($p < 0.05$).

with the CTL group ($p < 0.001$). In contrast, M-Obese rats showed higher Lee index (6%; *ES* = 2.59) compared with CTL animals, confirming elevated adiposity ($p < 0.05$). Moreover, the M-Obese group presented elevated plasma values of triglycerides (44%; *ES* = 2.44) and hyperinsulinemia (48%; *ES* = 1.60) compared with CTL rats ($p < 0.05$). The TyG index was approximately 6% higher in the M-Obese (*ES* = 2.01) group than in CTL animals. Although Delta's glass analysis has pointed medium ES to fasting glucose (−0.78), IL10 plasma levels (0.68) and stomach weight (0.55) in M-Obese rats did not significantly differ (Student's t-test, $p > 0.05$) from CTL animals (**Table 1**).

High fat content was observed in WAT-M (128%; *ES* = 7.89) and WAT-I (103%; *ES* = 3.12) of M-Obese rats compared with the CTL group (**Table 1**; $p < 0.0001$). M-Obese rats displayed adipocyte hypertrophy in WAT-I (86%; *ES* = 2.19) and WAT-M (209%; *ES* = 17.67) depots, where we consequently observed a reduction in the number of adipocytes (WAT-I: 72%; *ES* = −5.96 and WAT-M: 68%; *ES* = −7.36) in comparison with adipocytes from CTL group (**Figures 2A–D**; $p < 0.0001$). Additional representative photomicrographs of WAT-I and WAT-M depots in the CTL and M-Obese groups are showed in **Supplementary Figures 1a–d**.

Vagus Nerve Ablation Reduces Body Weight Gain and Adiposity in Non-obese Rats (CTL) in a Spleen-Influenced Manner

As a consequence of the SV surgery, the stomach weight was 53% and 52% higher in CTL_{SV} and CTL_{SV+SPL} groups, respectively, in comparison with CTL_{SHAM} animals ($p < 0.0001$; **Table 2**). The SV surgery affected body weight gain [$F_{(1.40)} = 0.0823$; $p = 0.0001$] and NAL [$F_{(1.30)} = 11.10$; $p < 0.0023$]. Thus, the body weight gain was reduced by 34.2% and 34.6% in CTL_{SV} and CTL_{SV+SPL}, respectively, compared with CTL_{SHAM} group ($p < 0.05$). Similar results were also observed when vagotomized rats were compared with the CTL_{SPL} group (**Table 2**). Moreover, the CTL_{SV} and CTL_{SV+SPL} rats also displayed significant reductions in NAL when compared with non-vagotomized groups (CTL_{SHAM} and CTL_{SPL}; $p < 0.05$). However, the Lee index was not affected by SV surgery. In contrast, spleen ablation did not affect body weight gain or NAL, but influenced the Lee index [$F_{(1.28)} = 5.033$; $p = 0.0330$]. The spleen weight was similar between CTL_{SHAM} and CTL_{SV} groups (**Table 2**; $p < 0.05$).

SV surgery modified the fasting glycemia [$F_{(1.31)} = 13.33$; $p = 0.0010$] with interaction between SV surgery and spleen ablation in this variable [$F_{(1.31)} = 81.96$; $p = 0.075$]. Thus, CTL_{SV+SPL} rats showed hyperglycemia when compared with other experimental groups (**Table 2**; $p < 0.001$). Insulin and total cholesterol during fasting were influenced by SV [$F_{(1.24)} = 9.616$; $p = 0.0049$] and SPL [$F_{(1.27)} = 5.22$; $p = 0.0304$] surgeries, respectively. However, we did not observe significant difference in Tukey's post-test (**Table 2**; $p > 0.05$). In contrast, SV surgery modified triglycerides plasma levels [$F_{(1.24)} = 12.32$; $p = 0.0018$], which were reduced in CTL_{SV+SPL} animals in comparison with CTL_{SPL} rats ($p < 0.05$). Although the combination of SV and SPL surgeries [$F_{(1.24)} = 7.783$; $p = 0.0102$] influenced TyG values, as shown in **Table 2**, no statistical difference between the groups was identified by Tukey's post-test. Plasma levels of IL10 were influenced by SPL factor [$F_{(1.16)} = 5.922$; $p = 0.0271$],

as well as by the interaction between SPL and SV surgeries [$F_{(1.16)}$ = 12.56; p = 0.0027]. CTL_{SV+SPL} rats showed lower IL10 plasma levels in comparison with CTL_{SV} group, while CTL_{SV} animals presented higher IL10 plasma levels when compared with other experimental groups ($p < 0.05$).

The impact of SV and SPL surgeries on adiposity content and histology from non-obese rodents (CTL) are presented in **Figures 3A–F**, **4A–F**. The adiposity content was significantly influenced by SV surgery in both WAT-I [$F_{(1.29)}$ = 7.163; p = 0.0121] and WAT-M [$F_{(1.28)}$ = 16.50; p = 0.0004] depots. Thus, CTL_{SV} animals presented lower WAT-I weight in comparison with CTL_{SPL} rats (**Figure 3D**; $p < 0.05$). In addition, in WAT-I depots from the CTL_{SV} group, we observed a reduction in adipocyte size (**Figure 3E**; $p < 0.05$) and a consequent increase in adipocytes number (**Figure 3F**; $p < 0.05$) in relation to other groups, as shown in the representative photomicrography (**Figure 3A**). The weight of WAT-M in CTL_{SV} group was also reduced by 35 and 40%, respectively, in relation to CTL_{SHAM} and CTL_{SPL} rats ($p < 0.05$; **Figure 4A**). Thus, in WAT-M depots from CTL_{SV} rats, adipocyte size was reduced (**Figure 4E**; $p < 0.05$), and adipocytes numbers were consequently increased (**Figure 4F**; $p < 0.05$) in comparison with CTL_{SHAM} animals, as shown in the representative photomicrography (**Figure 4A**).

Spleen ablation alone did not alter the content of WAT-I and WAT-M depots when compared with CTL_{SHAM} groups (**Figures 3D**, **4D**); $p > 0.05$). However, CTL_{SPL} rats were found to have higher WAT-I and WAT-M mass weight in relation to CTL_{SV} animals (**Figure 3D**; $p < 0.05$). Moreover, interaction between SV-SPL surgeries influenced size [$F_{(1.65)}$ = 6.613; p = 0.0124; **Figure 3E**] and number [$F_{(1.65)}$ = 20.63; p = 0.0001 **Figure 3F**] of adipocytes in WAT-I, and also affected WAT-M adipocytes size [$F_{(1.20)}$ = 14.54; p = 0.0011]. Thus, CTL_{SV+SPL} animals show that size and number of adipocytes in WAT-I and WAT-M depots are higher than CTL_{SV} rats and similar to CTL_{SHAM} rats (**Figures 3E,F**, **4E,F**). Additional representative photomicrographs of WAT-I and WAT-M depots from CTL rats submitted to SV or SPL surgeries rats are showed in **Supplementary Figures 1e–l**). Both SV and SPL surgeries did not modify IL10 expression neither in WAT-I (**Figures 3B,C**) nor in WAT-M depots (**Figures 4B,C**) in CTL groups.

Vagus Nerve Ablation Induces Preferential White Adipose Tissue Visceral Content Reduction in M-Obese Rats, Improving Insulin Sensitivity Without Changes in IL10 Plasma Levels or Expression in White Adipose Tissue

The VN ablation induced significant increases in stomach weight in $M\text{-}Obese_{SV}$ and $M\text{-}Obese_{SV+SPL}$ rats in relation to $M\text{-}Obese_{SHAM}$ groups, confirming gastric stasis (**Table 3**; p = 0.0001). In the M-Obese groups, SV [$F_{(1.27)}$ = 69.31; $p < 0.0001$] and SV + SPL combination [$F_{(1.27)}$ = 4.461; p = 0.0441] affected stomach weight. $M\text{-}Obese_{SV}$ and $M\text{-}Obese_{SPL}$ groups showed significant reduction in approximately 24% in body weight gain in comparison with $M\text{-}Obese_{SHAM}$ rats ($p < 0.05$). Moreover, $M\text{-}Obese_{SV}$ rats had lower body weight gain in relation to $M\text{-}Obese_{SPL}$ animals ($p < 0.05$). Neither SV nor SPL surgeries altered NAL and spleen weight in M-Obese animals. However, SV surgery affected the Lee index [$F_{(1.26)}$ = 7.933; p = 0.0091] resulting in smaller Lee index value in $M\text{-}Obese_{SV+SPL}$ rats compared with $M\text{-}Obese_{SHAM}$ animals (**Table 3**; $p < 0.05$). Fasting values of glucose and total cholesterol were not modified by SV and/or SPL surgeries in M-Obese groups ($p > 0.05$). In contrast, SPL surgery affected fasting insulin plasma levels [$F_{(1.23)}$ = 7.862; p = 0.0101] resulting in lower levels in $M\text{-}Obese_{SV}$ animals when compared with the $M\text{-}Obese_{SV+SPL}$ groups (**Table 3**; $p < 0.05$). Moreover, SV

TABLE 2 | Effects of subdiaphragmatic vagotomy (SV) and/or splenectomy (SPL) surgeries on biometric and biochemical parameters from non-obese rats (CTL).

					p-value		
	CTL_{SHAM}	CTL_{SPL}	CTL_{SV}	CTL_{SV+SPL}	SPL	SV	I
Body weight gain (g)	149 ± 5.1[c,d]	129 ± 4.8[c,d]	98 ± 7.4[a,b]	98 ± 10[a,b]	0.2290	0.0001	0.2098
NAL (cm)	23.16 ± 0.14[c]	23.00 ± 0.26	22.12 ± 0.28[a]	22.44 ± 0.25	0.7521	0.0023	0.3186
Lee index	0.32 ± 0.002	0.31 ± 0.004	0.32 ± 0.003	0.31 ± 0.002	0.0330	0.7143	0.8095
Stomach (g/100 g)	0.48 ± 0.01[c,d]	0.52 ± 0.03[c,d]	1.04 ± 0.06[a,b]	1.02 ± 0.05[a,b]	0.8762	< 0.0001	0.4618
Spleen (g/100 g)	0.14 ± 0.01	N/A	0.13 ± 0.01	N/A		0.2828	
Glucose (mg/dl)	93 ± 8[d]	74 ± 5[d]	103 ± 11[d]	152 ± 17[a,b,d]	0.2055	<0.0010	0.0075
Insulinemia (ng/ml)	0.09 ± 0.02	0.10 ± 0.03	0.19 ± 0.03	0.19 ± 0.02	0.9126	0.0049	0.8911
Cholesterol (mg/dl)	113 ± 3.8	202 ± 35.2	147 ± 23	179 ± 28	0.0304	0.8226	0.2928
Triglycerides (mg/dl)	120 ± 13	129 ± 10[d]	88 ± 5.7	78 ± 14[b]	0.9433	0.0018	0.4220
TyG	2.03 ± 0.03	1.97 ± 0.02	1.96 ± 0.03	2.05 ± 0.02	0.6856	0.9318	0.0102
IL10 (pg/ml)	30.17 ± 3.29[b]	36.17 ± 4.04[a,d]	56.43 ± 10.72	25.11 ± 2.18[b]	0.0271	0.1537	0.0027

Data are expressed as mean ± SEM (n = 8–11 rats/group). ANOVA two-way F-values are shown in SPL, SV, and I column. Letters above numbers indicate statistical difference among groups—(a) CTL_{SHAM}; (b) CTL_{SP}; (c) CTL_{SV}; (d) CTL $_{SV+SPL}$ in Tukey post hoc test ($p < 0.05$).

SPL, splenectomy; V, subdiaphragmatic vagotomy; I, Interaction; NAL, naso-anal length; TyG, Triglycerides and glucose index; IL-10, Interleukin 10; g, grams; cm, centimeters; mg, milligrams; dl, deciliters; ng, nanograms; ml, milliliters; N/A, not applicable. CTL_{SHAM}, surgical simulation control; CTL_{SPL}, splenectomized control; CTL_{SV}, subdiaphragmatic vagotomy control; CTL_{SV+SPL}, subdiaphragmatic vagotomy + splenectomized control; WAT-I, white adipose tissue–inguinal; IL-10, Interleukin 10; SPL, splenectomy; SV, subdiaphragmatic vagotomy; I, interaction.

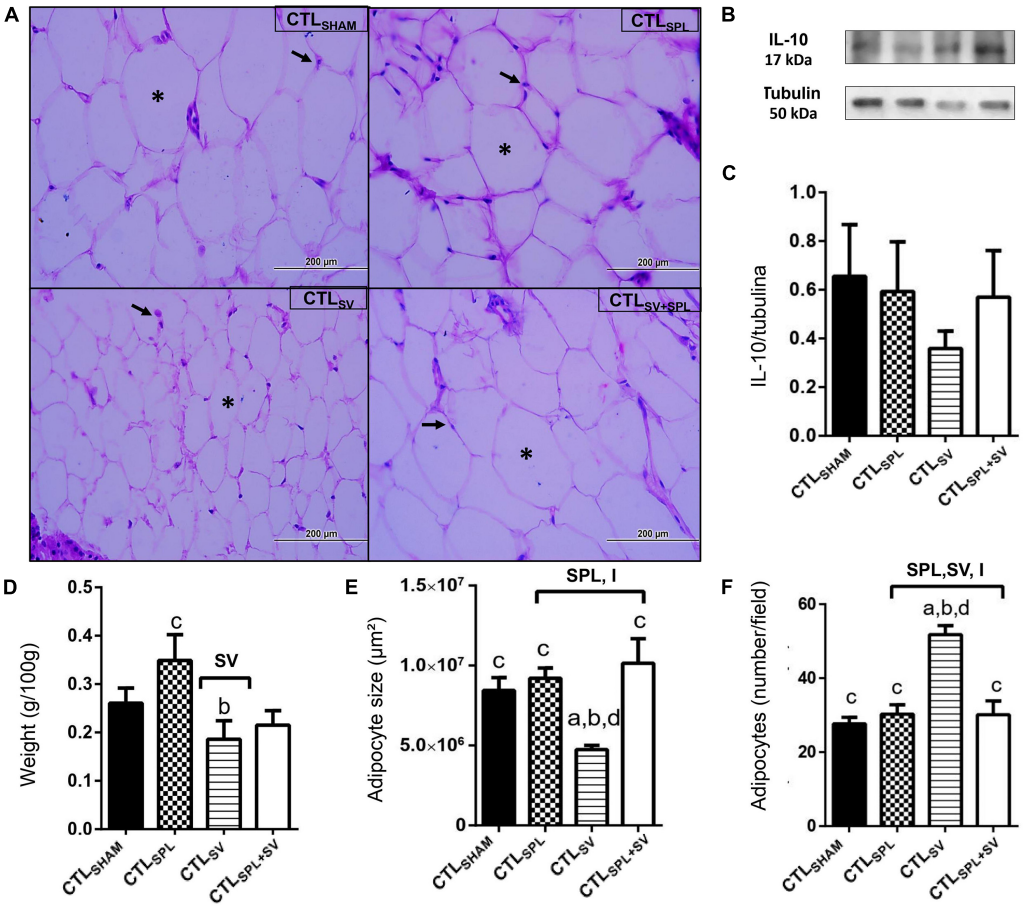

FIGURE 3 | Subdiaphragmatic vagotomy induces a reduction in WAT-I content in non-obese (CTL) rats with splenic participation and without changes in IL10 protein expression. **(A)** Representative photomicrographs of the broad WAT-I, stained with H&E, magnification 40×; adipocyte nuclei (arrow) and fat deposition (asterisk). **(B)** Representative WB band densitometry; 50 kDa region (tubulin); 17 kDa region IL10; In graphical, data are mean ± SEM **(C)** IL-10 expression (n = 4 rats/group); **(D)** weight of WAT-I (n = 6 rats/group); **(E)** size of adipocytes; **(F)** number of adipocytes (n = 6 rats/group). Line and symbols (SPL, SV, and I) above bars show significant F effect for two-way ANOVA. Letters represent statistical difference among groups–(a) CTL_{SHAM}; (b) CTL_{SPL}; (c) CTL_{SV}; (d) CTL $_{SV+SPL}$ in Tukey *post hoc* test ($p < 0.05$). Legend: CTL_{SHAM}, simulated surgery control; CTL_{SPL}, splenectomized control; CTL_{SV}, subdiaphragmatic vagotomy control; CTL_{SV+SPL}, subdiaphragmatic vagotomy + splenectomized control; WAT-I, white adipose tissue–inguinal; IL-10, Interleukin 10; SPL, splenectomy; SV, subdiaphragmatic vagotomy; I, interaction.

surgery affected plasma triglycerides levels [$F_{(1.17)}$ = 6.573; p = 0.0201], but without a difference in Tukey's post-test between M-Obese groups (**Table 3**; $p > 0.05$). Thus, the TyG index was influenced by SV surgery [$F_{(1.15)}$ = 25.67; p = 0.0001] in M-Obese groups. Therefore, we found smaller values of TyG index in M-$Obese_{SV}$ and M-$Obese_{SV+SPL}$ groups in relation to M-$Obese_{SHAM}$ rats (**Table 3**; $p < 0.05$). M-Obese rats did not show significant changes in IL10 plasma levels in any of the surgical procedures.

The influence of SV and/or SPL surgeries on adiposity content and histology of M-Obese rats are shown in **Figures 5A–F**; WAT-I) and **Figures 6A–F**; WAT-M). The content of WAT-I was not affected by SV or SPL in M-Obese rats (**Figure 5D**; $p > 0.05$). However, the adipocytes size [$F_{(1.16)}$ = 13.83; p = 0.0019] and number [$F_{(1.17)}$ = 16.25; P = 0.0009] were modified by SV factor in M-Obese animals. Adipocyte size was significantly smaller in M-$Obese_{SV}$ and M-$Obese_{SV+SPL}$ groups in relation to M-$Obese_{SHAM}$ rats (**Figures 5E,F**; $p < 0.05$). The number of adipocytes was elevated in WAT-I from M-$Obese_{SV}$ group in comparison to M-$Obese_{SHAM}$ animals (**Figure 5F**; $p < 0.05$). The WAT-M content was influenced by SV surgery in M-Obese rats [$F_{(1.29)}$ = 29.84; $p < 0.0001$], resulting in lower weight of WAT-M in M-$Obese_{SV}$ rats when compared with M-$Obese_{SHAM}$ and M-$Obese_{SPL}$ groups (**Figure 6D**; $p < 0.05$). Moreover, adipocytes size [$F_{(1.20)}$ = 9.012; p = 0.0070] and number [$F_{(1.20)}$ = 12.42; p = 0.0021] in WAT-M depots were modified by SV factor, since adipocytes size was smaller, while adipocytes numbers were increased in M-$Obese_{SV+SPL}$ rats in comparison with M-$Obese_{SHAM}$ group (**Figure 6F**; $p < 0.05$). Representative photomicrography shows the effects of the surgical procedures in WAT-I (**Figure 5A**) and WAT-M (**Figure 6A**). Additional representative photomicrographs of WAT-I and WAT-M depots from M-Obese rats submitted to SV or SPL surgeries rats are showed in **Supplementary Figures 1m–t**. In contrast, IL10

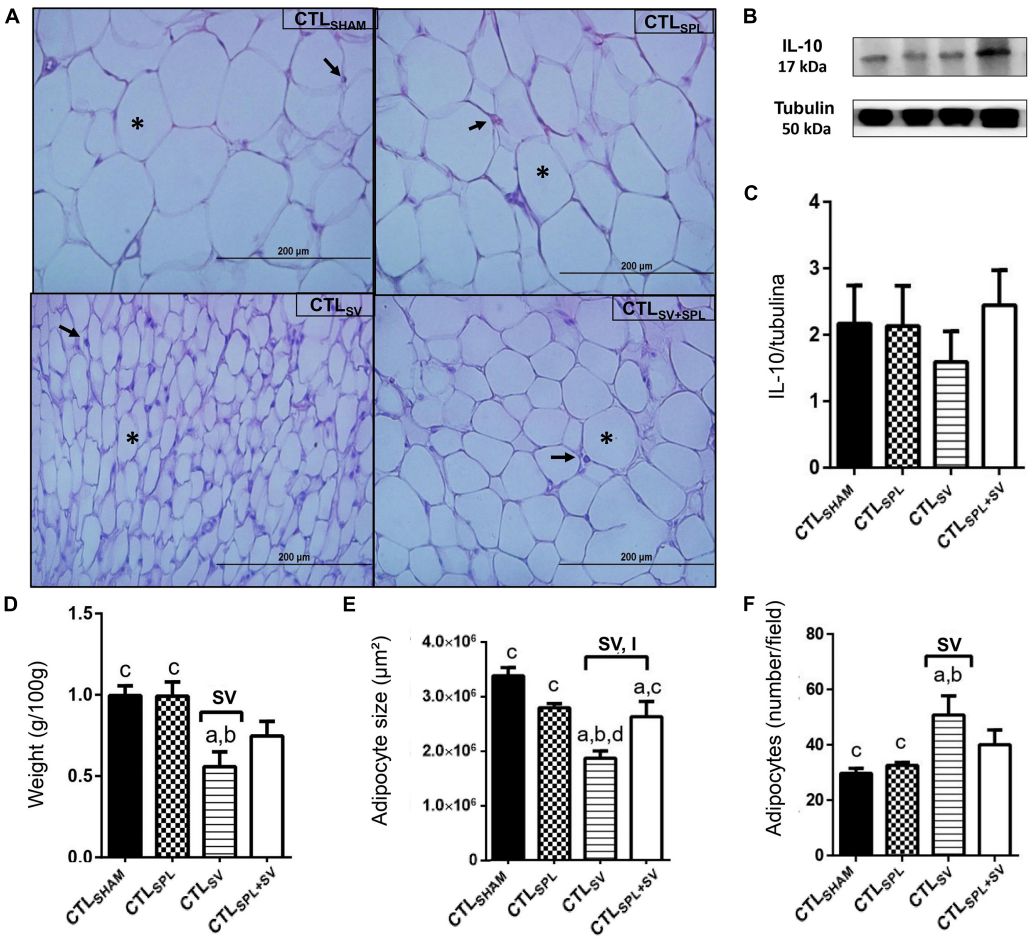

FIGURE 4 | Subdiaphragmatic vagotomy induces reduction in WAT-M content in non-obese (CTL) rats with splenic participation and without changes IL10 protein expression. **(A)** Representative photomicrographs of the broad WAT-M, stained with H&E, magnification 40×; adipocyte nuclei (arrow) and fat deposition (asterisk). **(B)** Representative WB band densitometry; 50 kDa region (tubulin); 17 kDa region IL10; In graphical, data are mean ± SEM **(C)** IL-10 expression (n = 4 rats/group); **(D)** weight of WAT-I (n = 6 rats/group); **(E)** size of adipocytes; **(F)** number of adipocytes (n = 6 rats/group). Line and symbols (SPL, SV, and I) above bars show significant F effect in Two-way ANOVA. Letters represent statistical difference among groups—(a) CTL_{SHAM}; (b) CTL_{SPL}; (c) CTL_{SV}; (d) CTL $_{SV+SPL}$ in Tukey *post hoc* test ($p < 0.05$). Legend: CTL_{SHAM}, simulated surgery control; CTL_{SPL}, splenectomized control; CTL_{SV}, subdiaphragmatic vagotomy control; CTL_{SV+SPL}, subdiaphragmatic vagotomy + splenectomized control; WAT-I, white adipose tissue—inguinal; IL-10, Interleukin 10; SPL, splenectomy; SV, subdiaphragmatic vagotomy; I, interaction.

expression was not modified by SV or SPL ablation in WAT-I (**Figure 5B**; $p > 0.05$) and WAT-M (**Figure 6B**; $p > 0.05$) depots.

Effect Size of Subdiaphragmatic Vagotomy and Splenectomy Surgeries Presented Different Impact in Non-obese and M-Obese Rats

Glass's delta analysis is a measure that enabled us to calculate the effect size (ES) of SV and/or SPL surgeries on CTL and M-Obese rats, considering their respective SHAM groups as internal controls. A schematic summary of ES is shown in **Table 4**, while ES values are presented in **Supplementary Table 1**. In CTL rats, VN ablation led to more pronounced effects, such as a reduction in body weight gain, anti-adiposity actions and a reduction in triglycerides plasma levels and TyG index, indicated by large negative ES values in SV groups. In contrast, CTL_{SV} groups presented elevated fasting insulin and IL10 plasma levels, since these variables show large positive *ES*-values (**Supplementary Table 1**). Excluding large positive *ES*-values for cholesterol plasma levels, SPL surgery showed, in general, a minor impact in the CTL group, with smaller or medium *ES*-values for other variables (**Supplementary Table 1**). Importantly, in CTL rats (non-obese), the spleen ablation changes the impact of vagotomy in adiposity and metabolic variables. For example, the positive small *ES*-value of SV in Lee index appears as large negative ES in the CTL_{SV+SPL} group. Similarly, the large negative ES of SV in TyG, WAT-I adiposity and IL10 plasma levels disappear in CTL_{SV+SPL} groups. On the other hand, glucose and cholesterol plasma levels were elevated by SV, and these effects were accented by splenectomy, having large *ES*-values in CTL_{SV+SPL} (**Table 4** and **Supplementary Table 1**).

In M-Obese rats a reduction in body weight gain and adiposity was also noted in vagotomized rats, with large negative *ES*-values (**Table 4** and **Supplementary Table 1**). However, in contrast to the CTL group, in M-Obese rats, the SV increased NAL and reduced Lee index, resulting in larger negative *ES*-values. Importantly, in M-Obese groups, SV surgery modified insulin and triglycerides plasma levels, since these variables presented large negative ES, with repercussions in TyG index (**Table 4** and **Supplementary Table 1**). The anti-adiposity impact of SV surgery was also noted in M-Obese groups with several *ES*-values in WAT-M and WAT-I depots. Thus, SV surgery in M-Obese rats provoked greater reduction in WAT-M content (larger negative *ES*-values; **Supplementary Table 1**) in comparison with WAT-I content. In both WAT depots from M-Obese rats were noted larger negative *ES*-values in size of adipocytes and a consequent large positive *ES*-values in numbers of adipocytes (**Table 4** and **Supplementary Table 1**). In contrast, spleen ablation did not modify this effect of SV in M-Obese groups. Moreover, spleen absence in M-Obese rats also reduced adipocytes size in WAT, with larger negative *ES*-values (**Table 4** and **Supplementary Table 1**).

DISCUSSION

It is widely accepted that ANS and immunological functions are differently modulated in obese and non-obese states and that changes in neuro-immune axis explain many comorbidities related to WAT mass expansion (Balbo et al., 2016; Mauer et al., 2016; Gotoh et al., 2017). Herein, we confirmed that SV surgery exerts anti-adiposity actions, promoting a reduction in body weight gain, WAT content, and adipocyte size, especially in non-obesity conditions. Importantly, the impact of VN ablation in adiposity in non-obese animals may be dependent on the presence of spleen and changes in IL10 plasma levels. On the other hand, in M-Obese rats, the response to VN ablation involves the restoration of insulin sensitivity, primarily reducing WAT visceral adipocyte hypertrophy and content.

Initially confirming previous studies by us (Grassiolli et al., 2007) and (Leite et al., 2015), we showed that neonatal administration of MSG promoted massive adiposity, insulin resistance, hypertriglyceridemia, and hyperinsulinemia. Moreover, excessive expansion of WAT in M-Obese rats was characterized by adipocyte hypertrophy in both visceral and subcutaneous WAT depots. Autonomic unbalance, with higher vagal hyperactivity and lower sympathetic tonus, is an evident phenomena in M-Obese rodents, which contributes to elevated WAT mass (Torrezan et al., 2019). In addition, in this obese rodent model, several hormonal and metabolic abnormalities contribute to elevated WAT mass and adipocyte hypertrophy, such as greater insulin lipogenic action (Kulyte et al., 2017), reduced lipolytic responses, higher cortisol and reduced growth hormone (GH) levels. Herein, we also confirmed the reduction of spleen weight in M-Obese rats, which may be related with histological alterations in white and red pulp in this organ, as previously demonstrated by our research group (Guareschi et al., 2019). Despite the spleen atrophy observed in the present study, we did not observe significant reductions in IL10 plasma levels in M-Obese rats, in contrast to a previous study (Caetano et al., 2017).

Our data corroborate previously published studies, showing that SV surgery reduces body weight gain and lowers adiposity in CTL and M-Obese animals (Balbo et al., 2007; Dezfuli et al., 2018), an effect that may relate to changes in food intake. The impact of VN ablation on food intake presents contradictory results, and time and technique surgery procedure are important aspects to consider when interpreting these results (Inoue and Bray, 1977; Louis-Sylvestre, 1983; Andrews et al., 1985). However, SV surgery did not alter food intake in non-obese or M-Obese animals (no published data). In this regard,

TABLE 3 | Effect of SV and/or SPL surgeries on biometric and biochemical parameters of M-Obese animals.

					p-value		
	M-Obese$_{SHAM}$	**M-Obese$_{SPL}$**	**M-Obese$_{SV}$**	**M-Obese$_{SV+SPL}$**	**SPL**	**SV**	**I**
Weight gain (g)	$132 \pm 9^{c,d}$	117 ± 3^{c}	$88 \pm 5^{a,b}$	100 ± 5^{a}	0.7855	0.0001	0.0322
NAL (cm)	19.3 ± 0.28	19.8 ± 0.3	20.1 ± 0.4	19.8 ± 0.4	0.8143	0.3359	0.3096
Lee index	0.343 ± 0.003^{d}	0.334 ± 0.003	0.329 ± 0.005	0.327 ± 0.003^{a}	0.1047	0.0091	0.3600
Stomach (g/100 g)	$0.49 \pm 0.02^{c,d}$	$0.45 \pm 0.01^{c,d}$	$0.85 \pm 0.06^{a,b}$	$1.04 \pm 0.10^{a,b}$	0.2008	<0.0001	0.0441
Spleen (g/100 g)	0.102 ± 0.002	N/A	0.100 ± 0.003	N/A		0.6974	
Glucose (mg/dl)	75 ± 6	81 ± 5	85 ± 6	89 ± 2	0.3650	0.0961	0.8604
Insulinemia (ng/ml)	0.18 ± 0.02	0.21 ± 0.01	0.13 ± 0.02^{d}	0.23 ± 0.02^{c}	0.0101	0.4976	0.1309
Cholesterol (mg/dl)	146 ± 16	131 ± 33	116 ± 26	126 ± 32	0.9420	0.6015	0.7203
Triglycerides (mg/dl)	217 ± 31	236 ± 32	145 ± 27	150 ± 23	0.6945	0.0201	0.8149
TyG	$2.16 \pm 0.03^{c,d}$	2.10 ± 0.02	2.00 ± 0.02^{a}	2.00 ± 0.02^{a}	0.2878	0.0002	0.2017
IL10 (pg/ml)	53.58 ± 5.85	57.56 ± 7.62	61.53 ± 8.67	61.37 ± 14.95	0.5635	0.5042	0.8985

Data are expressed as mean ± SEM (n = 8–11 rats/group). ANOVA two-way, F-values are shown in SPL, SV, and I columns. Letters above numbers indicate statistical difference among groups–(a) M-Obese$_{SHAM}$; (b) M-Obese$_{SPL}$; (c) M-Obese$_{SV}$; (d) M_Obese$_{SV+SPL}$ in Tukey post hoc test ($p < 0.05$).

SPL, splenectomy; SV, subdiaphragmatic vagotomy; I, interaction; NAL, nasoanal length; TyG, triglycerides and glucose index; IL-10, interleukin 10; g, grams; cm, centimeters; mg, milligrams; dl, deciliters; ng, nanograms; ml, milliliters; N/A, not applicable; M-Obese$_{SHAM}$, surgical simulation, MSG; M-Obese$_{SPL}$, splenectomized MSG; M-Obese$_{SV}$, subdiaphragmatic vagotomy MSG; M-Obese$_{SV+SPL}$, subdiaphragmatic vagotomy + splenectomized MSG.

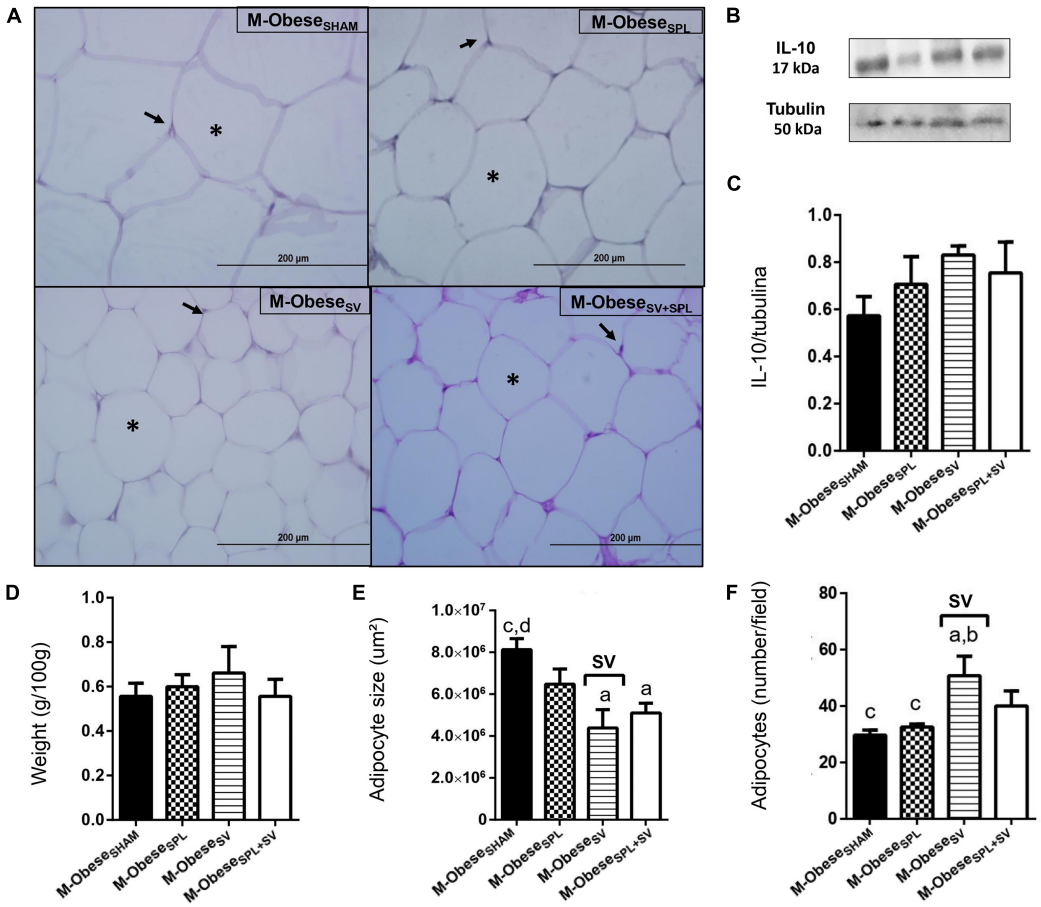

FIGURE 5 | Subdiaphragmatic vagotomy does not change WAT-I content in M-Obese rats but reduces adipocytes hypertrophy, without altering IL10 expression, regardless of spleen ablation. **(A)** Representative photomicrographs of the broad WAT-I, stained with H&E, magnification 40×; adipocyte nuclei (arrow) and fat deposition (asterisk). **(B)** Representative WB band densitometry; 50 kDa region (tubulin); 17 kDa region IL10; In graphical, data are mean ± SEM **(C)** IL-10 expression (n = 4 rats/group); **(D)** weight of WAT-I (n = 6 rats/group); **(E)** size of adipocytes; **(F)** number of adipocytes (n = 6 rats/group). Line and symbols (SPL, SV, and I) above bars show significant F effect in Two-way ANOVA. Letters represent statistical difference among groups—(a) M-Obese$_{SHAM}$; (b) M-Obese$_{SPL}$; (c) M-Obese$_{SV}$; (d) Obese$_{SV+SPL}$ in Tukey *post hoc* test ($p < 0.05$). Legend: M-Obese$_{SHAM}$, simulated surgery MSG; M-Obese$_{SPL}$, splenectomized MSG; M-Obese$_{SV}$, subdiaphragmatic vagotomy MSG; M-Obese$_{SV+SPL}$, subdiaphragmatic vagotomy + splenectomized MSG; WAT-I, white adipose tissue–inguinal; IL10, Interleukin 10; SPL, splenectomy; SV, subdiaphragmatic vagotomy, I, interaction.

SV causes a reduction in stomach motility and consequent gastric stasis, with higher food accumulation in this organ (Louis-Sylvestre, 1983; Andrews et al., 1985). Herein, the stomach weight from vagotomized rats (M-Obese and CTL) was significantly higher in relation to respective SHAM groups, indicating SV surgery efficacy.

Vagotomized CTL rats showed significant reduction in growth, suggesting an impact of VN in GH action or secretion in the non-obese state. Interestingly, ghrelin is a GH-releasing factor, which is altered by gastric stasis (Date, 2012). Our findings support a study (Al-Massadi et al., 2011) demonstrating that vagotomized animals downregulate GHRH mRNA in the ARC and downregulate mRNA of both GHRH and GHS receptors at the pituitary level, which are essential for the full GH-releasing effect of ghrelin. These responses were not observed in M-Obese animals probably due to extensive ARC lesions and a well-recognized reduction in GH release in M-Obese treated rats (Olney, 1969).

The VN plays an important role in glucose homeostasis, in particular, by modulating insulin secretion by the pancreas (Balbo et al., 2007), a response altered in obesity. In the present work we noted that fasting metabolic parameters were differently modulated by SV and SPL surgery in non-obese (CTL) and M-Obese animals. Thus, fasting glucose elevation was a consequence of VN ablation in CTL rats—an effect which was accentuated by spleen ablation—suggesting the participation of both the VN and the spleen in glucose homeostasis in the healthy state. Supporting this hypothesis, cervical VN stimulation (VNS) causes a rise in fasting glucose, reducing glucose tolerance in lean rats (Stauss et al., 2018), while hyperglycemia was more frequently observed in splenectomized humans (Ley et al., 2012).

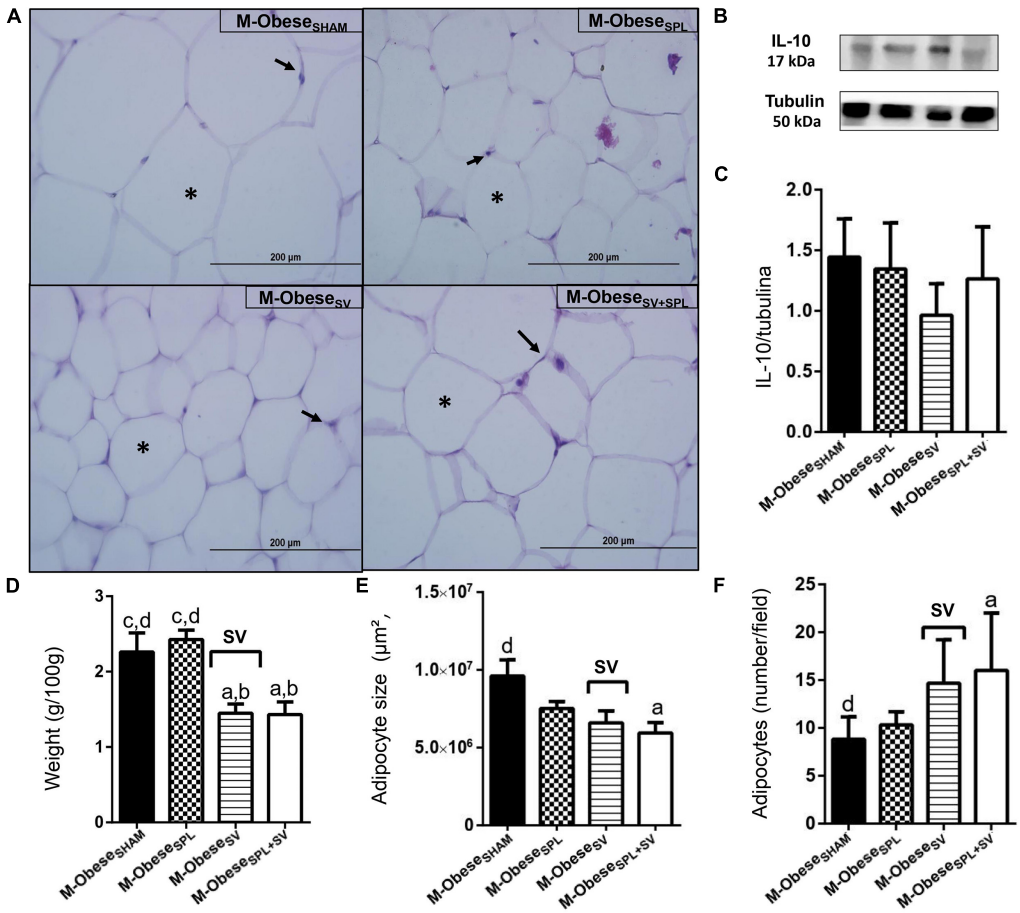

FIGURE 6 | Subdiaphragmatic vagotomy induces a reduction in WAT-M content in M-Obese rats, which is enhanced in the absence of the spleen, without leading to changes in IL10 expression. **(A)** Representative photomicrographs of the broad WAT-M, stained with H&E, magnification 40×; adipocyte nuclei (arrow) and fat deposition (asterisk). **(B)** Representative WB band densitometry; 50 kDa region (tubulin); 17 kDa region IL10; In graphical, data are mean ± SEM **(C)** IL-10 expression (n = 4 rats/group); **(D)** weight of WAT-I (n = 6 rats/group); **(E)** size of adipocytes; **(F)** number of adipocytes (n = 6 rats/group). Line and symbols (SPL, SV, and I) above bars show significant F effect in two-way ANOVA. Letters representing statistical difference among groups—(a) M-Obese$_{SHAM}$; (b) M-Obese$_{SPL}$; (c) M-Obese$_{SV}$; (d) Obese$_{SV+SPL}$ in Tukey *post hoc* test ($p < 0.05$). Legend: M-Obese$_{SHAM}$, simulated surgery MSG; M-Obese$_{SPL}$, splenectomized MSG; M-Obese$_{SV}$, subdiaphragmatic vagotomy MSG; M-Obese$_{SV+SPL}$, subdiaphragmatic vagotomy + splenectomized MSG; WAT-I, white adipose tissue–inguinal; IL-10, Interleukin 10; SPL, splenectomy; SV, subdiaphragmatic vagotomy; I, interaction.

In the present study, SV surgery reduced fasting insulin and improved insulin sensitivity in M-Obese rats, but not in CTL animals. As mentioned above, vagal hyperactivity is associated with hyperinsulinemia and insulin resistance in M-Obese rodents (Grassiolli et al., 2007; Balbo et al., 2007). The VN also exerts an impact in lipid metabolism, and VNS causes a reduction in plasma triglycerides in rats (Chen et al., 2018). According to our data, SV surgery also reduced triglyceride plasma levels in non-obese and M-Obese animals. However, it is important to note that the vagotomy-induced reduction in triglycerides was more pronounced (by *ES*-values) in M-Obese rats, confirming that vagal dysfunction has a role in dyslipidemia in this obese model (Lubaczeuski et al., 2015). In addition, vagotomy in M-Obese rats restored insulin sensitivity. Moreover, we have previously demonstrated that spleen ablation at 60 days of life did not alter glucose tolerance or insulin levels in CTL animals, but significantly reduces insulin levels and improves insulin sensitivity in M-Obese rats (Souza et al., 2020). Thus, ARC lesions in M-Obese rats possibly lead to VN hyperactivity and splenic dysfunction (Cohen et al., 2013) and therefore, the ablation of the VN and the spleen exerts a positive impact on glucose and lipid metabolism in this obese model.

The reduction in body weight gain and triglyceride levels observed in vagotomized, CTL and M-Obese rats may be partially explained by the lower adiposity found in these groups. However, the anti-adiposity effects of SV on WAT were more evident in CTL animals, in which we observed a greater reduction in adipocyte size in both visceral and subcutaneous WAT depots. These data suggested that, in the healthy state, the VN has a greater impact in fat mass regulation. Insulin is a central hormone for adipocyte lipogenesis and proliferation (Gustafson et al., 2015). Considering that in CTL rats, neither insulin levels nor insulin sensibility were affected by SV surgery, we believe that VN ablation in this case favors SNS lipolytic action in WAT.

TABLE 4 | *Glass's delta* effect size in CTL (non-obese) and M-Obese rats.

	CTL (non-obese)			M-Obese		
	SHAM X SPL	**SHAM X SV**	**SHAM X SPL + SV**	**SHAM X SPL**	**SHAM X SV**	**SHAM X SPL + SV**
Biometric	**ES**	**ES**	**ES**	**ES**	**ES**	**ES**
BW gain	**↓ Large**	**↓Large**	**↓Large**	↓Medium	**↓Large**	**↓Large**
NAL	↓Small	**↓Large**	**↓Large**	↑Medium	**↑Large**	↑Medium
Lee index	**↓Large**	↑Small	**↓Large**	**↓Large**	**↓Large**	**↓Large**
Stomach w.	**↑Large**	**↑Large**	**↑Large**	↓Medium	**↑Large**	**↑Large**
Spleen w.	N/A	↓Small	N/A	N/A	↓Small	N/A
Plasma						
Glucose	**↓Large**	↑Small	**↑Large**	↑Small	↑Medium	↑Medium
Triglycerides	↑Small	**↓Large**	**↓Large**	↑Medium	**↓Large**	**↓Large**
TyG	↓Medium	**↓Large**	↔	**↓Large**	**↓Large**	**↓Large**
Insulin	↔	**↑Large**	**↑Large**	↑Small	**↓Large**	↑Medium
Cholesterol	**↑Large**	**↑Large**	**↑Large**	↓Small	↓Medium	↓Small
IL10	↑Medium	**↑Large**	↓Medium	↑Small	↑Medium	↑Medium
WAT-I						
Weight	**↑Large**	**↓Large**	↓Medium	↑Small	↑Medium	↔
Cell size	↑Small	**↓Large**	↑Medium	**↓Large**	**↓Large**	**↓Large**
Cell number	↑Small	**↑Large**	↑Small	↓Medium	**↑Large**	**↑Large**
IL10 protein	↔	↓Medium	↓Small	**↑Large**	**↑Large**	**↑Large**
WAT-M						
Weight	↔	**↓Large**	**↓Large**	↑Small	**↓Large**	**↓Large**
Cell size	**↓Large**	**↓Large**	**↓Large**	**↓Large**	**↓Large**	**↓Large**
Cell number	↑Medium	**↑Large**	**↑Large**	↑Medium	**↑Large**	**↑Large**
IL10 protein	↔	↓Medium	↑Small	↔	↓Medium	↓Small

Glass's delta effect size (ES) = small (d = 0.2–0.49), medium (0.5–0.79), and large (≥ 0.8). ES numeric values are shown in ***Supplementary Table 1****. Legend: ↑ rise, ↔ no effect and ↓ reduction; Bold: Large ES; BW, body weight; NAL, naso-anal length; w, weight; WAT, white adipose tissue inguinal; WAT-M, white adipose tissue mesenteric; IL10, interleukin 10.*

The VN appears to be able to affect peripheral SNS flux by a modulatory action on the nucleus of the solitary tract (NTS) and hypothalamic nucleus (Bonaz, 2020). In this regard, auricular VNS elevates norepinephrine levels in WAT depots (Chen et al., 2018). Moreover, as demonstrated by another study (Holland et al., 2019), the VN exerts effect on lipogenic pathways in WAT via melanocortin system responses at the hypothalamic level, suggesting that increased VN activity may have a role in the gain of fat mass.

In M-Obese animals, insulin fasting and insulin sensibility were improved by VN ablation, explaining the adipocyte size reduction. Thus, it is likely that SV surgery in M-Obese rats corrects vagal hyperactivity, restoring insulin action and exerting anti-adiposity effects, as suggested in other studies (Balbo et al., 2007). Similarly, clinical studies have demonstrated that surgical ablation of the abdominal VN can result in considerable reduction of body weight and vagal denervation has also been linked to increased weight loss following gastrectomy (Miyato et al., 2012). In the present work, we performed a total SV surgery, making it impossible to distinguish afferent from efferent vagal signals. However, in obesity, vagal afferent signals are also disturbed, suggesting that vagal blocking therapy can provide significant weight loss in obese patients (Ikramuddin et al., 2014; Apovian et al., 2017). Moreover, VNS is able to increase brown adipose tissue thermogenesis and promote brightening in WAT depot of obese rodents, favoring elevated energy expenditure and fat reduction (Van Meijel et al., 2019). Importantly, afferent vagal signals are conducted to the ARC nucleus (Miller, 2017), which is damaged in M-Obese rats. Thus, the interruption of peripheral vagal hyperactivity in M-Obese is a central anti-adiposity effect of SV in this obese model. Herein, we also noted that, in M-Obese animals, the visceral WAT depot was more responsive to VN ablation, presenting higher reduction in content in comparison to WAT-I. Corroborating this finding, selective VN denervation in obese animals submitted to gastrectomy resulted in preferential reduction of visceral WAT, indicating that VN locally regulates the amount of intra-abdominal fat tissue (Miyato et al., 2012).

The neuro-immunological axis is related with ANS innervation to immune organs, such as the spleen. Interestingly, the VN and spleen are involved in common responses, especially anti-inflammatory activities (DiSpirito and Mathis, 2015; Ai et al., 2018; Serhan and Levy, 2018). However, we Souza et al. (2020) and Wu et al. (2014) and Rosas-Ballina et al. (2015) have previously demonstrated that the spleen can also participate in glucose homeostasis and fat mass distribution. The results shown in the present work indicate, for the first time, that vagal–splenic signals could be participating in adiposity control, particularly in non-obese condition. Thus, we demonstrated that spleen ablation avoided an SV-induced reduction in WAT mass and adipocytes size suggesting that the vagal effects on WAT could be dependent on splenic activity in health state.

The spleen has been reported as an important site of IL10 production (Gotoh et al., 2017) and splenectomized rats showed reduction in IL10 plasma levels, associated with a pro-inflammatory effect on WAT. Moreover, splanchnic nerve stimulation regulates IL10-related splenic anti-inflammatory responses (Bonaz et al., 2016) via the beta adrenergic receptor; a VN-mediated process. In our study, however, splenectomy did not promote any significant reduction in IL10 plasma levels neither in CTL nor in M-Obese rats, suggesting that other sites may be contributing to IL10 plasma concentrations. Although the spleen is essential for anti-inflammatory reflex, other abdominal organs, such as the adrenal gland, may be involved in this response (Martelli et al., 2019). Interestingly, we observed that in CTL vagotomized animals, there was an increase in IL10 plasma levels in the presence, but not in the absence of a spleen. Thus, we speculated that in vagotomized CTL animals there is an augmented SNS firing rate to spleen, stimulating IL10 production. This hypothesis is supported by another study that found higher levels of norepinephrine in the spleen after vagotomy (Pongratz et al., 2012).

In addition, we also demonstrated that increased plasma levels of IL10 observed in vagotomized CTL animals does not appear to be dependent on WAT, since the expression of IL10 protein in visceral or subcutaneous WAT were not influenced by SV or SPL. The role of IL10 in WAT is largely unknown. Some studies have suggested that IL10 might create an anti-inflammatory milieu by promoting the activity of M2 macrophages (Lumeng et al., 2007; Almeida et al., 2019; Steen et al., 2020). In contrast, IL10 adipogenic and pro-inflammatory effects have also been reported (Acosta et al., 2019).

In our study, neither SV nor SPL ablation promoted significant alterations in IL10 plasma levels or IL10 expression in WAT from M-Obese rats, suggesting that the vagal-splenic circuits are interrupted in obesity. Similarly, other studies have not supported an anti-obesity role for IL-10 (Pongratz et al., 2012; Bonaz et al., 2016). In this sense, loss of IL10 expression in mice increased energy expenditure and protected against diet-induced obesity (Rajbhandari et al., 2018). We have previously demonstrated that the spleen of M-Obese rats display altered histological distribution of white splenic pulp (Guareschi et al., 2019), suggesting splenic dysfunction in this obesity model. However, to date, IL10 production in the spleen of M-Obese rats has not been studied. In addition, the participation of other cytokines in adipocyte vagal-splenic responses cannot be discharged. For example, the IL17 response by spleen cells has been demonstrated to be dependent on the presence of adipocyte-derived mediators (Silvana et al., 2016), suggesting that multiple cytokines participate in cross-directional interactions between spleen and adipocytes. Importantly, VN-splenic axis also modulates plasma levels of pro-inflammatory cytokines, such as, IL6 and TNFα, which are altered in this M-Obese rodent model, but have not been assessed in the present study.

In summary, we demonstrated that VN ablation has anti-adiposity effects on obese and non-obese rats. However, in non-obese animals, anti-adiposity effects of vagotomy on WAT are dependent on increased IL10 plasma levels and the presence of the spleen, suggesting that the vagal-splenic axis modulates the metabolism in health state. In contrast, in M-Obese animals, VN ablation restores insulin sensitivity and consequently reduces WAT visceral mass, without the participation of the spleen or IL10, pointing out a disrupted vagal-splenic axis in hypothalamic obesity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comitê de Ética no Uso de Animais (CEUA) da Universidade Estadual do Oeste do Paraná.

AUTHOR CONTRIBUTIONS

JK: data analysis and interpretation, design of the work, critical revision of the article, and final approval of the version to be published. BSS: data collection, conception, design of the work, and final approval of the version to be published. VC: data analysis and interpretation, critical revision of the article, and final approval of the version to be published. FC, IM, and BFS: data collection, histological technique, and final approval of the version to be published. JV: molecular technique, critical revision of the article, and final approval of the version to be published. SB: surgical technique, critical revision of the article, and final approval of the version to be published. SG: conception, drafting the article, data analysis and interpretation, critical revision of the article, and final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.672027/full#supplementary-material

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BRIEF RESEARCH REPORT
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Cerebral Hemodynamic Correlates of Transcutaneous Auricular Vagal Nerve Stimulation in Consciousness Restoration: An Open-Label Pilot Study

Yutian Yu[1,2,3*†], *Yi Yang*[4,5*†], *Shuoqiu Gan*[6,7†], *Shengnan Guo*[3], *Jiliang Fang*[8], *Shouyan Wang*[9], *Chunzhi Tang*[10], *Lijun Bai*[6†], *Jianghong He*[4,5†] *and Peijing Rong*[3†]

[1] *Acupuncture Department, Beijing Shijitan Hospital, Capital Medical University, Beijing, China,* [2] *Ninth School of Clinical Medicine, Peking University, Beijing, China,* [3] *Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China,* [4] *Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing, China,* [5] *Department of Neurosurgery, PLA Army General Hospital, Beijing, China,* [6] *The Key Laboratory of Biomedical Information Engineering, Ministry of Education, Department of Biomedical Engineering, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, China,* [7] *Department of Medical Imaging, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China,* [8] *Department of Radiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing, China,* [9] *Institute of Science and Technology for Brain-Inspired Intelligence, Fudan University, Shanghai, China,* [10] *Clinical Medical College of Acupuncture, Moxibustion and Rehabilitation, Guangzhou University of Chinese Medicine, Guangzhou, China*

This study aimed to preliminarily illustrate the cerebral hemodynamic correlates of transcutaneous auricular vagal nerve stimulation (taVNS) in consciousness restoration. Arterial spin labeling (ASL) was adopted with functional magnetic resonance imaging (fMRI) to measure cerebral blood flow (CBF) changes before and after taVNS in 10 qualified patients with disorders of consciousness (DOC). Before taVNS, five patients responded to auditory stimuli (RtAS), and five did not respond to auditory stimuli (nRtAS). The RtAS DOC patients obtained favorable prognoses after the 4-week taVNS treatment, whereas the nRtAS ones did not. Simultaneously, taVNS increased CBF of multiple brain regions in the RtAS DOC patients, but hardly in the nRtAS ones. In conclusion, the preserved auditory function might be the prior key factor of the taVNS responders in DOC patients, and taVNS might alleviate RtAS DOC by activating the salience network, the limbic system, and the interoceptive system.

Keywords: functional magnetic resonance imaging (fMRI), arterial spin labeling (ASL), cerebral blood flow (CBF), transcutaneous auricular vagal nerve stimulation (taVNS), disorders of consciousness (DOC), responded to auditory stimuli (RtAS), non-responded to auditory stimuli (nRtAS)

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Stanford University, United States
Ian Spence,
The University of Sydney, Australia

****Correspondence:***
Yutian Yu
yutianyu@bjsjth.cn
Yi Yang
yangyi_81nk@163.com

† These authors have contributed equally to this work

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INTRODUCTION

Emergency healthcare and reanimation techniques have decreased the mortality of patients with severe traumatic brain injury (TBI) and hypoxic-ischemic encephalopathy (HIE) significantly in recent decades. However, some patients with TBI or HIE manifest with very poor prognoses and finally suffer from disorders of consciousness (DOC), a medical condition changed from complete self-awareness

to inhibited or absent self-awareness and arousal (1), either temporary or permanent. Increasing numbers of DOC patients are laying an enormous burden on families, economies, and societies worldwide. Thus, the exploration of a novel method to restore consciousness is urgently needed.

Although new neuromodulation techniques, such as transcranial direct current stimulation (tDCS) (2), transcranial magnetic stimulation (TMS) (3), and low intensity focused ultrasound pulse (LIFUP) (4), have been introduced (5), deep brain stimulation (DBS), and spinal cord stimulation (SCS) remain two mostly employed neurostimulation techniques for DOC patients and show some promises in restoring consciousness (6, 7). However, high costs, complicated processes, and potential surgical side effects limited their applications.

Corazzol et al. applied vagal nerve stimulation (VNS) to treat a patient lying in the vegetative state (VS) for 15 years following TBI (8). After 1 month of stimulation, clinical examination revealed reproducible, stable improvements in general arousal, sustained attention, body motility, and visual pursuit. Scores on the Coma Recovery Scale-Revised (CRS-R) test were improved, indicating a transition from a vegetative to minimally conscious. The results challenged the belief that DOC persisting longer than 12 months is irreversible (9). However, the same as DBS and SCS, VNS is expensive and invasive with irreversible implants. The disadvantages block its clinical application in more DOC patients.

The vagal nerve carries somatic and visceral efferents and afferents distributed throughout the brain, either monosynaptically or *via* the nucleus tractus solitarii (NTS) (10). The vagal nerve has a branch of afferent projections in the auricular concha and external ear channels (11). Thus, transcutaneous auricular vagal nerve stimulation (taVNS) was developed based on this anatomical characteristic (12, 13) and was previously found to produce comparable efficacy with classic VNS (14), which means taVNS is a promising form of classic VNS (15).

Before the French team's publication (8), our team also has a peer-reviewed case report on the beneficial effects of taVNS on the consciousness level of a 73-year-old female single patient who developed into DOC after cardiopulmonary resuscitation for 50 days (16). After a 4-week taVNS treatment, her CRS-R scores rose from 6 to 13, and her diagnosis was changed from VS to minimally conscious state (MCS). The BOLD functional magnetic resonance imaging (fMRI) outcomes also showed improved brain functional connectivity (FC). This was the first case of taVNS in a DOC patient and the first report of encouraging results from clinical conditions to brain FC.

After the case report, we then showed that only auditory-function-preserved patients with DOC are reversible by taVNS (17). In the same survey, we also focused on arterial spin labeling (ASL) with fMRI in the brain of these DOC patients, which uses magnetically labeled arterial blood water protons as an endogenous tracer and is a non-ionizing and non-invasive measurement of cerebral blood flow (CBF) (18, 19), and the most effective approach uses magnetically labeled arterial blood water (20). Arterial spin labeling perfusion is commensurate with other more invasive methods such as PET and dynamic susceptibility contrast-enhanced MRI (DSC-MRI) perfusion (21), with higher accuracy and acceptance, and without specific ethics requirements in humans (22, 23). For DOC patients, especially the ones in VS, the increment of CBF is the basis of their brain functional recovery (24). Nevertheless, the ASL-fMRI results of the study were neither sufficiently illustrated nor adequately discussed previously. Thus, in this study, we aimed to preliminarily illustrate the cerebral hemodynamic correlates of taVNS in consciousness restoration.

METHODS

Ethics

This is an open-label pilot study within a clinical trial (Trial registration: ChiCTR-INR-16008745). The study was reviewed and approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Approval Number: 2016062001). Written informed consent to participate in this study and to publish the work was provided by the legal guardians of the patients.

Participants and Scales Assessing

Patients were recruited from the Department of Neurosurgery, PLA Army General Hospital, Beijing, China, for this study. Inclusion criteria were patients in VS, MCS, and coma following severe brain damage after the acute brain insult for at least 2 days. Patients were excluded when there was a contraindication for MRI (e.g., presence of ferromagnetic aneurysm clips and pacemakers), MRI acquisition under sedation or anesthesia, uncertain clinical diagnosis (25), and data could not be further processed in the further ASL-fMRI analysis.

The patients' consciousness states were assessed twice, before (T0) and after (T1) the taVNS treatment, using the JFK Coma Recovery Scale (JFK CRS-R) (26), which includes six subscales addressing auditory, visual, motor, oromotor, communication (language), and arousal processes. The elapsed time between eligibility and baseline assessments was within 24 h.

Also, their prognoses were judged *via* the Glasgow Outcome Scale (GOS). Glasgow Outcome Scale provides a measurement of outcome ranging from 1 to 5 (1, dead; 2, vegetative state/severe disability; 3, able to follow commands/unable to live independently/moderate disability; 4, able to live independently/unable to return to work or school; 5, good recovery/able to return to work or school) (7, 27). In this study, any GOS score $\leq$ 2 was defined as "unfavorable prognosis," whereas a score from 3 to 5 was defined as "favorable prognosis" (7, 28, 29).

No other treatments, including drugs that could modify cortical excitability, were administered. And we followed the CONSORT checklist in this study.

The taVNS Treatment

The points for taVNS were placed in the concha area, where there are principal vagal nerve branch distributions (11, 14, 30). Both the cymba concha (100% auricle branch of the vagal nerve) and the cavity of concha (45% auricle branch of the vagal nerve and 55% great auricular nerve) (11) were

stimulated (**Supplementary Figure 1**). After the stimulation areas were sterilized, ear clips with plate electrodes were attached to the area (auricular concha) at the stimulation site (**Supplementary Figure 1**) of both ears. Stimulation parameters were adjusted according to our previous study (16): (1) density wave to 20 Hz, and pulse width to 0.5 ms; (2) intensity: 4–6 mA (this intensity will cause slight pain to the ears in conscious people, which is mostly tolerable). The treatment lasted 30 min continuously and was carried out twice a day (8:00 and 16:00) for 4 weeks.

Functional MRI Data Acquisition

Patients received fMRI scanning sessions before and after the treatment. The elapsed time between the MRI scans and treatment was within 24 h. Most of the scans were carried out at around 15:00. A 3.0 T MR scanner (HD750, General Electronic Co., USA) was used for this study equipped with an eight-channel head coil. A wedge-shaped foam padding was used to minimize head motions.

Structure imaging included 3D-T1-weighted. Arterial spin labeling sequences were obtained during the eyes-open status. Patients' ASL sequences with significant motion degradation were excluded from the analysis.

The raw ASL images were acquired twice by three-dimensional ASL sequences (31–33). The 3D ASL, including M0 image and perfusion different image, was obtained with the parameters as follows: TR = 4,632 ms, TE = 10 ms, slice thickness = 4 mm, field of view (FOV) = 24 × 24 mm^2, post labeling delay (PLD) = 1,500 ms. In addition, T1-weighted three-dimensional high-resolution structural images were obtained using a sagittal BRAin Volume imaging (BRAVO) sequence with TR = 7.8 ms, TE = 3.0 ms, TI = 600 ms, flip angle of 9°, and 186 slices with a voxel size of 1 × 1 × 1 mm^3.

ASL Pre-processing

Arterial spin labeling pre-processing used ASLtbx based on SPM12 (Statistical Parametric Mapping, available at www.fil.ion.ucl.ac.uk/spm/software/Spm12) on the Matlab platform (R2013b; Math Works, Natick, MA).

Before calculating the CBF map, the orientation of each 3D ASL and 3D T1 image should be reset to the center of the image matrix at the midpoint of the AC-PC line. The CBF (ml/100 g/min) map of 3D ASL was calculated using the mean Perf difference image, and M0wm (M0wm was extracted from a white matter mask) by using batch scripts provided in ASLtbx (34). Perfusion difference image was registered to individual T1 image so that they could be later normalized to Montreal Neurological Institute (MNI) template space for next smoothing. We normalized CBF within the ASL images to avoid major variations in CBF due to cardiac blood flow and pressure liabilities. The normalized CBF map was smoothed with an isotropic Gaussian filter with a full-width-at-half-maximum (FWHM) = 6 mm^3 to filter noise for later group analysis. We then performed whole-brain voxel-wise analyses of the images within the general linear model framework using SPM12. The analyses were constrained to gray matter tissue only by thresholding the analysis mask to 40% of the mean gray matter image of our sample.

ASL Statistics Analysis

After pre-processing, we extracted mean CBF from 92 regions of interest using the AAL parcellation toolbox. The following data analysis processing was conducted on SnPM13, alongside

TABLE 1 | Patients' demographics and clinical characteristics.

ID	State	Grouping before taVNS treatment	Gender	Age	Course	Cause	Prognosis	GOS	CRS-R (T0) before the taVNS treatment	CRS-R (T1) after the taVNS treatment
P01	VS	RtAS	Male	41	10 days+	HIE	Favorable	3	A1V0M2O1C0Ar2	A2V3M2O1C0Ar2
P02	VS	nRtAS	Male	43	50 days+	HIE	Unfavorable	2	A0V0M2O1C0Ar2	A1V1M2O1C0Ar2
P03	VS	nRtAS	Male	31	90 days+	cerebral hemorrhage	Unfavorable	2	A0V0M2O1C0Ar2	A1V1M2O1C0Ar2
P04	MCS	RtAS	Male	23	90 days	HIE	Favorable	4	A2V3M2O1C0Ar2	A4V5M5O2C2Ar3
P05	MCS	RtAS	Female	27	300 days	Brain injuries due to traffic accident	Favorable	3	A3V3M3O1C0Ar3	A3V3M3O1C0Ar3
P06	MCS	RtAS	Male	42	50 days+	Cerebral hemorrhage	Favorable	5	A1V0M3O1C0Ar2	A4V1M6O3C2Ar3
P07	VS	nRtAS	Male	39	30 days+	Brainstem hemorrhage	Unfavorable	2	A0V0M2O0C0Ar0	A0V0M2O0C0Ar0
P08	VS	nRtAS	Male	29	60 days+	HIE	Unfavorable	2	A0V0M1O0C0Ar2	A1V2M2O0C0Ar2
P09	VS	nRtAS	Female	19	15 days	Brain injuries due to traffic accident	Unfavorable	2	A0V1M2O0C0Ar1	A1V1M2O1C0Ar2
P10	VS	RtAS	Female	73	90 days+	HIE	Favorable	3	A1V1M1O1C0Ar2	A3V3M3O2C0Ar2

VS, vegetative state; MCS, minimally conscious state; RtAS, response to auditory stimulus; nRtAS, non-response to auditory stimulus; HIE, hypoxic-ischemic encephalopathy; GOS, Glasgow Coma scale; CRS-R, Coma Recovery Scale-Revised. The subscales for the CRS-R are Auditory Function (A), Visual Function (V), Motor Function (M), Oromotor Function (O), Communication (C), and Arousal (Ar).

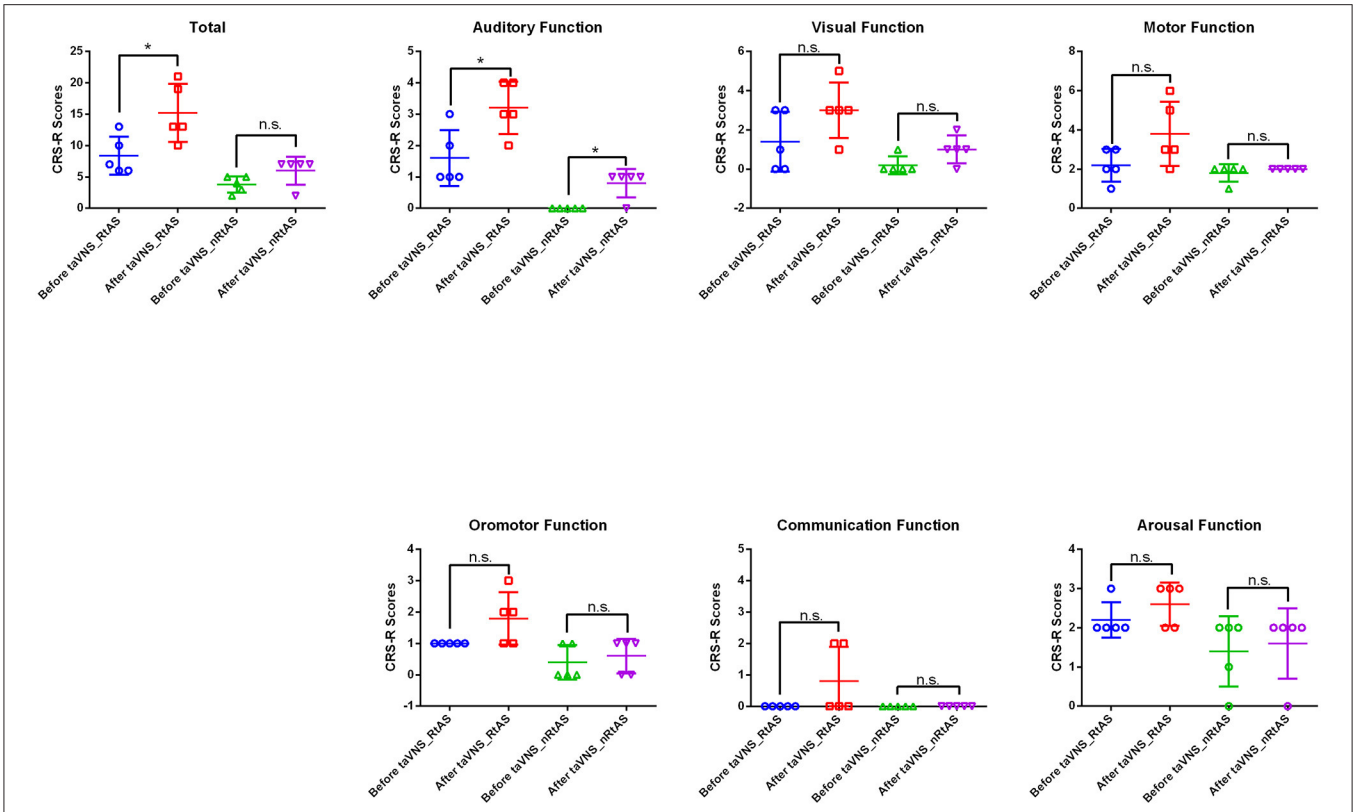

FIGURE 1 | Patients' CRS-R total and subitems scores before and after taVNS. *$P < 0.05$, before taVNS vs. after taVNS; n.s., not significant; $P > 0.05$; before taVNS vs. after taVNS.

SPM12b installation. SnPM refers to an implementation of Statistical non-Parametric Mapping by Andrew Holmes and Tom Nichols (35). The voxel-wise comparison was used in SnPM to examine CBF increment from pre-taVNS to post-taVNS, with 5,000 permutations. Regions with significant CBF changes were defined as ROIs, and the ROI-based CBF change ratio [CBF change ratio = (post_CBF – pre_CBF)/pre_CBF] was calculated.

Clinical Data Analysis

To compare the consciousness recovery state before and after the taVNS treatment, the patients' CRS-R total scores and six subscales' scores of each group were analyzed with *t*-tests, respectively. GraphPad Prism 6 software was used to analyze the data. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Demographic Information

The demographic information was shown in **Table 1**. Briefly, we recruited seven males and three females, aged from 19 to 73 years old; three of them were with MCS, while seven of them were with VS. In these patients, five of them were caused by HIE, two by brain injuries due to traffic accidents, two by a cerebral hemorrhage, and one by brainstem hemorrhage.

Clinical Characteristics

According to the first auditory score (**Table 1**), five patients fell into the responded to auditory stimuli (RtAS) group [subscale Auditory Function (A) ≥ 1: Auditory startle], and the other five fell into the non-responded to auditory stimuli (nRtAS) group [subscale Auditory Function (A) = 0: None].

Through the GOS assessment (**Table 1**) (7, 29), after the taVNS treatment, five patients can be classified as favorable prognoses (GOS > 2), while the other five patients can be classified as unfavorable prognoses (GOS ≤ 2).

The data illustrated that patients, who responded to auditory stimuli [RtAS, CRS-R (T0) subscale Auditory Function (A) ≥ 1] before the taVNS treatment, were going to have favorable prognoses (GOS > 2) after the treatment. Simultaneously, patients, who did not respond to auditory stimuli [nRtAS, CRS-R (T0) subscale Auditory Function (A) = 0] before the taVNS treatment, were going to have unfavorable prognoses (GOS ≤ 2) after the treatment.

All patients' CRS-R subitems and total scores before and after the taVNS treatment were presented in **Table 1** and **Figure 1**. The data showed that taVNS only improved the total scores of the RtAS group significantly ($P < 0.05$). As a result, we reported that only the RtAS DOC patients were responsive to taVNS (17).

TABLE 2 | CBF increased brain regions by taVNS.

	CBF improved regions	MNI (mm)			T-score	Voxel size
		X	Y	Z		
RtAS	Thalamus (R)	18.29	−11.97	3.22	2.90	76
	Superior Temporal Gyrus (L)	−35.62	6.64	−25.75	5.10	81
	Superior Frontal Gyrus (L)	−24.93	66.99	−14.26	5.03	262
	Precentral Gyrus (R)	42.35	−5.83	45.45	3.14	53
	Precentral Gyrus (L)	−64.67	−4.73	42.94	3.86	178
	Postcentral Gyrus (L)	−32.50	−16.64	46.28	2.54	54
	Hippocampus (R)	33.16	−43.34	−11.74	10.16	64
	Orbital Gyrus (L)	−8.07	31.81	−41.40	4.57	131
	Middle Temporal Gyrus (R)	73.64	−1.92	−16.82	3.06	92
	Middle Frontal Gyrus (R)	29.52	−1.48	47.09	2.43	71
	Middle Frontal Gyrus (L)	−50.28	48.99	7.57	3.49	227
	Midbrain (R)	15.89	−19.31	−18.89	5.49	188
	Medulla (R)	2.78	−39.72	−43.98	10.61	64
	Medulla (L)	0.62	−40.00	−45.80	2.79	116
	Occipital Lobe (R)	5.66	−100.33	1.17	3.38	77
	Occipital Lobe (L)	−2.87	−95.73	4.40	3.88	113
	Insula (L)	−31.20	19.01	−12.56	3.93	112
	Inferior Frontal Gyrus (L)	−31.32	8.96	−24.28	6.67	92
	Cerebellum (R)	15.61	−48.40	−46.67	22.86	811
	Cerebellum (L)	−39.97	−61.17	−45.69	9.80	147
	Caudate (R)	11.86	9.38	6.32	5.66	115
	Caudate (L)	−5.26	15.76	7.70	3.97	83
	Lentiform Nucleus (R)	33.11	−15.89	−10.23	4.06	107
	Lentiform Nucleus (L)	−33.14	−19.69	−4.75	2.58	69
nRtAS	Cerebellum (L)	−54.70	−71.41	−28.72	3.79	61

R, right; L, left; MNI, Montreal Neurological Institute. Voxel < 50 were excluded in this study.

CBF Changes

As shown in **Table 2** and **Figure 2**, taVNS increased CBF of multiple brain regions in the RtAS DOC patients; the CBF increment in the nRtAS group with the taVNS treatment is relatively weak, which was merely prominent in the left cerebellum. Cerebral blood flow changes ratio graphs, and some ROI-based CBF changes between pre-taVNS and post-taVNS in the RtAS group can be seen in **Supplementary Figure 2** and **Supplementary Table 1**.

DISCUSSION

VNS (8) and taVNS (16) have been identified as therapeutic strategies for DOC (5). Signs of consciousness recovery after taVNS were recorded in some severe TBI patients (36). It was also suggested that taVNS is a feasible, safe, and effective tool for DOC patients (37).

Initially, we designed a clinical trial, planning to use taVNS and transcutaneous non-vagal nerve stimulation (tnVNS, as a sham control group) in DOC patients. However, previous studies (36, 37) and this one found that only a portion of DOC patients is responsive to taVNS, which implied that designing a tnVNS group is not yet necessary presently (and that the clinical trial might have failed); however, finding the prior key factor(s) of the taVNS responders in DOC patients is more valuable and instructive to future studies. It has been reported that the auditory network FC has good correspondence with the level of consciousness (25), which is also estimated to be capable of predicting the prognoses of DOC (38). Intriguingly, we found that only RtAS DOC patients were responsive to taVNS (17). Therefore, the evidence provides a possible answer that the preserved auditory function might be the key factor.

The discovery of the key factor is indeed accidental. We originally assumed that all DOC patients are responsive to taVNS. Thus, this work formerly intended to reveal more detailed neuroimaging evidence of how taVNS alleviates DOC. And the results are also fortuitous and interesting.

Previous neurophysiologic taVNS/fMRI studies in healthy subjects majorly focused on the BOLD signal (15, 39–41). Because they applied different stimulating methods (sites and parameters), they have different findings. In this study, we deployed taVNS in DOC patients, in which both the cymba concha and the cavity of concha (11) were stimulated (**Supplementary Figure 1**). We found some overlapping brain regions in RtAS DOC patients with the previous studies, such as the thalamus, the caudate, the insula, and the frontal cortex. Nevertheless, this study majorly focused on the altered ASL signal in DOC patients. Meanwhile, neural networks and systems based on the brain regions need to be interpreted to deepen the study's insight. In the former two works (8, 16), the primary discoveries were the default mode network (DMN), the thalamo-cortical network, and the centro-posterior network. A hypothesis article has proposed three new possible neural networks (42): the external fronto-parietal network (ExN), the salience network, and the Mesocircuit model. This work provides some new evidence and may confirm the salience network hypothesis.

Impaired consciousness was proportional to the reduction in mean CBF regardless of pathology types (43). Therefore, increment in CBF is the basis of consciousness recovery. The results of this study showed that taVNS increased CBF of multiple brain regions in the RtAS DOC patients, but hardly in the nRtAS ones (**Table 2** and **Figure 2**). Here, we disclose the details.

The First Level: Through the Vagal Nerve, taVNS Might Modulate the Salience Network and the Limbic System

The afferent vagal fibers connect to the NTS in the medulla, which in turn projects connections to other locations in the brain (44, 45). Previous studies confirmed that taVNS precisely activated the NTS in healthy subjects (41). The results of this study showed that taVNS increased CBF of the medulla in the RtAS DOC patients, which indicated that taVNS might also modulate the NTS directly in these patients. With the modulation of NTS, the peripheral nerve stimulations can pass through the vagal nerves and reach the thalamus (46). The thalamus is the large mass of gray matter in the dorsal part of the brain with several functions such as relaying of sensory signals,

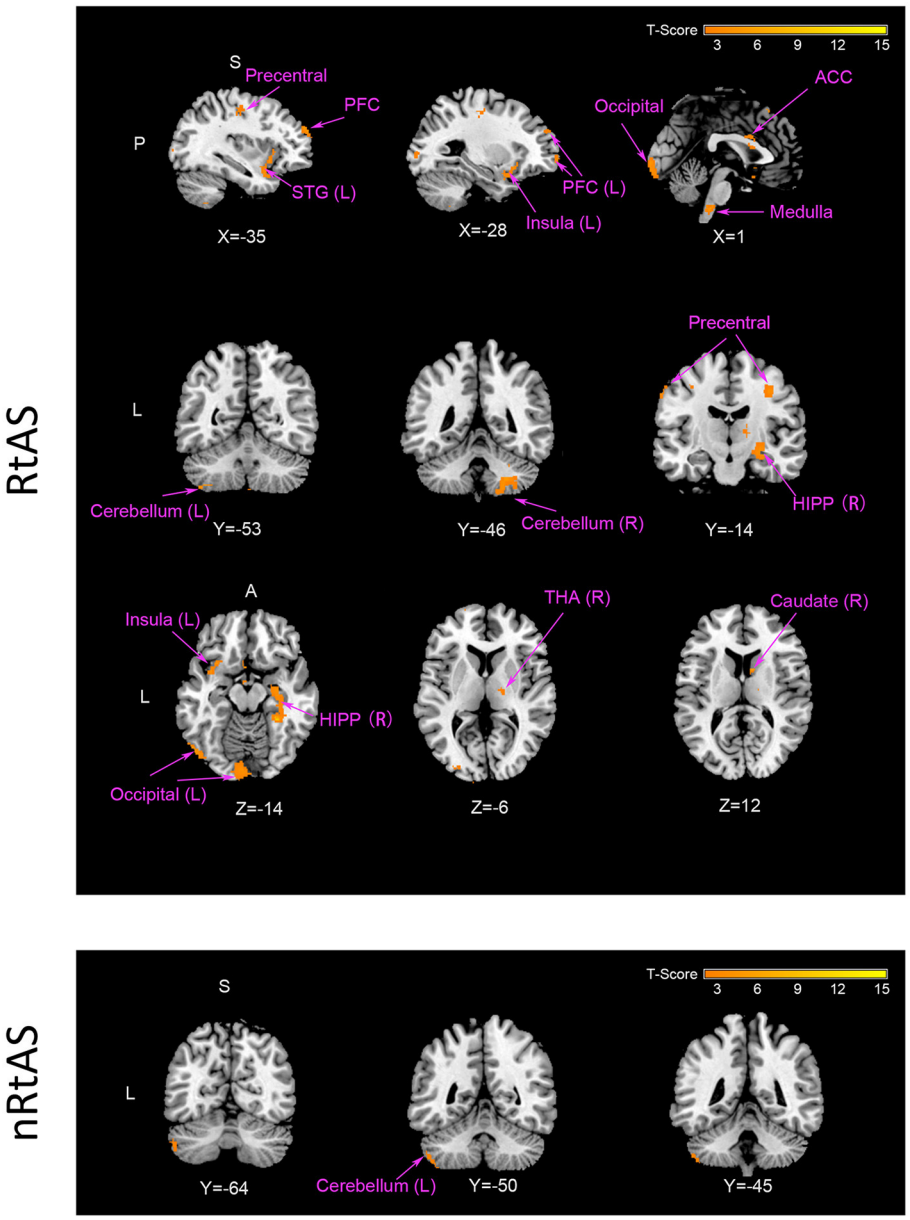

FIGURE 2 | CBF increment by taVNS. The RtAS group: taVNS increased the RtAS DOC patients' CBF in this study in many brain regions presented in this figure: STG (L) of temporal lobe; PFC (L) including SFG (L), MFG (L), and IFG (L); medulla; cerebellum (L) and cerebellum (R); precentral; caudate (R); HIPP (R); insula (L); occipital (L); THA (R). Images displayed in Montreal Neurological Institute standard stereotaxic space, and coordinates are provided for each slice. STG (L), left superior temporal gyrus; PFC (L), left prefrontal cortex including left superior frontal gyrus [SFG (L)], left middle frontal gyrus [MFG (L)], and left inferior frontal gyrus [IFG (R)]; cerebellum (L), left cerebellum; cerebellum (R), right cerebellum; precentral, precentral gyrus; caudate (R), right caudate; HIPP (R), right hippocampus; insula (L), left insula; occipital (L), left occipital lobe; THA (R), right thalamus. The nRtAS group: taVNS increased the nRtAS DOC patients' CBF only in the left cerebellum. Images displayed in Montreal Neurological Institute standard stereotaxic space and coordinates are provided for each slice. Cerebellum (L), left cerebellum. Uncorrected $P < 0.05$, $T > 1.97$.

including motor signals, to the cerebral cortex (47, 48) and the regulation of consciousness, sleep, and alertness, which also plays a vital role in arousal and awareness (49). Through the medulla and the thalamus, taVNS might modulate patients' left insula, which plays an essential role in consciousness (50). It has been proposed that primates possess a unique mapping of autonomic interoceptive information within the insula that forms the substrate of conscious feelings (51). The insula is also one of the core brain regions that anchor the salience network (52), which segregates the most relevant internal and extrapersonal stimuli (52) and is associated with individual differences in interoceptive accuracy (53). This study showed that taVNS increased CBF of

the insula in the RtAS DOC patients, indicating that taVNS might activate these patients' consciousness by improving their salience network connectivity (42). Cerebral blood flow upregulations of the insula directly accentuated some limbic-related areas such as the hippocampus, which is also recognized as a critical structure for autonoetic consciousness (54–56). taVNS also increased CBF of other limbic-related regions such as the caudate, the middle temporal gyrus (MTG), the orbitofrontal cortex (OFC), the inferior frontal gyrus (IFG). Significantly, the IFG is involved in evaluating linguistic, interoceptive, and emotional information (57), including visuospatial attention (58), and the improvement of these cognitive functions by taVNS might be one basis of consciousness recovery in these RtAS DOC patients.

The Second Level: Through the Up-Conducting Pathway of the Interoceptive System, taVNS Might Modulate the Cerebral Cortex

The improved thalamic fundamental metabolic level leads to the CBF upregulations of the cerebral cortex [somatosensory cortex (occipital lobe, superior temporal gyrus—STG, and MTG) and executive control cortex (prefrontal areas)] through the up-conducting pathway of the interoceptive system (51, 59). Meanwhile, the improved insula fundamental metabolic level also leads to metabolic upregulations of the somatosensory cortex and the prefrontal areas, which are also involved in the interoceptive system (51, 60). The interoceptive system is crucial for maintaining homeostatic conditions (61) in the body and, potentially, aiding in self-awareness (62) and is fundamental in human emotional well-being (59) and consciousness (51). Traceably, interoceptive signals are transmitted to the brain *via* multiple pathways, and the vagal nerve is one of them (51). Thence, taVNS might trigger the consciousness restoration effects in the RtAS DOC patients by activating the interoceptive system. It is worth mentioning that the STG is not merely involved in auditory processing, including language, but also has been implicated as a critical structure in social cognition (63, 64). Therefore, CBF upregulations of the STG by taVNS might also help to restore consciousness by improving these RtAS DOC patients' cognitive functions.

The Third Level: taVNS Might Modulate the Thalamo-Cortical Loop

Previously, a consensus has been reached that disconnections in long-range thalamo-cortical pathways are involved in DOC's situation (65). The caudate is one of the structures that make up the dorsal striatum, a basal ganglia component (66). The increment of CBF in these RtAS DOC patients' caudate illustrated that taVNS might modulate the (ganglia-)thalamo-cortical loop (67), which might reconnect the very disconnections.

LIMITATIONS

Considering the small sample size and the lack of a control group, we should interpret our data cautiously. One of the limits of this open-label pilot study is that there were only 10 qualified DOC patients enrolled, with only 5 being responsive to taVNS, which can hardly illustrate all detailed mechanisms of the consciousness restoration by taVNS. Moreover, before taVNS, patients in the RtAS group already had better clinical conditions than those in the nRtAS group: three patients of the RtAS group were in MCS, and two were in VS, whereas all five of the nRtAS subjects were in VS (**Table 1**); this indicated that the observed differences in response between RtAS and nRtAS might have been significantly influenced by differences in these patients' baseline clinical conditions. It is also unclear whether a DOC patient being responsive to auditory stimuli would respond to any extended procedures, which presumably include auditory stimuli rather than the actual procedure, such as taVNS. Also, the benefits of taVNS require time to emerge in DOC patients; however, we set the duration of the taVNS treatment for 4 weeks in this study (17), limiting its effects for the patients (the French team monitored the effects of VNS for more than 6 months in that particular case) (5, 8). In addition, to map more detailed mechanisms, other monitoring methods, such as functional near-infrared spectroscopy (fNIRS), electroencephalography (EEG), and magnetoencephalography (MEG), need to be considered in upcoming studies. Eventually, physiological parameters, such as blood pressure, pulse rate, heart rate variability, and the baroreflex, need to be tested in the future to measure tolerability and parasympathetic activity.

CONCLUSION

This study newly demonstrated that taVNS might primarily activate the salience network, the limbic system, and the interoceptive system, which better illustrates how taVNS alleviates RtAS DOC than the previous studies. The results also indicated that the preserved auditory function might be the prior key factor of the taVNS responders in DOC patients. Therefore, future time-limited controlled trials applying taVNS and tnVNS (as a sham control group) on DOC patients should probably avoid enrolling the nRtAS ones.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences. Written informed consent to participate in this study was provided by the patients legal guardians.

AUTHOR CONTRIBUTIONS

YYu and YYa designed the study and drafted the manuscript. YYa and JH recruited participants and performed data collection. SGa and LB analyzed the data. PR, CT, JF, and SW provided resources and general assistance. YYu and SGu localized the electrodes. All authors provided feedback on the manuscript, contributed to the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.684791/full#supplementary-material

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Auricular Neuromodulation for Mass Vagus Nerve Stimulation: Insights From SOS COVID-19 a Multicentric, Randomized, Controlled, Double-Blind French Pilot Study

Claire-Marie Rangon[1], Régine Barruet[2], Abdelmadjid Mazouni[3], Chloé Le Cossec[4], Sophie Thevenin[4], Jessica Guillaume[4], Teddy Léguillier[4], Fabienne Huysman[5] and David Luis[5,6]*

[1] Pain and Neuromodulation Unit, Neurosurgery Department, Hôpital Fondation Adolphe de Rothschild, Paris, France, [2] Infectious Diseases Department, Centre Hospitalier Simone Veil, Beauvais, France, [3] Pain Unit, Centre Hospitalier Simone Veil, Beauvais, France, [4] Clinical Research Department, Hôpital Fondation Adolphe de Rothschild, Paris, France, [5] Clinical Research Department, Centre Hospitalier Simone Veil, Beauvais, France, [6] Intensive Care Unit, Centre Hospitalier Simone Veil, Beauvais, France

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***Correspondence:**
Claire-Marie Rangon
cmrangon@for.paris

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Importance: An exacerbated inflammatory response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is believed to be one of the major causes of the morbidity and mortality of the coronavirus disease 2019 (COVID-19). Neuromodulation therapy, based on vagus nerve stimulation, was recently hypothesized to control both the SARS-CoV-2 replication and the ensuing inflammation likely through the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway and could improve the clinical outcomes as an adjunct treatment. We proposed to test it by the stimulation of the auricular branch of the vagus nerve, i.e., auricular neuromodulation (AN), a non-invasive procedure through the insertion of semipermanent needles on the ears.

Objective: The aim of this study was to assess the effect of AN on the clinical outcomes in patients affected by COVID-19.

Design, Setting, and Participants: A multicenter, randomized, placebo-controlled, double-blind clinical trial included 31 patients with respiratory failure due to COVID-19 requiring hospitalization. Within 72 h after admission, patients received either AN ($n = 14$) or sham neuromodulation (SN, $n = 15$) in addition to the conventional treatments.

Main Outcome and Measures: The primary endpoint of the study was the rate of a clinical benefit conferred by AN at Day 14 (D14) as assessed by a 7-point Clinical Progression Scale. The secondary endpoint of the study was the impact of AN on the rate of transfer to the intensive care unit (ICU) and on the survival rate at D14.

Results: The AN procedure was well-tolerated without any reported side effects but with no significant improvement for the measures of both primary ($p > 0.3$) and secondary

($p > 0.05$) endpoints at the interim analysis. None of the AN-treated patients died but one in the SN group did (81 years). Two AN-treated patients (73 and 79 years, respectively) and one SN-treated patient (59 years) were transferred to ICU. Remarkably, AN-treated patients were older with more representation by males than in the SN arm (i.e., the median age of 75 vs. 65 years, 79% male vs. 47%).

Conclusion: The AN procedure, which was used within 72 h after the admission of patients with COVID-19, was safe and could be successfully implemented during the first two waves of COVID-19 in France. Nevertheless, AN did not significantly improve the outcome of the patients in our small preliminary study. It is pertinent to explore further to validate AN as the non-invasive mass vagal stimulation solution for the forthcoming pandemics.

Clinical Trial Registration: [https://clinicaltrials.gov/], identifier [NCT04341415].

Keywords: auricular neuromodulation, vagus nerve stimulation, COVID-19, pandemics, NF-κB, cholinergic anti-inflammatory pathway, non-invasive neuromodulation

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has overwhelmed the sanitary capacity. Additional therapeutic arsenals that could reduce the morbidity rate, although untested in the given context but previously proven to be efficacious in a related clinical context, are urgently needed. The role of the nervous system in respiratory failure in patients with COVID-19 has been recently emphasized (Li et al., 2020; Manganelli et al., 2020; Tassorelli et al., 2020). The heavy viral infection within the brain stem of deceased patients suggests that the neuroinvasive potential of SARS-CoV2 is likely to be partially responsible for COVID-19 acute respiratory failure. This finding favors treatment modalities involving the vagus nerve and the cholinergic anti-inflammatory pathway (CAP), which was supported by several research teams (Bara et al., 2020; Bonaz et al., 2020; De Virgiliis and Di Giovanni, 2020; Leitzke et al., 2020; Mazloom, 2020; Pomara and Imbimbo, 2020; Rangon et al., 2020; Staats et al., 2020; Tornero et al., 2020; Azabou et al., 2021; Mastitskaya et al., 2021).

In fact, the key role of the vagus nerve in controlling inflammation through the so-called "inflammatory reflex" was highlighted almost 20 years ago by Tracey (Tracey, 2002), with the concept constantly being refined, particularly with the description of the CAP (Czura et al., 2003; Pavlov and Tracey, 2005; Oke and Tracey, 2008; Andersson and Tracey, 2012; Olofsson et al., 2012; Pereira and Leite, 2016; Chavan and Tracey, 2017; Serhan et al., 2018, 2019; Bonaz, 2020a). It is now well-acknowledged that the immune-inflammatory processes are modulated by the vagus nerve in a significant manner. Therefore, the vagus nerve modulation appears to be a good candidate to tackle the COVID-19-associated cytokine storm.

In fact, in animal models, it was demonstrated that the stimulation of the vagus nerve modulates immune response through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (O'Mahony et al., 2009; Sun et al., 2013; Leitzke et al., 2020). NF-κB, a family of evolutionarily conserved transcription factors, is a double-edged sword capable of inducing the expression of both antiviral host factors and viral genes in a context-dependent manner (Deng et al., 2018). It has previously been shown that the respiratory syncytial virus (RSV) (Masaki et al., 2011), the porcine reproductive and respiratory syndrome virus (PRSSV) (Wang et al., 2013), and a variety of coronaviruses were shown to divert the NF-κB to the benefit of their own replication (Poppe et al., 2017). Recently, the hyperactivation of the NF-κB pathway has been implicated in the pathogenesis of severe/critical COVID-19 phenotype (Hariharan et al., 2020; Hirano and Murakami, 2020). Thus, the modulation of the NF-κB pathway in favor of host defense, through vagus nerve stimulation (VNS), is particularly attractive against viral infections.

Interestingly, the vagal tone, which modulates the activity of the inflammatory reflex in humans, can be monitored through the measurement of the heart rate variability (HRV) (Thayer, 2009; Williams et al., 2019). The HRV constitutes a physiological marker of the vagal tone, quick to measure and non-invasive, due to the continuously monitored ECG or even an assessment by a handy smartphone (Chen et al., 2020; Shaffer et al., 2020).

Several epidemiological studies have shown that reduced HRV is a risk factor for all-cause mortality and morbidity (Liao et al., 2002), not only in cardiovascular diseases (Fang et al.,

Abbreviations: ABVN, Auricular Branch of the Vagus Nerve; ACTH, Adrenocorticotropin Hormone; ANS, Autonomic Nervous System; AN, Auricular Neuromodulation; ARDS, Acute Respiratory Distress Syndrome; CAP, Cholinergic Anti-inflammatory Pathway; COVID-19, Coronavirus Disease 2019; CRP, C-Reactive Protein; HRV, Heart Rate Variability; ICU, Intensive Care Unit; NF-κB, Nuclear Factor Kappa-light-chain-enhancer of activated B cells; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SN, Sham Neuromodulation; SPN, Semipermanent Needle; VNS, Vagus Nerve Stimulation; tVNS, transcutaneous VNS; taVNS, transcutaneous auricular VNS; tcVNS, transcutaneous cervical VNS.

2020), metabolic diseases (Pavlov, 2021), and neurodegenerative diseases (Rangon et al., 2020), but also in acute respiratory distress syndrome (ARDS) (Chen et al., 2018), sepsis (De Castilho et al., 2018; Barnaby et al., 2019), and the severe infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Hasty et al., 2020; Leitzke et al., 2020; Aragon-Benedi et al., 2021; Pan et al., 2021).

Given this, an ideal therapeutic "all-in-one" approach for the COVID-19 pandemic should be able to (1) increase the HRV significantly, (2) be readily operational, (3) target not only the "regular" SARS-CoV-2 but also the current and emerging virulent variants, (4) provide minimal adverse events given the frailty of the target population, and (5) be less time-consuming and cost-effective.

Vagus nerve stimulation is presently achieved either by pharmacological or by neuromodulatory approaches. Regrettably, the non-pharmacological therapeutic strategies that target the immune-inflammatory processes and thereby could potentially improve the outcome of the patients with COVID-19 have not so far been sufficiently highlighted (Azabou et al., 2021). In fact, the vagus nerve holds a specific and important area in bioelectronic medicine, an evolving field, in helping diagnosis and treatment of the disease (Pavlov et al., 2020).

Invasive VNS (iVNS), using a specifically designed surgically implantable electrode cuff (for selective activation of the CAP) wrapped around the cervical vagus nerve, has recently been suggested for neuroimmunomodulation in COVID-19 (Mastitskaya et al., 2021). Nevertheless, implanting a VNS device in patients who are critically ill can be challenging, and hence, non-invasive transcutaneous VNS received particular attention as evidenced by the launch of several clinical trials (i.e., NCT04368156, NCT04379037, NCT04382391, NCT04638673, and NCT04514627). Among the two options, one is transcutaneous cervical VNS (tcVNS), where the stimulating electrodes are applied to the skin surface over the sternocleidomastoid muscle in the neck, and the other, the auricular VNS (taVNS or aVNS), which targets the auricular branch of the vagus nerve (ABVN) that innervates part of the skin of the outer ear, mainly the auricular concha and most of the area around the auditory meatus (Peuker and Filler, 2002; Butt et al., 2020). Both tcVNS and taVNS have been shown to elicit comparable therapeutic effects as VNS (for a review, Yap et al., 2020).

Remarkably, taVNS, the bioelectronic medicine approach targeting only the afferent arm of the vagus nerve, makes it easier to read into HRV, a marker for efferent vagal activation (Burger et al., 2020). Besides, the taVNS was proven not only to modulate the activity of NF-κB in animal models (Zhao et al., 2012) but also to increase the HRV values in healthy humans, to reduce sepsis, and to increase the survival rate, both significantly, in experimental models (for a review, Rangon et al., 2020). Hence, the taVNS is opted in four out of the five ongoing clinical trials assessing the impact of bioelectronic non-invasive VNS in COVID-19 (i.e., NCT04379037, NCT04382391, NCT04638673, and NCT04514627). Nevertheless, the bioelectronic approach, which is still in the development phase, requires additional investigations to establish the parameters for optimum stimulation, especially in the crucial pandemic situation (Bonaz, 2020b; De Virgiliis and Di Giovanni, 2020).

As we are overwhelmed (more than 3 million deaths worldwide) by the present context, also potentially in the near future by more and more emerging virulent variants, it is time to take advantage of scientifically assessed complementary medicine that has the benefit of having some hindsight. From 2009, Tracey highlighted the need to learn from the acquired knowledge in acupuncture technics (Oke and Tracey, 2009). In fact, in 2021, VNS through acupuncture was suggested as a feasible approach to activate the CAP to control the COVID-19-associated inflammatory burst (Qin et al., 2021).

Interestingly, the non-electrical stimulation of the ABVN through auricular acupuncture or acupressure, using either needles, seeds, or beans, is also able to increase the HRV, both in rats (Gao et al., 2012a) and in humans (Hsu et al., 2007; Gao et al., 2012b; Arai et al., 2013). It is quite understandable that the physical stimulation of the external acoustic meatus (innervated by the ABVN) has been known since the nineteenth century to elicit a cough reflex induced by vagal regulation, the so-called "Arnold's reflex" (He et al., 2012).

Given this, auricular acupuncture using semipermanent needles (SPNs, **Figure 1**), i.e., auricular neuromodulation (AN), can be conceived to provide sustained and personalized vagus stimulation. The rationale resides in the histology of acupoints that of course exhibits intersubject variability (i.e., age, gender, diseases, etc.). These acupoints have relatively lower electrical impedance than the non-acupoints, the former further depending on the architecture of the so-called "neurovascular complex," which is formed by a combination of myelinated and unmyelinated nerve fibers, small arterial and venous capillaries, and a small lymphatic vessel (Rabischong and Terral, 2014). Thus, the SPN remains on the acupoints of the ears (from a few days to several weeks) and falls spontaneously, depending on each individual.

Therefore, the protocol of taVNS with SPN is a "ready-to-go" procedure with no need of presetting the parameters of stimulation (Bonaz, 2020b), and it is a rapid one (i.e., it takes <10 s to put one SPN on the ear, **Figure 1D**). The only parameters to determine on the spot are the number and the localization of the SPNs on the ear. Such user-friendliness may explain the enthusiasm of the American soldiers on the battlefield to use five SPNs per ear directly to alleviate severe acute pain in the past 20 years (Niemtzow, 2018). Studies on the mechanism of action suggested that afferent projections from the ABVN to the nucleus of the solitary tract constitute the anatomical basis for the vagal regulation and the analgesic effects in the battlefield (i.e., auricular) of acupuncture (He et al., 2012; Usichenko et al., 2017). Since the COVID-19 pandemic has often been metaphorically represented as a war situation, auricular vagus nerve modulation with SPN is very likely to be successfully implemented in the emergency department or at the clinic (Niemtzow, 2020).

Due to its role in modulating NF-κB pathway, AN could lead to a decrease in the host inflammatory response and to a decrease in the SARS-COV-2 replication. Thus, modulation of this signalling pathway could result in both a decrease in the number of admissions to intensive care units (ICU) and

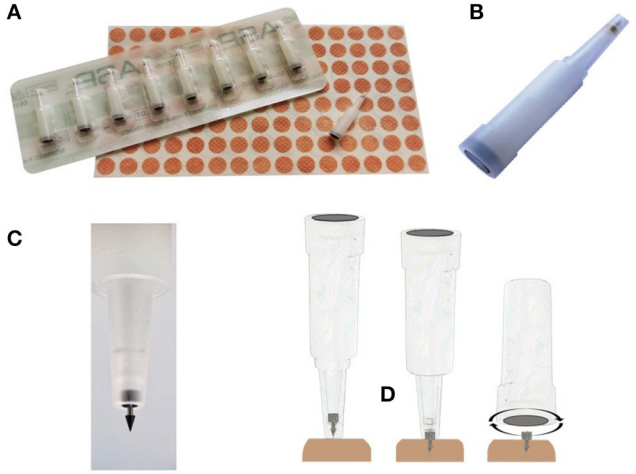

FIGURE 1 | The semipermanent needles (SPNs). **(A)** SPN packaging. **(B)** Close-up of a SPN inside its sterile blister. **(C)** Close-up of a SPN ready to be inserted in the skin of the outer ear. **(D)** The three-step procedure for SPN insertion: (1) the blister is placed on the disinfected skin point, (2) a pressure exerted on the blister delivers the SPN inside the skin, and (3) the SPN is once again sinked using the bottom end of the blister (images from Sedatelec^{R}, Irigny, France).

a decrease in mortality (Li et al., 2020). In this pilot trial, we investigated the impact of AN through SPNs on the short-term outcome (i.e., 14 days) of inpatients with respiratory failure due to COVID-19.

MATERIALS AND METHODS

The study followed the CONSORT checklist (http://www.consort-statement.org).

The study was approved by the CPP SUD-Est II, an ethical board affiliated with the French Ministry of Health.

Patients and Treatments

Adults of both sex (over 18 years old) with confirmed COVID-19 ARDS, based on the positive PCR for SARS-CoV2 and/or a suggestive chest scan and the following clinical criteria (i.e., abnormal lung auscultation OR SpO2 < 94% OR oxygen supplementation OR non-invasive ventilation), who were admitted to the Hôpital Fondation Adolphe de Rothschild (Paris) or the Hôpital Simone Veil (Beauvais, France) were proposed, within the first 72 h, to participate in the randomized controlled double-blind trial. In case of cognitive disorders or measures of legal protection, informed consent was obtained by phone from a trustworthy person designed in the medical file. Pregnant or breastfeeding women were excluded. Former critical inpatients could secondarily be included within 72 h after their transfer to a non-ICU.

During the first semester of 2020, there were not enough data available in the literature to calculate the number of patients needed to treat. Thus, 60 patients (30 per arm) were planned for inclusion in our pilot study. If the results proved to be interesting, they would allow to calculate the number to treat secondarily to set a broader trial. A futility intermediate analysis was planned at midcourse, allowing the study to continue, provided that the *P*-value was inferior to 0.3 in favor of the verum group.

Patients were randomized to receive either verum auricular vagus nerve (AN) or sham (SN) neuromodulation. After disinfection of the ears with Chlorhexidine^{R}, the trained physician wearing gloves carried out neuromodulation treatment, beginning with the right ear and then the left ear of the patient, without the presence of the nursing staff in the room. In the verum group, four semipermanent sterile needles (Classic ASP, Sedatelec^{R}, Irigny, France) were implanted on each auricle, i.e., eight needles per patient, following an order and a precise localization (surrounding the concha): upside, bottom side, external side, and internal side, corresponding to the following acupoints: (1) master point of endoderm, (2) master point of reticular formation, (3) thymic plexus, and (4) adrenocorticotropin hormone (ACTH) point (ear maps according to Alimi, 2017; **Figure 2A**). Hydrogen peroxide (Herouville Saint Clair, France) was then applied on each implanted SPN to stop potential bleeding. No electrical stimulation was subsequently applied on the needles. The concha was subsequently hidden by an opaque and waterproof Band-Aid (i.e., made with a compress under a Tegaderm^{R}, fixed thanks to Steril-Strips^{R}, **Figure 2B**). In the sham group, no needle was implanted. Instead, the trained physician pressed the empty needle applicator against the four acupoints of each concha before placing the opaque Band-Aid. In fact, the pressure applied on each auricle with the needle applicator on the selected acupoints was constantly painful by itself, and then, it could yield the same subjective perception as true needles to the patients with hypoxic-ischemic encephalopathy. Moreover, undertaking the sham stimulation outside the auricular region innervated by the ABVN, such as the ear lobe, called "location sham," was recently advised against (Rangon, 2018; Borges et al., 2021; Verma et al., 2021). The patients, their physicians, and nursing staff were blind about randomization because the ears were hidden by the opaque Band-Aid throughout the hospitalization. Patients of both groups received the regular drugs used for COVID-19 pneumonia (i.e., corticosteroids, antibiotics, etc.) in accordance with the current practice guidelines at the hospital at that time, in addition to neuromodulation treatment. An industrial partner (Sedatelec^{R}) has proposed to provide the SPNs for the procedure to run this clinical trial.

Clinical Status Assessment

Clinical status was assessed using a 7-category ordinal scale (Cao et al., 2020): (1) not hospitalized with the resumption of normal activities, (2) not hospitalized, but unable to resume normal activities, (3) hospitalized, not requiring supplemental oxygen, (4) hospitalized, requiring supplemental oxygen, (5) hospitalized, requiring nasal high-flow oxygen therapy, non-invasive mechanical ventilation, or both, (6) hospitalized, requiring extracorporeal membrane oxygenation (ECMO), invasive mechanical ventilation, or both, and (7) death.

(1) not hospitalized with the resumption of normal activities;
(2) not hospitalized, but unable to resume normal activities;

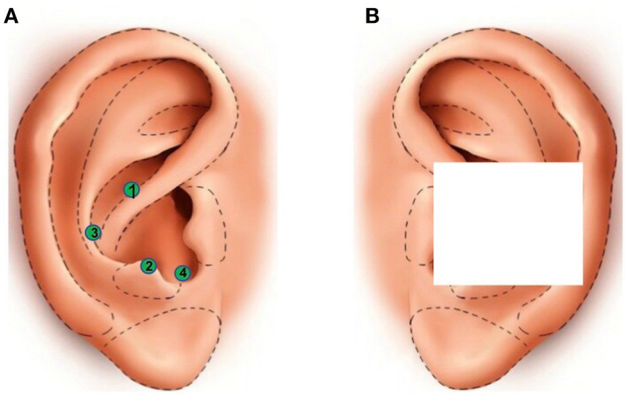

FIGURE 2 | Neuromodulation with SPNs on the concha. **(A)** Localization of the four selected acupoints, represented as green circles, of the concha, following the order indicated by the number in the circle: (1) master point of endoderm, (2) master point of reticular formation, (3) thymic plexus, and (4) ACTH point (i.e., ear maps according to Alimi, 2017). Each acupoint is either implanted with SPNs (verum group) or pressed on with the empty needle applicator (sham group). **(B)** The white square symbolizes the opaque Band-Aid allowing double-blind treatment. Both ears of the patients receive same neuromodulation treatment (i.e., verum or sham group). With the permission of Sedatelec[R].

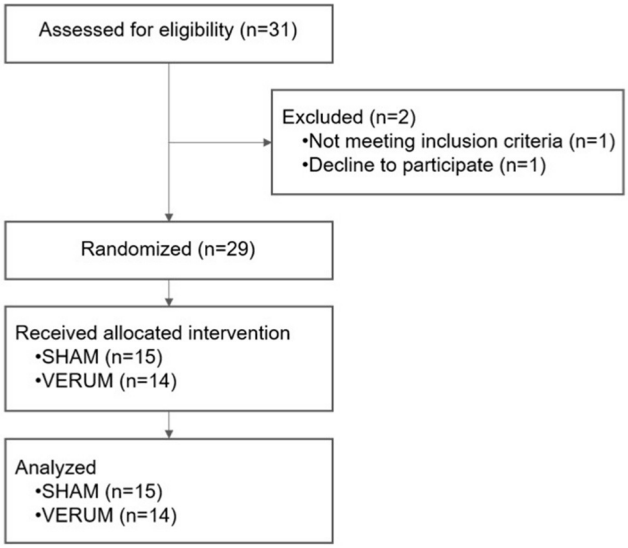

FIGURE 3 | Study flowchart.

(3) hospitalized, not requiring supplemental oxygen;
(4) hospitalized, requiring supplemental oxygen;
(5) hospitalized, requiring nasal high-flow oxygen therapy, non-invasive mechanical ventilation, or both;
(6) hospitalized, requiring extracorporeal membrane oxygenation (ECMO), invasive mechanical ventilation, or both; and
(7) death.

Laboratory Tests

The collection of biological assessments [in particular, C-reactive protein (CRP) blood tests] and imaging were decided by the physicians of the COVID unit (this protocol does not). Ethics approval for analysis of all the data collected was waived by the hospital Institutional Review Board since all the data of the patients collected conformed to the policies.

Statistical Analysis

Statistical analyses were performed using the R software (version 4.0.3). The descriptive analyses of the qualitative variables were presented as number and percentage and that of the quantitative variables were presented as mean and SD. Two means of quantitative variables were compared using the Student's *t*-test if the assumptions were verified, if not the Wilcoxon–Mann–Whitney non-parametric test was used. The time until clinical improvement and the CRP at recruitment was analyzed using the Wilcoxon–Mann–Whitney test, and the age at recruitment was analyzed using the Student's *t*-test. For all the comparisons of categorical variables, the Fisher's exact test was used since the assumption for the Pearson's chi-square test was not valid for any of them. All statistical tests were two-sided, and the significance level fixed for interim analyses was 30%.

TABLE 1 | Characteristics of study patients at recruitment.

Variables	SHAM ($n = 15$)	VERUM ($n = 14$)	*P*
Gender, male, *n* (%)	7 (47)	11 (79)	0.17[a]
Age (mean ± SD, years)	68.5 ± 15.6	73.4 ± 11.6	0.34[b]
Age (median, years)	65.0	75.5	
Initial hospitalization in a recruiting center, *n* (%)	9 (60)	7 (50)	0.87[a]
Oxygen therapy required, *n* (%)	14 (93)	14 (100)	1[a]
High flow nasal oxygen or MV required, *n* (%)	1 (6.7)	0 (0)	
CRP (mean ± SD, mg/L)	71.0 ± 43.1	89.96 ± 77.5	0.77[c]
Comorbidities			
Hypertension (%)	8 (47)	8 (57)	0.40[c]
Diabetes (%)	8 (47)	5 (36)	0.78[c]
Asthma (%)	0 (0)	2 (14)	
Dyslipidemia (%)	4 (24)	2 (14)	0.54[c]
Renal disease (%)	2 (12)	0 (0)	
Cardiovascular disease (%)	2 (12)	1 (7)	0.93[c]
Vascular disease (%)	2 (12)	3 (21)	0.49[c]
Obesity (%)	4 (24)	3 (21)	0.91[c]

CRP, C-reactive protein; MV, mechanical ventilation.
[a] *Fisher's exact test.*
[b] *Student's t-test.*
[c] *Wilcoxon–Mann–Whitney test.*

RESULTS

Patient Characteristics

Thirty-one patients with respiratory failure due to COVID-19 requiring hospitalization for non-invasive oxygen supplementation were included in this study. Among them, 29 have been analyzed (i.e., study flowchart, **Figure 3**). Within 72 h after admission, patients received either auricular (verum

TABLE 2 | Clinical evolution of patients during the medical follow-up.

Outcome measures	SHAM ($n = 15$)	VERUM ($n = 14$)	*P*
7-category ordinal Clinical Progression Scale score improvement or Hospital discharge, *n* (%)	12 (80)	9 (64)	0.43[a]
Time until clinical improvement (mean ± SD, days)	8.8 + 3.5	8.9 + 3.3	0.86[c]
Transfers to ICU, *n* (%)	1 (6.7)	2 (14.3)	0.60[a]
14-day all-cause mortality, *n* (%)	1 (7)	0 (0)	1[a]

[a] *Fisher's exact test.*
[b] *Student's t-test.*
[c] *Wilcoxon–Mann–Whitney test.*

group, $n = 14$) or placebo neuromodulation therapy (sham group, $n = 15$) in addition to their usual treatments. The sham group was composed of 7 males and 8 females (mean age = 68.5 ± 15.6 years), and the verum group was composed of 11 males and 3 females (mean age = 73.4 ± 11.6 years). Whereas, there was no statistically significant difference between sham and verum groups regarding mean age (**Table 1**), the median age of the AN arm was roughly 10 years old (75.5 vs. 65 years). Moreover, gender was not distributed in a balanced way between both groups, with 47 and 79% of males in the sham and the verum group, respectively. Regardless of the group considered, all patients required oxygen therapy with one patient requiring mechanical ventilation in the Sham group. Regarding CRP levels, all patients presented a proinflammatory state, and no statistical difference between groups was observed for this parameter.

Patient Clinical Evolution

We first evaluated the effects of AN inpatient outcomes regarding the 7-category ordinal scale evolution or hospital discharge. Concerning these parameters, we did not observe any clinical improvement in the verum group compared to the sham group (**Table 2**). Even if we adjusted these parameters to gender, age, or CRP levels, no statistical difference was found between study groups (i.e., data not shown). We then evaluated the time until clinical improvement and the transfers to the ICU for the study patients. Again, there was no statistically significant difference between groups. Finally, for the 29 patients in both groups, one case in the sham group died.

DISCUSSION

The COVID-19 infection has rapidly spread throughout the world causing a major healthcare crisis. About 20% of patients with COVID-19 develop severe disease requiring hospitalization. Among them, a high mortality rate of up to 97% is observed with respiratory failure as the leading cause of death. Despite many therapeutic strategies under investigation, there is still no curative treatment available. With the increasing rates of infection, there is an urgent need for new therapeutic approaches to counteract the infection. The excessive inflammatory response to SARS-CoV-2 is thought to be a major cause of acute respiratory failure in those patients. As the nervous system has shown to be a strong modulator of respiratory function and the immune response, we suggested as others that neuromodulation could be used to improve patient outcomes. As a result, we raised the hypothesis that AN could be used as a potential adjunct treatment to modulate inflammatory response in patients with COVID-19 and improve their clinical outcomes. Thus, we explored the clinical effects of AN in patients with COVID-19 using a 7-category ordinal Clinical Progression Scale (Cao et al., 2020), or hospital discharge as primary study outcome and the transfers to the ICU and survival rate as secondary study outcomes.

In our pilot study, patients of both arms are likely to be as severely infected by SARS-CoV-2, as shown by the identical median of the CRP (Deng et al., 2020; Terpos et al., 2020).

Regardless of the endpoint considered, AN does not appear to improve inpatients with COVID-19 outcomes.

This result could be explained by three major limitations. First, we hypothesized that taVNS was able to improve the patient outcome by modulating the excessive inflammatory response due to SARS-CoV-2 infection. However, we failed to show any anti-inflammatory effect of AN using CRP as a biomarker. Other more specific markers such as tumor necrosis factor-alpha (TNF-α) or interleukin-1 beta (IL-1β) should be used in further studies to highlight the potent anti-inflammatory effects of AN in those patients but would imply additional invasive assessment for the patient.

Second, age and gender differences in immune responses have been reported in infectious diseases such as COVID-19 with more elderly men than young women dying from the disease. By pure coincidence (due to non-paired randomization), AN and PN small population are not set on equal footing regarding COVID-19 prognosis factors. Therefore, our two groups are evidently not comparable regarding age and gender, while those remain comparable regarding comorbidities (**Table 1**), CRP (**Table 1**), and smoking status (i.e., all the patients included were non-smokers). The age issue is considered as the strongest prognosis factor for COVID-19 (Izcovich et al., 2020; O'Driscoll et al., 2021). Our study is in line with this result, as the improvement in the respiratory status of the patients of both arms is significantly correlated to their age ($p < 0.03$). Considering that the case fatality rate of the 70- to 79-year-old patients was shown to be roughly two times higher than the case fatality rate of the 60- to 69-year-old patients (Signorelli and Odone, 2020), whatever the country considered (Chen et al., 2021), the AN group, showing a median age 10 years older than the PN group (75.5 vs. 65 years old), was expected to count more deaths than the placebo arm. Moreover, in our study, 80% of the verum population consisted of men, contrary to the sham group (i.e., <50%). Considering that the male gender is correlated to a bad outcome in the COVID-19 pandemic, it is noteworthy that there are fewer deaths in the AN arm than in the placebo arm. Such age and gender differences between groups may represent the major source of bias in our study, and in particular given the small size.

Third, we acknowledged that our cohort is too small to draw any firm conclusion, but this preliminary study provides some leads and merits further exploration with a much larger study

population to assess if AN-reduced inflammation could confer potential health benefits to the patients with COVID-19. In fact, the results of our pilot study provide us clues to optimize the design of the relatively larger upcoming clinical trials.

In fact, first, the choice of primary and secondary endpoints can be better defined by this experience. In the chaotic situation of the first COVID-19 wave, we wanted to select the most convenient primary endpoint, which is easy to acquire from medical files, i.e., the clinical improvement on a pragmatic validated scale (Cao et al., 2020). Nevertheless, contrary to the known effect of fast pain relief, taVNS through SPN might require a relatively longer period (i.e., more than 14 days) to elicit a significant clinical improvement in severe SARS-CoV-2 infection (as suggested by Pan et al., 2021). In fact, iVNS is known to be a slow-acting therapy as reported in epilepsy (Panebianco et al., 2015) and inflammatory bowel diseases (Sinniger et al., 2020). It might have been more relevant to assess the mortality rate at Day 28 (D28) instead of Day 14 (D14) (Cao et al., 2020; Hermine et al., 2021)), as well.

In contrast, choosing the rate of transfer to the ICU as a primary endpoint might have been more appropriate, as it generally happens within 10 days after hospital admission (Cheng et al., 2020). Nevertheless, contrary to the Beauvais General hospital, the Rothschild Foundation Hospital is not a frontline hospital for patients with COVID-19, only admitting transfers from other centers. Therefore, a significant percentage (i.e., half) of our small population might not be suitable for assessing AN efficiency in COVID-19.

Besides fatality and the rates of transfer to the ICU, HRV increase should be selected as a primary endpoint, allowing a more discerning assessment of AN efficiency with small patient samples. In fact, HRV eases bias induced by comorbidities, age, gender, drugs, and nowadays vaccines since the latter factors influence the basal HRV values (Hasty et al., 2020; Wee et al., 2020). Regrettably, in this pilot study, we did not monitor vagal tone through HRV. Such monitoring is not at all cumbersome as the patients are continuously monitored. In fact, in a recent study, a Holter monitor was used to record, collect, and analyze the dynamic ECG data over 24 consecutive hours for all 34 patients (Pan et al., 2021). Nevertheless, most non-ICU patients were regularly, but only occasionally, monitored, and the medical-grade accuracy of HRV by the smartphone apps was not available at that time in our hospitals. However, we believed that the HRV parameter constitutes an optimal primary endpoint for the upcoming trials (Pan et al., 2021).

Second, AN should be dispensed earlier. As AN does not elicit noteworthy side effects, patients should receive AN as soon as they are presumed to be COVID-19 positive, ideally at the clinic, for instance, long before receiving the PCR results of SARS-CoV-2, or at least upon arrival at the emergency ward. This time-saving process would help decrease the replication of the virus and the inflammatory response.

Finally, AN efficiency might be optimized regarding the choice of the localization of the SPNs on the ear. In particular, one localization has drawn attention, i.e., outside of the ear concha (Volf et al., 2020). The main barrier lies in the fact that double-blinding gets more difficult, requiring a larger Band-Aid, less easy and comfortable to wear for patients during several days.

CONCLUSION

Auricular neuromodulation with Semi Permanent Needles was successfully implemented in two hospitals during the COVID-19 pandemic and was well-tolerated by oxygen-requiring patients with COVID-19. However, in our preliminary study, this non-invasive vagus nerve neuromodulation technique did not significantly improve the outcome of the patients with COVID-19 when applied within the first 72 h of hospitalization. Other studies are necessary to clarify these results and to assess if reduced inflammation induced by AN is sufficient to induce potential health benefits in those patients.

Contrary to conventional approaches, autonomic neuroimmunology, whereby immune functions can be modulated by the vagus nerve, targets a common hallmark of immune dysregulation across infectious diseases and improves the homeostasis potential of the host. As the vagally driven CAP can stop the action of NF-κB, adequate vagal signaling might modulate the severity of several viral infections, thus supporting complementary non-invasive vagal neuromodulation use in one-size-fits-all antiviral strategy, now in case of vaccine shortage or poor efficiency or after, for the upcoming pandemics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP SUD-Est II affiliated to the French Ministry of Health. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-MR, the principal investigator, did all the neuromodulation treatments (auricular and sham treatments) in Hôpital Fondation Rothschild and part of them in Centre Hospitalier de Beauvais and wrote the manuscript. RB and DL selected the patients of Beauvais Hospital for inclusion. AM did some part of the neuromodulation treatments in Beauvais Hospital. ST coordinated the study between the two hospitals (Head project). CL, TL, and FH collected and analyzed the data from medical records. JG and CL did the statistical analysis. DL made it possible to have a multi centric center (Director of Clinical Research Department in Beauvais Hospital). All authors contributed to the article and approved the submitted version.

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Decreased Autonomic Reactivity and Psychiatric Comorbidities in Neurological Patients With Medically Unexplained Sensory Symptoms: A Case-Control Study

*Victoria Ruschil[1,2], Nazar Mazurak[3], Martin Hofmann[3], Ekaterina Loskutova[3], Paul Enck[3], Tobias Freilinger[1,4] and Katja Weimer[3,5]**

[1] Department of Neurology and Epileptology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany, [2] Department of Psychiatry and Psychotherapy, University of Tübingen, Tübingen, Germany, [3] Department of Psychosomatic Medicine and Psychotherapy, University Medical Hospital Tübingen, Tübingen, Germany, [4] Department of Neurology, Klinikum Passau, Passau, Germany, [5] Department of Psychosomatic Medicine and Psychotherapy, Ulm University Medical Center, Ulm, Germany

Up to 48% of patients with medically unexplained symptoms seen in neurological practice suffer from sensory symptoms, which could be of functional nature or secondary to psychiatric disorders. These patients show high medical care utilization causing elevated healthcare costs. Despite the high prevalence, little is known about clinical characteristics and pathophysiological mechanisms. For functional disorders such as irritable bowel syndrome, a reduction of heart rate variability (HRV) has been shown, suggesting a dysfunction of the autonomic nervous system (ANS). The aim of this study was to investigate psychological data and functional changes of the ANS in patients with medically unexplained sensory symptoms (MUSS). In this exploratory pilot study, 16 patients (11 females, 31.6 ± 11.9 years) with MUSS, who were recruited at a single tertiary neurological center, underwent a structured clinical interview (SCID) to evaluate psychiatric comorbidities. Patients and age- and sex-matched healthy volunteers filled in questionnaires, and individual sensory thresholds (perception, pain) were detected by quantitative sensory testing (QST). HRV was assessed at baseline and under three different experimental conditions (tonic pain stimulus, placebo application, cold-face test). All tests were repeated after 6–8 weeks. SCID interviews revealed clinical or subclinical diagnoses of psychiatric comorbidities for 12 patients. Questionnaires assessing somatization, depression, anxiety, and perceived stress significantly discriminated between patients with MUSS and healthy controls. While there was no difference in QST, reduced ANS reactivity was found in patients during experimental conditions, particularly with regard to vagally mediated HRV. Our pilot study of neurological patients with MUSS reveals a high prevalence of psychiatric comorbidities and provides evidence for altered ANS function. Our data thus give insight in possible underlying mechanisms for these symptoms and may open the door for a better diagnostic and therapeutic approach for these patients in the future.

Keywords: autonomic nervous system, functional disorders, clinical neurophysiology, heart rate variability, medically unexplained sensory symptoms

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***Correspondence:**
Katja Weimer
katja.weimer@uni-ulm.de

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INTRODUCTION

Medically unexplained symptoms (MUS) have a high prevalence of up to 49% in primary medical care (1). Patients with MUS often suffer from the insecurity that an organic origin of their symptoms could be missed and complicate the communication with their treating physicians (2). As a consequence of being more subjectively distressed by their symptoms, patients with MUS have more frequent contacts to physicians, a higher utilization of secondary care, and a higher probability to receive more costly investigations (3–6).

For neurological outpatient clinics in particular, patients with MUS make up a relevant number of about 30% (7). Medically unexplained sensory symptoms (MUSS) are among the most common neurological presentations of MUS next to non-epileptic seizures and functional motor symptoms (8, 9). MUSS can include different sensations such as numbness or paresthesia and either occur in a hemisensory pattern or are distributed unsystematically involving different parts of the body. However, a precise definition of MUS and MUSS is difficult because standardized terms for this heterogenic group of patients are missing. Even by established diagnostic classification systems, e.g., the *International Classification of Diseases, Tenth Revision* (ICD-10) and *Diagnostic and Statistical Manual of Mental Disorders V* (DSM-V), these conditions are not represented sufficiently. On the other hand, the term MUS, which is used to describe symptoms that do not refer to a specific etiology and cannot be explained by another underlying disorder, has also been subject to much criticism for being purely descriptive and negatively defined by the absence of another disorder (10, 11). MUS can include functional disorders that exclusively concentrate on a single organic system (e.g., irritable bowel syndrome) as well as somatoform disorders which are characterized by several persistent and changing symptoms without sufficient organic origin for at least 2 years. At the same time, patients with MUS are often positive for psychiatric diagnoses classified by ICD-10 such as depression or anxiety disorders that only become apparent by elaborate psychological diagnostics and structured clinical interviews (12). The symptomatic overlap of these definitions makes it difficult for treating physicians to differentiate between the terms.

Recently, research started to focus on the biological basis of MUS such as somatoform disorders. So far, neuroendocrinological, immunological, and functional brain imaging findings have been postulated to be involved in the central and peripheral processing of afferent stimuli in patients with MUS (13). Next to environmental factors (14–16), increased sensitization to peripheral stimuli could be at least partially responsible for triggering and maintaining MUS. Another regulatory mechanism for this sensitization could involve the autonomic nervous system (ANS). The link between autonomic regulation and central processing mechanisms has been demonstrated most convincingly for the perception of pain. On the one side, vagal activity has an inhibitory effect on descending pain pathways, whereas sympathetic activity leads to an increased perception of pain (17). On the other side, acute as well as chronic pain is accompanied by elevated sympathetic and reduced parasympathetic activity. Parasympathetic activity correlates with pain experience in daily life as well as with experimentally induced pain in healthy probands (18–20).

For some functional as well as somatoform disorders, such as irritable bowel syndrome and fibromyalgia syndrome, measurable changes in the regulation of the ANS have become apparent (21–23). As the ANS is in permanent control of the heart rhythm, it is responsible for short-term (parasympathetic) or medium-term (baroreflex, sympathetic) changes of beat-to-beat intervals. In comparison with healthy controls, patients with somatoform disorders showed a reduction of heart rate variability (HRV) as a marker for reduced parasympathetic activity. Based on these findings, the reduction of HRV has previously been discussed as a potential biomarker for somatoform as well as functional disorders (24).

Although patients with MUS in general and MUSS in particular form a relevant group of neurological patients, there is still little knowledge on their clinical characteristics and potential physiological biomarkers such as HRV. However, improving the diagnostic approach would be a crucial first step toward a better management of these patients. We hypothesized that patients with MUSS show certain psychological and biophysiological clinical features in comparison with healthy controls. In assumption that MUSS are stress-related disorders, we raised the question if patients with MUSS differed from healthy controls by reduced parasympathetic activity. Therefore, the aim of this explorative pilot study was (1) to examine the eligibility and economic use of psychological questionnaires to detect psychiatric disorders in neurological patients with MUSS and (2) to test HRV as a surrogate marker for ANS function in this specific group. We performed structured clinical interviews, psychological questionnaires, quantitative sensory testing (QST), and HRV analysis in order to generate specific clinical data and to evaluate the regulation of their ANS under different conditions such as pain stimuli, placebo application, and the cold-face test.

MATERIALS AND METHODS

Study Design and Participants

Patients were prospectively recruited from the Department of Neurology of the University Hospital Tübingen, Germany between February 2016 and August 2017. They all received a comprehensive diagnostic work-up during inpatient hospital evaluation. Inclusion criteria were persistent sensory symptoms for a minimum of 4 weeks in patients above 18 years. Underlying organic diseases had been excluded prior to the study according to state-of-the-art neurological standards by experienced neurologists (VS, TF). All patients underwent imaging of the brain and/or parts of the spine. Other diagnostic measures, if considered necessary, included lumbar puncture, evoked potentials, nerve conduction studies, EEG, and sympathetic skin reaction. Exclusion criteria were relevant medical conditions (e.g., diabetes, severe psychiatric disorder, substance-dependence, suicidal ideation, cold urticaria, pregnancy, or breastfeeding) or medication with potential influence on heart rate, peripheral nervous system (PNS), or central nervous system (CNS) (e.g., antiarrhythmic, antiepileptic, antipsychotic,

analgesic, etc.) Patients as well as controls were instructed to refrain from smoking for 2 h, from caffeine for 8 h, and from alcohol and any medication for 24 h before testing.

Age- and sex-matched healthy controls were recruited *via* email and advertisements from the catchment area of the University of Tübingen. For practical reasons, we allowed age to differ for ±3 years from the matched patient's age. All participants provided written informed consent before study inclusion. This study was approved by the Ethics Committee of the Medical Faculty of Tübingen University (Project No. 765/2015BO2) and was conducted in accordance with the Declaration of Helsinki.

Patients visited the lab three times for a diagnostic interview (T0) and two experimental sessions (T1, T2). Healthy controls visited the lab at two experimental sessions only (T1, T2). To avoid circadian fluctuations, most T1 and T2 sessions were conducted between 8 a.m. and 2 p.m. and at the same time of the day with small deviations (0 ± 2.5 h) within persons. Controlling for daytime did not affect the results. We planned to investigate all participants again after 6 weeks but had to allow some variance due to time and organizational issues. The time interval between both experimental sessions was 6–8 weeks. Results of T2 are presented in the **Supplementary Material**.

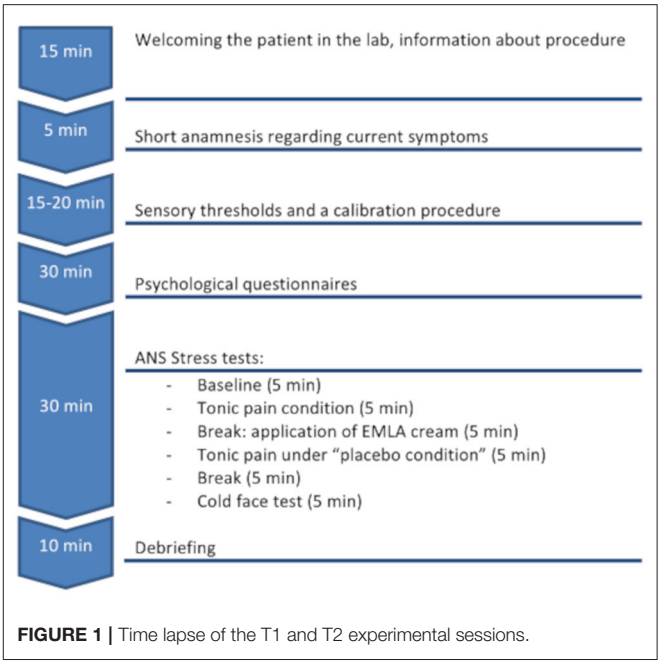

FIGURE 1 | Time lapse of the T1 and T2 experimental sessions.

Procedures

Diagnostic Interview

During the initial visit (T0), patients underwent a structured clinical interview (SCID) according to the *Diagnostic and Statistical Manual of Mental Disorders IV* (DSM-IV) (25) to diagnose mental disorders. All interviews were performed by trained psychologists (KW, EL). Diagnoses were noted when criteria were fully met, and subclinical diagnoses were noted when two criteria at most were not met. Furthermore, patients filled in an adapted version of the German Pain Questionnaire (26) in which "pain" was replaced by "symptoms" to assess location, onset, severity, and course of sensory symptoms.

Sensory Threshold and Calibration

The two experimental sessions (T1, T2) were identical for all patients and controls (see also flowchart **Figure 1**).

First, sensory thresholds and a calibration procedure were performed with a Peltier thermode. Three squares of 3 × 3 cm for the positioning of a thermode (Thermal Sensory Analyzer, TSA-II, Medoc Ltd., Israel) were drawn on the volar forearm of the non-dominant hand. The middle square was used for the assessment of the warmth detection threshold (WDT) and the heat pain threshold (HPT) according to the quantitative sensory testing (QST) protocol (27) as well as a calibration procedure. The other two squares were used for a pain and an "analgesic" condition (see below) for which the order of squares was randomized between participants and lab visits. For QST, participants were instructed to press a button as soon as they felt the temperature changes (for WDT) or immediately after the heat stimulus became painful (for HPT). After the button was pressed, the heat sensor returned immediately to the initial temperature of 32°C. The procedure was repeated three times with random breaks (between 3 and 10 s) to avoid learning effects, and means were calculated as outcome measures. During the calibration procedure, six stimuli with a temperature between HPT-1°C and HPT + 1.5°C were applied for 15 s with 10-s breaks in between. The participants were instructed to rate the intensity of pain on a numeric rating scale (NRS) with 0 set for "no pain" and 10 for "the most intensive pain ever." The temperature that was rated as "4" was used for the further experimental procedure.

Psychological Questionnaires

From the Patient Health Questionnaire (PHQ-D) (28), the modules PHQ-15, PHQ-9, "anxiety," and "stress" were used to assess somatization, depression, anxiety, and psychosocial stress as continuous outcomes (sum scores) as well as binary outcomes for the presence of a somatization disorder, major depression, other depressive syndrome, general anxiety disorder, and panic disorder according to the ICD-10 criteria. The Somatic Symptom Disorder Scale (SSD-12) (29) determined psychological characteristics such as cognitive, affective, and behavioral aspects according to the B criteria of the DSM-V somatic symptom disorder. Sum scores were calculated for all three subscales. The German version of the Perceived Stress Questionnaire-20 (PSQ-20) (30) measured three dimensions of internal stress reactions with the subscales "worries," "tension," "joy," and "demands" as external stressors. Means were calculated for all four subscales and an overall stress mean score.

Electrocardiogram and ANS Challenges

Participants were connected to a 3-channel ECG (skin Ag-AgCl electrodes). All data were collected using a TaskForce® Monitor (CNSystems Medizintechnik AG; Graz, Austria). Data

were collected continuously during the whole subsequent experimental procedure at a sampling rate of 1,000 Hz. Participants were invited to sit in a comfortable chair and instructed to avoid rapid or intensive movements during the testing procedure. They were asked to sit relaxed for 5 min to collect baseline ECG data.

To evaluate HRV reactivity under different conditions, first a tonic pain stimulus with a heat intensity of "4" on the NRS was used. Stimuli were applied 10 times for a duration of 20 s with 10-s breaks in-between (overall 5 min) to induce a physiological stress response. After each 20 s period, participants were asked to rate the intensity of pain on the same NRS scale as described above.

During the following 5-min break, an application of an inert topical analgesic cream (EMLA cream, AstraZeneca GmbH, Wedel, Germany) was applied to the last marked square on participants' forearm. Participants were told about the nature of EMLA and its ability to modify their perception of heat pain stimuli. However, participants were unaware of the fact that EMLA comes into effect after at least 30 min only (31), to examine a possible placebo effect (32). After 5 min, the skin was thoroughly cleaned and the thermode was put on the pre-treated skin square. The same series of pain stimuli with the assessment of intensity ratings was used as described above.

Finally, after a 5-min break, a cold pack (T $\approx$ 4°C) was placed on the participants' foreheads for 5 min. This so-called cold-face test (CFT) induces an automatic activation of the parasympathetic nervous system in healthy participants (33).

Analysis of Heart Rate Variability

Raw ECG data were exported in the form of the interbeat intervals and stored locally for further processing. Data on autonomic activity were derived from analyses of the HRV based on mathematical transformations of time differences between consecutive heartbeats [interbeat intervals (IBI)]. The detailed background can be found elsewhere [e.g., in (33, 34)]. Analyses were performed with the software Kubios HRV 3.0 (Kubios Oy, Kuopio, Finland) by an experienced researcher (NM). First, 5-min IBI were extracted from the continuous recording for each of the experimental conditions: "baseline," "tonic pain," "placebo pain," and "cold-face test." IBI were screened for artifacts (by NM) and, if necessary, corrected using the in-built algorithms (threshold-based artifact correction algorithm). Thirty intervals were corrected and six were excluded from further analyses due to insufficient quality of signal. Four of these six intervals were the measures of one control participant (female, 32 years) at T1 and the other two intervals were from one patient (male, 29 years) from both T1 and T2. Consequently, data of those two participants were not considered in repeated measures ANOVAs of HRV. Exclusion of data of those two participants did not influence any other analysis. We evaluated mean IBI in milliseconds as marker of general HRV as well as logarithmized root mean square of successive differences (RMSSD) and logarithmized high-frequency power (HF) as proxies for parasympathetic activity. HF power was obtained using fast Fourier transformation procedure (256 s window width and 50% window overlap).

Statistical Analysis

Statistical analyses were performed with SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.), and p-values of <0.05 were considered statistically significant. Normal distribution of outcome measures was assessed with Shapiro-Wilk tests. Between-group comparisons were performed with Mann-Whitney U-tests and with Wilcoxon tests for within-subject comparisons as data were not normally distributed in at least one group. When variances were unequal, adaption of df was applied according to Welch and corrected p-values are reported only. As HRV parameters (IBI, RMSSD, HF) were normally distributed, they were analyzed with 4 × 2 repeated measures ANOVAs (periods × group) and t-tests as *post-hoc* tests. p-Values were adjusted according to Greenhouse-Geisser when Mauchly's sphericity test was significant (unadjusted df values are reported for reasons of readability).

RESULTS

Overall, 45 patients were screened for study inclusion. Twenty-two patients were excluded due to several reasons, and seven patients took part at T0 only as shown in **Figure 2**.

Participants' Characteristics and Baseline Data

Sixteen patients, five male and 11 females between 19 and 63 years, completed the study. Time since the onset of sensory symptoms varied between 6 weeks and 36 years and 7 months (Md = 10.25 months). Patients reported to have visited 0 to 18 healthcare professionals (e.g., general practitioners, specialists, psychologists, hospitals, or alternative practitioners) because of the sensory symptoms before contacting our neurological clinic (**Table 1**). All of them had received (mainly magnetic resonance) imaging of the brain and/or parts of the spine. Further diagnostics included lumbar puncture (n = 14), sensory evoked potentials (n = 13), nerve conduction studies (n = 7), EEG (n = 1), and sympathetic skin reaction (n = 2). None of the diagnostic interventions had shown abnormal results.

Patients rated the temporal characteristics of their symptoms as follows: 25% described their symptoms as constant with minor fluctuations over time, 12.5% had constant symptoms with strong fluctuations, 31.3% had symptom attacks with symptom-free periods in between, and 25% had constant symptoms with symptom attacks (one patient could not describe the temporal characteristics). The localizations of sensory symptoms were reported differently by each patient; however, a predominance of symptoms was located to the left side of the body (**Figure 3**).

Based on the SCID interviews, 12 patients fulfilled either one or more full clinical or subclinical psychiatric diagnoses defined by the DSM-IV criteria: five patients fulfilled one and two patients fulfilled two diagnoses; four patients met criteria for one subclinical diagnosis, three patients for

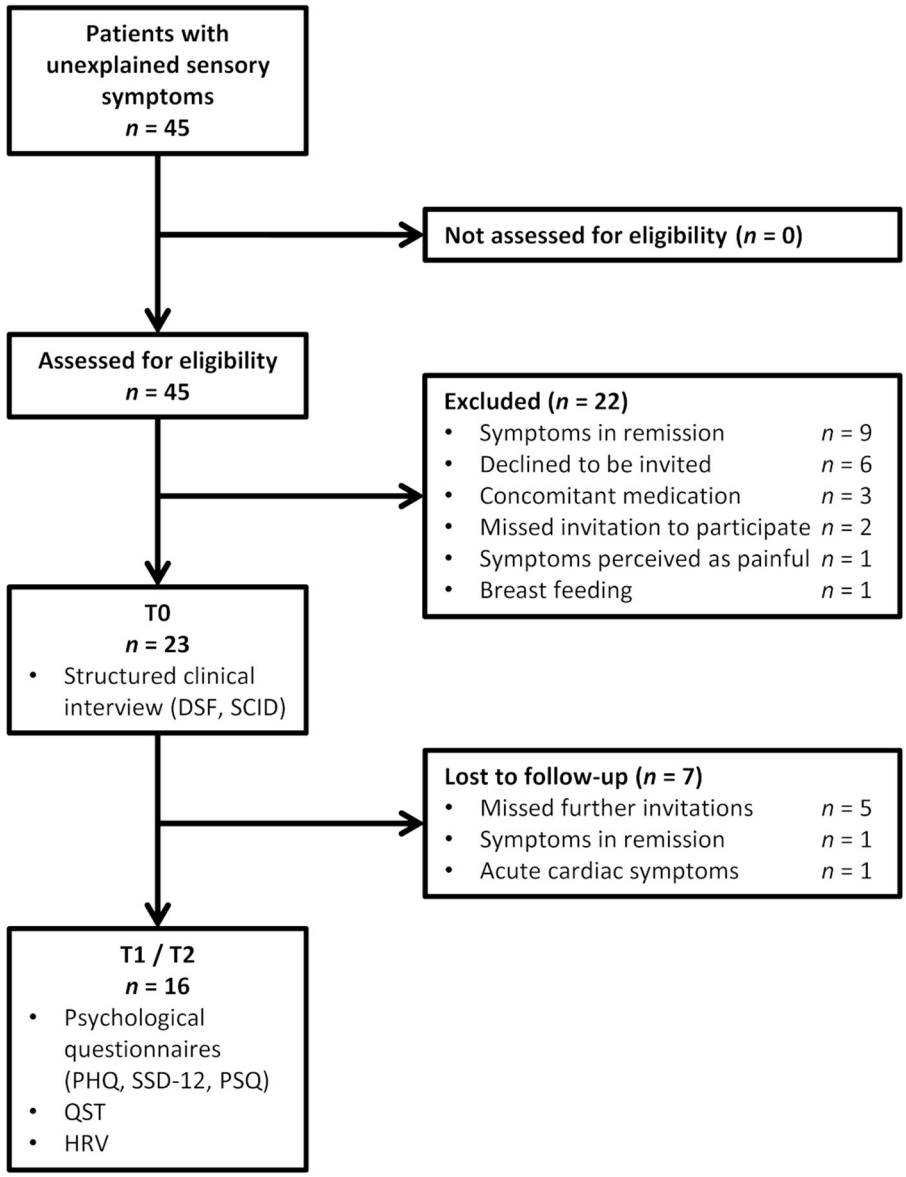

FIGURE 2 | Flowchart for patient inclusion (SCID, Structured clinical interview for mental disorders; DSF, modified German pain questionnaire; PHQ, Patient Health Questionnaire; SSD-12, somatic symptom disorder questionnaire; PSQ, perceived stress questionnaire; QST, quantitative sensory testing; HRV, heart rate variability assessment).

two, and one patient for four subclinical diagnoses. Next to somatization disorder, pain and panic disorders were represented most prominently (**Figure 4**). Patients who fulfilled DSM-IV diagnosis were given the advice to consult the Department of Psychosomatic Medicine and Psychotherapy or a local physician/psychotherapist.

Psychological Questionnaires

A significant discrimination between patients and controls was possible with the scales for somatization, depression, and anxiety of the PHQ and SSD-12 scales. Furthermore, binary outcomes of the PHQ identified patients. The PSQ showed significantly higher values for stress, tension, and worries in patients compared with controls (**Table 2**).

Quantitative Sensory Testing

The evaluation of the individual WDT and HPT by QST as well as of the applied test temperature according to a VAS rating of 4 showed no difference between patients and healthy controls (**Table 3**). In addition, no significant difference between patients and controls was found for the intensity of pain

TABLE 1 | Demographic and clinical characteristics of patients and controls [data presented as *n* (%), mean (SD), or median (1st quartile−3rd quartile)].

	Patients ($n = 16$)	Controls ($n = 16$)
Age (years)	31.63 (11.93)	30.31 (9.39)
Sex (*n*, %)		
- Female	11 (68.8%)	11 (68.8%)
- Male	5 (31.2%)	5 (31.2%)
BMI (kg/m²)	24.77 (3.81)	23.57 (2.35)
Age at symptom onset (years)	27.69 (7.76)	
Duration since first symptom (months)	10.25 (3.88–31.50)	
Consulted healthcare practitioners	4.0 (2.0–8.0)	

based on VAS during tonic pain and after application of a local placebo.

Autonomic Reactivity

Repeated measures ANOVAs showed a significant change in IBI between recording periods [$F_{(3,84)} = 8.910$, $p < 0.001$], but no difference between groups [$F_{(3,84)} = 1.790$, $p = 0.170$]. There was no change in RMSSD [$F_{(3,84)} = 2.093$, $p = 0.124$], but groups differed significantly [$F_{(3,84)} = 5.033$, $p = 0.007$]. HF significantly changed between periods [$F_{(3,84)} = 4.441$, $p = 0.013$], and this change was different between groups [$F_{(3,84)} = 3.288$, $p = 0.039$] (**Figure 5**).

Post-hoc repeated measures ANOVAs for each group separately showed a significant difference between periods in patients for IBI [$F_{(3,42)} = 4.405$, $p = 0.009$] but not for RMSSD [$F_{(3,42)} = 0.986$, $p = 0.409$] and HF [$F_{(3,42)} = 1.198$, $p = 0.322$]. In contrast, changes over time were significant in all three variables in controls [IBI: $F_{(3,42)} = 5.697$, $p = 0.010$; RMSSD: $F_{(3,42)} = 5.525$, $p = 0.003$; HF: $F_{(3,42)} = 5.488$, $p = 0.003$]. However, there is no significant difference between patients and controls at any single time point (**Table 4**).

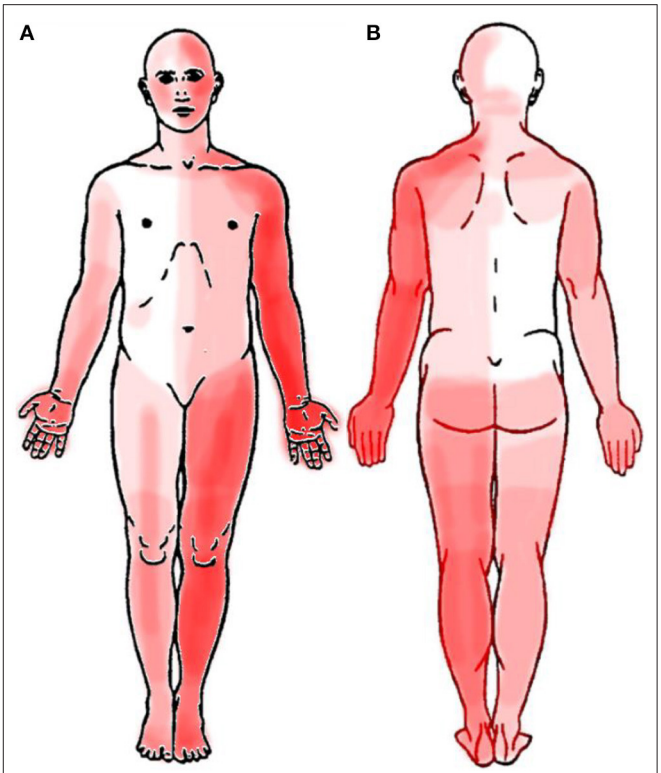

FIGURE 3 | Localization of sensory symptoms in front **(A)** and back **(B)** views in patients [frequency between 0 (white) and 15 (dark red)].

DISCUSSION

With this explorative pilot study, we could discover measurable deviations of psychophysiological features in a small and well-characterized group of neurological patients with medically unexplained sensory symptoms (MUSS). HRV analysis revealed reduced HRV reactivity pointing at an autonomous dysfunction in 16 patients with MUSS compared with healthy controls (HC). Our findings support a strong psychophysical component of MUSS in line with comparable studies in other somatoform disorders (35). Most patients (12 of 16) fulfilled at least one clinical or subclinical psychiatric diagnosis according to DSM-IV. Among these, somatization, panic, and anxiety disorders were the most common. This finding was supported by further psychological questionnaires (e.g., PHQ and SSD-12) revealing significant differences between patients and controls with regard to somatization, depression, and anxiety. Patients also reported significantly more tension and worries, and less joy. The high prevalence of psychiatric diagnoses in neurological in- and outpatients was comparable with previous studies, especially regarding patients with MUS (12, 36). As the patients scored repeatedly higher in psychological questionnaires at T1 and T2, the data can be considered reliable.

Despite the clinical impression that patients with MUSS are more sensitive for their body functions, there were no differences between patients and healthy controls regarding pain and perception thresholds. This fact was confirmed at T2 where within-subject comparisons showed similar pain and perception thresholds as at T1. However, tonic pain was considered worse in T2 than in T1 by the patients, but not by healthy controls. This could be interpreted as reduced tolerance toward repeated pain stimuli of patients which might be caused by negative expectations. The lack of difference in placebo response following a standard placebo analgesia test (32) supports the concept of normal sensory and pain perception in MUSS as compared with healthy volunteers.

Heart rate responded to different test conditions in both patients and controls; however, vagally mediated HRV did not differ between test conditions in patients on both occasions. It should particularly be mentioned that HRV of patients even did not respond to the cold-face test, a challenge test of the vagus

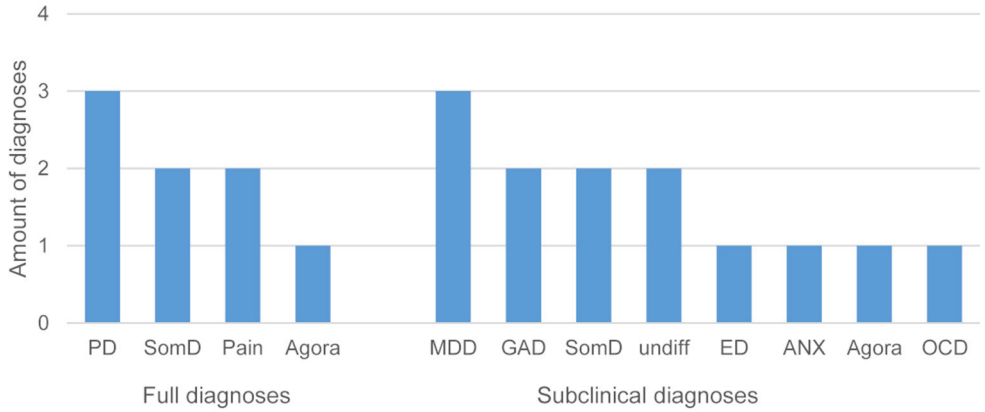

FIGURE 4 | Frequency of fully met and subclinical diagnoses in patients (PD, panic disorder; SomD, somatization disorder; Pain, pain disorder; Agora, agoraphobia without panic disorder; MDD, major depression disorder; GAD, generalized anxiety disorder; undiff, undifferentiated somatoform disorder; ED, eating disorder (binge eating or anorexia nervosa); ANX, anxiety (not otherwise specified); OCD, obsessive-compulsive disorder).

TABLE 2 | Results of psychological questionnaires in patients and controls at T1 [reported as median (1st quartile−3rd quartile)].

	Patients	Controls	*p*-value (U)
Continuous outcomes			
PHQ somatization	10.50 (5.25–16.00)	3.50 (2.00–5.00)	0.002
PHQ depression	5.00 (1.25–9.50)	2.00 (1.00–2.00)	0.019
PHQ anxiety	4.50 (2.50–7.00)	2.00 (1.00–2.75)	0.007
PHQ stress	4.00 (1.25–6.00)	1.50 (1.00–3.75)	0.056
SSD12 cognitive	5.50 (3.25–8.75)	0.00 (0.00–1.75)	< 0.001
SSD12 affective	6.50 (3.00–10.00)	1.00 (0.00–1.00)	< 0.001
SSD12 behavioral	3.50 (1.00–7.25)	0.00 (0.00–0.00)	< 0.001
PSQ worries	1.90 (1.40–2.80)	1.30 (1.20–1.55)	0.002
PSQ tension	2.40 (1.80–3.10)	1.40 (1.20–1.60)	< 0.001
PSQ joy	2.78 (1.80–3.20)	3.50 (3.25–3.80)	< 0.001
PSQ demands	2.30 (1.45–2.60)	1.80 (1.45–2.35)	0.324
PSQ sum	2.13 (1.75–2.98)	1.53 (1.31–1.70)	0.001
Binary outcomes			
PHQ somatization	7 (43.8%)	0 (0%)	
PHQ major depression	2 (12.5%)	0 (0%)	
PHQ other depression	2 (12.5%)	0 (0%)	
PHQ panic disorder	1 (6.3%)	0 (0%)	
PHQ other anxiety disorder	2 (12.5%)	0 (0%)	

TABLE 3 | Warmth detection and heat pain thresholds and pain perception in patients and controls at T1 [reported as median (1st quartile−3rd quartile)].

	Patients	Controls	*p*-value (U)
WDT (°C)	33.17 (33.08–33.68)	33.20 (32.89–33.78)	0.637
HPT (°C)	45.63 (43.48–47.82)	45.58 (43.50–46.18)	0.692
Test temperature (according to VAS4) (°C)	45.80 (45.43–46.73)	46.15 (45.13–47.08)	0.763
Tonic pain rating	3.85 (2.23–5.60)	4.75 (4.18–5.38)	0.181
Placebo pain rating	4.60 (3.20–6.60)	4.95 (4.20–5.98)	0.692

nerve. Thus, patients with MUSS showed a significantly lowered reactivity of the parasympathetic branch of the ANS as compared with healthy controls.

This finding supports the assumption that decreased HRV could work as a surrogate marker for somatoform or "functional disorders" also in these patients as it has previously been discussed for other disorders (21–24). However, a major limitation of our study is the small sample size and a high number of dropouts and exclusions. There was also an imbalance of female to male participants that may weaken the results of the study; however, considering that MUS predominantly occur in female sex, it reflects the normal distribution of these patients (7, 12). The inconsistent level of significance in the evaluation of HRV data could be explained by small group sizes due to the difficult patient collective with many lost in follow-up between T0 and T1. To confirm the effect, studies with larger groups would be necessary.

The results of this pilot study give further insights into the close relationship between MUSS, psychiatric disorders, and the reduction of HRV. As shown in previous studies, depression and anxiety are related to a reduction in HRV independently as well as in combination with physical somatoform symptoms (37). Our results further support the assumption that MUSS add to the group of stress-related disorders involving a complex dysregulation of cognitive appraisal, emotional features, and biophysical symptoms with symptoms interacting and influencing one another.

Regardless of how advanced and subspecialized neurological diagnostics have become during the past decades, patients with different MUS are still a not clearly defined heterogenous group from the neurological as well as a psychosomatic or psychiatric point of view. However, our results indicate that it is possible to further differentiate this group by psychophysiological

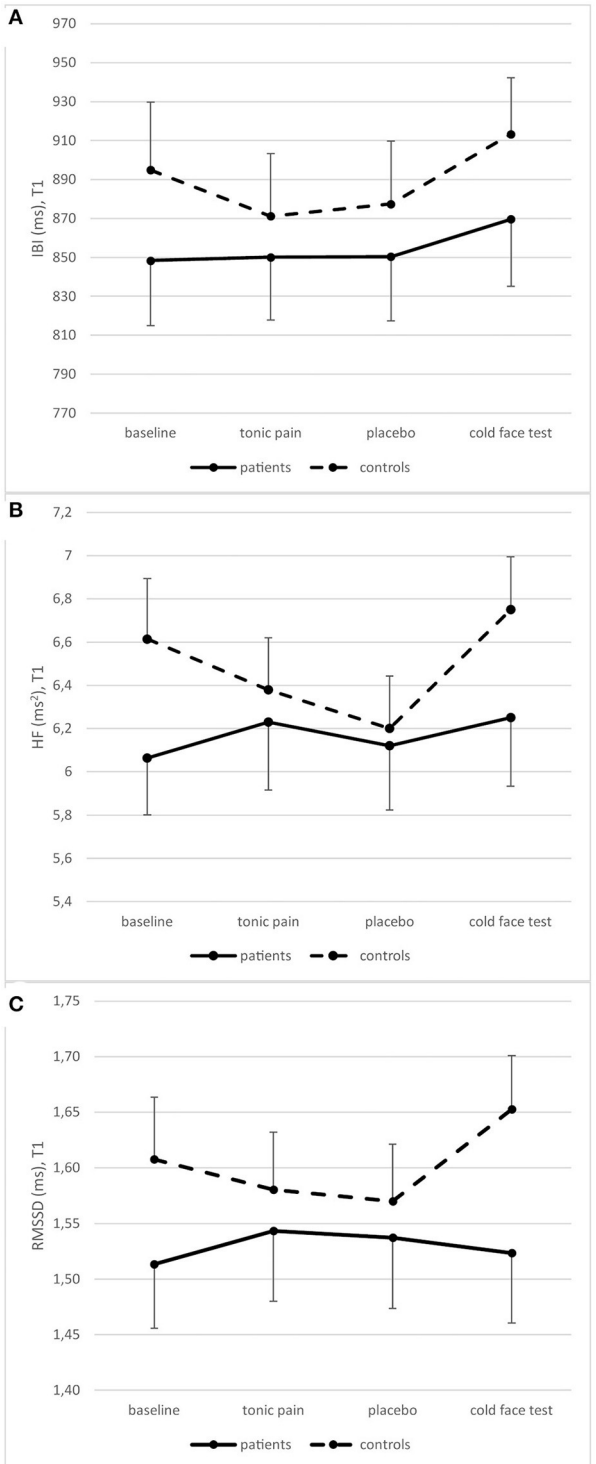

FIGURE 5 | Heart rate variability measured as IBI **(A)**, HF **(B)**, and RMSSD **(C)** in patients and controls at T1 (means and standard errors).

TABLE 4 | HRV parameters in patients and controls at T1 (reported as mean ± SD; results of *t*-tests).

	Patients	Controls	*p*-value
IBI baseline	848 ± 130	895 ± 135	0.345
IBI pain	850 ± 125	871 ± 124	0.646
IBI placebo	850 ± 128	877 ± 125	0.563
IBI cold-face test	870 ± 134	913 ± 112	0.342
RMSSD baseline	1.51 ± 0.22	1.61 ± 0.22	0.250
RMSSD pain	1.54 ± 0.24	1.58 ± 0.20	0.656
RMSSD placebo	1.54 ± 0.25	1.57 ± 0.20	0.692
RMSSD cold-face test	1.52 ± 0.24	1.65 ± 0.19	0.113
HF baseline	6.06 ± 1.01	6.61 ± 1.08	0.162
HF pain	6.23 ± 1.21	6.38 ± 0.93	0.707
HF placebo	6.12 ± 1.15	6.20 ± 0.94	0.836
HF cold-face test	6.25 ± 1.22	6.75 ± 0.95	0.222

criteria. Subdividing patients with MUS is of high scientific and clinical relevance and can be a first step toward a better diagnostic access to this difficult group of patients and gives way to potential individualized therapeutic approaches. Psychoeducation and techniques to improve HRV, such as HRV biofeedback (e.g., slow paced breathing), should get more into therapeutic focus for patients with MUS/MUSS in the future (38).

Even though this pilot study might be of little impact considering the small group size, it gives an impetus to consider that neurological patients with MUSS are not only characterized by the absence of another disorder. In our sample, the patients also showed characteristics that could be positively identified by standardized clinical interviews (e.g., depression, anxiety disorders) and physiological measures (reduction of HRV). It is of high practical relevance that treating physicians are sensitized for the psychophysical mechanisms underlying MUS, because it is crucial to identify these patients at an early stage of disease and direct them to a suitable therapeutic intervention in order to prevent chronification of symptoms and unnecessarily high healthcare utilization. To sharpen the view for underlying mechanisms of MUSS and answer the question if HRV analysis would be an eligible clinical tool in the diagnostics of patients with MUSS, larger-scaled studies are necessary.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the medical faculty of Tübingen University (Project No. 765/2015BO2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TF, PE, and KW contributed to the study design. NM, EL, MH, and KW assessed and analyzed the data. VR and KW did the literature search, created the figures, and wrote the first draft. All authors contributed to the data collection and interpretation of results, reviewed and critically revised the manuscript, and approved the final version for submission.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.713391/full#supplementary-material

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A Review on the Vagus Nerve and Autonomic Nervous System During Fetal Development: Searching for Critical Windows

Francesco Cerritelli[1], Martin G. Frasch[2], Marta C. Antonelli[3,4], Chiara Viglione[1], Stefano Vecchi[1], Marco Chiera[1] and Andrea Manzotti[1,5,6]*

[1] Research and Assistance for Infants to Support Experience Lab, Foundation Center for Osteopathic Medicine Collaboration, Pescara, Italy, [2] Department of Obstetrics and Gynecology and Center on Human Development and Disability, University of Washington, Seattle, WA, United States, [3] Facultad de Medicina, Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis", Universidad de Buenos Aires, Buenos Aires, Argentina, [4] Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany, [5] Department of Pediatrics, Division of Neonatology, "V. Buzzi" Children's Hospital, Azienda Socio-Sanitaria Territoriale Fatebenefratelli Sacco, Milan, Italy, [6] Research Department, Istituto Osteopatia Milano, Milan, Italy

The autonomic nervous system (ANS) is one of the main biological systems that regulates the body's physiology. Autonomic nervous system regulatory capacity begins before birth as the sympathetic and parasympathetic activity contributes significantly to the fetus' development. In particular, several studies have shown how vagus nerve is involved in many vital processes during fetal, perinatal, and postnatal life: from the regulation of inflammation through the anti-inflammatory cholinergic pathway, which may affect the functioning of each organ, to the production of hormones involved in bioenergetic metabolism. In addition, the vagus nerve has been recognized as the primary afferent pathway capable of transmitting information to the brain from every organ of the body. Therefore, this hypothesis paper aims to review the development of ANS during fetal and perinatal life, focusing particularly on the vagus nerve, to identify possible "critical windows" that could impact its maturation. These "critical windows" could help clinicians know when to monitor fetuses to effectively assess the developmental status of both ANS and specifically the vagus nerve. In addition, this paper will focus on which factors—i.e., fetal characteristics and behaviors, maternal lifestyle and pathologies, placental health and dysfunction, labor, incubator conditions, and drug exposure—may have an impact on the development of the vagus during the above-mentioned "critical window" and how. This analysis could help clinicians and stakeholders define precise guidelines for improving the management of fetuses and newborns, particularly to reduce the potential adverse environmental impacts on ANS development that may lead to persistent long-term consequences. Since the development of ANS and the vagus influence have been shown to be reflected in cardiac variability, this paper will rely in particular on studies using fetal heart rate variability (fHRV) to monitor the continued growth and health of both animal and human fetuses. In fact, fHRV is a non-invasive marker whose changes have been associated with ANS development, vagal modulation, systemic and neurological inflammatory reactions, and even fetal distress during labor.

Keywords: fetal development, autonomic nervous system, vagus nerve, cholinergic anti-inflammatory pathway, heart rate variability, critical window, maternal health

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***Correspondence:**
Marco Chiera
marco.chiera.90@gmail.com

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INTRODUCTION

The autonomic nervous system (ANS) is one of the main biological systems that regulates the body's physiology: in particular, the interaction between its two branches—the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS)—allows the organism to efficiently cope with endogenous and exogenous stressors (Goldstein, 2006; Bonaz et al., 2021).

Regarding the PNS, the vagus nerve has been recognized as a fundamental afferent pathway for sensing the milieu interior in peripheral tissues, thus allowing the organism to respond by activating complex networks involving the whole ANS, endocrine, metabolic, innate, and adaptive immune systems (Frasch et al., 2018b; Bonaz et al., 2021).

One of such responses, able to perform homeokinetic adjustments, is the cholinergic anti-inflammatory pathway (CAP). Briefly, through the vagus nerve, the brain receives information about the body's inflammatory milieu and, thus, activates neuroimmune responses to dampen the detected inflammation (Garzoni et al., 2013; Frasch, 2020; Bonaz et al., 2021). Very little is known, however, about the central representation, the information processing network, of the vagus nerve. An idea of neuroimmunological homunculus has been proposed and requires further research (Conway et al., 2006; Tracey, 2007; Diamond and Tracey, 2011; Frasch et al., 2018a; Castel et al., 2020).

The ANS regulatory capacity begins before birth as the sympathetic and parasympathetic activity contributes significantly to the fetus' development (Hoyer et al., 2017; Mulkey and du Plessis, 2018; Zizzo et al., 2020). Indeed, several studies have shown the vagus nerve is involved in many vital processes during fetal, perinatal, and postnatal life: from the regulation of inflammation through the CAP, which may affect each organ (Herry et al., 2019; Frasch, 2020), to the production of hormones involved in bioenergetic metabolism (Bystrova, 2009).

On the other hand, the ANS activity, as measured through heart rate variability (HRV)—a non-invasive biomarker evaluating the variability of the time intervals between successive heartbeats—is affected by the developmental and health conditions of both fetus and infant. Heart rate variability analysis can give paramount hints about the fetus' or newborn's well-being and allow clinicians to predict the occurrence of even deadly complications (**Table 1**) (Stone et al., 2013; Al-Shargabi et al., 2017; Massaro et al., 2017; Chiera et al., 2020; Frasch, 2020). Indeed, the first findings relating HRV alterations to fetal death, thus hinting to a potential predictive role of HRV, date back to the early sixties of the twentieth century (Hon and Lee, 1963).

Nonetheless, the importance of ANS and vagus nerve regulation in fetal and neonatal development is still underrepresented (Sadler, 2012; Moore et al., 2016). Moreover, except the third trimester (Mulkey and du Plessis, 2018, 2019), literature usually lacks an analysis of potential "critical windows"

TABLE 1 | Summary of the HRV metrics cited in the main text (Brändle et al., 2015; Massaro et al., 2017; Oliveira et al., 2019; Patural et al., 2019; Frasch et al., 2020a).

Parameter	Definition
TIME-DOMAIN	
SDNN	Standard deviation of NN intervals. Related to both SNS and PNS activity and equivalent to Poincaré SD2.
RMSSD	Root mean square of consecutive RR interval differences. Related to vagal modulation and equivalent to Poincaré SD1.
FREQUENCY-DOMAIN	
LF	Low-frequency band: 0.04–0.2 Hz for newborns and 0.04–0.15 Hz for infants. It can be expressed as LF peak, LF power or LFn (normalized power in relation to total power). Related to both sympathetic and vagal modulation and baroreceptor reflexes.
HF	High-frequency band: 0.20–2.00 Hz for newborns and 0.20–1.40 Hz for infants. It can be expressed as HF peak, HF power or HFn (normalized power in relation to total power). Related to both vagal modulation and respiratory cycle.
LF/HF	Ratio of LF-to-HF power. Related to both SNS and PNS activity.
TP	Total power of the electrocardiography spectrogram.
NON-LINEAR	
SD1	Poincaré plot standard deviation perpendicular to the line of identity. Equivalent to RMSSD.
SD2	Poincaré plot standard deviation along the line of identity. Equivalent to SDNN.
CSI	Cardiac Sympathetic Index (ratio of SD2-to-SD1).
HRV Index	Number of all RR intervals divided by the number of RR intervals at the highest point of the RR histogram.
Parseval Index	Ratio between the square root of the sum of LF and HF powers and the value of SDNN
PE	Permutation entropy.
DFA α1	Detrended fluctuation analysis, which describes short-term fluctuations. Usually used to assess brain injury or pathology severity.
DFA α2	Detrended fluctuation analysis, which describes long-term fluctuations. Usually used to assess brain injury or pathology severity.
RMS1	Root mean square from detrended fluctuation analysis, which describes short-term fluctuations. Usually used to assess brain injury or pathology severity.
RMS2	Root mean square from detrended fluctuation analysis, which describes long-term fluctuations. Usually used to assess brain injury or pathology severity.

Abbreviations: ABP, arterial blood pressure; ANS, autonomic nervous system; ASD, autism spectrum disorders; CAM, cardiac autonomic modulation; CAN, central autonomic network; CAP, cholinergic anti-inflammatory pathway; CNS, central nervous system; CRH, corticotropin-releasing hormone; CS, cesarean section; CTG, cardiotocography; ECG, electrocardiography; EEG, electroencephalography; EFM, electronic fetal monitoring; EMF, electromagnetic field; ENS, enteric nervous system; FASD, fetal alcohol spectrum disorders; fHR, fetal heart rate; fHRV, fetal heart rate variability; FM, fetal movement; GM, general movement; HIE, hypoxic-ischemic encephalopathy; HPA, hypothalamic-pituitary-adrenal; HR, heart rate; HRV, heart rate variability; NAS, neonatal abstinence syndrome; NC, neural crest; NEC, necrotizing enterocolitis; NOWS, neonatal opioid withdrawal syndrome; PAE, prenatal alcohol exposure; PGP, pelvic girdle pain; PNE, prenatal nicotine exposure; PNS, parasympathetic nervous system; REM, rapid eye movements; SDB, sleep-disordered breathing; sGC, synthetic glucocorticoid; SIDS, sudden infant death syndrome; SNS, sympathetic nervous system; sOT, synthetic oxytocin; SSRI, selective serotonin reuptake inhibitor; VAS, vibroacoustic stimulation; WGA, week gestational age; ZIKV, Zika virus.

in the perinatal period that may give information about ANS development. Defining such critical windows during gestation could help clinicians know exactly when to monitor fetuses to effectively assess the developmental status of both ANS and vagus nerve.

Indeed, several animal studies and even some human studies have shown that many factors (e.g., malnutrition, high level of stress, toxic exposure) during early, mid, and late gestation can impair brain and ANS development/regulation, thus inducing biological and behavioral alterations. Moreover, some studies have highlighted that the same factors can differently affect development based on the exposure period (i.e., early vs. late gestation) (Schulz, 2010; Gartstein and Skinner, 2018; Kasahara et al., 2020, 2021). As a consequence, by relying on appropriate animal models, several authors have defined potential critical windows related to human brain development (see, for instance, Figure 1 in Morrison et al., 2018).

Therefore, the present paper aims to review the fetal and perinatal development by focusing on the development of the ANS and, in particular, of the vagus nerve to identify possible critical windows that could impact their maturation. This paper will also focus on which factors—i.e., fetal characteristics and behaviors, maternal lifestyle and pathologies, placental health and dysfunction, labor, incubator conditions, and drug exposure—may affect the development of the vagus.

THE FETAL ANS DEVELOPMENT: THE SEARCH OF "CRITICAL WINDOWS"

As the ANS is controlled by several brain areas (e.g., amygdala, hypothalamus, insula, cingulate cortex, and several brainstem nuclei) that constitute the central autonomic network (CAN), paying attention to their development is fundamental for studying the fetal ANS. In the same way, due to the CAP, it is also relevant to review the development of the organs potentially connected to the vagus nerve (Sklerov et al., 2019; Frasch, 2020). As the vagus nerve affects the body metabolism in a pleiotropic manner, such a review could help better understand the networks underlying this effect and how to harness them for improving fetal and neonatal health (Castel et al., 2020).

Since this review focuses on the role of ANS in human fetuses and newborns, the discussed time spans regarding gestational age are related to human development. As aids for the readers, **Table 1** contains the meaning of the several HRV metrics cited in the following sections, whereas **Figure 1** and **Table 2** summarize the main findings of the current review.

A Review of ANS Development

The Development During the 1st Trimester

The nervous system differentiates from the ectoderm approximately around 3 weeks gestational age (WGA), when part of the ectoderm develops into the neural plate. At 4 WGA, neurulation occurs: the neural folds arise from the neural plate and begin to fuse in both cranial and caudal direction, thus forming the neural tube, that is, the future central nervous system (CNS). During neurulation, some neuroectodermal cells detach from the neural folds and differentiate into the neural crests (NCs), the specialized cells that will form the future ANS (Moore et al., 2016).

By 4 WGA, the forebrain, midbrain, and hindbrain (the primary brain vesicles) are already visible, as are the spinal ganglia that come from the NCs. In the next weeks, the nervous system rapidly grows: the five secondary brain vesicles, along with the four ventricles and the meninges form, while the glioblasts (the glial cells precursors) start to differentiate and migrate throughout the developing nervous system. The neural tube also begins to thin to form the central canal of the spinal cord (Moore et al., 2016).

During this period (4–8 WGA), from the NCs several derivatives arise: the dorsal root ganglia, the sympathetic ganglia, the renal, celiac, and intestinal ganglia and plexus, the afferent ganglia of cranial nerves, and the suprarenal medulla (Newbern, 2015; Moore et al., 2016). In particular, by 6 WGA, the first parasympathetic ganglia can be detected (Müller and O'Rahilly, 1989) and, by 10 WGA, the sympathetic trunks have appeared throughout the vertebral column (Kruepunga et al., 2021) and, apart from the colonic loop, the extrinsic innervation of the enteric nervous system (ENS) is complete (Kruepunga et al., 2020). Neural crest cells also contribute to spleen development (Barlow-Anacker et al., 2017). The spleen begins to develop at 5 WGA as a lobulated organ, appears clearly around 8 WGA, and initially functions as a hematopoietic center; then, during fetal development, it gradually loses both this role and its lobules (Moore et al., 2016; Hill, 2021a).

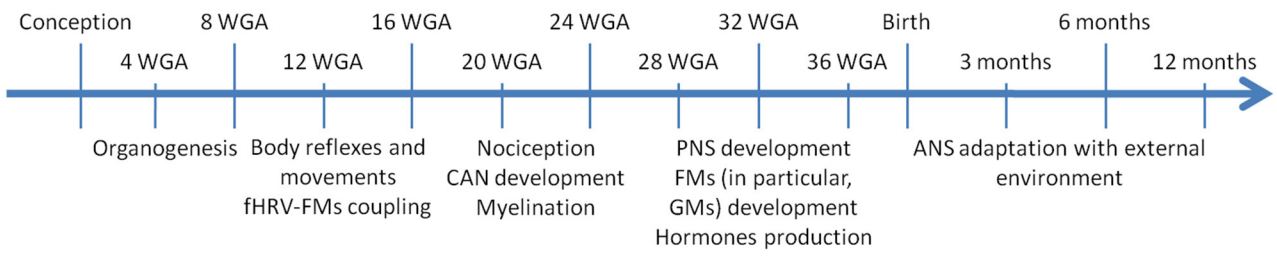

FIGURE 1 | Timeline of fetal and postnatal development that shows the main events related to ANS development in order to define the main critical windows clinicians should pay attention to. ANS, autonomic nervous system; CAN, central autonomic network; fHRV, fetal heart rate variability; FM, fetal movement; GM, general movement; PNS, parasympathetic nervous system; WGA, weeks gestational age.

TABLE 2 | Summary of the finding of the present review regarding ANS development and HRV.

	1st–2nd trimester	3rd trimester	Birth	Postnatal period
Physiological development	The onset of fetal breathing movements increases RMSSD and HF power and decreases LH/HF power. Variations are also observed for SDNN, SDNN/RMSSD, LFn, and HFn. As behavioral states become more recognizable, fHRV-FMs coupling increases. From the 2nd trimester, HRV metrics analysis can estimate fetal autonomic brain age.	Vagus nerve myelination and development of vagal control by the nucleus ambiguus. Appearance of short-term variability in frequency-domain HRV metrics (e.g., HF) and reduction of long-term variability metrics (e.g., LF). fHRV patterns after VAS stimulation (after 32 WGA) or transabdominal fetal stimulation with halogen light can be used to assess ANS and vagal development. As revealed by specific metrics (e.g., AsymI, SLDE α, dlmax, dlmean, pL, sgridAND), *in utero* hypoxia decreases short-term predictability of ifHRV and increases its long-range similarity.	Steep rise in PNS tone around 37–38 WGA. Right before birth, SNS outflow from CAN greatly increases to support the fetus during labor. Before and after birth, the vagus and CAP functionality can be assessed through specific HRV metrics (e.g., AsymI, DFA α1, KLPE, and SDLE α). These metrics can also predict whether fetuses may recover after surgery and whether inflammation is under control.	Several HRV metrics (e.g., SDNN, CSI, HFn, LFn, TP, SD2, HRV Index, and Parseval Index) increase after birth, with the PNS modulation that takes predominance (e.g., lower LF/HF and higher RMSSD and HF) during the following 2 years. Measuring HRV during different sleep states helps assess ANS development: quiet sleep is characterized by PNS predominance and active sleep is characterized by SNS predominance. Social interactions, including skin-to-skin contact, increase ANS maturation.
Environmental factors affecting ANS development	Sex-specific differences in the non-linear properties of HRV, especially in the weeks before birth. Lower fHR, altered Fhrv, and more fHR decelerations, which point out to impaired vagal development, sympatho-vagal balance, and neurodevelopment can be due to: • maternal stress; • maternal pain (through higher cytokine production); • maternal malnutrition; • maternal sleep disorders (which reduce fetal breathing movements); • maternal pathologies (chronic hypoxia, infections, autoimmune, metabolic, neuroendocrine, and mood disorders); • placental dysfunction (due to neuroendocrine, immune, and cardiovascular alterations). Many fHRV are reduced by nicotine and/or alcohol exposure, starting from the 1st trimester. These perturbations last after birth, given the fact that alcohol, in particular, impairs CAN development. Pathogens such as Zika virus can hinder the communication between CAN and the cardiac pacemaker cells. Extreme temperature variations alter cerebral blood flow and, thus, fHRV. Noise, light, pollution, and EMF can affect brain and ANS development. Among drugs: • sGCs transiently induce SNS suppression, but can affect neurodevelopment (e.g., altered R-R interval variability lasting until adulthood); • in the 1st trimester, ACE inhibitors may induce cardiovascular and CNS malformations; • in the 3rd trimester, SSRIs reduce FMs and fHRV reactivity after VAS; • opioids increase many fHRV metrics throughout pregnancy and impair FMs and fetal breathing movements in the 3rd trimester; • prolonged administration of MgSO4, especially with sGCs, reduce fetal breathing movements and affect fHRV dose-dependently. IUGR reduces FMs and increases HR decelerations.		sOT may alter vagal control since it can bind to neurons in the vagal dorsal motor nucleus. Full lateral position of the mother in case of epidural analgesia seems to change fHRV less than wedged supine position. A difficult labor is revealed by alterations in fHR and fHRV, in particular, in PNS-related metrics. Compared to CS, spontaneous labor without analgesia increases PNS-related metrics and decreases SNS-related ones. Difficult delivery or CS without labor may induce hypoxia and oxidative stress that can cause cell death in the ANS brainstem centers. Hyperactive uterine contractions, perinatal hypoxia, and fetal acidemia greatly increase RMSSD and induce HR decelerations due to hypotensive pattern of pathological arterial blood pressure and cardiovascular decompensation. Preterm birth or lack of labor reduce cortisol release, which may alter the CAP development.	Sudden increase in RMSSD could pinpoint to an acute inflammatory response. Neuroinflammation alters global ANS functioning as revealed by entropic HRV metrics. Sepsis, NEC, and other diseases, which can be predicted by HRV metrics such as RR intervals, sample asymmetry and SampEn, impair ANS regulation centers through cytokines. Brain injury affects ANS postnatal adaptation, with several DFA metrics (i.e., α1, α2, RMS1, and RMS2) associated with specific brain injuries. Postnatal complications induce lower ANS tone. The incubator may also affect HRV: • warm incubators tend to increase SNS tone and reduce PNS activity, whereas the opposite is true for incubators 2°C colder than newborns' temperature; • excessive noise (>45 dB) can alter ANS development; • excessive light due to incubators not properly covered can heighten SNS tone; • EMF coming from the incubator power increases LF/HF and decreases HF; • lack of sleep due to medical procedures can dampen vagal modulation; • prone position increases PNS-related HRV metrics but decreases short-term variability. Supine and side position seem to induce more stable HR and oxygenation.

(Continued)

TABLE 2 | Continued

	1st–2nd trimester	3rd trimester	Birth	Postnatal period
Consequences of ANS development disruption	Impairment in FMs and uncoupling between FMs and fHRV can reveal altered fetal sensorimotor capacities. Impaired vagal balance, as indexed by low PNS-related metrics (e.g., RMSSD), can induce alteration in stress response and inflammation regulation (impairment in CAP development). Impaired neurodevelopment and myelination and altered neurotransmitter signaling throughout the CNS. Lower non-linear HRV metrics reveal higher risk of morbidity and death during or after birth. Reduced fetal growth.		Several pathologic conditions: apnoea, hypoxemia, sleep disruption, decreased growth, immunity impairment, and neuroendocrine alterations. Impaired autonomic regulation and brain development revealed through DFA metrics, i.e., α1, α2, RMS1, and RMS2. Alteration in CAP development and difficulties in regulating postnatal inflammation (e.g., NEC) as revealed by lower RMSSD and altered non-linear metrics. Whereas low RMSSD, SDANN, and SDNN may reveal poor neurological development, it seems neurological complications (e.g., cerebral palsy) and behavioral alterations (e.g., autism) can be investigated (even predicted) by specific HRV patterns. HRV alteration (lower HR, SNS-, and PNS-related metrics) could also reveal risk of SIDS. Hypertension, metabolic impairment, immune disorders, depression, and behavioral disorders in infancy, adolescence, and adult life as revealed by HRV metrics too low or high compared to peers.	

TECHNICAL CONSIDERATIONS REGARDING HRV ANALYSES

The reproducibility of HRV properties in a given physiological setting depends on various factors that—due to their variety—are often neglected when comparisons and generalizations are made, such as:

1) The biosensors used to acquire the raw signal (e.g., PPG, BCG, ECG),
2) The sampling rate of the signal (for best precision in capturing HRV, we advise 1,000 Hz for the derivation of the raw RRI signal),
3) Pre-processing pipeline steps (preceding actual HRV computation):
   a) RRI → HRV computation window length, 5 min is the standard, but this number varies widely in publications;
   b) Sliding window settings impact smoothing HRV dynamics; when several 5 min segments are analyzed and the HRV values averaged, this can be non-overlapping or overlapping to variable degrees, usually 50% overlap;
   c) Other filter settings such as frequency bands chosen to reflect VLF, LF, and HF bands of HRV or embedding dimensions, time delays, and time scales for non-linear metrics.

ACE, angiotensin-converting enzyme; α1, a short-term fluctuations metric in detrended fluctuation analysis; α2, a long-term fluctuations metric in detrended fluctuation analysis; ANS, autonomic nervous system; AsymI, multiscale time irreversibility asymmetry index; BCG, ballistocardiogram; CAN, central autonomic network; CAP, cholinergic anti-inflammatory pathway; CNS, central nervous system; CS, cesarean section; CSI, cardiac sympathetic index; dlmax, max length of diagonal structures in recurrence quantification analysis; dlmean, average length of the diagonal structures in recurrence quantification analysis; ECG, electrocardiogram; DFA, detrended fluctuation analysis; EMF, electromagnetic field; fHR, fetal heart rate; fHRV, fetal heart rate variability; FM, fetal movement; GM, general movement; HF, high frequency; HFn, high frequency normalized power; HR, heart rate; HRV, heart rate variability; IUGR, intrauterine growth restriction; KLPE, Kullback-Leibler permutation entropy; LF, low frequency; LFn, high frequency normalized power; NEC, necrotizing enterocolitis; pL, percentage of laminarity; PNS, parasympathetic nervous system; PPG, photoplethysmogram; RMSSD, root mean square of consecutive RR interval differences; RMS1, root mean square from detrended fluctuation analysis; RMS2, root mean square from detrended fluctuation analysis; RRI, R-R interval; SampEN, sample entropy; SDANN, standard deviation of the average NN; SDLE α, scale-dependent Lyapunov exponent slope; SDNN, standard deviation of NN intervals; SD2, Poincaré plot standard deviation along the line of identity; sGC, synthetic glucocorticoid; SIDS, sudden infant death syndrome; SNS, sympathetic nervous system; sOT, synthetic oxytocin; SSRI, selective serotonin reuptake inhibitor; TP, total power; VAS, vibroacoustic stimulation; VLF, very-low frequency; WGA, weeks gestational age.

Among all the NC cells, the vagal NC cells are of particular interest due to their derivatives. Vagal NC cells emerge adjacent to the somites 1–7, rostral to the head NCs and caudal to the trunk and sacral ones, and are the ENS precursors (Hutchins et al., 2018; Simkin et al., 2019). But these cells also contribute to the formation of organs such as the heart (especially, the intrinsic cardiac nervous system), thyroid and parathyroids, thymus, lung, and pancreas ganglia. Vagal NC cells then give rise to the parasympathetic, sympathetic, and sensory ganglia (Hutchins et al., 2018; Simkin et al., 2019; Fedele and Brand, 2020).

Several animal studies in vertebrates and mammals show that vagal NC cells ablation heavily impairs the development and function of the aforementioned tissues. For example, neurons and glial cells in the pancreas do not appear and the thymus loses both its vasculature and its capacity of developing T-cells (Hutchins et al., 2018).

At 5–6 WGA, the 12 cranial nerves appear (Moore et al., 2016). As the parasympathetic system is constituted by the oculomotor, facial, glossopharyngeal, and vagus nerves, and since the trigeminal, facial, glossopharyngeal, spinal accessory, and vagus nerves lay the foundations for social interaction especially in newborns—these nerves are central to sensing environmental stimuli, including the human voice, and to carrying out behaviors such as head-turning, smiling, sucking, and swallowing—(Porges, 2011), the cranial nerves maturation may be paramount for an optimal ANS growth and, thus, for developing regulatory adaptive responses. Regarding the vagus nerve, at this stage, the dorsal motor nucleus is the predominantly active effector center (Porges, 2011).

At 4–6 WGA the primitive diaphragm appears (Hill, 2021d) and, by 7 WGA, the four chambers of the heart have formed, thus connecting the heart to the aorta and the pulmonary trunk. By 10 WGA, fetal circulation passively exchanges gas mainly with the placenta and breathing begins. As gestation proceeds, blood entering the pulmonary circulation increases (Morton and Brodsky, 2016; Tan and Lewandowski, 2020).

From 7 to 8 WGA gray and white matter differentiate and, by the end of the 1st trimester, after the embryo has become a fetus, it is possible to recognize all the main brain structures, including the choroid plexus, thalamus, hypothalamus, amygdala, basal ganglia, corpus callosum, brainstem nuclei, cerebellum, and the neural plate of the insula (Moore et al., 2016; Hill, 2021b). Around the transition between the 1st and 2nd trimester, even the circumventricular organs, in particular, the area postrema, appear: they are organs devoid of the brain-blood barrier that, hence, allow the brain to be directed informed about the biochemical status of the internal milieu (Gokozan et al., 2016).

Interestingly, at 6 WGA, the embryo begins to show spontaneous movements and can "respond" to touch and light, as the motor spinal nerve fibers have appeared around 4 WGA (Moore et al., 2016): indeed, at 8 WGA the embryo is capable of activating a spinal reflex in response to touch, and between 7 and 11 WGA all facial reflexes have developed (Borsani et al., 2019). At 10 WGA the corticospinal connectivity begins to develop, together with an increase in synaptogenesis, and, as a result, body reflexes in the upper limbs start to appear (Borsani et al., 2019). Since fetal movements (FMs) tend to synchronize with fetal heart rate variability (fHRV), their detection can give important insights on ANS development, even in the 1st trimester (DiPietro et al., 2007; Zizzo et al., 2020). From 12 to 13 WGA, fHRV-FMs coupling becomes heavily affected by external factors: therefore, its analysis could shed more light on how the fetus is affected by maternal and environmental factors (DiPietro et al., 2015).

During this period, yawning develops (Borsani et al., 2019). Since yawning has been related to thermoregulatory behaviors and the ability of "switching" between arousal and rest states, both of which are strictly dependent on ANS functionality, the detection of yawning at this early age could represent a useful indicator of ANS development or, as suggested, brainstem functionality (Walusinski, 2006, 2010, 2014; Gallup and Eldakar, 2013). This hypothesis could be reinforced by the evidence that yawning seems to rely on interoception, the inner sense that constantly monitors the internal milieu and for which the vagus nerve plays a central role, and to be processed by the CAN (in particular, insula, hypothalamus, locus coeruleus, and reticular activating system) (Walusinski, 2006). Lastly, yawning amount and duration seem to correlate with neuronal development in mammals (Gallup et al., 2016).

The Development During the 2nd Trimester

At the beginning of the 2nd trimester, the cerebral cortex creates more and more sulci and gyri to increase its surface area and allow brain development. In particular, the subplate (a central hub for cortical connectivity) becomes visible and begins to receive connection from the thalamus, basal forebrain, and brainstem. Around 17–19 WGA, the cerebellum development can be assessed through magnetic resonance imaging (Hill, 2021d), the corticospinal tracts toward the whole body appear, and at 20 WGA the spinothalamic tract is completed (Borsani et al., 2019).

At 18–19 WGA, nociceptive neurons and the typical hormonal response to pain appear (Borsani et al., 2019; Chen et al., 2021), thus hinting that primitive pain perception might begin at this stage. Although thalamocortical connections, which strongly increase around 20–22 WGA, are usually required for pain experience and the hemodynamic-behavioral responses to nociceptive stimuli are usually detected around 26 WGA (Verriotis et al., 2016; Chen et al., 2021), several authors argue that fetuses could feel pain at least from 12 WGA, as the first superficial thalamic-subplate connections seem to be functionally equivalent to the later thalamocortical ones (Derbyshire and Bockmann, 2020).

Around 20 WGA and for the rest of the 2nd trimester, myelination occurs (Borsani et al., 2019). From the NCs, neurilemma cells arise and form the myelin sheaths that wrap both the axons of somatic motor neurons and the preganglionic autonomic motor neurons (Moore et al., 2016). Also, fetal microglia from the bone marrow invade the CNS, while gray and white brain matters keep on developing: the cerebral lobes with all their sulci and gyri, cortexes such as the insula, the cerebellum, the pons, and many other structures increasingly resemble their adult counterparts (Moore et al., 2016). From 17 to 24 WGA, the spleen almost completely develops its reticular

framework (Satoh et al., 2009; Hill, 2021a). Regarding the ANS, brainstem, and hypothalamic centers form several connections with the other CAN structures, that is, insula, anterior cingulate gyrus, hippocampus, hypothalamus, and amygdala (Mulkey and du Plessis, 2018; Schlatterer et al., 2021).

During the 2nd trimester, the body becomes covered with lanugo, a fine downy hair that plays a paramount role in neurodevelopment. Indeed, through lanugo, the flow of amniotic fluid and FMs stimulate the low-threshold unmyelinated C afferent fibers (Bystrova, 2009), which have been found to be essential for both nociception and interoception. This stimulation reaches the brainstem, in particular the nucleus of the solitary tract—the main afferent nucleus of the vagus nerve—but also other nuclei including the parabrachial ones, and then more cortical structures such as the hypothalamic paraventricular nucleus and the posterior insular cortex. As a result, several motor and neuroendocrine responses are induced: for instance, gastrointestinal and anabolic hormones such as cholecystokinin, insulin, and insulin-like growth factor-1 are released. On the other hand, the hypothalamic-pituitary-adrenals (HPA) axis and vagal activation become more finely tuned (Bystrova, 2009).

Regarding sensorimotor development, around 15 WGA a complex set of movements (e.g., breathing movements, general body movements, isolated limb, head, and neck movements, startle and twitch movements, and swallowing) are available, whereas, by 18 WGA, stable handedness can be detected. Then, as gestation progresses, the amount of movement, both spontaneous and in response to vibroacoustic stimuli, gradually increases: indeed, mothers begin to feel their babies' movements (Moore et al., 2016; Borsani et al., 2019). At about 18–20 WGA, rapid eye movements (REM) can be detected (Borsani et al., 2019) as well as quiet and active sleeping states (Dereymaeker et al., 2017), although fetal behavioral states do not show particular coherence with ANS tone (Brändle et al., 2015).

As several vital systems are forming around the end of the 2nd trimester (e.g., alveolar ductal development and surfactant secretion begin at 24 WGA), babies born prematurely before 26 WGA have a higher chance of dying or developing neurodevelopmental disability (Moore et al., 2016; Morton and Brodsky, 2016; Hill, 2021c). Nevertheless, neonatal intensive care units have improved so much in the years that babies born as early as 20–22 weeks can survive if properly treated (Moore et al., 2016).

It is worth noting that, from the 2nd trimester, HRV metrics can successfully estimate fetal autonomic brain age (Hoyer et al., 2012, 2013b, 2015, 2017, 2019). Interestingly, gestational age can also be precisely estimated by measuring the opening and closing time of fetal cardiac valves (Marzbanrad et al., 2017), finding that could highlight the contribution of intrinsic fHRV or, at least, heart activity, to global HRV.

The Development During the 3rd Trimester

At 26–29 WGA, fetuses become able to efficiently control body temperature (Moore et al., 2016). By 29 WGA, all the major corticospinal, spinothalamic, and spinocortical tracts as well their myelination are completed, whereas from 24 WGA functional connectivity patterns in the fetal brain are recognizable. Indeed, at 32 WGA the cerebral cortex resembles the adult one and, in the next week, synchronicity between the hemispheres develops, as well as complex connections among several brain areas and networks (Moore et al., 2016; Borsani et al., 2019). As the 3rd trimester advances, specific nociceptive event-related potentials in the brain increasingly develop until 35–37 WGA (Verriotis et al., 2016).

From 25 WGA until birth, the vagus nerve myelination, the vagal control by the nucleus ambiguus, and, thus, the PNS rapidly develop, as testified by the appearance of short-term variability in frequency-domain HRV metrics (e.g., HF) and the proportional reduction of long-term variability metrics (e.g., LF), more related to SNS and baroreflex functions (Mulkey and du Plessis, 2018, 2019; Schlatterer et al., 2021). In particular, a steep rise in PNS tone can be detected around 37–38 WGA and basal fetal cardiovascular function becomes more influenced by the PNS. Nevertheless, from 30 WGA, cortisol, thyroxine, and catecholamines levels rise to prepare the fetus for birth. In the case of preterm birth, the ANS remains predominantly affected by both the SNS and the primitive vagal dorsal motor nucleus, with consequences on the stress response regulation toward environmental stimuli (e.g., excessive tachycardia or bradycardia) (Morton and Brodsky, 2016; Mulkey et al., 2018; Mulkey and du Plessis, 2019).

Lastly, whereas general movements (GMs) tend to increase until 32 WGA and then decrease as birth approaches, facial movements increase more and more (Borsani et al., 2019). During the 3rd trimester, distinct fetal behavioral states (i.e., quiet sleep, active sleep, quiet awake, and active awake) become increasingly recognizable through the analysis of heart rate (HR), eye movement, and GMs, as corticothalamic and brainstem areas develop more complex interactions (Brändle et al., 2015; Borsani et al., 2019). Interestingly, the analysis of specific HRV metrics, for instance, SDNN (more related to global neuroautonomic regulation), RMSSD (more related to vagal modulation), and PE, can help identify the actual behavioral state and, therefore, detect potential problems in neurodevelopment whether incoherence between HRV and body movements is detected (Schneider et al., 2008; Brändle et al., 2015).

The Development During Labor

Birth, as the transition from fetal to neonatal physiology, is a major challenge for the fetus and requires an ANS able to efficiently adapt the cardiovascular, respiratory, thermoregulatory, and metabolic (e.g., glycemic regulation) systems to the new environment. For this reason, before birth neuroendocrine signaling (e.g., catecholamines, cortisol, thyroid hormones, and renin-angiotensin) surges, and SNS outflow from CAN (e.g., hypothalamus, cortical, and brainstem centers) greatly increases. During labor, however, the nervous system is highly vulnerable to injuries due to hypoxia, energy deprivation, or oxidative stress (Morton and Brodsky, 2016; Mulkey and du Plessis, 2018; Mulkey et al., 2021).

Compared to cesarean section (CS), spontaneous labor without analgesia seems to improve cardiovagal modulation as shown by HF increase and LF decrease, despite several HRV metrics tend to increase in the postnatal days regardless

of delivery mode (Kozar et al., 2018). A well-developed ANS responds efficiently to umbilical cord clamping by inducing the fetus to commence spontaneous breathing. Otherwise, especially in case of a difficult delivery or CS without labor, the fetus may experience hypoxia and have difficulties in secreting pulmonary surfactant, thus developing respiratory pathologies that can backfire, in particular, on ANS brainstem centers through oxygen deprivation and hinder autonomic regulation (Hillman et al., 2012; Morton and Brodsky, 2016; Mulkey and du Plessis, 2018).

It is noteworthy that all the adaptations required to pass from intrauterine to extrauterine life seem to rely on cortisol, whose release is markedly reduced in case of preterm birth or lack of labor (Hillman et al., 2012; Morton and Brodsky, 2016). Since cortisol is an efferent agent in anti-inflammatory neural reflexes like the CAP (Bonaz et al., 2021), its reduced production may alter the CAP development itself. Indeed, low cortisol levels could fail in regulating postnatal inflammation, e.g., necrotizing enterocolitis (NEC), which could induce cell death in the vagal brainstem centers (Fritze et al., 2014).

The Development in the Postnatal Life

After labor, pulmonary vascular resistance decreases compared to systemic vascular resistance as lungs inflate and fetal circulation becomes active (Tan and Lewandowski, 2020), whereas the nervous system integrates sensory, motor, and regulatory functions in more complex behaviors (Moore et al., 2016). In particular, centers that are paramount to neurotransmitters (e.g. noradrenaline) biosynthesis and autonomic functions (e.g., temperature, breathing, and satiety regulation) including the area postrema greatly develop around birth (Gokozan et al., 2016).

As revealed by HRV analysis, the ANS constantly develops. Indeed, several metrics related to both SNS and PNS (e.g., SDNN, CSI, HFn, LFn, TP, SD2, HRV Index, and Parseval Index) change right after birth and in the following months, with the PNS modulation that takes predominance (e.g., lower LF/HF and higher RMSSD and HF) during the following 2 years (Oliveira et al., 2019; Patural et al., 2019) to better regulate the increasingly social interactions the infant encounters (Porges, 2011).

It is worth remembering that the two ANS branches are anything but antagonists: in fact, the regulation of many organic functions (Goldstein, 2006), including the immune and inflammatory responses (Bonaz et al., 2021), strictly depends on the synergy between the two branches. Should one of them be damaged, the other one alone could not regulate the body's homeostasis efficiently anymore.

At birth, the higher cortical networks controlling the ANS increase their connectivity (Schlatterer et al., 2021) and, in low-risk newborns at around 1 day of age, coherence between electrocortical activity and brainstem-mediated autonomic tone, especially parasympathetic, can be easily detected (Mulkey et al., 2021). Social interactions, including skin-to-skin contact between mother and newborns, help increase ANS maturation as well as stress responsivity and circadian rhythms, in particular in preterm infants (Ulmer Yaniv et al., 2021).

As newborns spend most of their time sleeping, ANS development correlates also with sleep development, with quiet sleep (absence of movements and slow rhythmic breathing) characterized by PNS predominance and active sleep (myoclonic twitching, facial, eye, and head movements, and irregular heart and breath rates) characterized by SNS predominance. Hence, measuring HRV during different sleep states can help better assess ANS development (Yiallourou et al., 2012; Dereymaeker et al., 2017).

How Disruptions in "Critical Windows" Can Affect ANS Development

In the previous section, we described fetal development with particular attention to ANS, CAN, sensorimotor development, and some organs. We have shown how alterations in CAN activity and sensorimotor behaviors can give hints about the developing ANS function. Similar hints can also be gained by monitoring organ development due to the research regarding the CAP and the systemic interactions of the vagus nerve (Bonaz et al., 2021).

However, the intrinsic spatiotemporal complexity of ANS development and the complex species differences in the timelines of neural development from which inferences are often drawn to human development make it difficult to identify specific or exclusive critical windows, although we might point to particular periods: 4–8 WGA, due to organogenesis; 12 WGA, due to subplate and fHRV-FMs coupling appearance; 18–24 WGA, due to nociception, CAN, and spleen development and myelination; the 3rd trimester, due to PNS development and change in FMs and hormones production; and birth, due to ANS adaptation to interact directly with the external environment.

Therefore, the next two sections will discuss short-term and long-term consequences of disruptions in ANS development.

Short-Term Consequences

Fetal or birth complications affecting ANS development may have consequences on organic functions and mortality right after birth and in the following months.

Autonomic nervous system impairment occurring *in utero* due to congenital heart disease, chronic hypoxemia, fetal growth restriction, acidemia, the trial of labor, bacterial, and viral infections or maternal factors can lead to labor complications (even death) and prematurity (Mulkey and du Plessis, 2018). Since the ANS requires 37 weeks *in utero* to fully develop, preterm newborns can experience difficulties in regulating their internal milieu in the face of environmental factors (Praud et al., 2017). Moreover, preterm birth often involves invasive treatments (e.g., invasive respiratory support) or drugs that can stress even more the immature ANS. Indeed, ANS responsiveness seems to remain quite low even when preterm infants reach term-equivalent ages compared to full-term newborns (Mulkey et al., 2018; Patural et al., 2019; Schlatterer et al., 2021).

Health conditions may, however, affect postnatal ANS maturation more than gestational age at birth: in the 3 months after birth, infants with a higher number of postnatal complications (e.g., infections, patent ductus arteriosus requiring treatment, mechanical ventilation) show a lower ANS tone measured through DFA metrics, i.e., $\alpha 1$, $\alpha 2$, RMS1, and RMS2. Whereas, $\alpha 1$ differs already at birth, the other metrics show different trajectories after 30–40 days, hinting at different ANS

development due to the experienced postnatal comorbidities (Schlatterer et al., 2021). Asymptomatic toddlers born to mothers infected with Zika virus (ZIKV) during pregnancy, show alterations in their non-linear HRV measures reflecting chaotic behavior and recurrence plot properties. These changes were suggested to result from the effects of chronic hypoxia—which ZIKV infection is known to cause—on the interactions between the ANS and the cardiac pacemaker cell development which generates intrinsic HRV (Frasch et al., 2020a; Herry et al., 2021).

It is widely known that comorbidities such as NEC or hypoxic-ischemic encephalopathy (HIE) can negatively affect ANS (Schlatterer et al., 2021). For instance, stroke can easily alter both sympathetic and parasympathetic tone, thus impairing the ANS regulatory capacities (Reich et al., 2019). Specific kinds of brain injuries seem also to specifically affect ANS: for instance, DFA revealed that white matter injury, watershed stroke, basal ganglia injury, or global injury can show characteristic HRV signatures (Metzler et al., 2017). Prematurity increases the risk of these adverse events (Mulkey and du Plessis, 2018). Fetal gut injury due to inflammation in a fetal sheep model of NEC etiology leaves a specific HRV signature and preterm neonates show abnormalities in HRV 1–3 days before clinical symptoms (Stone et al., 2013; Liu et al., 2016).

However, we have yet to fully understand ANS development, even after birth. Despite knowing that the CAN coordinates autonomic function and that left and right hemispheres modulate PNS and SNS tone, respectively (Mulkey et al., 2021), we have conflicting results on how the brain precisely controls ANS in newborns. For instance, whereas hypoxic-ischemic injuries in the right hemisphere seem to depress SNS tone, vaso-occlusive strokes in the same hemisphere appear to increase SNS tone. Whereas, vaso-occlusive strokes in the left hemisphere seem to increase PNS tone, hypoxic-ischemic injuries seem to have no effect on PNS tone. In some cases, insular injuries do not significantly change ANS tone; or, despite the effects on ANS tone, systemic hemodynamic meters (e.g., diastolic and systolic blood pressure) do not seem affected by brain injuries (Schneebaum Sender et al., 2017; Reich et al., 2019).

Many postnatal pathologies—e.g., hyperbilirubinemia, congenital heart disease, respiratory distress syndrome, respiratory syncytial virus, NEC, and sepsis—can lead to ANS impairment, probably due to inflammatory cytokines and toxins that can bypass the brain-blood barrier and induce neural death in areas paramount to ANS control (Fritze et al., 2014; Al-Shargabi et al., 2017; Bonaz et al., 2021).

As pathologies can affect ANS development, altered ANS function can make newborns more vulnerable to those same pathologies, due to failure in activating efficient anti-inflammatory reflexes including the CAP (Yiallourou et al., 2012; Mulkey et al., 2018; Bonaz et al., 2021). Indeed, dysfunctional CAP can fail in regulating inflammation, thus letting the immune system go unchecked and free to induce tissue damage, shock, and even death, whereas, on the other hand, a functional CAP can prevent microglia activation, thus showing a neuroprotective effect (Garzoni et al., 2013; Frasch et al., 2016).

Autonomic nervous system impairment, maybe through impaired cardiocirculatory and ventilatory regulation, can increase the risk of apparent life-threatening events or sudden infant death syndrome (SIDS) and, thus, of death in the first 6–12 months of postnatal life. Indeed, newborns that died from SIDS seemed to have brainstem and cerebellar impairments and to fail in arousing from sleep, which could highlight the importance of assessing HRV development during pregnancy (Yiallourou et al., 2012; Horne, 2018; Mulkey et al., 2018).

It is interesting that fetal ANS impairment can also have consequences on infant temperament at both 3 and 6 months (Howland et al., 2020).

Long-Term Consequences

Autonomic nervous system impairment may have consequences on the global development that persist during infancy, adolescence, and adult life.

Although preterm infants may reach good ANS maturation after 2–3 years (De Rogalski Landrot et al., 2007), ANS impairment tends to remain even later in life and to be accompanied by SNS and HPA regulation impairment, both during wakefulness and sleep (Haraldsdottir et al., 2018; Urfer-Maurer et al., 2018).

Autonomic nervous system dysfunction in the first year of life could have consequences on cardiovascular regulation even during adolescence, which makes paramount monitoring ANS development in the 1st and 2nd year to discover infants at risk. Indeed, cardiovascular regulation impairment could lead to higher mortality due to cardiovascular events (Patural et al., 2019). An immature ANS due to inflammation, prematurity, or fetal growth restriction can also lead to other pathologies such as type 2 diabetes (Mulkey and du Plessis, 2018). These and other metabolic or immune pathologies that could arise later in life might have their origin in alteration of CAP functioning, that is, in inflammation regulation by the ANS (Pavlov and Tracey, 2012; Burgio, 2015; Bonaz et al., 2021). Furthermore, ANS alterations in newborns can lead to neurological dysfunctions including cerebral palsy at 2 years of age (Dimitrijević et al., 2016).

Since ANS development is coupled with FMs development, ANS alterations can heavily affect CNS and neurobehavioral growth: on the one hand, FMs are usually considered the base for the fight-or-flight response, thus influencing the stress response (Schmidt et al., 2014); on the other hand, ANS immaturity correlates with altered cerebral and psychomotor development (Hoyer et al., 2014; Schmidt et al., 2014; Schneider et al., 2018), and even with altered cognitive, language, playing, and social skills (Doussard-Roosevelt et al., 1997, 2001; Bornstein et al., 2002; DiPietro et al., 2007; Siddiqui et al., 2017).

Autonomic nervous system immaturity due to fetal or postnatal complications (e.g., maternal pathologies, birth difficulties) could then impair the whole brain development and, therefore, behavior, stress response, and mood regulation, with negative consequences—even serious neurological or psychological pathologies—in infants, adolescent, and adult life (Mulkey et al., 2018; Mulkey and du Plessis, 2019). Even painful procedures during hospitalization seem to induce alteration in stress regulation and brain development in newborns (Holsti et al., 2006). Maternal stress during pregnancy alters fetal ANS by entraining fetal heart rate (fHR) with maternal HR decelerations

during respiratory effort (Lobmaier et al., 2020). Eight-years old children with autism spectrum disorders (ASD), depression or conduct disorder showed, respectively, distinct signatures of HRV compared to controls and it has been proposed that, at least for ASD etiology, such abnormalities in HRV may be detected as early as *in utero* where they may be induced by asymptomatic inflammatory events altering ANS, among other systems (Frasch et al., 2019, 2021).

Due to the negative consequences ANS impairment can have later in life, it is paramount to rely on any protective factor that can sustain ANS maturation, starting from taking care of maternal health and lifestyle, which can greatly affect fetal development and, then, breastfeeding. In the same way, stress relief during pregnancy, skin-to-skin contact, and social interactions between newborns and parents/caregivers can help newborns tune their ANS, reduce stress, increase resilience, and improve their neural plasticity (Horne, 2018; Mulkey and du Plessis, 2018; Manzotti et al., 2019; Antonelli et al., 2021).

THE ENVIRONMENTAL FACTORS AFFECTING THE ANS DEVELOPMENT

Whereas, in the previous section we described the fetal development in relation to ANS maturation, showing potential short-term and long-term alteration in case something should negatively affect pregnancy or labor, this section will now detail what factors can alter ANS development and in which ways.

Prenatal Life Influence on the ANS

Fetal Factors

Incorrect Fetal Movements

Around 8–9 WGA, it is possible to clearly record GMs (Prechtl, 1990). General movements analysis can differentiate specific movement patterns, concerning a specific part of the fetal body, from non-specific movement patterns, involving more than one body segment (de Vries et al., 1982, 1985). General movements quality, as well as their variability and modulation in intensity and velocity, could be valid indicators of fetal neurodevelopment, in particular concerning supra-spinal control on motor activity (Moreira Neto and Porovic, 2018). Different methods could be used to evaluate the quantity and quality of FMs, such as mother perception, acceleration, and variability at cardiotocography (CTG), real-time ultrasound, myography, actocardiography (Zizzo et al., 2020), and the Non-stress Test, which relates FMs to fHR accelerations (Bryant et al., 2020).

Both gross motor activity and breathing FMs, particularly during early gestation, represent an important evaluative parameter for fetal neurobehavior and to predict future neurodevelopmental disorders. Indeed, respiratory sinus arrhythmia appears to be strictly related and modulated by efferent vagal activity. Fetal motor activity, therefore, results closely tied to breathing and cardiovascular patterns, which are modulated by the ANS (Zizzo et al., 2020).

According to a recent systematic analysis, FMs can affect the ANS as revealed through modifications in the time-domain and frequency-domain fHRV metrics. The onset of fetal breathing movements, in particular, induces a variation in the main vagal nerve activity parameters: RMSSD and HF power increase, while LH/HF power decreases. Variations are also observed for SDNN, SDNN/RMSSD, LFn, and HFn (Zizzo et al., 2020). In this sense, monitoring the quality and the evolutionary pattern of FMs could reveal useful insight regarding ANS, in particular, vagal maturation.

Fetal movements patterns could be limited as a consequence of an inappropriate ratio between fetal and uterine dimension (Shea et al., 2015; Verbruggen et al., 2018), as well as a consequence of an inadequate amount of amniotic fluid (Sival et al., 1990). Since we have seen that amniotic flow and fetal growth restriction can affect vagal development, their effects could then be mediated by FMs. Indeed, fetuses with intrauterine growth restriction (IUGR) frequently present an oxygenation impairment, with a decrease of breathing movements and GMs, and an increasing number of HR decelerations (Bekedam et al., 1991).

Fetal motor capacities, through the implied neuronal activity, is paramount for differentiation, migration, synaptogenesis, and development of neuronal networks. Environmental stimuli, *per se*, induce motor reactions, as the sensory system maturation proceeds. General movements allow fetuses to experience their bodies and the surrounding space, hence exploring the consequences their movements have on their bodies and environment (Fagard et al., 2018). In summary, the FMs make it possible to start developing a primitive body map. The repetitive movements, with the consequent repetitive sensory afferents induced, produce habituation in the fetus, that is manifested also in fHR changes: the ANS trains to adapt the body systems' response to environmental stimuli, even through the movements (Lecanuet et al., 1992; Kisilevsky and Low, 1998).

It is therefore legitimate to argue that a decreased possibility/capacity to express movements by the fetus, that is related to an impairment of the sensory stimulation, could interfere with the normal neurodevelopmental pattern of ANS and vagus nerve itself.

Maternal perception of FMs, in conclusion, is one of the factors that could influence in a relevant way the emotional state of the mother. Despite the fact that there are contrasting results about the sensitivity of fetal activity reported by the mother (Sorokin et al., 1982; Schmidt et al., 1984), it appears that there is an association between the increase of force and frequency of perceived movements and a reduced risk of stillbirth and negative outcomes (Heazell et al., 2018). In some cases, the healthcare providers recommend around 28 WGA the "count to 10" method: the mother counts the number of FMs every day, at the same hour, verifying that they occur not $<$10 times in a 2–3-h period (Bryant et al., 2020). Moreover, it seems that monitoring FMs could contribute to creating a primitive maternal–child bonding, even before birth (Flenady et al., 2019). Diminished perception of this motor activity, could therefore be worrisome for the mother, leading to an increased amount of stress, which in turn may have an impact on fetal neurodevelopment (Bryant et al., 2020).

Fetuses' Sex

A variety of different ANS and vagus nerve pattern activities are now well-established between different sexes, which can

be well-represented by HRV tracking even during fetal life. Regarding vagal activity in fetuses, not all the authors agree in observing significative sex-related differences (Oguch and Steer, 1998; DiPietro et al., 2004; Lange et al., 2005; Bhide and Acharya, 2018), even when the same methodologies were used. The discrepancy between these studies may be partly due to the different experimental variables.

Male fetuses show a more reactive ANS and less complexity in control systems than female ones, with a more linear and significantly less complex fHR activity (Bernardes et al., 2008). Moreover, an exploratory study conducted on twins, highlighted that sex-specific differences in fetuses can be noticed both in linear and non-linear fHR metrics. This seems to be significantly influenced also by the combination between twins of different sexes, both in intrapair average and absolute differences (Tendais et al., 2015).

The regulatory patterns of sympathovagal balance show sex-specific differences as pregnancy progresses (Buss et al., 2009). During the 1st trimester, there are no significant differences in HRV track between male and female fetuses (McKenna et al., 2006). Both sexes demonstrate an evolutionary pattern with increased entropy indexes until 34 WGA, with a slight decrease after 37 WGA. With the passing of the weeks, however, sex-specific differences in the sympathovagal balance start to be detected. From 34th WGA, female fetuses show indeed a higher mean fHR and entropy, as well as a lower short-term variability and sympathovagal balance, compared to the males (Gonçalves et al., 2017). This could suggest a different developmental pattern of the vagus nerve, both sex- and time-specific.

In this sense, hormones may play a crucial etiological role, as literature shows that gonadal hormones have a significant impact on the sex-dependent differentiation of the entire nervous system (Chung and Auger, 2013). During pregnancy, the maturation pattern of fetal gonads must be taken into account. In the first weeks, these glands are undifferentiated between male and female fetuses and complete the cellular differentiation process around the 44th post-conceptional day (Baker and Scrimgeour, 1980). The production of testosterone in male fetuses begins during the 2nd month of pregnancy and reaches the maximum in the 2nd trimester (Chung and Auger, 2013), just before when vagal tone starts to rise (Mulkey and du Plessis, 2019).

The different patterns of ANS development could also be due both to a different sex-specific susceptibility to environmental stimuli and a different neurodevelopmental trajectory of male and female fetuses, which could lead to sex-specific developmental intervals of maximum susceptibility to environmental exposures (Buss et al., 2009; Bale, 2016).

Moreover, considering the role of the vagus nerve in the CAP, which involves immune cells such as macrophages (Borovikova et al., 2000; Tracey, 2007; Fairchild et al., 2010; Frasch et al., 2016), and the sex-related neurodevelopment impact of microglial functions (Hanamsagar and Bilbo, 2016), the different characteristics of the developmental pattern of vagus nerve between male and female fetuses could predispose for sex-specific susceptibility and outcomes in many neurodevelopmental disorders (Berntson and Cacioppo, 2004).

Maternal Factors

Maternal Distress

The definition of stress has evolved through time, but it is now accepted that a stressor is an uncontrollable environmental demand that exceeds the physiological imbalance of the organism (Selye, 1950; Koolhaas et al., 2011). Several uncontrollable situations such as natural disasters, relational or financial problems, bereavement, and/or stressful daily hassles can threaten a woman's life during her pregnancy. These situations increase the risk of impairment of fetal brain development resulting in emotional, behavioral, and/or cognitive problems in later life (Glover, 2015; Antonelli et al., 2021).

Historically, research findings oriented to understand the mediators that underlie the basis of fetal programming pointed out the HPA axis disregarding the role of ANS. However, more recently, several papers brought to light the importance of the ANS highlighting the fact that the early developmental disruption of the connections between the ANS and the limbic system might restrict the capacity of the ANS to respond to the environment and can be later implicated in neuropsychiatric disorders of the infant (Montagna and Nosarti, 2016; Frasch et al., 2018a, 2020b). Since, according to the Polyvagal theory (Beauchaine et al., 2007), the social/emotional development is related to the vagal system, any insult such as maternal distress suffered during the autonomic trajectory development will interfere with the maturation of the cerebral cortices structures and of the ANS disrupting the development of the Social Engagement System of the child (Porges and Furman, 2011).

Moreover, an impairment in vagal balance, particularly a decreased tone, might be implicated in depression, anxiety, post-traumatic stress disorder, and schizophrenia (Thayer and Brosschot, 2005). We have recently demonstrated that maternal stress affects the coupling of maternal HR to fHR showing that the fetuses of stressed mothers show significant decreases in fHR, probably representing a fetal stress memory that may serve as a novel non-invasive biomarker (Lobmaier et al., 2020).

Maternal Pain

Maternal pain during pregnancy is a symptom that should be seriously taken into account by clinicians, since it could underlie severe pathological conditions, which, in turn, can lead to negative outcomes, both for the mother and the fetus (Brown and Johnston, 2013). Many pathologies, even pre-existing before conception, may induce an acute exacerbation of the chronic pain during the pregnancy, including rheumatoid arthritis, sickle cell disease, Ehlers-Danlos syndrome, and vulvodynia, tuberous sclerosis, and irritable bowel syndrome (Ray-Griffith et al., 2018).

The most frequently observed symptoms are: low back pain, headache, pelvic girdle pain (PGP), leg cramps, breast tenderness, abdominal pain, and ligament pain (Davis, 1996; Jarrell, 2017; Lutterodt et al., 2019). Some symptoms, such as PGP and low back pain, could be physiological consequences of the musculoskeletal adaptations of the maternal body, due to an increasing uterine volume (Davis, 1996; Vermani et al., 2010; Casagrande et al., 2015; Sehmbi et al., 2017; Lutterodt et al., 2019). This could be a potential causing factor of the time-dependent onset of pain symptoms during the pregnancy.

Continued pain and discomfort throughout the pregnancy can induce both a sensitization of the maternal nervous system, with a lower pain threshold (Gintzler, 1980; Cogan and Spinnato, 1986; Eid et al., 2019), and a dysregulation in the inflammatory response, mediated by the CAP via the vagus nerve (Garzoni et al., 2013).

The patterns of cytokine response and the neuroimmune physiology of the fetus and the placenta mirror the maternal ones throughout pregnancy (Sherer et al., 2017, 2018; Prins et al., 2018; Peterson et al., 2020). These patterns are altered by pain and could hence not only be pre-emptive for negative outcomes for labor and birth (Goldenberg et al., 2008), but also suggest their influence on the neurodevelopment of the fetal vagal system itself (Borovikova et al., 2000; Tracey, 2009; Frasch et al., 2016).

The maternal pain perception, moreover, can undermine her emotional state, contributing to increased stress and worries (Persson et al., 2013; Clarkson and Adams, 2018; Mackenzie et al., 2018; Lutterodt et al., 2019), and this could additionally interfere with the vagus nerve development of the fetus.

Despite the evidence of positive effects of non-surgical and non-pharmacological treatments, such as physiotherapeutic interventions, acupuncture, or osteopathic manipulations (Gutke et al., 2015; Gallo-Padilla et al., 2016; Ray-Griffith et al., 2018; Smith et al., 2018; Cerritelli et al., 2020), the use of drugs for pain relief (e.g., low-dose aspirin, acetaminophen, non-steroidal anti-inflammatory drugs, and opioids) during pregnancy is still very common (Ray-Griffith et al., 2018). These therapies can affect fetal neurodevelopment: the increase prescriptions for opioids during pregnancy, for example, correlates to an increased incidence of neonatal abstinence syndrome (NAS), resulting in sleeping and feeding difficulties, as well as a dysregulation in newborns' CNS (Pryor et al., 2017; Ray-Griffith et al., 2018). Hence, further research is needed about these drugs' impact on fetal vagus nerve development.

Maternal Nutrition, Microbiota, and Metabolic Pathologies

Maternal nutrition is a substantial part of the "developmental or metabolic programming" of newborns (Koletzko et al., 2018, 2019), with short- and long-term consequences on health and a relevant impact on fetoplacental growth patterns (Morrison and Regnault, 2016).

The inadequate intake or absorption of nutrients, such as polyunsaturated fatty acids, folic acid, and vitamin B12, may result in altered fetal neurodevelopment (Starling et al., 2015; Wang et al., 2015), and lead to important consequences on neurobehaviour during infancy (Gernand et al., 2016; Bordeleau et al., 2021). Maternal malnutrition has an impact also on the endocrine system development: the fetus is overexposed to maternal cortisol, due to the lack of placental control systems (Allen, 2001; Seckl and Meaney, 2004), and the fetal HPA axis can be impaired, possibly leading to a dysregulation of sympathovagal balance and inflammatory set point (Christian and Stewart, 2010).

Even if further research is needed, maternal vitamin D intake deficit correlates with altered metabolic and contractile development of the heart and the control mechanisms of blood pressure in fetuses (Morris et al., 1995; Gale et al., 2008). Similarly, calcium, zinc, and iron assumption during pregnancy seems to affect fetal cardiovascular development and autonomic regulatory function (Christian and Stewart, 2010). Therefore, it may be assumed that vagus nerve development itself could be affected by maternal malnutrition.

Inadequate maternal nutrition could also lead to excessive weight gain and gestational diabetes, with negative outcomes for the fetus (AlSaif et al., 2015; Edlow, 2017; Peterson et al., 2020; Tong and Kalish, 2020). Maternal obesity, in particular, both before conception and during pregnancy, is associated with impaired fHRV and poorer ANS development (Christifano et al., 2020).

Maternal metabolic dysregulation seems to be related to gut dysbiosis (Hasain et al., 2020; Tong and Kalish, 2020) and, thanks to the direct link due to the feto-placenta-maternal unit (Nuriel-Ohayon et al., 2016; Laursen et al., 2017; Bordeleau et al., 2021), it could negatively influence the development of fetal microbiota (Zhou and Xiao, 2018; Tong and Kalish, 2020). Maternal metabolic dysfunctions and dysbiosis can affect the systemic inflammatory response of the mother and the fetus (Hasain et al., 2020; Peterson et al., 2020). Moreover, considering the correlation between the gut-brain-microbiome axis and immune system via neuroendocrine signaling (Aidy et al., 2015; Vitetta et al., 2018; Fuhler, 2020), these maternal affections can affect fetal neurodevelopment and potentially lead to adverse health outcomes later in life (Borre et al., 2014; Abdel-Haq et al., 2018; Tong and Kalish, 2020; Bordeleau et al., 2021).

Lastly, this feto-maternal pro-inflammatory state could interfere with the set point of the fetal vagal regulatory system through CAP dysregulation, thus impairing fetal intestinal permeability and integrity (Garzoni et al., 2013).

Circadian Clock and Vagal Activity

During the 3rd trimester of pregnancy, emerging fetal sleep states are detectable, with the onset of the functional organization of the sleep cycle around 28–30 WGA, and these patterns gradually consolidate around 36 WGA (Van den Bergh and Mulder, 2012). At this developmental stage, the fetus shows alternating behavioral states, passing through non-REM sleep and REM sleep, which lasts about 70–90 min (Visser et al., 1992), with usually a wake state duration under 10%.

Sleep cycles are essential for fetal neurodevelopment (Graven and Browne, 2008). The deprivation of the REM sleep phase during uterine life can lead to altered cortical and brainstem development and to the supersensitivity to the noradrenaline of the pyramidal cells in the hippocampus, impairing the receptors' sensitivity (Mirmiran et al., 2003). Consequently, the fetal and infant capacity to homeostatically adapt to the environment could be impaired (Van den Bergh and Mulder, 2012).

Fetal heart rate patterns, together with GMs and REM recording, are the main factors to define the passage through fetal sleep states (Van den Bergh and Mulder, 2012), pointing out the direct relationship between vagal activity and circadian rhythm.

Although the ANS and vagus nerve activity appear to have an endogenous circadian rhythm (Hilton et al., 2000; Kentish et al., 2013), with a pacemaker system that regulates its behavior regardless of sleep-awake states (Malpas and Purdie, 1990; Hilton

et al., 2000), a circadian variation pattern of vagal activity can be recorded via HR variations.

The circadian dynamics of fHR is synchronized with the maternal rhythm of the activity at rest, HR, cortisol, melatonin, and body temperature. It is possible, therefore, to assume that the mother, considering the limited light-dark variation during intrauterine life, entrains the developing circadian rhythm of the fetus to this cycle (Mirmiran et al., 2003). In this sense, dysregulations in mother circadian rhythm, due to disturbed sleep-awake patterns, could interfere with the development of circadian control systems of the fetus and, consequently, with the circadian profile of the vagus nerve, impacting the physiologic vagal neurodevelopment itself.

Several factors can normally affect the sleep states of the mother during pregnancy: the hormonal, biomechanical, and behavioral changes that occur during the three trimesters, often lead to increased sleep disturbances, particularly in the last trimester (Sahota et al., 2003; Pien and Schwab, 2004; Facco et al., 2010; Mindell et al., 2015).

It has been reported in these cases a correlation with deficit in fetal growth, adverse outcomes for labor and birth, and even preterm birth (Lee and Gay, 2004; Micheli et al., 2011; Zafarghandi et al., 2012; Warland et al., 2018). Especially in the 3rd trimester, it is frequently reported the onset of sleep-disordered breathing (SDB) (Santiago et al., 2001; Edwards et al., 2002; Pien and Schwab, 2004; Venkata and Venkateshiah, 2009). Sleep-disordered breathing can disturb not only the physiological patterns of maternal sleep, but it could also lead to nocturnal hypoxia, which produces an enhanced activation of the maternal ANS (Pien and Schwab, 2004; Sahin et al., 2008). There are also repercussions on the fetus, in response to these apneic events: Fetal heart rate decelerations and decreased breathing movements, which could affect neonates' neurobehavior, even if currently there are not enough data to support definitive conclusions about long-term outcomes (Pien and Schwab, 2004).

Lastly, maternal sleep deprivation seems to be related to the depressive state (Jarczok et al., 2018) and leads to increased systemic inflammation, with the increment in the production of pro-inflammatory serum cytokines (Chang et al., 2010). In this context, further research is needed to better clarify the relationship between maternal neuroendocrine dysregulation, due to chronobiological impairments, and the impact on fetal neurodevelopment, particularly on the CAP.

Maternal Diseases

Maternal pathologies influence the onset of altered neurodevelopment patterns during fetal life, which could lead to actual neurologic disorders during childhood (Faa et al., 2016; Vohr et al., 2017). In particular, metabolic and cardiovascular pathologies, like obesity, diabetes, hypertension, and preeclampsia, can disrupt vagus nerve development (Brown et al., 2008; Russell et al., 2016; Moors et al., 2020).

The pathologies that induce chronic hypoxemia in the mother, including SBD (Pien and Schwab, 2004) and anemia, may induce dysregulations in the vagal control systems. Such hypoxic states can also be secondary to infections, including chorioamnionitis, leading to several hemodynamic abnormalities, and cardiovascular compromise (Frasch, 2018). The endotoxemia, through lipopolysaccharide molecules, seems to interfere with the cardiac pacemaker synchronization system, altering the fHRV pattern (Frasch and Giussani, 2020).

Many viral infections, such as cytomegalovirus and other TORCH organisms or ZIKV infection, can lead to preterm birth and induce severe neurodevelopmental alterations in the fetus (Silasi et al., 2015; Vohr et al., 2017). These pathologies are associated with fetal inflammatory response syndrome, which manifests as increased pro-inflammatory markers, including IL-6, IL-1, and TNF-α (Gotsch et al., 2007). As a result, oligodendrocytes and their progenitors are damaged during a critical period of brain development, thus altering brain myelination and leading even to periventricular leukomalacia (Kadhim et al., 2001). The increase in cytokine production consequent to the inflammatory response can enhance the risk for cardiovascular disease, impairing CAN, autonomic reactivity (Harrison et al., 2013), and, therefore, affecting the vagal development. Moreover, a recent pilot study described that *in utero* ZIKV-affected babies display HRV metrics abnormalities. Even early prenatal exposure to the virus, indeed, seems to create a chronic hypoxic environment to the fetus, affecting vagus nerve developmental patterns, and imprinting postnatally the ANS cardiac pacemaker activity (Herry et al., 2021).

The implications of these infection-associated immunological events on fetal neurodevelopment seem to be time-dependent, thus supporting the clinical relevance of carefully evaluating the interfering factors throughout the entire pregnancy (Meyer et al., 2007).

Similarly, even autoimmune diseases can involve a dysregulation in maternal inflammatory response (Gordon, 2004; Lu-Culligan and Iwasaki, 2020; Han et al., 2021). Systemic lupus erythematosus and antiphospholipid antibody syndrome, for instance, entail the presence of IgG antiphospholipid antibodies, which can cross the placenta, interfering with the brain development and impacting the cardiovascular system of the fetus (Buyon, 1998; Nalli et al., 2017). These pathologies can dysregulate the neuroendocrine axis, altering, in particular, the cortisol production (Wilder, 1998; Sheng et al., 2020) and, thus, affecting sympathovagal balance development.

Even conditions that induce secondary hypertension or HPA dysregulation, such as pheochromocytoma, hyperaldosteronism, or Cushing syndrome, can affect fetal neurodevelopment (Morsi et al., 2018; Corsello and Paragliola, 2019).

Lastly, maternal mood disorders and depression seem to strongly impact the fetal neuroendocrine systems, in particular the amygdala (McEwen et al., 2016) and the hippocampus (Nemoda et al., 2015), strongly involved in the CAN (Harrison et al., 2013; Thome et al., 2017). Moreover, maternal psychopathology influences HRV tracking also after birth, showing a higher mean HR and lower vagal modulation and confirming the impact of this prenatal disorder on vagal neurodevelopment (Dierckx et al., 2009).

Nicotine/Alcohol Exposure

Smoke and alcohol exposure can induce in premature newborns higher sympathetic function, lower parasympathetic function,

and had less cardiac autonomic adaptability (Mulkey and du Plessis, 2019). During the 1st trimester, there are significant negative correlations between the amount of maternal cigarette smoking and fHRV metrics, highlighting the disrupted temporal organization of autonomic regulation before birth (Zeskind and Gingras, 2006; Kapaya et al., 2015).

Prenatal nicotine exposure (PNE) is associated with higher systolic blood pressure and altered HRV after birth (Nordenstam et al., 2019). Furthermore, the diminished vagal cardiovascular capacity to respond to hypoxic and hypercapnic states in the fetus, due to the abolishment of the serotonergic neurotransmission to cardiac vagal neurons caused by PNE, has been hypothesized as a likely link to SIDS (Kamendi et al., 2006, 2009).

The effects of prenatal alcohol exposure (PAE) on fetuses are collectively known as fetal alcohol spectrum disorders (FASD). Of the various organ systems affected by FASD, the brain is the most severely impacted: even if the hippocampus seems to be spared, other CAN structures show an impaired development, including the thalamus, hypothalamus, and prefrontal cortex, and also the spinal cord (Caputo et al., 2016).

Alcohol easily crosses the placenta and can disrupt maternal–fetal hormonal interactions, leading to long-term impairments on neuroendocrine and immune competence (Zhang et al., 2005). Prenatal alcohol exposure also impacts the limbic-HPA axis development and functioning, leading to disrupted cortisol response (Ouellet-Morin et al., 2011). Prenatal alcohol exposure, therefore, induces a dysregulated fetal immune response, increasing the risk for infections, chorioamnionitis, and/or placental abruption, which can favor premature delivery (Reid et al., 2019). The consequences on the long-term capacity of CAP inflammatory response are worthy of further investigations.

Lastly, early pain reactivity measured through HRV in alcohol-exposed newborns seems to be blunted, suggesting different responsiveness to environmental stimuli even after birth (Oberlander et al., 2010). This seems to confirm the PAE effects on HPA axis stress reactivity, possibly via changes in central serotonin levels and/or HPA axis functions, and on the vagus nerve development itself (Haley et al., 2006).

Placenta

Placental Dysfunctions

A growing body of evidence suggests the placenta's role in modulating fetal development. The placenta can be defined as a neuro-immune-endocrine secretive organ, with the essential role of conveying nutrients and developing modulatory signals to fetuses, limiting their exposure to any factors that could alter their physiologic developmental patterns.

Many factors can affect placental physiology, such as maternal nutrition, nicotine or alcohol exposure, drugs, pathologies, and stress (Nugent and Bale, 2015). This results in an overexposure of the fetus to maternal cortisol, that in turn may produce an overactive fetal cortisol response and may be a predisposing factor to develop disorders related to elevated HPA axis activity, which could lead to impairments in environmental stimuli adaptability by ANS in newborns (Nugent and Bale, 2015).

The dysregulation of placental functions can induce an insufficient intake of nutrients for the fetus, thus, especially during mid and late gestation, impairing the development of various brain areas, including the hypothalamus that seems to be particularly sensitive to the nutrient deficiency (Nugent and Bale, 2015).

Moreover, through the production of corticotropin-releasing hormone (CRH), the placenta stimulates adrenocorticotropic hormone release in the anterior pituitary of the fetus, affecting both fetal HPA axis and amygdala development (Seckl and Meaney, 2004). A dysregulation in CRH production, secondary to various maternal conditions, such as hypoxia, increased inflammatory cytokines and glucocorticoids, stress, preeclampsia, and eclampsia, may lead to altered neurodevelopmental patterns of these fetal structures (Allen, 2001; Bale, 2016; Sheng et al., 2020). It could also affect the role of placenta as the immune pacemaker of pregnancy, increasing the risk of preterm birth (Allen, 2001; Peterson et al., 2020) and thus impairing vagal development during the last weeks of gestation.

Recently, placenta calcifications samples collected at delivery have been speculated to act as a memory of prenatal maternal stress and diseases exposure, hence highlighting the potential role of placenta in regulating the offspring's future cardiovascular and metabolic health, even through the *in utero* modulation of the developing ANS (Wallingford et al., 2018).

In addition, even mild inflammatory states in the mother might affect the placental conversion of maternal tryptophan to serotonin upstream of the fetal brain (Goeden et al., 2016). This affection ultimately interferes with cerebral fetal neurodevelopment, including the nucleus accumbens and hippocampus, that are involved in CAN (Seckl and Meaney, 2004).

In conclusion, many diseases can impact the placental function, which in turn seems to have a role in regulating the neurodevelopment of the main actors that interact with the fetal vagus nerve, and the monitoring of placenta secretome could be a useful biomarker of diseases and potential developmental disruptions of the ANS in the fetus (Aplin et al., 2020).

Placental Microbiome

Although the womb was usually considered sterile, recently it has been suggested that the placenta harbors a unique microbiome, composed of non-pathogenic commensal microbes (Aagaard et al., 2014). The placental microbiota seems to display an evolutionary pattern during pregnancy and appears to be diversified in the various areas of the placenta (Aagaard et al., 2014; Cao et al., 2014; Pelzer et al., 2017).

Many authors substantiated the materno-fetal transmission of the microbiome through the placenta during prenatal life, identifying the presence of the microbiota both in the peripartum placenta (Zheng et al., 2015; Pelzer et al., 2017) and in the meconium (Walker et al., 2017), partially regardless of the delivery mode.

Placenta dysbiosis can be related to antepartum infections (Aagaard et al., 2014; Doyle et al., 2017; Pelzer et al., 2017), maternal nutrition (Aagaard et al., 2014), maternal weight gain (Antony et al., 2015; Zheng et al., 2015; Benny et al.,

2019), gestational diabetes mellitus (Bassols et al., 2016; Pelzer et al., 2017), use of probiotics/antibiotics (Pelzer et al., 2017), and periodontal pathogens (Fischer et al., 2019), and can lead to several adverse outcomes, such as preeclampsia and chorioamnionitis (Amarasekara et al., 2015; Doyle et al., 2017; Fischer et al., 2019; Olaniyi et al., 2020).

Moreover, it has been observed a correlation between placenta dysbiosis and preterm birth (Aagaard et al., 2014; Antony et al., 2015; Bassols et al., 2016; Prince et al., 2016; Pelzer et al., 2017; Fischer et al., 2019). More than the infections themselves, it would seem it is the dysregulation of the placental inflammatory response that could affect the production of placental CRH and stimulate the fetal HPA axis, leading to preterm labor and birth (Parris et al., 2021). The high immunoreactivity of the premature infants' gut could also lead to neurodevelopmental disorders (Cao et al., 2014).

The fetoplacental transmission of the placental microbiome may occur through the ingestion of the amniotic fluid and via umbilical cord blood supply (Cao et al., 2014; Walker et al., 2017). The matches collected between the placental and fetal gut and lung microbiomes seem to be associated with neonatal airway complications and enterocolitis (Al Alam et al., 2020; Parris et al., 2021), possibly affecting the ANS-mediated control mechanisms of these systems. Since the placental microbiome could also affect metabolic pathways, thus leading to a pro-inflammatory environment (Gomez-Arango et al., 2017; Fischer et al., 2019), it could also affect the ANS and vagus development of the fetus.

Despite this evidence, other authors claim that placental microbiome does not exist—the aforementioned results would be just due to methodological errors—or that there is only extremely low biomass, probably leading to minor clinical effects (Lauder et al., 2016; Leiby et al., 2018; Kuperman et al., 2020; Parris et al., 2021).

Nevertheless, the placental microbiome could be an interesting factor to be taken into account by the clinicians as a potential regulator of vagus nerve development.

Exogenous (Non-maternal) Factors

Environmental Stimuli

The variations in ambient and core temperature appear to impact newborns HRV (Massaro et al., 2017): in particular, extreme variations seem to affect the autoregulatory capacities of the cardiac system and, consequently, HRV, possibly due to a dysregulated release of excitatory neurotransmitters (Tsuda et al., 2018). Particularly warmer- or colder-than-average temperatures seem to be associated with increased systemic inflammation and alteration in placental blood flow (Martens et al., 2019; Sun et al., 2019), leading to dysregulation of metabolic and immune substrates of the fetus, and possibly influencing the regulatory functions of ANS.

Exposure to acoustic stimuli, through vibroacoustic stimulation (VAS), is one of the most used benchmarks for the assessment of fetal distress, since VAS induces a somato-cardiac effect mediated by the fetal behavioral states (Busnel et al., 1992). This cardiac-orienting response does not seem to be detectable before the functional maturity of the fetal autonomic system, which occurs around 32 WGA (Krueger and Garvan, 2019). The fetal responsiveness recorded via fHRV patterns after VAS stimulation (Abrams and Gerhardt, 2000) is refined as the pregnancy proceeds, being a useful parameter to evaluate the maturation of ANS and vagus nerve (Buss et al., 2009). As the nervous system of the fetus develops, around 36–39 WGA a different fHRV pattern is detectable according to different acoustic stimuli, as for example human speech (Lecanuet et al., 1992; Krueger and Garvan, 2019).

Even if the light-dark cycle does not seem to significantly affect the fetal circadian rhythm, there are age-dependent fHRV patterns after transabdominal fetal stimulation with halogen light, similarly to the VAS (Peleg and Goldman, 1980), which seem increasingly definite in the later stages of pregnancy, when the fetal optic pathways and ANS control on the cardiac activity reach maturity (Thanaboonyawat et al., 2006).

The persistent exposure to photic stimuli or high noise during pregnancy, for instance, due to the mother's occupation, seems to correlate with reduced fetal growth (Selander et al., 2019), increased incidence of congenital heart diseases (Gong et al., 2017), impaired development of cortical areas (Zhang et al., 1992; Wilson et al., 2008), and potential disruption of the vagus nerve maturation.

The occupational and environmental exposure to pollution may be another important disrupting factor for fetal ANS development. A growing body of evidence suggests that chemicals exposure can impact fetal growth (Dejmek et al., 1999; Snijder et al., 2012; Desrosiers et al., 2015) and is a risk factor for congenital heart diseases (Gong et al., 2017), preterm birth and severe neurodevelopmental abnormalities (Zhang et al., 1992; Lacasaña et al., 2006; Langlois et al., 2012; Yurdakök, 2012). Even if the effects on the vagus nerve are still to be defined, the hazards exposure may affect the fetal motor activity (DiPietro et al., 2014) and be related to delayed neurobehavioural development in neonates (Handal et al., 2008). It is instead well known that methylmercury from seafood impairs the higher centers of cardiac autonomic function (Karita et al., 2018), leading to severe ANS abnormalities (Sørensen et al., 1999; Grandjean et al., 2004; Murata et al., 2006; Gribble et al., 2015).

Lastly, even electromagnetic field (EMF) radiations during pregnancy, due to overexposure to mobile phones, diagnostic instruments, or occupation, seem to be a risk factor for preterm delivery (Roşu et al., 2016) and to have effects on ANS development (Rezk et al., 2008).

Drugs During Pregnancy and Labor

In the course of pregnancy, mother and fetus could undergo a variety of drug administrations, many of which can cross the placenta affecting fetal development in several ways (Miljković et al., 2001).

Drugs to Support Fetal Growth Synthetic glucocorticoids (sGCs) are the therapy of choice to support fetal maturation for the risk of preterm delivery. Usually, it is administered between 24 and 34 WGA, mainly to ensure optimal fetal pulmonary development

(Mulder et al., 2004; Committee on Fetus and Newborn and Section on Anesthesiology and Pain Medicine, 2016).

As excessive maternal cortisol, sGCs override the placenta mediator system (Sheng et al., 2020) and induce transient HRV modification toward sympathetic suppression (Multon et al., 1997; Senat et al., 1998; Subtil et al., 2003; Mulder et al., 2004; Schneider et al., 2010). These modifications seem to last at worst 4 days after the first dose (Mulder et al., 2004; Verdurmen et al., 2013) and the influence on ANS modulation seems minor (Verdurmen et al., 2018; Noben et al., 2019). However, the long-term impact is currently under investigation. There appears to be an association between multiple courses of sGCs prenatal administrations and infant neurodevelopmental abnormalities (Spinillo et al., 2004). Nevertheless, the presence of many confounding factors and the small sample sizes make unclear the mechanism of action of glucocorticoids on fHRV and consequently the impact on ANS development (Verdurmen et al., 2013).

Prenatal exposure to sGC may interfere both with the fetal endogenous cortisol sensitivity, which involves the hippocampus, amygdala, and HPA axis and with the fetal cardiovascular and metabolic regulatory systems (Seckl and Meaney, 2004). This could lead to profound epigenetic changes in the fetal nervous system development and specifically in the vagal functionality, with possible long-term consequences (Crudo et al., 2013; Chang, 2014). This *in utero* programming effect on the fetal HPA axis could last until adulthood: there are recent observations of altered R-R interval variability in adult offspring exposed to elevated fetal sGCs (Sheng et al., 2020), a result that deserves further investigations to better understand the sGCs potential role in neurodevelopmental altered pathways.

Drugs for Maternal Pathologies and Drug Abuse. Most of the medication administered to the mother during pregnancy can cross the placental barrier and reach the fetus.

Selective serotonin reuptake inhibitors (SSRIs) seem to induce alterations in the fetal limbic system (Lattimore et al., 2005), disrupting the central serotonin signaling (Oberlander et al., 2009). Exposure to SSRIs during the 3rd trimester is associated with blunted FMs and reactivity to VAS, as well as with reduced fHRV at 36 WGA (Oberlander et al., 2009; Nguyen et al., 2019). The fetuses, moreover, appear to have an increased risk of preterm birth (Morrison et al., 2005; Suri et al., 2007; Oyebode et al., 2012) and poor neonatal adaptation syndrome (Lattimore et al., 2005; Sivojelezova et al., 2005).

Neonates affected by *in utero* exposure to SSRIs display fewer HRV rhythms and changes in behavioral states, based on the time drugs were administered (Zeskind and Stephens, 2004). They may show altered pain reactivity and parasympathetic cardiac modulation during recovery after an acute noxious event (De las Cuevas and Sanz, 2006), which lasts up to two months, and also altered HPA stress response patterns and hippocampal plasticity (Morrison et al., 2005; Oberlander et al., 2009; Gemmel et al., 2019).

Among antihypertensive drugs, methyldopa does not seem to particularly affect fetal well-being. In contrast, the first-trimester exposure to angiotensin-converting enzyme inhibitors is associated with the greater incidence of malformations of the cardiovascular and CNS (Podymow and August, 2008, 2011), and the timing of exposure seems to be particularly relevant (Caton Alissa et al., 2009). The use of nifedipine concurrently with magnesium sulfate (MgSO4) seems associated with severe hypotension, neuromuscular blockade, and cardiac depression in the fetus (Khedun et al., 2000).

The effects of prenatal opioids exposure on the fetus are widely described. Although there are not evident depressive effects on non-stress test reactivity or variability in the case of daily opioid use for chronic pain (Brar et al., 2020), the current guidelines recommend a prescription at the lowest effective dose (Ray-Griffith et al., 2018).

The opioid prenatal overexposure has instead neurotoxic consequences on several fetal systems: cardiovascular, respiratory, neurobehavioral, metabolic, and neuroendocrine (Conradt et al., 2018). Early exposure of buprenorphine induces higher levels of fHRV, whereas at 32–36 GWA fetuses display less suppression of motor activity (Jansson et al., 2011; Conradt et al., 2018), indicating the involvement of the vagus nerve system. Opioid dependence, moreover, is associated with the increased risk for neonatal opioid withdrawal syndrome (NOWS) (McCarthy et al., 2017; Conradt et al., 2018, 2019).

After birth neonates with NOWS show increased levels of norepinephrine, corticotropin, noradrenaline, and acetylcholine, and lower dopamine and serotonin levels (Conradt et al., 2018), thus suggesting a possible disruption in vagal and CAP activity. Exposure to opioids prenatally could result in programming effects on stress response systems, leading also to long-term consequences (Conradt et al., 2018, 2019).

Opioids are often used as analgesic therapy during labor. There are many observed effects of this medication on fHRV and fetal breathing patterns, mostly transient and dose-/time-dependent (van Geijn et al., 1980; Kariniemi and ÄMmälä, 1981; Low et al., 1981; Viscomi et al., 1990; Capogna, 2001; Mattingly et al., 2003; Shekhawat et al., 2020). To better understand the real implications of intrapartum opioids administration on fetal vagus nerve development, however, it is essential that future research distinguishes its effects from that produced by other substances and associated environmental stressors, illustrating the role of the timing of exposure in specific windows of fetal neurodevelopment, as well as the potential long-term outcomes (Conradt et al., 2018, 2019).

It is also in common use during the peripartum period to administer the mother antibiotic prophylaxis. This seems to be associated with an increased risk for antibiotic-resistant neonatal sepsis (Mercer et al., 1999; Hantoushzadeh et al., 2020). Moreover, prophylactic antibiotic use during vaginal or cesarean delivery may indirectly lead to epigenetic changes in the fetus, with possible implications in the immune system and stress response (Tribe et al., 2018), consequently involving vagus nerve development and suggesting, therefore, the utility to consider potential alternative approaches (Ledger and Blaser, 2013).

Drugs Used to Manage the Timing of Delivery. MgSO4, which is often administered between 23 and 27 WGA or intrapartum for its tocolytic effect to delay labor onset in pregnancy at risk for preterm birth (Ramsey and Rouse, 2001; Ingemarsson and Lamont, 2003), does not seem to show a significant change in

fHRV (Stallworth et al., 1981; Nensi et al., 2014). These results do not vary in co-administration with opioids (Cañez et al., 1987), whereas the association with sGC should deserve greater attention by the clinicians (Verdurmen et al., 2017). Moreover, the prolonged administration of MgSO4 seems to be associated with a change in fetal breathing movements (Peaceman et al., 1989) and to affect fHRV dose-dependently (Cardosi and Chez, 1998; Kamitomo et al., 2000).

The Labor/Delivery Influence on the ANS

Labor is divided into three stages. The 1st stage begins when significant and regular contractions start and ends when there is a full cervical dilation of 10 cm. The 2nd stage of labor begins with complete cervical dilation and ends with the delivery of the fetus. The 3rd stage commences when the fetus is delivered and ends with the delivery of the placenta (afterbirth) (Hutchison et al., 2021).

In this section, we will review the influences that the events that can occur in the first two stages of labor can have on the development of the vagus nerve and ANS. In particular, we will study what events can interfere with the correct development of these two systems by analyzing the evidence from preclinical and clinical studies in this regard, with the aim of preventing potential complications with long-term consequences.

Changes in fHR and fHRV during labor can reveal alterations in fetal reserve to survive the trial of labor and in ANS development, specifically, also in vagus nerve modulation (Chiera et al., 2020). However, it is important to highlight that so far fHR monitoring has not been enough to prevent fetal injury during labor and, consequently, long-term complications. In fact, the role of fHR monitoring (electronic fetal monitoring, EFM, to be precise), despite being used in over 90% of hospitals during delivery, remains controversial (Schifrin, 2020). There is no clarity on its usefulness in decreasing perinatal mortality or cerebral palsy when measured by a CTG which has been explained by the incorrect focus of EFM on prediction of acidemia, a poor correlate to fetal injury, instead of predicting fetal cardiovascular decompensation *per se* as well as the limitations of CTG technologies in capturing the short-term time scale fluctuations of HRV reflecting vagal modulations (Alfirevic et al., 2017; Frasch et al., 2017; Frasch, 2018; Gold and Frasch, 2021). Consequently, EFM fails to identify fetuses at risk of brain injury; this is because there is no clear correlation between fetal brain injury and acidemia (Westerhuis et al., 2007; Cahill et al., 2017). Additional modalities of EFM have been proposed such as intrapartum maternal transabdominal electrocardiography (ECG) and electroencephalography (EEG) which will increase the ability of EFM to predict fetal brain injury. Fetal EEG, while feasible, has not yet made its way into clinical practice. Fetal scalp electrodes can also be used to directly measure pH during childbirth, but this technique, being even more invasive, is not yet fully accepted and also has some complications (Sabir et al., 2010).

Preclinical Studies

Through preclinical studies on animals and using fHRV, which remains the best method to monitor the fetus, researchers are trying to find solutions to improve and increase the prevention of specific complications. The preclinical studies analyzed in this review were selected from among those carried out on the ovine species. Sheep are in fact an important model for simulating human pregnancy. Research on the ovine species, reproducing the conditions of human labor, is indeed leading to the discovery and evidence of different algorithms to prevent pathological complications (Frasch et al., 2011; Wang et al., 2014).

In this paragraph, we will consider the studies that simulate the 1st stage of labor.

During this stage, uterine contractions expose the fetus to continuous and significant hypoxic stress. Perinatal hypoxia contributes significantly to the possibility of perinatal brain injury (Gotsch et al., 2007). Statistically, severe fetal acidemia ($pH < 7.00$) has been shown to occur in between 0.5 and 10% of human births. Of the neonates in this series, 20% will have neurological consequences including cerebral palsy and HIE (Goldaber et al., 1991; DuPont et al., 2013).

Among the possible fHRV metrics, RMSSD is a metric that allows researchers to assess what influence labor may have on the vagus nerve. Indeed, it is known to increase with worsening acidemia (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Frasch et al., 2007, 2009a,b; Durosier et al., 2014).

Gold et al. hypothesized, through the use of an algorithm based on RMSSD, the possibility of identifying the precise moment, during labor, when fetuses begin to lose maintenance of cardiac output, that is, when they show a hypotensive pattern of pathological arterial blood pressure (ABP). This hypothesis is highly clinically relevant because, in the case of repetitive umbilical cord occlusions with worsening acidemia, fetuses may easily develop cardiovascular decompensation, a phenomenon that occurs through ABP responses to HR decelerations. Such a failure in maintaining cardiac output during labor could facilitate brain injury in newborns and can be detected on the individual basis using fHRV analysis from EFM (Gold et al., 2021; Roux et al., 2021).

The above-mentioned studies show us what impact labor can have on the ANS and the vagus nerve. In fact, the brain, through the CAN, controls the sympathetic and parasympathetic preganglionic motoneurons and regulates the activity of the vagus nerve (Benarroch, 1993). Consequently, a cerebral alteration, caused by a brain injury, affects the ANS including the vagus. This is the reason why a measure of vagal modulation like RMSSD could help predict a similar event.

Another common complication during labor is given by cardiac repercussions that can affect the fetus. Hypoxia can induce heart failure which is revealed by accelerations and decelerations in fHRV (Rivolta et al., 2014). This event shows how the fetal ANS quickly reacts in the face of a stressor. Further research is required to tie these findings with the recent discovery of the intrinsic fHRV produced by the heart's sino-atrial node (Frasch et al., 2020a). The evidence so far shows that the intrinsic fHRV is influenced by the fetal ANS activity and, conversely, also shapes the ANS activity into the postnatal period, at least in part via the afferent fibers of the vagus nerve (Herry et al., 2021).

Vagal modulation, as revealed by RMSSD, and global ANS functioning, as revealed by entropic HRV metrics, can also be affected by the neuroinflammation *in utero* that can occur during labor due to short-term hypoxic acidemia. A key role in the control of inflammation is played by the CAP and a functional neuroimmunological link in the fetus has been shown to improve postnatal health and brain injury (Frasch et al., 2016; Liu et al., 2016). Cholinergic anti-inflammatory pathway also plays a very important role in preventing and ameliorating NEC cases (Garzoni et al., 2013). During labor, especially in cases of prematurity, there are several conditions that can cause NEC: two of these are certainly hypoxia and acidemia (Sharma and Hudak, 2013). Since the vagus is closely related to the intestinal system, severe intestinal inflammation such as NEC can destroy the cells of the vagal nuclei in the brainstem and inhibit CAP (Fritze et al., 2014).

Clinical Studies

Labor in humans is certainly associated with physiological changes, most of which are regulated by ANS (Sanghavi and Rutherford, 2014; Soma-Pillay et al., 2016). In this paragraph, we will focus on clinical studies done during both the 1st stage of labor and the 2nd stage of labor.

The 1st Stage of Labor

During this stage, various components come into play that create an alternate activity between SNS and PNS (Norman et al., 2011; Musa et al., 2017). For example, the maternal pain and anxiety due to uterine contractions induce an activation of the sympathetic division (Jones and Greiss, 1982), whereas the release of the hormone oxytocin instead activates the parasympathetic division (Gamer and Büchel, 2012).

A possible critical event during the first stage of labor is certainly the risk of cardiac events. By examining cardiac autonomic modulation (CAM), some authors hypothesized that there might be a predominance of activation of the SNS over the PNS. However, SNS hyperactivity during labor is unlikely to manifest like myocardial infarction, as sympathetic CAM increases simultaneously with HRV metrics related to PNS (Musa et al., 2017).

An important factor that is present in several cases of labor is epidural analgesia (Bautista and George, 2020). Its influence on the development of the ANS and the vagus nerve can be understood through a careful examination of fHRV changes. In fact, epidural analgesia itself does not show precise or significant changes in fHRV (Lavin, 1982), but it can interact with other aspects already existing in the mother. Hypotension and uterine hypertonia, for instance, can create significant imbalances in the fHRV during epidural analgesia in labor (Lavin, 1982). Several studies have also shown a major change in fHRV in women given an analgesic agent such as lidocaine (Boehm et al., 1975; Lavin, 1982).

An aspect that should not be underestimated in the administration of epidural analgesia is the position of the mother. fHRV undergoes fewer changes with the use of the full lateral position, compared with the use of wedged supine position (Preston et al., 1993). The reason behind this evidence could be the greater prevention of aortocaval compression given by the full lateral position (Preston et al., 1993). The 1st stage of labor does not always occur naturally and without complications: consequently, there may be an artificial intervention that causes the induction of labor to increase uterine contractions. Labor is usually induced through the administration of synthetic oxytocin (sOT). As previously mentioned, oxytocin plays an important role in activating the PNS and it has been shown to dampen SNS (Gamer and Büchel, 2012). However, the induction of oxytocin did not reveal cardiac complications in women with heart disease (Dogra et al., 2019).

The 2nd Stage of Labor

Although the induction of the hormone oxytocin has no cardiac repercussions, it can still influence the development of the vagus nerve and the ANS in the last hours of labor. It has been shown that a pattern of hyperactive uterine contractions (often caused by the administration of sOT), during the last 2 h of labor, is strongly associated with acidemia and hypoxia at birth and with fHR and fHRV alterations (Freidman and Sachtleben, 1978; Woodson et al., 1979; Cibils and Votta, 1993; Ladfors et al., 2002; Jonsson et al., 2008; Verspyck and Sentilhes, 2008; Aye et al., 2014). As previously mentioned, acidemia can induce NEC and consequently inhibit the CAP (Garzoni et al., 2013).

The long-term consequences of developing cardiac control systems and ANS are hence under investigation (Frasch and Giussani, 2020). Moreover, sOT infusion may decrease spontaneous oxytocin release (Jonas et al., 2009), elevating days after birth fetal plasmatic oxytocin concentration, not dampening the HPA axis response, and increasing the CAN system control activity (Yee et al., 2016).

Exposure to sOT may therefore lead to epigenetic remodeling in the infant (Tribe et al., 2018; Uvnäs-Moberg et al., 2019), and the documented presence in animal models of specific binding for oxytocin in the vagal dorsal motor nucleus during early embryonic life (Tribollet et al., 1989) suggests a strict link between oxytocin and vagus nerve.

Drugs aside, the most relevant aspect to be evaluated during the 2nd stage of labor is certainly the mode of delivery.

The major critical events that can affect vagus nerve and ANS development occur in premature births and delivery by CS at term (Hillman et al., 2012; Tribe et al., 2018; Mulkey et al., 2019).

The case of premature births is probably the most challenging. Preterm newborns have an immature ANS, which has great repercussions on cardiac and respiratory functions (Mulkey and du Plessis, 2018). In infants born before 36 WGA, there is also a 90% incidence of NEC, as the premature gastrointestinal tract has increased permeability (Garzoni et al., 2013).

Although CS delivery is far faster than a vaginal birth modality, the fetus is still subjected to significant stress that can create changes in the development of some systems, including the ANS (Tribe et al., 2018). An important difference should be moreover emphasized between the CS delivery following a period of labor and CS delivery prior to the onset of labor. Infants born with CS delivery following a period of labor have a higher and more developed ANS tone compared to those born with CS without labor (Mulkey et al., 2019). Furthermore, labor pain

before CS helps prevent respiratory disorders and avoid umbilical cord acidemia, thus protecting the developing ANS (Senturk et al., 2015).

Delivery without labor or preterm birth is associated with lower cortisol, angiotensin, and catecholamines levels compared to labored or full-term delivery. This situation puts newborns at risk of cardiovascular and metabolic dysfunctions, which can then negatively affect the developing brain by inducing inflammation or glucose deficiency (Hillman et al., 2012; Morton and Brodsky, 2016; Mulkey and du Plessis, 2018).

The Incubator Influence on the ANS

Once born, newborns have to deal with the extrauterine environment that, especially for preterm babies, is constituted by the incubator. Here, environmental factors such as temperature, light, noise, procedures could induce a high amount of stress, which could become toxic, that is, induce severe alterations in the brain and organic development, with long-lasting complications (De Jonckheere and Storme, 2019; Weber and Harrison, 2019).

Temperature

As thermoregulation is one of the most important ANS functions, the temperature can easily affect ANS development. Indeed, warm incubators tend to increase SNS tone and reduce PNS activity, whereas the opposite is true for incubators 2°C colder than newborns' temperature (Franco et al., 2003; Stéphan-Blanchard et al., 2013). Therefore, temperature that constantly deviates from newborns' one can lead to excessive ANS activation. Indeed, extreme variations of environmental or core temperature can impair the ANS, with all due complications (Fox and Matthews, 1989; Mowery et al., 2011).

Nonetheless, when used correctly, the temperature can also help newborns to correctly develop: indeed, therapeutic hypothermia in newborns with 36 WGA in case of HIE can help reduce brain and injury brain inflammation, thus having positive effects on neurodevelopment (Massaro et al., 2017; Lemyre and Chau, 2018).

Noise

If born prematurely, newborns lose the low-frequency maternal sounds that are essential for the correct maturation of their hearing system, with negative consequences on brain maturation and speech development (McMahon et al., 2012).

Furthermore, without uterus protection, newborns can be particularly sensitive to sounds, which usually overcome the 45 dB limit recommended by the American Academy of Pediatrics (Almadhoob and Ohlsson, 2020), thus resulting in toxic stress (Weber and Harrison, 2019). The consequences can be quite severe: from apnea to hypoxemia, from sleep disruption to decreased growth, from immunity impairment to neuroendocrine alterations (Almadhoob and Ohlsson, 2020).

Therefore, every possible procedure aimed to protect babies from harmful noises or to introduce positive sounds including the human voice, especially maternal voice (even recorded), and songs, can result in important neuroprotective effects (McMahon et al., 2012; Weber and Harrison, 2019). It is noteworthy that earmuffs show ambiguous results, that is, they can both help oxygenation and sleep and provoke stress (Zahr and de Traversay, 1995; Aita et al., 2013; Almadhoob and Ohlsson, 2020).

Light

As newborns pass the majority of their time sleeping, excessive light due to incubators not properly covered can negatively affect the newborns' cardiorespiratory regulation, thus increase stress level and altering ANS development (Ozawa et al., 2010; Weber and Harrison, 2019), in addition, to induce all the negative effects of sleep deficiency. Moreover, excessive light can impair visual development, thus increasing SNS and HPA axis activation (Weber and Harrison, 2019). On the other side, properly covering incubators or using eye masks facilitate a quiet sleep state characterized by a stable respiratory function (Shiroiwa et al., 1986; Venkataraman et al., 2018).

Electromagnetic Fields

Newborns can also be subject to EMFs, from the incubator or the care unit environment. Electromagnetic field coming from the incubator power influences the ANS as revealed by HRV analysis as LF/HF increases, whereas HF decreases (Bellieni et al., 2008). The farther away newborns stay from the incubator power, the less the ANS is affected (Bellieni et al., 2008; Passi et al., 2017).

Although incubator EMF seems to be under the normative value, EMFs could become dangerous when the incubator, and thus the newborn, is surrounded by other instruments or staff's devices that, through their own EMF, can give rise to electromagnetic interference (Besset et al., 2020). This phenomenon has the potential of altering ANS in three ways, especially in preterm or vulnerable infants. Firstly, by directly affecting brain development, as the skull has a low bone density that fails in adequately protecting newborns' brain from EMF (Besset et al., 2020). Secondly, by suppressing melatonin secretion, thus altering the immune system and, potentially, the CAP, with all the negative consequences already mentioned. Lastly, instrumental malfunctioning can induce life-threatening conditions (Carvajal de la Osa et al., 2020).

Incorrect Oxygenation

Correct oxygenation is paramount for the newborn's development and survival (Ho et al., 2020). Indeed, in preterm infants, both too high or too low oxygenation can be dangerous for the nervous system. For instance, too high oxygenation can easily induce lung injury, thus increasing inflammation and stressing the already immature CAP, whereas too low oxygenation can cause neurodevelopmental impairment and HIE (Rantakari et al., 2021).

It is noteworthy that delayed umbilical cord clamping, that is, delaying the procedure by 60–180 s seems to improve both peripheral and cerebral oxygenation, in addition to several hemodynamics parameters, which could reveal a positive effect on ANS function and development (Bruckner et al., 2021).

Posture

Babies' position is another factor that could impact ANS.

Indeed, a prone position improves oxygenation, increases PNS tone, and lowers both salivary cortisol and stress behavior

(Gomes et al., 2019). Nevertheless, prone sleeping is considered a risk factor for SIDS and it shows a lower short-term variability in HRV (Lucchini et al., 2016). It is thus noteworthy that, in the case of prone sleeping position, between the 2nd and 4th months after birth, both blood pressure and cerebral oxygenation seem to decrease, whereas there is a propensity for cortical arousal, maybe to counter the fall in pressure and oxygenation. This would explain why ANS impairment, for instance, due to prematurity, can make newborns more vulnerable to SIDS (Horne, 2018). As supporting evidence, complications including fetal growth restriction alter sleep stages by reducing active sleep (Aldrete-Cortez et al., 2019).

Supine position with manual restraint for flexion seems to have positive effects on ANS as measured through HRV (Gomes et al., 2019). In the same way, side position during feeding seems to have stabilizing effects on both ANS, as revealed by HR, and oxygenation in preterm newborns (Thoyre et al., 2012), although results are conflicting (Raczyńska and Gulczyńska, 2019).

Respect for the Baby's Sleep

Sleep impairment due to prematurity or environmental factors can lead to global neurodevelopmental complications (e.g., attention deficit, motor disability, and low cognitive abilities), as newborns show weaker functional connectivity networks during such impaired sleep (Uchitel et al., 2021).

Drugs including theophylline, caffeine, and methylxanthine, used to support respiratory functions have the potential of altering sleep quality and induce drowsiness. In the same way, improper light and noise can disrupt newborns' sleep duration, especially in preterm and very preterm ones (Gogou et al., 2019). But maybe, more than anything, medical procedures tend to impair babies' sleep the most, in particular since in preterm newborns many interventions are perceived as noxious and painful, thus heightening the stress response (Holsti et al., 2006; Weber and Harrison, 2019). Luckily, applying gentle touch, an "intervention" that stimulates the vagus nerve via C-tactile fibers, to medical care has the potential to regulate ANS function (Manzotti et al., 2019) and, thus, it is conceivable it could have protective effects on sleep. Otherwise, even clustered-care could help reduce the time the babies' sleep is disrupted (Holsti et al., 2006).

CLINICAL IMPLICATIONS FOR THE MANAGEMENT OF FETUSES AND NEWBORNS

"The newborn is not new, and neither is the fetus".

Paraphrased from a personal communication with Dr. Barry Schifrin

Our current clinical management of fetal and maternal health is not individualized and intermittent, suffering from a pattern of catching up with clinical symptoms instead of using biometric data to predict and steer developmental trajectories and health outcomes for the pregnant mother and her fetus during the delivery and after to their fullest potential. The fear of missing fetal injury during labor has led to soaring CS rates and the failure of the EFM has resulted in the misguided dissent over its utility in general throwing the proverbial baby with the bathwater.

What is amiss is the understanding and acting upon the fact that each fetus and newborn are not truly new, but, rather, come with weeks and months of experience *in utero*. Can we learn to assess this individual experience?

In this review, we highlighted the growing understanding of the vagus nerve's developmental physiology, the largest and perhaps most important homeostatic (or, better, homeokinetic) regulatory system in our body (Macklem and Seely, 2010; Herry et al., 2019). The exciting take-home message is that much of this development can be gauged non-invasively during pregnancy using readily available bioelectric sensors such as ECG or, less qualitatively well, but still useful, using ultrasound-derived HR estimation, the mainstay of today's CTG and EFM technologies that is in sore need of technological disruption.

What could and should we do to disrupt this status quo and enable individualized insights into the various developmental effects the vagus nerve maturation and pleiotropic physiology provide?

Research is increasingly pointing toward the existence of an "HRV code," that is, specific HRV spatio-temporal alterations related to specific organic alterations (e.g., hypoxia, cerebral, cardiac, or gut pathologies) (Herry et al., 2019; Frasch, 2020), to the impact of external factors (e.g., smoking, physical activity, diabetes), and to the fetal brain and nervous development (Hoyer et al., 2012, 2013a, 2015, 2017, 2019). Indeed, fHRV can be affected by both neural factors (i.e., ANS) and non-neural factors, e.g., metabolic and organic functions (DiPietro et al., 2015).

Therefore, knowing that the ANS-organ connections are established so early in the embryo could offer useful insight for comprehending the complex role of the vagus nerve and autonomic ganglia in the developing embryo/fetus. Currently, ANS development is usually assessed during the 3rd trimester, when vagus nerve myelination begins (Mulkey and du Plessis, 2018). However, the literature shows that the ANS can be assessed through fHRV from 15 to 20 WGA (Hoyer et al., 2012, 2013b, 2015, 2019; Shuffrey et al., 2019). Moreover, the heartbeat can be heard through ultrasound as early as the 6th WGA, the HR accelerates constantly during the first 8 WGA (Rodgers et al., 2015) and it was recently discovered that an intrinsic fHRV (i.e., due to sino-atrial node rhythms) exists—it can affect the global fHRV and be influenced by pregnancy events (e.g., hypoxia) (Frasch et al., 2020a) and it shows a specific "signature," that is, specific HRV metrics (Herry et al., 2019). Maternal and transabdominal ECG recorded in the early third trimester carry information about the chronic exposure of the mother–fetus dyad to stress detectable by deep learning, a form of artificial intelligence techniques (Sarkar et al., 2021). Therefore, it would be worth it to improve fetal cardiac monitoring technology in order to detect ECG and fHRV even in the first weeks of pregnancy.

Such an advancement could improve the embryo's health assessment during early pregnancy, thus going well-beyond detecting embryonic–fetal malformations and toward individualized prospective functional assessments. For instance, although we know that folate deficiency can impede the

neural tube closure or that alcohol can induce dramatic brain modifications (Moore et al., 2016), we still lack knowledge about more subtle influences, that is, the precise effects of just some alcohol during the first weeks of pregnancy or the early effects of maternal stress (Avalos et al., 2014; Caspers Conway et al., 2014; Antonelli et al., 2021). On the other hand, we are learning that certain factors (e.g., choline supplementation) may protect the fetus from excessive HPA axis activation and have long-lasting (into adulthood) effects on cognition through neural and epigenetic modifications (Korsmo et al., 2019). Furthermore, some studies have already shown the feasibility of correlating external factors related to maternal lifestyle or "internal" factors such as FM to fHRV changes as early as 20–28 WGA (DiPietro et al., 2007; Hoyer et al., 2019; Shuffrey et al., 2019).

As these findings are available for the 2nd and 3rd trimester, assessing fHRV in the 1st trimester may shed more light on what can protect or damage the ANS in these early formative stages of development and the consequences of its alterations.

Taken together, non-invasive assessment of maternal and fetal health via HRV monitoring throughout the pregnancy, from its earliest stages, will enable an individualized risk profiling and outcome projection during the latter stages of gestation and during delivery, a true disruption of the status quo that will improve maternal and perinatal health outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

FC, MC, SV, CV, and AM conceptualized the paper. FC, MC, SV, CV, MA, and MF wrote the first draft. All authors reviewed, edited and approved the final version of the paper.

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Transcutaneous Auricular Vagus Nerve Stimulation Improves Spatial Working Memory in Healthy Young Adults

Jin-Bo Sun[1,2†], Chen Cheng[2†], Qian-Qian Tian[1], Hang Yuan[2], Xue-Juan Yang[1,2], Hui Deng[1,2], Xiao-Yu Guo[1], Ya-Peng Cui[1], Meng-Kai Zhang[1], Zi-Xin Yin[1], Cong Wang[2] and Wei Qin[1,2]*

[1] Engineering Research Center of Molecular and Neuro Imaging of the Ministry of Education, School of Life Sciences and Technology, Xidian University, Xi'an, China, [2] Intelligent Non-invasive Neuromodulation Technology and Transformation Joint Laboratory, Xidian University, Xi'an, China

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Vitor Engracia Valenti,
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Peking University Sixth Hospital, China
Dahua Yu,
Inner Mongolia University of Science and Technology, China

***Correspondence:**
Wei Qin
wqin@xidian.edu.cn

† These authors have contributed equally to this work and share first authorship

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Working memory (WM) is one of the core components of higher cognitive functions. There exists debate regarding the extent to which current techniques can enhance human WM capacity. Here, we examined the WM modulation effects of a previously less studied technique, transcutaneous auricular vagus nerve stimulation (taVNS). In experiment 1, a within-subject study, we aimed to investigate whether and which stimulation protocols of taVNS can modulate spatial WM performance in healthy adults. Forty-eight participants performed baseline spatial n-back tasks (1, 3-back) and then received online taVNS, offline taVNS, or sham stimulation before or during (online group) the posttest of spatial n-back tasks in random order. Results showed that offline taVNS could significantly increase hits in spatial 3-back task, whereas no effect was found in online taVNS or sham group. No significant taVNS effects were found on correct rejections or reaction time of accurate trials (aRT) in both online and offline protocols. To replicate the results found in experiment 1 and further investigate the generalization effect of offline taVNS, we carried out experiment 2. Sixty participants were recruited and received offline taVNS or offline earlobe stimulation in random order between baseline and posttests of behavioral tests (spatial/digit 3-back tasks). Results replicated the findings; offline taVNS could improve hits but not correct rejections or aRT in spatial WM performance, which were found in experiment 1. However, there were no significant stimulation effects on digit 3-back task. Overall, the findings suggest that offline taVNS has potential on modulating WM performance.

Keywords: taVNS, working memory, n-back task, cognitive enhancement, non-invasive neuromodulation

INTRODUCTION

Working memory (WM), a core component of higher cognitive functions, is a system that combines attentional control with temporary storage and information manipulation (Chiesa et al., 2011). The field of cognitive psychology has underlined the importance of the ability to maintain and manipulate information over a period of seconds in WM and the vital role of WM for complex mental abilities, including problem solving, reasoning, and learning (Baddeley and Hitch, 1974). Specifically, one of the central limitations of human cognition is the restricted amount

of information that can be kept in WM (Cowan, 2001), and the differences in WM capacity among individuals are associated with variation in several important abilities such as academic performance (Gathercole et al., 2003), non-verbal reasoning ability (Kyllonen and Christal, 1990), and control of attention (Kane et al., 2007). Many clinical populations, including individuals with schizophrenia, attention-deficit/hyperactivity disorder (ADHD), stroke, and traumatic brain injury, also exhibit a lower WM capacity. Moreover, deficits in WM play a crucial role in normal neurocognitive aging and the rapid cognitive deterioration associated with dementias, such as Alzheimer's disease (Park and Reuter-Lorenz, 2009; Grady, 2012). Fortunately, researches at the beginning of the 2000s showed that the WM was an ability that could be increased by training or psychosocial inventions rather than an immutable individual characteristic (for review, see Constantinidis and Klingberg, 2016). Therefore, the available ways to improve the capacity of WM are urgently needed.

At present, non-invasive brain stimulation techniques, such as transcranial direct current stimulation (tDCS; Andrews et al., 2011; Arkan, 2019; Živanović et al., 2021), transcranial alternating current stimulation (tACS; Ermolova et al., 2019; Benussi et al., 2021), and transcranial magnetic stimulation (TMS; Chen et al., 2015; Hulst et al., 2017), have become one of the mainstream clinical treatment approaches to moderate the WM because of their potential, convenience, and safety. Although some of previous studies have demonstrated the availability of transcranial current stimulation on modulating WM by altering the activity of neurons through changing the resting membrane potential of neurons (Bindman et al., 1962; Nitsche and Paulus, 2000), some recent studies found limited positive effects of tDCS on WM accuracy with a minor reaction time enhancement in healthy cohorts (e.g., Koshy et al., 2020; Shires et al., 2020; for meta-analysis, see Hill et al., 2016; Medina and Cason, 2017). Indeed, the effect of tDCS on WM heavily relies on the stimulation form (online/offline), stimulation duration, current density, and stimulation area (right or left dorsolateral/ventral lateral prefrontal cortex, posterior parietal cortex, or premotor cortex, etc.), which have been further studied (e.g., Nikolin et al., 2018; Živanović et al., 2021). The same problems also appeared in tACS and TMS studies (Chung et al., 2018; Pavlov et al., 2020). At the same time, transcutaneous auricular vagus nerve stimulation (taVNS), as an emerging cranial nerve stimulation method, represents a promising alternative (van Leusden et al., 2015).

The cranial nerves are a specialized part of the peripheral nervous system that emerges directly from the brain rather than through the spine. For each cranial nerve, there is a relatively accessible portion, and each of them is intimately linked to perception and regulation of central nervous system, with "bottom-up" functions in cognition and clinical disorders, which makes them a special target for neuromodulation. The vagus nerve, which is made up of approximately 80% sensory afferent fibers, is the longest cranial nerve (Agostoni et al., 1957). It projects to the nucleus tractus solitarii (NTS) in the medulla, before it is relayed further to other brainstem nuclei and higher-order structures, including the thalamus, hippocampus, amygdala, and insula (Goehler et al., 2000; Saper, 2002). Since the end of the last century, multiple studies in clinical populations have found the special enhancement of vagus nerve stimulation (VNS) on cognition and memory (e.g., Clark et al., 1999; Schachter, 2004; Ghacibeh et al., 2006; Merrill et al., 2006). Recently, some brain imaging studies found that, taVNS, a non-invasive neurostimulation technique that targets the auricular branch of the vagus nerve, produced increased blood oxygen level–dependent signal in the contralateral postcentral gyrus, bilateral insula, frontal cortex, right operculum, and left cerebellum (Badran et al., 2017). Yakunina et al. (2017) suggested that taVNS could modulate the activities in the locus coeruleus (LC) and the areas innervated by this region, including the insula, hippocampus, amygdala, and thalamus. As frontal cortex, hippocampus, and the neurotransmitters, such as norepinephrine (NE), which is released by LC, are known to be important for many cognitive functions, including WM (Gu, 2002; Duffau, 2006; Funahashi, 2017), taVNS gains ever-increasing scientific interest in cognition modulation. In healthy volunteers, several clinical studies have demonstrated that taVNS could modulate a series of cognitive processes, such as emotion recognition (Colzato et al., 2017), divergent thinking (Colzato et al., 2018), inhibitory control processes (Beste et al., 2016), response selection functions (Steenbergen et al., 2015), conflict adaptation (Fischer et al., 2018), attentional processes (Ventura-Bort et al., 2018), and post-error slowing (Sellaro et al., 2015), and so on. In addition, after the first study to explore the effect of taVNS on memory performance (Jacobs et al., 2015), studies have investigated the enhancement of taVNS on verbal memory (Mertens et al., 2020), high-confidence recognition memory (Giraudier et al., 2020), memory reinforcement (Hansen, 2019), long-term emotional episodic memory (Ventura-Bort et al., 2021), and associative memory (Jacobs et al., 2015).

Nevertheless, most of these studies did not refer to WM performance; thus, the effect of taVNS on WM is still unknown. Although direct evidence has been scant, the findings from invasive VNS have shown that the VNS over the left cervical vagus nerve improved immediate WM of epilepsy patients (Sun et al., 2017). Therefore, it is valuable to explore the immediate regulatory effect of taVNS on WM. In the current study, we aimed to investigate the effects of taVNS on WM in healthy volunteers by using n-back tasks. There were two specific questions: first, is there any difference between online and offline taVNS protocols in modulation effect on WM? Up to now, both online (e.g., Jacobs et al., 2015; Colzato et al., 2018; Giraudier et al., 2020) and offline (e.g., Alicart et al., 2020; Warren et al., 2020) taVNS could be seen in researches, and to the best of our knowledge, none of the studies have compared their efficiency. Meta-analyses of tDCS studies have suggested that for healthy population the significant effect could be found only in offline stimulation (see Hill et al., 2016), which might be caused by different neurobiological processes; namely, the online effects might result from resting membrane potential alterations, whereas the offline effects appear to result from modulation of synaptic plasticity (Stagg and Nitsche, 2011; Medeiros et al., 2012; Hill et al., 2016). In addition, the neurotransmitter release needs time to take effect, which might lead to a stronger effect of offline protocol

than online stimulation. However, several studies, such as that of Neuser et al. (2020), reported a significant online taVNS effect on motivation. Thus, we compared the effects of online and offline taVNS in the first experiment. Second, are there some generalization effects of taVNS on modulating WM performance, namely, does taVNS have effects on more than one modality of WM tasks? As taVNS has extensive activation on cerebral cortex (Yakunina et al., 2017) and the neurotransmitters released by taVNS might affect a series of cognition, it might have a comprehensive effect on WM performances. However, according to previous studies, there were different neural bases for verbal (like digit) and non-verbal (like spatial) WM tasks (Owen et al., 2005). Thus, the specific effects of taVNS on different modalities and WM tasks are valuable to investigate. To testify this question, we used spatial WM tasks in the first experiment because it has been heavily investigated, and numerous studies have found that it could be improved by the increased activity of prefrontal neurons and dopaminergic transmission (for review, see Constantinidis and Klingberg, 2016) and then tested the corresponding taVNS effects on digit WM tasks in the second experiment. To sum up, in this study, we aimed at (1) investigating the taVNS effects on spatial WM performance and choosing the optimal stimulation protocol between online and offline stimulation and (2) replicating the taVNS effects on spatial WM performance and further investigating its influence on digit WM tasks.

MATERIALS AND METHODS

Experiment 1

In this experiment, we aimed to investigate the enhancement of taVNS on spatial WM by using online taVNS, offline taVNS, and sham groups with two n-back tasks, namely 1-back and 3-back tasks. The behavioral changes between baseline and posttest per condition (online taVNS, offline taVNS and sham) were calculated to evaluate the effects of taVNS on WM.

Participant

Forty-eight healthy students at Xidian University were included in this experiment. Each of them had to participate in three sessions, including online taVNS, offline taVNS, and sham. All participants were right-handed, with no smoking, neurological disease, or brain damage history. No participants reported ear injuries, drinking, or taking drugs 48 h before the experiment. Before the experiment, participants were provided with information about the stimulation procedure and experimental protocols and written informed consent. Participants were instructed that they could withdraw from the experiment at any time if they did not wish to continue, and all of them could receive corresponding remuneration. All research procedures were conducted in accordance with the Declaration of Helsinki and approved by the institutional research ethics committee of Xijing Hospital of the Air Force Medical University (KY20192008-X-1). Finally, 46 participants completed the experiment successfully (25 female, average age = 20.39 $\pm$ 1.96 years, range = 18–25 years), whereas two subjects were excluded from the data analysis because of withdrawing.

Design

The experiment was a within-subjects design, with each participant completing three separate sessions, which were different in stimulation conditions [i.e., online taVNS, offline taVNS, and sham (stimulation equipment placebo); **Figure 1A**]. In the online taVNS condition, participants first tested the baseline of behavioral tasks and then had a 25-min rest. Then, they received a taVNS stimulation at the beginning of the posttest of behavioral task until the end, which lasted approximately 15 min. In the offline taVNS section, participants completed 15-min baseline test of the behavioral tasks, a 25-min taVNS stimulation, and a 15-min posttest of behavioral tasks in turn. The process of sham condition was similar with offline taVNS, except that the 25-min stimulation was instead by a 30-s stimulation at the beginning and end time. The three sessions were separated by a period of at least 2 days (M_{days} = 3.46 $\pm$ 1.50), and the stimulation orders were counterbalanced between participants. One or 2 days before the formal experiment sessions, participants needed to come to the laboratory to familiarize themselves with the experimental procedure, practice the behavioral tasks (completed whole tasks until reached an accuracy rate of 60%), and test the acceptability of taVNS.

taVNS Stimulation Equipment and Parameters

The electrical stimulation equipment used in this study was made by our joint laboratory (XD-Kerfun BS-VNS-001), an upgrade version of the one that has been successfully used in previous researches (e.g., Shen et al., 2021; Sun et al., 2021). The taVNS channel was connected to two silver chloride electrodes (outer diameter 7 mm). The anode and cathode of taVNS were both placed in the left cymba conchae with the cathode inside and 0.5 cm apart from the anode. The electrical stimulation waveform was a single-phase rectangular pulse with a pulse width of 500 μs and frequency of 25 Hz. The current was delivered with a cycle of 30 s on and 30 s off to avoid habituation.

As perceived and tolerated stimulation intensity varies across participants, the current intensity was determined by each participant by using the threshold method to match the subjective experience of the stimulation. Before formal test of each session, there was a threshold test. In the threshold test section, participants were asked to give direct feedback on their feeling of each stimulation intensity on a 10-point scale ranging from (1) no perception to (3) light tingling to (6) strong tingling to (10) intense pain. The stimulation started with an intensity of 0.1 mA and increased stepwise in 0.1-mA increments until the subject reported a slight feeling of pain (corresponding to $\geq$7 on the subjective sensation scale) and then decreased in 0.1-mA increments until 0.1 mA below the light tingling threshold (corresponding to $\leq$3 on the subjective sensation scale). The protocol was repeated twice, and the average of the intensities rated as 5 (mild tingling) was used as the stimulation threshold (Neuser et al., 2020). The individual stimulation intensities varied from 0.1 to 1.3 mA for the online taVNS group (M_{online} taVNS = 0.7 $\pm$ 0.36 mA) and from 0.2 to 1.6 mA for the offline taVNS group (M_{offline} taVNS = 0.69 $\pm$ 0.38 mA). For the sham group, all the participants tested only the threshold that varied from 0.3 to 1.5 mA and received an intensity at

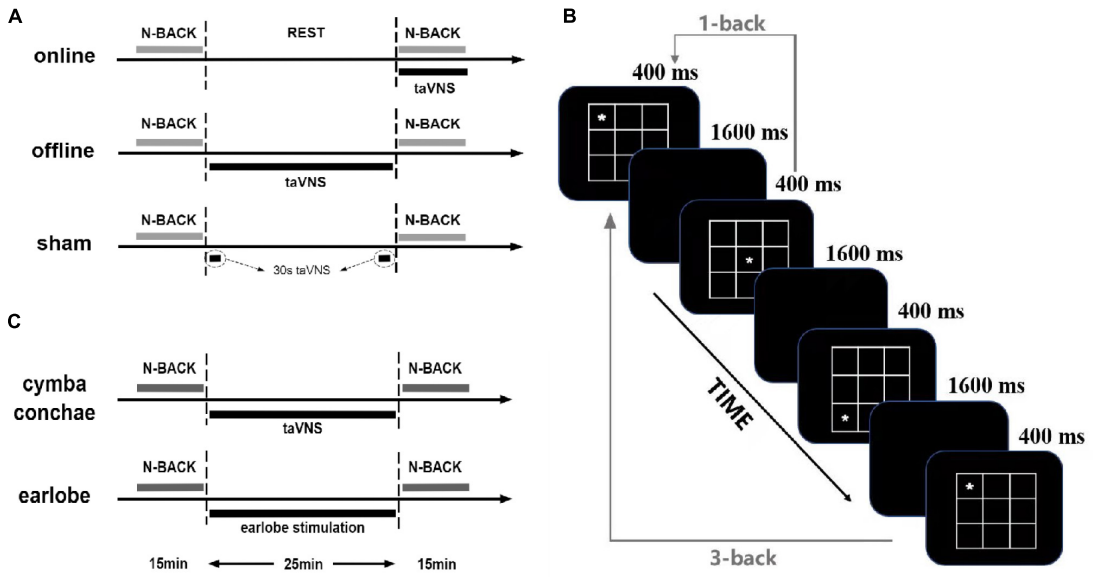

FIGURE 1 | Overview of the study and n-back tasks: **(A)** the protocol of experiment 1; **(B)** the paradigm of spatial n-back tasks, changing the spatial stimuli by digits is the digit 3-back task; **(C)** the protocol of experiment 2.

the beginning and the end of the stimulation section for 30 s (M_{sham} = 0.73 ± 0.27 mA).

Working Memory Tasks

The n-back task is one of the most frequently used paradigms in the assessment of WM capacity, which needs continuous updating of the transient memory storage with novel stimuli in order to compare the new stimuli with previously presented ones (Jarrold and Towse, 2006). In this experiment, we used 1- and 3-back tasks with spatial stimuli. There were four blocks (1-back, 3-back, 1-back, 3-back) with 72 experiment trials in each block. Each block was separated by a 30-s rest period. Before these four blocks, there was a training block with 16 trials for 1-back and 3-back tasks, respectively. Participants were instructed to press "F" when the site of the symbol ("*") was the same as in one or three trials earlier (namely, "matching" trial), but otherwise pressed "J" (namely, "mismatching" trial, **Figure 1B** for detailed parameters). Each trial was inserted as picture format with 257 × 257 pixels of width and height. One-third of the trials were matching. Training trials were before experiment trials, and participants could not start the formal experiment unless their training accuracy rate reached more than 75% and the average reaction time was less than 1,000 ms. Psychology experiment computer program E-Prime version 3.0 was used to administer the tasks and record response accuracy and reaction time of all the participants.

Experiment 2

Based on the design in experiment 1, in experiment 2 we used an active sham group (stimulation placebo), that is, earlobe stimulation group, which was used widely in taVNS modulation studies (e.g., Giraudier et al., 2020; Mertens et al., 2020; Neuser et al., 2020) to further replicate the results that were found in experiment 1. Besides, we added a 3-back task of digit to explore the generalization effect of taVNS on different WM tasks.

Participants

Sixty healthy students at Xidian University were included. The inclusion criteria were the same as in experiment 1, mainly including the right handedness, no smoking, no neurological disease, and no brain damage history. No participants reported ear injuries, drinking, or taking drugs 48 h before the experiment. One subject was excluded because of confusing matching and mismatching response, and another subject was excluded because of low baseline accuracy (<30%). Finally, there were 58 students in data analysis (24 female students; average age = 19.90 ± 1.49 years, range = 18–23 years). Each participant was provided written informed consent, and all research procedures were conducted in accordance with the Declaration of Helsinki and approved by the institutional research ethics committee of Xijing Hospital of the Air Force Medical University (KY20192008-X-1).

Design

The experiment was a within-subjects design, too, with each participant completing two separate sessions, which were different in stimulation conditions [i.e., offline taVNS and offline earlobe stimulation (offline ES); **Figure 1C**]. Despite the stimulation site, all the other conditions were the same in the two groups. There was at least a 2-day period (M_{days} = 2.93 ± 0.49) between two sessions.

taVNS Stimulation Equipment and Parameters

All the information of taVNS and stimulation intensity threshold was the same as in experiment 1, except that the anode and

cathode of taVNS were both placed in the left earlobe for the active sham group with anode front side and cathode back side. The stimulation intensity threshold was tested in the same way as in experiment 1, with stimulation intensities varying from 0.3 to 2.7 mA for the taVNS group (M_{off}line taVNS = 0.74 mA ± 0.37) and from 0.3 to 2.4 mA for the earlobe group ($M_{earlobe-sham}$ = 0.84 mA ± 0.39). Both offline taVNS and offline ES groups would receive a 25-min stimulation between baseline test and posttest.

Working Memory Tasks

Both spatial and digit 3-back tasks were used in this experiment with two blocks of each form. In total, there were four blocks (spatial, digit, spatial, and digit) with 72 trials in each block. The spatial 3-back paradigm was the same as in experiment 1. The procedure of digit 3-back task was the same as in spatial 3-back task, but the stimuli were changed from the site of "*" to nine Arabic numbers (1, 2, 3, 4, 5, 6, 7, 8, 9). The font of each number was Times New Roman, and the font size was 72. Participants were instructed to press "F" when the number was the same as in three trials earlier, but otherwise pressed "J." One-third of the trials were matching, and there was a 30-s period between each block. The paradigms and requirement of training blocks were the same as in experiment 1.

Data Analysis

There are four indicators that are often used, that is, hits (the accuracy of matching trials), correct rejections (the accuracy of mismatching trials) or false alarms (one minus correct rejections), *d* prime (*d*', hits minus false alarms), and reaction time (e.g., Jongkees et al., 2017). *d*' was first introduced based on signal detection theory to avoid distorted hits by false alarms (Haatveit et al., 2010). However, it is more of a receptivity indicator than a WM memory ability indicator, which mainly focused on participants' reaction tendency (Macmillan and Creelman, 1991). In a task with unbalanced matching and mismatching trials, there might be different change tendencies of hits and false alarms, whereas *d*' might weaken or even conceal these changes (e.g., Haatveit et al., 2010). As the present study was implemented in a healthy cohort whose improvement potential of WM is small, we used both hits and correct rejections (has similar power with false alarms), rather than *d*', as indicators to avoid missing any changes. Besides, we used the mean reaction time for accurate trials (aRT). Thus, three indicators were calculated for both experiments in the baseline (T0) and during stimulation/poststimulation (T1) of each condition for each participant. For each experiment, the trial if participant missed to response was regarded as a response error. One-way repeated-measures analysis of variance (ANOVA) and paired *t* test were used to check whether the indicators in T0 matched across conditions. The statistical analyses were performed in SPSSv26 (IBM Corp., Armonk, NY, United States) and MATLAB2019b (The MathWorks, Natick, MA, United States).

In experiment 1, the effect of taVNS on indicators were first assessed by using 2 × 3 repeated-measures ANOVA, with both time (T0, T1) and condition (online taVNS, offline taVNS, and sham) as within-subjects factors. *Post hoc* effect analysis was used in significant interaction effects via the paired *t* test for time (T0 vs. T1). In addition, one-way repeated-measures ANOVAs were used to directly compare the change scores from baseline (Δ score = T1 - T0) between conditions. Bonferroni correction was used to explore any significant effects.

In experiment 2, the effects of taVNS on indicators were first assessed using 2 × 2 repeated-measures ANOVAs, with both time (T0, T1) and condition (offline taVNS, and offline ES) as within-subjects factors. *Post hoc* effect analysis was used in significant interaction effects via the paired *t* test for time (T0 vs. T1). In addition, the paired *t* test was used to directly compare the change scores from baseline (Δ score = T1 - T0) between conditions. Bonferroni correction was used to explore any significant effects.

RESULTS

Experiment 1

There was no significant difference in baseline performance among the three conditions. There were no obvious feeling difference and adverse reactions of both online and offline taVNS, compared with the sham group (see **Supplementary Material** for detailed information).

Effects of taVNS on Spatial 1-Back Task

There was no significant main effect of conditions for hits [$F(2,90) = 0.18$, $p = 1.00$, $\eta_p^2 = 0.004$], correct rejections [$F(2,90) = 0.94$, $p = 1.00$, $\eta_p^2 = 0.02$] and aRT [$F(2,90) = 0.03$, $p = 1.00$, $\eta_p^2 = 0.001$]. The main effect of time was not significant in hits [$F(1,45) = 0.31$, $p = 1.00$, $\eta_p^2 = 0.01$] and correct rejections [$F(1,45) = 2.98$, $p = 0.55$, $\eta_p^2 = 0.06$], but was significant in aRT [$F(1,45) = 9.93$, $p = 0.02$, $\eta_p^2 = 0.18$]. The aRT at posttest was significantly shorter than that at baseline. The two-way interaction between time and groups was not significant in hits [$F(2,90) = 0.93$, $p = 1.00$, $\eta_p^2 = 0.02$], correct rejections [$F(2,90) = 1.04$, $p = 1.00$, $\eta_p^2 = 0.02$], and aRT [$F(2,90) = 0.01$, $p = 1.00$, $\eta_p^2 < 0.001$]. It suggested that both online and offline taVNS had no significant modulation on 1-back spatial WM (**Table 1** and **Figure 2**).

Effects of taVNS on Spatial 3-Back Task

There was no significant main effect of conditions for hits [$F(2,90) = 1.08$, $p = 1.00$, $\eta_p^2 = 0.02$], correct rejections [$F(2,90) = 3.09$, $p = 0.30$, $\eta_p^2 = 0.06$], and aRT [$F(2,90) = 0.11$, $p = 1.00$, $\eta_p^2 = 0.002$]. The main effect of time was not significant in hits [$F(1,45) = 5.39$, $p = 0.15$, $\eta_p^2 = 0.11$] and correct rejections [$F(1,45) = 0.72$, $p = 1.00$, $\eta_p^2 = 0.02$], but was significant in aRT [$F(1,45) = 32.46$, $p < 0.001$, $\eta_p^2 = 0.42$]. The reaction at posttest was much faster than that at baseline. The two-way interaction between time and groups was not significant in correct rejections [$F(2,90) = 1.20$, $p = 1.00$, $\eta_p^2 = 0.03$] and aRT [$F(2,90) = 0.54$, $p = 1.00$, $\eta_p^2 = 0.01$], but was significant in hits [$F(2,90) = 5.58$, $p = 0.03$, $\eta_p^2 = 0.11$]. The detailed information is presented in **Table 1**. *Post hoc* analysis showed that there were no differences between baseline and posttests in online taVNS [$t(45) = 0.07$, $p = 1.00$] and sham [$t(45) = -0.45$, $p = 1.00$] groups, whereas there was a significant improvement in the offline aVNS group

TABLE 1 | Accuracy and reaction time for n-back tasks per condition of experiment 1.

	Online group				Offline group				Sham group				Statistical test
	Baseline		Post		Baseline		Post		Baseline		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*(2,90)
1-Back													
Hits	0.870	0.012	0.869	0.013	0.866	0.013	0.871	0.013	0.872	0.011	0.855	0.018	0.93
Correct rejections	0.970	0.003	0.969	0.007	0.967	0.005	0.973	0.004	0.971	0.003	0.977	0.003	1.04
Accurate RT (ms)	540.86	17.91	511.39	17.31	538.32	17.76	510.96	14.35	537.88	15.93	507.78	13.39	0.008
3-Back													
Hits	0.757	0.021	0.766	0.021	0.751	0.023	0.808	0.018	0.758	0.021	0.751	0.026	5.58*
Correct rejections	0.918	0.021	0.940	0.008	0.930	0.021	0.958	0.007	0.930	0.023	0.945	0.008	1.25
Accurate RT (ms)	665.93	26.03	590.60	19.01	663.13	27.27	605.81	20.05	660.51	22.69	612.58	24.21	0.54

Hits means accuracy in matching trials; correct rejections means accuracy in mismatching trials; accurate RT means specifically referring to reaction time in all correct trials. F values referred to the two-way interaction. One asterisk indicates a corrected p < 0.05.

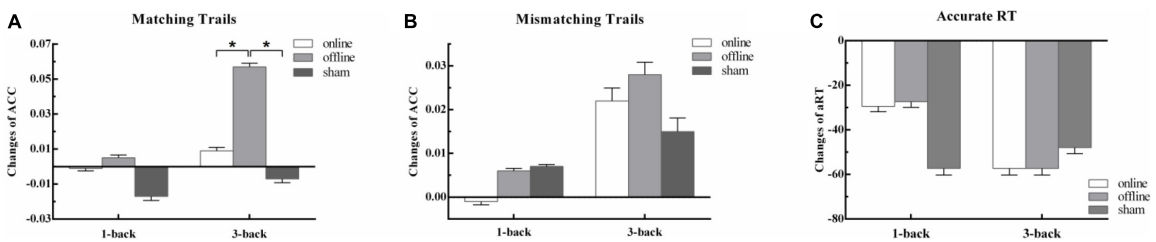

FIGURE 2 | The behavior outcomes of experiment 1. Task outcome changes (T1 - T0): **(A)** the change of accuracy rate for per condition in matching trials; **(B)** the change of accuracy rate for per condition in mismatching trials; **(C)** the change of mean reaction time of correct trials for per condition, and the quantities' units were ms. One asterisk indicates a corrected *p* value less than 0.05.

[$t(45) = 4.04$, $p = 0.001$]. One-way repeated-measures ANOVA of the Δ score (T1 - T0) among the online taVNS, offline taVNS, and sham groups found a significant difference [$F(2,90) = 5.59$, $p = 0.005$], which showed that the Δ score of the offline taVNS group was significantly higher than that of the online group ($d = 0.05$, $p = 0.02$) and sham group ($d = 0.06$, $p = 0.01$). The results are shown in **Figure 2**.

Experiment 2

There was no significant difference in baseline performance between the two conditions. There were no obvious feeling difference and adverse reactions of both offline taVNS and offline ES (see **Supplementary Material** for detailed information).

Effects of taVNS on Spatial 3-Back Task

The main effect of stimulus site for hits [$F(1,57) = 3.78$, $p = 0.34$, $\eta_p^2 = 0.06$], correct rejections [$F(1,57) = 0.81$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.42$, $p = 1.00$, $\eta_p^2 = 0.01$] was not significant. The main effect of time was significant in aRT [$F(1,57) = 15.87$, $p = 0.001$, $\eta_p^2 = 0.22$] and was marginal significant in hits [$F(1,57) = 7.36$, $p = 0.05$, $\eta_p^2 = 0.11$] and correct rejections [$F(1,57) = 6.73$, $p = 0.07$, $\eta_p^2 = 0.11$]. The aRT of posttest was shorter than that at baseline, and the hits at posttest were higher than those at baseline, whereas the hits and correct rejections at posttest were higher than those at baseline. The two-way interaction between time and groups was not significant in aRT [$F(1,57) = 0.07$, $p = 1.00$, $\eta_p^2 = 0.001$], but was significant in hits [$F(1,57) = 11.32$, $p = 0.006$, $\eta_p^2 = 0.17$] and correct rejections [$F(1,57) = 9.36$, $p = 0.02$, $\eta_p^2 = 0.14$]. The detailed information was presented in **Table 2**. *Post hoc* analysis of hits showed that there were no differences between baseline and posttests in earlobe group [$t(57) = 0.41$, $p = 1.00$], whereas there was a significant improvement in the taVNS group [$t(57) = 4.25$, $p < 0.001$]. The paired *t* test of the Δ score of hits between the two groups showed a significant difference [$t(57) = 3.36$, $p = 0.001$], which suggested that the Δ score of the taVNS group was significantly higher than that of the earlobe group (**Figure 3**). The paired *t* test of the Δ score of correct rejection between the two groups showed a significant difference [$t(57) = -2.22$, $p = 0.03$], which suggested that the Δ score of the taVNS group was significantly lower than that of the earlobe group (**Figure 3**).

Effects of taVNS on Digit 3-Back Task

There was no significant main effect of conditions for hits [$F(1,57) = 0.25$, $p = 1.00$, $\eta_p^2 = 0.004$], correct rejections [$F(1,57) = 0.50$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.63$, $p = 1.00$, $\eta_p^2 = 0.01$]. The main effect of time was not significant in hits [$F(1,57) = 1.76$, $p = 1.00$, $\eta_p^2 = 0.03$] and correct rejections [$F(1,57) = 0.55$, $p = 1.00$, $\eta_p^2 = 0.01$], but was significant in aRT [$F(1,57) = 7.99$, $p = 0.04$, $\eta_p^2 = 0.12$]. The aRT at posttest was significantly shorter than that at baseline. The two-way interaction between time and groups was not significant in hits [$F(1,57) = 0.04$, $p = 1.00$, $\eta_p^2 = 0.001$], correct rejections [$F(1,57) = 0.32$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.01$,

TABLE 2 | Accuracy and reaction time for spatial and digit 3-back tasks per condition of experiment 2.

	taVNS group				Earlobe group				Statistical test
	Baseline		Post		Baseline		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*(1,57)
Spatial 3-back									
Hits	0.825	0.018	0.870	0.014	0.828	0.021	0.832	0.017	11.32**
Correct rejections	0.965	0.005	0.967	0.005	0.955	0.006	0.970	0.004	9.36*
Accurate RT (ms)	612.22	21.92	555.42	19.81	618.12	25.61	567.06	22.07	0.07
Digit 3-back									
Hits	0.871	0.015	0.885	0.013	0.868	0.020	0.878	0.016	0.04
Correct rejections	0.972	0.004	0.975	0.005	0.976	0.003	0.976	0.003	0.32
Accurate RT (ms)	525.66	19.42	496.91	17.81	533.56	19.98	506.53	17.09	0.01

Hits, correct rejections, accurate RT, and F values have the same meaning with ***Table 1****. One asterisk indicates a corrected p < 0.05, and two asterisks indicate a corrected p < 0.01.*

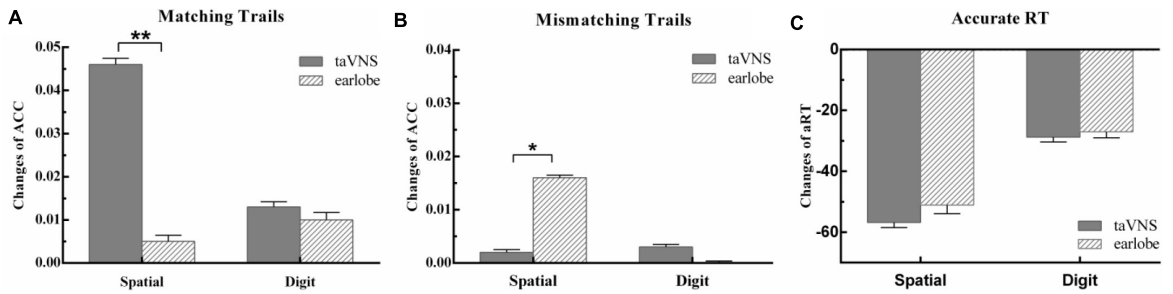

FIGURE 3 | The behavior outcomes of experiment 2. Task outcome changes (T1 - T0): **(A)** the change of accuracy rate for per condition in matching trials; **(B)** the change of accuracy rate for per condition in mismatching trials; **(C)** the change of mean reaction time of correct trials for per condition, and the quantities' units were ms. One asterisk indicates a corrected *p* value less than 0.05, and two asterisks indicate a corrected *p* value less than 0.01.

$p = 1.00$, $\eta_p^2 < 0.001$]. It suggested that both taVNS and earlobe groups had no significant modulation on 3-back digit WM (**Table 2** and **Figure 3**).

DISCUSSION

The current study assessed the effects of taVNS on WM performance under varying conditions: online and offline protocols, stimulation equipment sham and active sham (earlobe stimulation), 1-back and 3-back spatial WM tasks, and spatial and digit modalities of WM tasks. Overall, the experiments yielded relatively robust findings about the improvement of taVNS on offline spatial WM performance, no matter compared with online protocol, equipment sham, or active sham group. However, the enhancement of taVNS specifically appeared in offline 3-back spatial WM tasks, but not in online 1-back spatial or 3-back digit tasks.

To the best of our knowledge, this is the first study to investigate the immediate enhancement of WM by taVNS in healthy adults. In the first experiment, we discovered the improvement of offline taVNS on spatial WM capacity by comparing with online taVNS and sham groups, whereas in the second experiment, we replicated the results in experiment 1 with an active sham (offline ES) group. With the exploration and replication samples, we put relatively robust results about the improvement of offline taVNS on WM. There might be three reasons for the improvement. First, as we know, the vagus nerves project to the NTS in the medulla, before being relayed further to other brainstem nuclei and higher-order structures, including the thalamus, hippocampus, amygdala, and insula (Goehler et al., 2000; Saper, 2002). When the vagus nerve projects to the NTS and activates the noradrenergic neurons in the LC and cholinergic neurons in the nucleus basalis, NE and acetylcholine consequently release in wide areas of the cortex (Gu, 2002; Hassert et al., 2004; Roosevelt et al., 2006; Nichols et al., 2011). Subsequently, α1-adrenergic receptors in the dorsal raphe nucleus are activated by NE and release serotonin (Manta et al., 2009). These neurotransmitters can enhance behavioral and cognitive processes, including WM capacity by facilitating neural plasticity (Gu, 2002; Duffau, 2006). Second, long-term potentiation (LTP) as a process involving persistent strengthening of synapses that leads to a long-lasting increase in signal transmission between neurons is widely recognized as a cellular mechanism of memory formation (Bliss and Collingridge, 1993; Bear and Malenka, 1994). As NE is known to facilitate this early LTP through activating β-noradrenergic receptors, the VNS-induced LC-NE release system has been proposed as another possible mechanism of modulating memory performance (Harley, 2007; Mueller et al., 2008). Third, attentional mechanisms might contribute to the improvement of taVNS on WM performance. WM and attention

are interacting constructs and tightly intertwined, as attention provides the basis for selecting what information will be encoded in WM (Awh et al., 2006). Previous studies found that VNS could increase early visual N1 amplitude, which is similar to what is seen with increased level of attention (Mangun and Hillyard, 1991; Luck and Ford, 1998). Sun et al. (2017) further discovered that VNS could increase the WM capacity of epilepsy patients by attentional mechanisms.

However, the improved effect of taVNS on WM was absent in the online stimulation group. One would argue that the absence of enhancement in online condition can be attributed to the shorter stimulation time (approximately 15 min) compared with the offline group (25 min). This is possible, but not highly plausible, as no studies have found compelling evidence that increasing stimulation time led to stronger effects on cognitive performance. In fact, the stimulation time of online taVNS in previous studies was highly dependent on the length of behavioral tasks, which varied from 13 to 75 min (e.g., Giraudier et al., 2020; Neuser et al., 2020; Tona et al., 2020), whereas the positive results did not increase with the increase in stimulation time. Besides, if the timing was crucial, there is probably some systemic difference between the 1- and 3-back tasks, which are completed in turn, but this was not the case. Further researches are needed to investigate the specific effects of stimulation time. However, the most likely explanation in the current studies lies in different mechanism behind online and offline taVNS effects, which need more researches. However, as discussed previously, the effect of taVNS on WM mainly depends on the LC-NE release system, which need time to take effect, and this might be the first possible explanation. Furthermore, researches from tDCS showed that there was a trend toward improvement for offline WM performance but was not on online performance in the healthy subjects, whereas the neuropsychiatric cohort exhibited an opposite pattern (e.g., Živanović et al., 2021; for review, see Hill et al., 2016). As online tDCS alters neuronal firing by changing membrane potential, whereas the aftereffects of tDCS stem from changes in synaptic strength, these authors attributed their findings to the optimal cortical excitation/inhibition balance and insufficient neuronal excitability changes in online stimulation in healthy adults. The same pattern appeared here, as Sun et al. (2017) found an online VNS effect in epilepsy patients, whereas this study showed an offline taVNS effect in healthy participants. Thus, we consider that there might be a similar reason that the insufficient vagus nerve excitability changes during online taVNS in healthy adults restrict behavioral changes. Finally, the distracting effect of online stimulation might cover up the modulation effect of online taVNS, and this should be taken into account in further studies.

Beyond the stimulation protocols, the modulation of taVNS might also depend on the properties of the output measure used. Namely, although n-back is a typical paradigm for WM assessment, the numbers of steps back, for example, 1-back (Sandrini et al., 2012), 2-back (Keshvari et al., 2013; Hill et al., 2019), and 3-back (Hill et al., 2019; Živanović et al., 2021), as well as the stimuli of the task, for example, spatial (Živanović et al., 2021), letters (Hill et al., 2019), digits (Nozari and Thompson-Schill, 2013), and objects (Keshvari et al., 2013), are highly variable in the literature. The current study specifically found the taVNS effect on 3-back spatial WM task but not on spatial 1-back or digit 3-back tasks. A meta-analysis suggested that the verbal n-back, like the digit n-back task, was associated with enhanced activation in the left ventrolateral prefrontal cortex, whereas the non-verbal location n-back task was associated with enhanced activation in a set of regions that have been described as a spatial attention network, including right dorsolateral prefrontal, lateral premotor, and posterior parietal cortex (Owen et al., 2005). Given the discussion above, we know that the attentional mechanisms might be one of the reasons for the taVNS effect on WM performance. The improved selective attention induced by VNS (Sun et al., 2017) might have a larger effect on the spatial attention network and contributed to the difference in the improvement of taVNS on spatial and digit WM performance. Besides, the researches in tDCS found that the modulation of electric field on WM depends on the baseline performance (e.g., Assecondi et al., 2021). The individuals or tasks with lower baseline outcome were more likely to have a higher improvement. For 1-back spatial task, the high baseline performance might restrict the modulation of taVNS. It should be noted that the baseline performance of the digit 3-back task was similar to that of the spatial 1-back task, and it might be another probable reason for the uselessness of taVNS. Unfortunately, the present study did not use a digit WM with higher difficulty, and further researches are needed.

Finally, except the difference between the effective stimulation protocol by Sun et al. (2017; online stimulation) and the current study (offline stimulation), these two studies found that for both epilepsy patients and healthy adults, the increase in WM outcome appeared only in hit reactions but not in reaction time, missing response, or correct rejections. These results show consistency between the clinical study and laboratory investigation, which make these results more convincing. According to Sun et al. (2017), the improvement of selective attention increased accuracy in target trials, that is, hits, whereas the unchanged general level of attention was attributed to the unchanged aRT and correct rejection rate. Besides, the high baseline of correct rejections might restrict the increased potential of posttest, and the improved familiarity of the tasks leads to a comprehensive main effect of aRT in all groups. However, as shown in the meta-analysis, tDCS could improve the reaction time in healthy adults (Hill et al., 2016); the synergistic effects of tDCS and taVNS might lead to a more comprehensive improvement in WM, which has been proven in neuroimaging study (Sun et al., 2021), whereas the effects on behavior need to be investigated in the future. Another interesting finding that should be noted is that the offline ES increased participants' correct rejection rate. It might be caused by the special effect of nervus auricularis magnus, which was activated by earlobe stimulation. The further effect and mechanism of earlobe stimulation need more studies.

Nevertheless, there are still some limitations to the current study. First, the optimal condition offline taVNS, especially the stimulation time, was not clear in the present study. Although the stimulation time in this study has led to a strong effect, we know little about the effects in longer or shorter stimulation conditions. As the stimulation time influences the convenience

and safety and is important for standard protocol, it needs more investigation. Besides, the modulation effect of online taVNS might also depend on stimulation time more or less. The optimal stimulation time might lead to a stronger and more efficient online taVNS effect on behavior performance, which needs more researches in the future. Second, if the taVNS modulation effects exist only in the offline protocol, it is valuable to investigate the potential mechanism difference between online and offline taVNS, such as the excitability changes of vagus nerve, which might put new perspective about the effects of taVNS and need further researches. Third, as mentioned previously, although we failed to identify the generalization effects of taVNS from spatial WM to digit WM task, the absent improvement of taVNS on digit WM performance is not clear in detailed reasons, namely, whether it is caused by the specificity of taVNS or the high baseline performance. Thus, further studies with more difficult digit/verbal WM tasks or some subjects with lower WM capacity, such as aging people or patients, are needed. Fourth, beyond n-back task, there are many other tasks that require WM capacity, like Sternberg task. Therefore, studies with other tasks are needed to further verify the generalization effects of taVNS on WM ability. Lastly, although the immediate improvement of WM was strong after offline taVNS, it is unknown to date whether acute improvements can predict the sustained therapeutic effects of potential taVNS-based treatment. Translation to clinical settings remains as a vital question and urgently needs more researches.

CONCLUSION

To summarize, although the vagus nerve is known to play a vital role in the regulation of cognition, the immediate modulatory effects of vagal afferent signals on WM in healthy cohort are largely elusive to date. Here, using taVNS, we demonstrate that stimulation of the vagus nerve increases performance of offline spatial WM tasks in healthy populations, whereas the evidence of improvement of taVNS on digit WM tasks was absent and needs further researches. In general, our results shed light on the role of peripheral physiological signals in regulating WM and highlight the potential for non-invasive cranial nerve stimulation techniques to improve a person's cognition and behavior.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Research Ethics Committee of the Xijing Hospital of the Air Force Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-BS, Q-QT, and WQ: conception and study design. J-BS, Q-QT, X-YG, Y-PC, and CW: data collection or acquisition. J-BS, CC, and Q-QT: statistical analysis. J-BS, CC, HY, X-JY, and HD: interpretation of results. J-BS and CC: drafting the manuscript work or revising it critically for important intellectual content. All authors approval of final version to be published and agreement to be accountable for the integrity and accuracy of all aspects of the work.

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SUPPLEMENTARY MATERIAL

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Vagus Nerve Stimulation Reduces Indomethacin-Induced Small Bowel Inflammation

April S. Caravaca[1,2,3], Yaakov A. Levine[1,2,3,4]*, Anna Drake[3], Michael Eberhardson[1,2,5,6†] and Peder S. Olofsson[1,2,4†]*

[1] Laboratory of Immunobiology, Department of Medicine, Karolinska University Hospital, Solna, Sweden, [2] MedTechLabs, BioClinicum, Stockholm Center for Bioelectronic Medicine, Karolinska University Hospital, Solna, Sweden, [3] SetPoint Medical, Inc., Valencia, CA, United States, [4] Institute of Bioelectronic Medicine, The Feinstein Institutes for Medical Research, New York, NY, United States, [5] Department of Gastroenterology and Hepatology, University Hospital of Linköping, Linköping, Sweden, [6] Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden

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***Correspondence:**
April S. Caravaca
april.caravaca@ki.se
Yaakov A. Levine
ylevine@setpointmedical.com

† These authors have contributed equally to this work

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Crohn's disease is a chronic, idiopathic condition characterized by intestinal inflammation and debilitating gastrointestinal symptomatology. Previous studies of inflammatory bowel disease (IBD), primarily in colitis, have shown reduced inflammation after electrical or pharmacological activation of the vagus nerve, but the scope and kinetics of this effect are incompletely understood. To investigate this, we studied the effect of electrical vagus nerve stimulation (VNS) in a rat model of indomethacin-induced small intestinal inflammation. 1 min of VNS significantly reduced small bowel total inflammatory lesion area [(mean ± SEM) sham: 124 ± 14 mm^2, VNS: 62 ± 14 mm^2, $p = 0.002$], intestinal peroxidation and chlorination rates, and intestinal and systemic pro-inflammatory cytokine levels as compared with sham-treated animals after 24 h following indomethacin administration. It was not known whether this observed reduction of inflammation after VNS in intestinal inflammation was mediated by direct innervation of the gut or if the signals are relayed through the spleen. To investigate this, we studied the VNS effect on the small bowel lesions of splenectomized rats and splenic nerve stimulation (SNS) in intact rats. We observed that VNS reduced small bowel inflammation also in splenectomized rats but SNS alone failed to significantly reduce small bowel lesion area. Interestingly, VNS significantly reduced small bowel lesion area for 48 h when indomethacin administration was delayed. Thus, 1 min of electrical activation of the vagus nerve reduced indomethacin-induced intestinal lesion area by a spleen-independent mechanism. The surprisingly long-lasting and spleen-independent effect of VNS on the intestinal response to indomethacin challenge has important implications on our understanding of neural control of intestinal inflammation and its potential translation to improved therapies for IBD.

Keywords: Crohn's disease, inflammatory reflex, vagus nerve stimulation, small bowel, indomethacin, cholinergic anti-inflammatory pathway, bioelectronic medicine, inflammatory bowel disease

INTRODUCTION

Crohn's disease and ulcerative colitis are two debilitating inflammatory bowel diseases (IBD) characterized by abdominal pain, weight loss, and frequent bowel movements. The hallmarks of Crohn's disease are the involvement of the small bowel and the transmural inflammation in contrast to ulcerative colitis, which is limited to the colon and presents as a more superficial intestinal inflammation with bloody stools. IBD often affects patients relatively early in life, commonly with onset between 15 and 30 years of age (Dignass et al., 2012; Gomollón et al., 2017). Many of the therapeutic options involve systemic immunosuppressive drugs with potential long-term adverse effects such as infections and malignancies (Dignass et al., 2012; Torres et al., 2019). A significant fraction of patients do not respond adequately to available treatment and approximately half of the people affected by Crohn's disease still require abdominal surgery (De Simone et al., 2021). Accordingly, there is an unmet need to improve effective treatment of Crohn's disease while minimizing risks of serious side effects. This is particularly important in young patients who will require years of immunosuppressive maintenance treatment to keep the disease in remission. Interestingly, there is increasing evidence that neural reflexes regulate gut inflammation in health and disease, suggesting that targeting neural circuits is a potential therapeutic option in IBD (Goverse et al., 2016; Stakenborg and Boeckxstaens, 2021).

The *inflammatory reflex* is an example of a neural circuit that involves signaling in the vagus nerve that regulates both organ-specific and systemic immune activity (Borovikova et al., 2000; Rosas-Ballina et al., 2008; Eberhardson et al., 2020). In the motor arc of this circuit, the vagus nerve, the splenic nerve, choline acetyltransferase ($ChAT^+$)-expressing T cells, and alpha7 nicotinic acetylcholine receptors (α7 nAChR) on innate immune cells are essential for inhibition of systemic release of TNF-α in inflammation (Wang et al., 2003; Rosas-Ballina et al., 2008, 2011; Olofsson et al., 2012; Tarnawski et al., 2018; Caravaca et al., 2019). The discovery and functional mapping of this reflex has supported progress to clinical trials of pharmacological or electrical activation of the inflammatory reflex for treatment of diseases characterized by excessive inflammation (Bonaz et al., 2016; Koopman et al., 2016; Consolim-Colombo et al., 2017; Olofsson and Bouton, 2019; Genovese et al., 2020; Yap et al., 2020).

In parallel to the spleen-dependent neural regulation of inflammation, a direct route to the gut has been proposed, in which efferent vagus nerve fibers functionally connect with the myenteric plexus in the intestinal wall (Berthoud et al., 1990, 1991), referred to as the *intestinal cholinergic anti-inflammatory pathway* (Goverse et al., 2016). This pathway also inhibits release of pro-inflammatory cytokines from macrophages and other cells by cholinergic signals (Rosas-Ballina et al., 2011; Tarnawski et al., 2018; Stakenborg and Boeckxstaens, 2021).

There are many different animal models of IBD that recapitulate certain aspects of Crohn's disease and ulcerative colitis, but none fully capture the complexity of either clinical disease (Kiesler et al., 2001; Mizoguchi, 2012). An anti-inflammatory effect of vagus nerve stimulation (VNS) was observed in a number of studies of rodent colitis, such as DSS-, oxazolone-, and TNBS-colitis (Ghia et al., 2006; Bai et al., 2007; Snoek et al., 2010; Meregnani et al., 2011; Meroni et al., 2021) and others previously reviewed (Levine et al., 2018a). However, experimental data on VNS effect on lesions in the small bowel are largely lacking. This is important since nerve-mediated treatment mechanisms and effects may differ between anatomic regions and disease models (e.g., ulcerative colitis, Crohn's disease, postoperative ileus) (Payne et al., 2019). Whether the reduction of inflammation observed after VNS in intestinal inflammation is mediated by direct innervation of the gut or if the signals are relayed through the spleen is not completely understood (Ji et al., 2014; Matteoli et al., 2014). Furthermore, the duration of VNS effects on reduction of intestinal inflammation is not known. A better understanding of the kinetics of VNS treatment on inflammatory lesions is important to inform both design of nerve stimulators and clinical trials in IBD (D'Haens et al., 2018; Tarnawski et al., 2018). Accordingly, we studied mechanism and kinetics of VNS in indomethacin-induced acute intestinal inflammation as a small bowel disease model.

MATERIALS AND METHODS

Ethics Statement

This study and the experimental protocols were approved under #2010-008 by the Institutional Animal Care and Use Committee (IACUC; Manhasset, NY).

Animals

Male Sprague Dawley rats (6–8 weeks old) were obtained from Taconic Farms, Inc., (Hudson, NY) and the study was conducted at The Feinstein Institutes for Medical Research (FIMR; Manhasset, NY). Animal care including room, cage and equipment sanitation conformed to the guidelines cited in the Guide for the Care and Use of Laboratory Animals, and the applicable standard operating procedures of FIMR. Animals were housed in a laboratory environment with temperatures ranging between 19.5 and 24.5°C and relative humidity between 30 and 70%. Automatic timers provided 12 h of light and 12 h of dark. Animals were allowed access *ad libitum* to Harlan Teklad Rodent Chow (Denver, CO) and fresh municipal tap water. This study was approved by FIMR's Institutional Animal Care and Use Committee.

Vagus Nerve Stimulation

The surgery and method used for VNS has been previously described (Olofsson et al., 2015; Kressel et al., 2020). The rats were anesthetized with a ketamine (100 mg/kg) and xylazine (10 mg/kg) intramuscular injection and secured in supine position. A ventral midline cervical incision was made between the mandible and sternum, and the subcutaneous tissue and salivary glands were bluntly separated and retracted laterally. The left cervical vagus nerve was isolated between the sternomastoid and sternohyoid muscles and secured with a custom-built bipolar cuff electrode (Microprobes, Gaithersburg, MD) with a silastic

coated platinum-iridium wire lead. Sham stimulated rats were handled similarly but without electrical stimulation.

Stimulator (SetPoint Medical Corp., Valencia, CA)-driven charge-balanced biphasic pulses were generated using a bipolar current source and were capacitively isolated with > 1 uF ceramic capacitors on both electrode outputs. Rats were stimulated with a pulse waveform amplitude of 1 mA, 200 μs pulse width, and 50 μs inter-pulse-interval, previously demonstrated to activate vagus nerve fibers (Olofsson et al., 2015), for 60 s at 10 Hz. Following electrical stimulation, the electrode was removed, and the incision sutured with 4–0 braided silk.

Splenic Nerve Stimulation

The surgery and method used for splenic nerve stimulation (SNS) has been previously described (Kressel et al., 2020). The rats were anesthetized and secured in a right lateral decubitus position. The spleen was carefully exposed through a 3 cm skin incision above the spleen, and the major splenic blood vessels were traced back to their convergence. The splenic nerve was isolated and suspended on a steel hook electrode (PlasticsOne, Roanoke, VA). Electrical SNS was performed with the same parameters as the VNS, 1 mA, 200 μs pulse width, and 50 μs inter-pulse-interval, for 60 s at 10 Hz. After stimulation, the hook electrode was removed, and the incision sutured in two layers with 4–0 braided silk. Sham SNS was performed by exposing the splenic nerve without electrode placement and electrical stimulation.

Vagus Nerve Stimulation in Splenectomized Rats

The rats were anesthetized and secured in supine position. A bipolar cuff electrode was secured around the left carotid sheath as described above. The rats were gently repositioned to a right lateral decubitus position and the spleen exposed by a 3 cm incision on the left abdomen above the spleen. The major splenic blood vessels were isolated, ligated with 4–0 silk suture, cut distally to the suture, and the spleen removed. The incision was subsequently sutured in two layers with 4–0 braided silk, and electrical or sham VNS was performed as described above.

Enteropathy Model

We used an enteropathy model known to produce lesions in the small intestine (Kent et al., 1969; Del Soldato et al., 1985; Yamada et al., 1993; Anthony et al., 2000). 0.5 h after VNS or sham procedure, the rats were injected subcutaneously on the back with 10 mg/kg indomethacin (5 mg/mL in 5% sodium bicarbonate) and returned to rack housing. For experiments evaluating the prolonged effect of VNS, indomethacin was injected either 0.5, 24, 48, or 72 h after the VNS or sham procedure. Evans Blue was administered intravenously under anesthesia (isoflurane; 3%) 0.5 h before euthanasia. Rats were euthanized by CO_2 asphyxiation 24 h post induction of enteropathy. Blood was collected via cardiac puncture and clotted at room temperature for 1 h. The intestine from the proximal jejunum to the cecum was removed, cleaned, and formalin-fixed. The distal jejunum was assessed for histopathology.

For analysis of inflammatory mediators in the intestine and serum, Evans blue was withheld from a set of animals. In these animals, a standardized 2.54 cm section of the region between the distal jejunum and proximal half of ilium was cleaned, snap frozen in liquid nitrogen, and homogenized in a tissue protein extraction reagent (Pierce Biotechnology, Waltham, MA) containing a protease/phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Lesion area in these rats was not quantitated.

Assessment of Enteropathy and Serum Assays

Fixed intestines were flat-mounted, photographed, and the total lesion area was quantitated by a blinded scorer using digital morphometry (Scion Image; Scion Corporation or ImageJ; NIH). Photomicrographs were acquired of fixed jejunal samples that were embedded in paraffin and stained with hematoxylin and eosin. Chlorination and peroxidation activity were determined using EnzChek Myeloperoxidase Activity Assay Kit (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Intestinal IL-23 normalized to protein and serum HMGB1 concentrations were measured by western blot. Serum TNF was measured by ELISA (R&D Systems, Minneapolis, MN). Concentrations of select other mediators of intestinal inflammation were measured by a quantitative multiplexed electrochemiluminescence assay (Rat Discovery Kit, Meso Scale Discovery, Rockville, MD).

Statistical Analysis

Differences in lesion areas between groups were analyzed by Student's *t*-test or ANOVA with Bonferroni's *post hoc* analysis. Hedges' *g* was used to calculate effect size for Student's *t*-test to measure magnitude of differences between groups[1]. Biomarker data was assumed to be normally distributed and Grubbs' outlier test ($\alpha = 0.05$) was used. Reduction from mean level of sham biomarkers in serum and tissue were analyzed with Student's *t*-test. Data are shown as percentage of sham (mean ± SEM) unless otherwise specified and number of animals (n) are reported per group. $p < 0.05$ was considered significant. Statistical calculations were performed using the Prism 8 software (GraphPad software, San Diego, CA).

RESULTS

Vagus Nerve Stimulation Reduced Small Bowel Inflammation in Indomethacin-Induced Enteropathy

Rats were subjected to cervical VNS or sham stimulation followed by subcutaneous injection of indomethacin. Small intestinal mucosal enteropathy was evaluated 24 h thereafter. Ulcers were observed almost exclusively in the distal portion of the small intestine (**Figure 1A**). Quantification by digital morphometric analysis showed a significantly smaller cumulative lesion area in the small intestine in VNS-treated animals as compared

[1]https://www.socscistatistics.com/effectsize

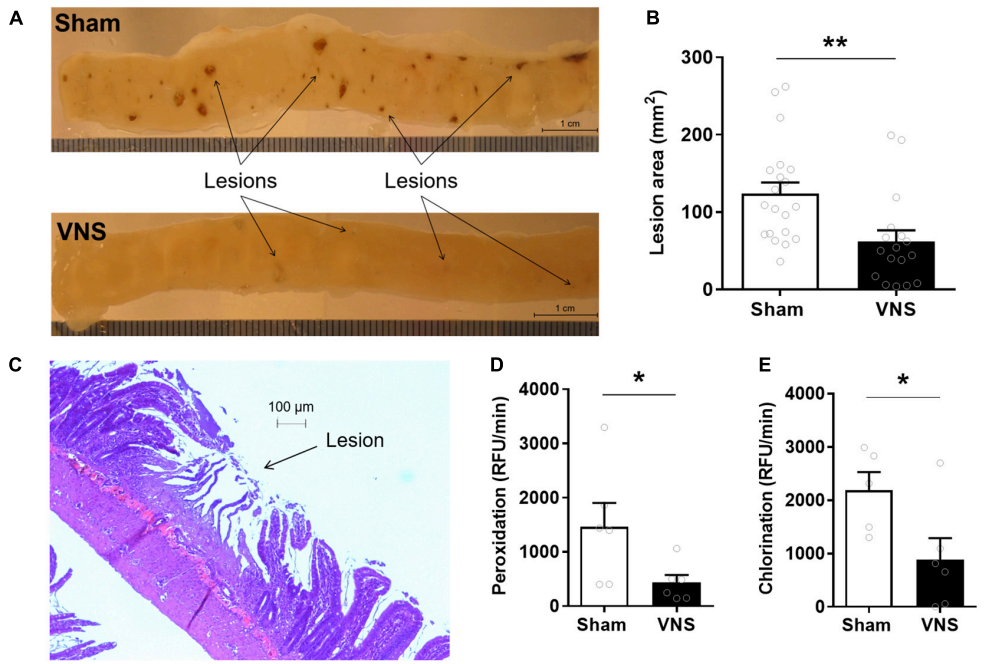

FIGURE 1 | VNS reduced intestinal ulcerations in indomethacin-induced enteropathy. Rats were subjected to VNS- or sham-treatment followed by subcutaneous injection of 10 mg/kg indomethacin in 5% sodium bicarbonate. **(A)** Representative photograph of a jejunum section in sham and VNS-treated rats. **(B)** Quantification of total lesion area. **(C)** Photomicrograph of jejunum with visible mucosal lesion after staining with hematoxylin and eosin. **(D,E)** Myeloperoxidase activity in the small intestine as measured by **(D)** peroxidation and **(E)** chlorination assays. Data is presented as mean ± SEM. $^{**}p < 0.01$ vs. sham **(B)**, $n = 17$–20 per group; $^{*}p < 0.05$ vs. sham **(D,E)**, $n = 6$ per group.

with sham ([mean lesion area ± SEM]: sham 124 ± 14 mm^2, VNS: 62 ± 14 mm^2, $p < 0.002$; effect size = 1.0) (**Figure 1B**). Microscopic analysis of ulcerated areas of the small intestine showed severe villus degradation (**Figure 1C**).

Myeloperoxidase (MPO) peroxidation activity, an established marker of neutrophil infiltration (Pozzoli et al., 2007), was significantly lower in VNS-treated as compared with sham-treated animals (**Figures 1D,E**). Peroxidation activity [**Figure 1D**; (RFU/min ± SEM) sham: 1,466 ± 439, VNS: 432 ± 142, $p = 0.02$; effect size = 1.3] and chlorination activity [**Figure 1E**; (RFU/min ± SEM) sham: 2,187 ± 342, VNS: 887 ± 402, $p = 0.02$; effect size = 1.3] were significantly reduced in VNS-treated compared with sham animals.

Serum TNF levels were significantly lower in VNS-treated rats compared with sham [(% of sham ± SEM) sham: 100 ± 16, VNS: 30 ± 8, $p = 0.003$; effect size = 2.2; **Figure 2A**]. Similarly, relative serum levels of the alarmin HMGB1 were significantly lower in VNS-treated animals as compared with sham animals [(% of sham ± SEM) sham: 100 ± 6, VNS: 53 ± 10, $p = 0.0006$; effect size = 2.3; **Figure 2B** and **Supplementary Figure 1**]. VNS-treated animals had significantly lower relative intestinal levels of IL-23 [(% of sham ± SEM) sham: 100 ± 17, VNS: 55 ± 16, $p = 0.04$; effect size = 0.9; **Figure 3A** and **Supplementary Figure 1**], IFN-γ, IL-1β, and IL-4 [(% of sham ± SEM) IFN-γ sham: 100 ± 27, VNS: 31 ± 11, $p = 0.03$; IL-1β sham: 100 ± 27, VNS: 31 ± 11, $p = 0.03$; IL-4 sham: 100 ± 34, VNS: 23 ± 11, $p = 0.04$; **Figure 3B** and **Supplementary Table 1**] as compared with sham. Mean relative levels of IL-5, KC, and TNF levels were lower, though not significant, in VNS vs. sham-treated animals [(% of sham ± SEM) IL-5 sham: 100 ± 21, VNS: 89 ± 37, $p = 0.4$; KC sham: 100 ± 36, VNS: 51 ± 15 $p = 0.1$; TNF sham: 100 ± 28, VNS: 55 ± 16, $p = 0.1$; **Figure 3B** and **Supplementary Table 1**]. IL-13-levels were at or below the lower limit of detection (2 pg/mL) and were not plotted.

Vagus Nerve Stimulation Effect on Small Bowel Inflammation Independent of Spleen

In certain contexts, the effects of VNS on cytokines and inflammation require the splenic nerve and an intact spleen

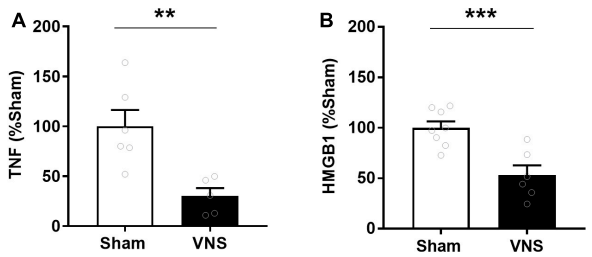

FIGURE 2 | VNS reduced serum TNF in rats with indomethacin-induced enteropathy. Blood was collected and **(A)** serum TNF was measured by ELISA. $n = 5$–6 per group. **(B)** Serum HMGB1 was measured semiquantitatively by western blot and normalized to sham. $n = 6$–8 per group. Data is presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. sham.

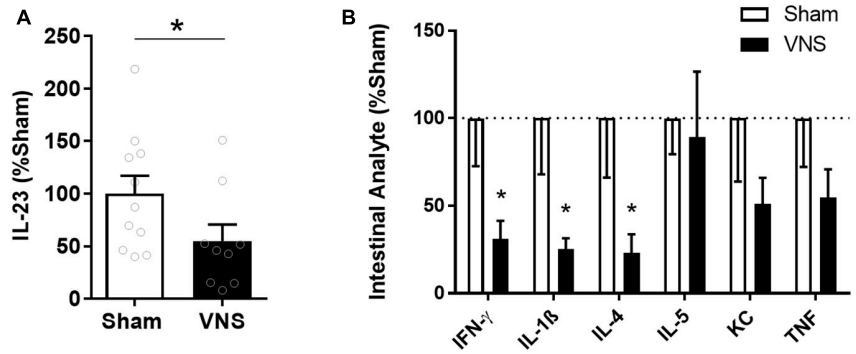

FIGURE 3 | Intestinal levels of select cytokines in VNS- or sham-treated animals with indomethacin-induced enteropathy. **(A)** Semi-quantitative measurements by western blot of intestinal IL-23 were normalized to the mean sham level. n = 9–11 per group. **(B)** Levels of select cytokines were measured by quantitative multiplexed electrochemiluminescence assay and normalized to the mean sham level. n = 5–6 per group. Data is presented as mean ± SEM. *$p < 0.05$ vs. sham.

(Rosas-Ballina et al., 2008, 2011; Ji et al., 2014; Matteoli et al., 2014). To determine whether the spleen was required for the VNS effect in this model, a set of animals was splenectomized immediately before VNS and indomethacin administration. Lesions in the small intestine were quantified 24 h after injection. The cumulative lesion area in the small intestine was significantly lower in VNS-treated than in sham-treated animals also in the absence of the spleen [(mean lesion area ± SEM) sham: 88 ± 28 mm^2, VNS: 15 ± 11 mm^2, $p = 0.02$; effect size = 1.1; **Figure 4A**]. Furthermore, relative serum HMGB1 levels were significantly lower in VNS-treated as compared with sham-treated animals [(mean lesion area ± SEM) sham: 100 ± 11, VNS: 62 ± 11, $p = 0.01$; effect size 1.2; **Figure 4B**]. We first confirmed that SNS was effective in reducing endotoxin-induced serum TNF in this rat strain in an independent group of rats (Kressel et al., 2020). To determine whether SNS alone reduces indomethacin-induced ulcers in the small intestine, we performed electrical or sham SNS followed by indomethacin injection and measured intestinal lesion area 24 h later. We observed no significant difference in lesion area between animals treated with SNS or sham SNS [(lesion size in mm^2 ± SEM) sham: 93 ± 13, SNS: 93 ± 22, $p = 0.5$; effect size = 0.01; **Figure 4C**]. In addition, SNS treated rats did not show significantly lower relative levels of serum HMGB1 levels [(% of sham ± SEM) sham: 100 ± 12, SNS: 153 ± 12, $p > 0.9$; effect size 1.3; **Figure 4D**]. Thus, SNS alone did not reduce small bowel lesion in indomethacin-induced enteropathy, nor was the spleen required for the protective effects of VNS in this model.

Protection Against Small Bowel Inflammation Sustained 48 h After Vagus Nerve Stimulation

In endotoxemia, a brief episode of stimulation, lasting seconds to minutes, has a sustained effect on the release of pro-inflammatory cytokines (Huston et al., 2007; Tarnawski et al., 2018). To investigate the duration of VNS effect on intestinal ulcer development in indomethacin-induced enteropathy, we performed VNS or sham surgery followed by a delayed induction of enteropathy, from 0.5 to 72 h. The area of intestinal ulceration was quantified at 24 h after indomethacin injection. We observed a significant decrease in intestinal lesion area when indomethacin was administered 0.5 or 24 h after VNS as compared with sham treatment [(lesion size in mm^2 ± SEM) sham: 124 ± 14, 24 h post-VNS: 62 ± 14, $p = 0.02$; vs. 48 h post-VNS: 49 ± 10, $p = 0.03$; **Figure 5**]. In animals injected with indomethacin at 48 h or 72 h after VNS, there was no significant reduction in lesion area as compared with sham-treated animals [(lesion size in mm^2 ± SEM) sham: 124 ± 14 vs. 72 h post-VNS: 130 ± 27, $p > 0.9$; vs. 96 h post-VNS: 108 ± 28, $p > 0.9$; **Figure 5**].

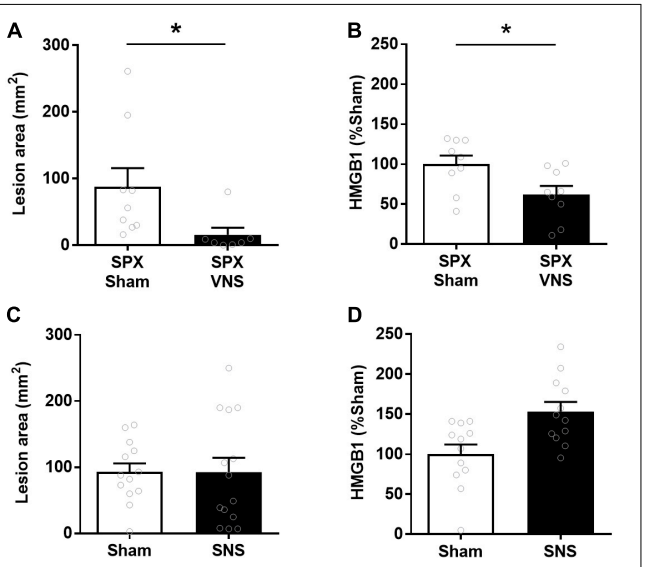

FIGURE 4 | VNS reduced intestinal ulcerations in indomethacin-induced enteropathy independent of spleen. **(A,B)** Rats were subjected to splenectomy (SPX), followed by VNS or sham treatment and indomethacin administration. The small intestine lesion area was quantified at 24 h. Mean and SEM values are plotted (**A**, n = 7–9 per group; **B** 9 per group). **(C,D)** Rats were subjected to splenic nerve stimulation (SNS) or sham SNS surgery followed by indomethacin injection. The small intestine lesion area was quantified at 24 h. Serum HGMB1 was measured semiquantitatively by western blot and normalized to mean sham values (**C**, n = 13–14 per group; **D**, n = 12 per group). Data is presented as mean ± SEM. * $p < 0.05$ vs. sham.

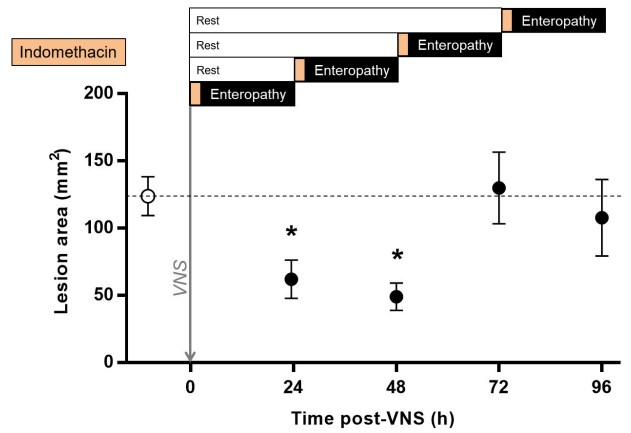

FIGURE 5 | Sustained protection against indomethacin-induced intestinal ulcerations after VNS. Rats were subjected to VNS or sham surgery followed by a rest period of 0.5, 24, 48, or 72 h and subsequent subcutaneous injection of 10 mg/kg indomethacin in 5% sodium bicarbonate. Animals were injected systemically with Evans blue prior to euthanasia at 24 h after indomethacin injection. The small intestine was formalin-fixed, photographed and digitized. Total lesion area was quantified by a blinded scorer using Scion Image or ImageJ. Data is presented as mean ± SEM. *$p < 0.05$ vs. sham; n = 5–20 per group. Dotted line specifies the mean of the sham group.

DISCUSSION

Here, we found that electrical stimulation of the vagus nerve reduced indomethacin-induced acute small bowel inflammation by a spleen-independent mechanism. Reduction of small bowel lesioning remained for 48 h after a single episode of VNS.

The findings here that electrical VNS reduced small bowel inflammation align with previous studies that show that signals in the vagus nerve regulate levels of pro-inflammatory cytokines and inflammation in a wide range of organs and scenarios (Steinberg et al., 2016). Reduction of the elevated levels of TNF that promote intestinal inflammation in Crohn's disease has been a therapeutic success (Hanauer et al., 2006). However, after 1 year of anti-TNF treatment, more than half of the patients have stopped the treatment due to inadequate response, loss of response, or intolerance (Roda et al., 2016). Therefore, the additional evidence of vagus nerve regulation of intestinal lesion development provided here may be useful to support further development therapeutic modalities that target the neural regulation of inflammation.

The mechanism for vagus nerve regulation of intestinal inflammation is not fully understood. The vagus nerve directly innervates the gastrointestinal tract and is a key conduit for the bi-directional communication between the enteric nervous system and the central nervous system (Prechtl and Powley, 1990). The main neurotransmitter of the vagus nerve is acetylcholine, known not only to regulate visceral functions such as gastrointestinal motility, but also the release of pro-inflammatory cytokines from innate immune cells (Cailotto et al., 2014). Both afferent and efferent signals in the vagus nerve participate in the regulation of pro-inflammatory cytokines in inflammation (Olofsson et al., 2015). Data from murine models suggest that the reflex control of gut inflammation is mediated by the efferent vagus nerve and sympathetic nerve signals that culminate in regulating the activity of macrophages that reside in the viscera where vagus innervation is abundant (Luckey et al., 2003; Ji et al., 2014; Matteoli et al., 2014; Stakenborg and Boeckxstaens, 2021; Zhang et al., 2021).

Available data indicate that signals in the vagus nerve regulate gut inflammation by different routes, spleen-independent and spleen-dependent. One spleen-independent conduit involves the vagus nerve, efferent sympathetic nerves and myenteric efferent fibers that regulate macrophages in the intestinal wall (Luckey et al., 2003; Matteoli et al., 2014). Evidence of spleen-independent routes for vagus-nerve-mediated regulation of inflammation is also found in other visceral organs such as the pancreas: VNS reduced pancreatitis severity by a spleen-independent mechanism (Zhang et al., 2021).

A spleen-dependent route of vagus nerve regulation of inflammation is the inflammatory reflex, which involves efferent vagus neurons, the celiac ganglion, the splenic nerve, and the spleen (Ji et al., 2014; Kressel et al., 2020). These signals within the inflammatory reflex are propagated to immune cells in the spleen capable of biosynthesizing acetylcholine (Olofsson et al., 2012). Both spleen-dependent and spleen-independent neural regulation of inflammation may reduce macrophage accumulation and release of pro-inflammatory cytokines in the gut through activation of α7 nAChR, α4β2 nAChR or other cholinergic receptors (Wang et al., 2003; de Jonge et al., 2005; Rosas-Ballina et al., 2008, 2011; van der Zanden et al., 2009; Costantini et al., 2012). Of note, $ChAT^{+}$ T cells, capable of acetylcholine biosynthesis, are found in the innervated Peyer's patches of the small intestine of rodents (Vulchanova et al., 2007; Dhawan et al., 2016; Willemze et al., 2019) and it is possible that these cells partake in local regulation of immune cell activity as has been observed in spleen (Rosas-Ballina et al., 2011). Considering this, the finding here that the spleen is not essential for VNS-mediated reduction of disease intensity in indomethacin-induced small bowel inflammation is important. The data imply that for treatment of inflammatory lesions in IBD with involvement of the small intestine, targeting the cervical or gut-directed subdiaphragmatic vagus may be superior to targeting the splenic nerve or spleen.

The observation here that VNS reduced small intestinal inflammation for up to 48 h aligns with our previous finding that VNS reduced systemic TNF release in endotoxemia for up to 48 h (Huston et al., 2007; Tarnawski et al., 2018) and offers support for refining therapeutic stimulation protocols. The first published clinical trial using VNS for treatment of Crohn's disease in biologic-naïve subjects was designed using the stimulation "duty cycle" (time on vs. time off) originally designed to treat epilepsy, stimulating up to 262 times per day (Bonaz et al., 2016). The findings here regarding the persistence of protection in the small bowel enabled a once per day VNS treatment in biologic-experienced subjects with Crohn's disease (D'Haens et al., 2018). The growing mechanistic insights on regional differences in the neural regulation of inflammation between and within organs such as the colon, the small bowel, and other visceral organs, will be valuable for design of future IBD trials using bioelectronic medicine.

An interesting molecule in persistent inflammation is the alarmin HMGB1, which is involved in or associated with the pathogenesis of many inflammatory diseases and regulated by cholinergic signals (Huston et al., 2007; Andersson and Tracey, 2011). HMGB1 is a "late mediator" of inflammation, and excessive levels of HMGB1 may be involved in sustaining inflammation, with associated inflammatory tissue damage, organ dysfunction, and cognitive impairment (Andersson et al., 2000; Valdes-Ferrer et al., 2016; Bruck et al., 2020). Of note, anti-HMGB1 therapy was effective in significantly reducing disease severity even when administered days after onset of severe inflammation (Huston et al., 2007), and anti-HMGB1 antibodies and HMGB1-targeted sorption beads have a protective effect in experimental intestinal inflammation (Maeda et al., 2007; Yang et al., 2009; Ju et al., 2014). Based on that knowledge, it will be interesting to consider whether the link between VNS and reduced HMGB1 levels may help explain how VNS may be effective in reducing not only inflammation onset, but also ongoing inflammation (Huston et al., 2007; Bonaz et al., 2016). Accordingly, our observation here that VNS reduced serum levels of HMGB1 along with molecular markers of inflammation in the gut and small bowel lesion area is noteworthy. The potential role of HMGB1 in IBD is being further explored (Andersson and Tracey, 2011) and a pathogenetic role has been proposed for HMGB1-dependent TLR4 activation in indomethacin-induced small intestinal damage (Nadatani et al., 2012). The available data supports further consideration and study of the vagus nerve regulation of HMGB1, its potential as a biomarker and therapeutic target in IBD (Palone et al., 2016).

The current study, using a rat indomethacin-induced enteropathy model, provides evidence that electrical activation of the cervical vagus nerve reduces inflammatory lesions in the small bowel, a pathology that is commonly a critical part of the pathogenesis in Crohn's disease (Liu et al., 2020). The rodent models of colitis that have previously been used to study vagus nerve and α7 nAChR-mediated regulation of disease severity are by many considered more relevant to ulcerative colitis than to Crohn's disease (Snoek et al., 2010; Galitovskiy et al., 2011; Ghia et al., 2011; Ji et al., 2014). In humans, previous surgical vagotomy was associated with later development of Crohn's disease, but not ulcerative colitis (Liu et al., 2020), suggesting that vagus nerve regulation of inflammation may be more important in small bowel inflammation than in colitis, although this remains to be comprehensively studied.

As described above, the first published study of VNS for the treatment of Crohn's disease used high duty cycle nerve stimulation (Bonaz et al., 2016; Sinniger et al., 2020). While no major safety concerns were reported, higher frequency and intensity of electrical nerve stimulation consume more energy, thus requiring greater battery capacity for implanted stimulators. Furthering our understanding of the VNS parameter requirements for clinical benefit can therefore allow for optimization of device design and stimulation protocols. Longer periods between stimulations may also reduce patient discomfort both by limiting the stimulation-associated laryngeal muscle contraction and temporary voice changes as well as enabling smaller devices that are easier to implant and maintain (Levine et al., 2018b,c). Therefore, the observation that a short VNS period of 60 s was followed by a prolonged anti-inflammatory effect provides important information for the development of future clinical therapeutics.

CONCLUSION

Electrical stimulation of the vagus nerve, but not of the splenic nerve, reduced indomethacin-induced small intestinal enteropathy for up to 2 days. The spleen was not required for the effect of VNS on small bowel lesion area. These observations are an important step toward better understanding of the mechanisms of vagus nerve regulation of inflammation in diseases with engagement of the small bowel.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Feinstein Institutes for Medical Research Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AC, AD, and YL planned and performed experiments. AC, AD, YL, ME, and PO analyzed and interpreted data. AC, YL, PO, and ME wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.730407/full#supplementary-material

Supplementary Figure 1 | VNS reduced IL-23 and HMGB1 in small bowel inflammation. Representative densitometry from western blot of **(A)** serum HMGB1 and **(B)** intestinal IL-23. Mean quantified integrated intensity (IKK count) of HMGB1 in the sham group was 0.69. Mean quantified integrated intensity (IKK count) of IL-23/Actin in the sham group was 0.25.

Supplementary Table 1 | VNS reduced levels of select cytokines in indomethacin-induced enteropathy. Levels of select cytokines were measured by quantitative multiplexed electrochemiluminescence assay and normalized to the mean sham level. Data is presented as mean ± SEM. * $p < 0.05$ vs. sham.

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Non-invasive Vagus Nerve Stimulation in Treatment of Disorders of Consciousness – Longitudinal Case Study

Albertyna Osińska[1], Andrzej Rynkiewicz[1]*, Marek Binder[2], Tomasz Komendziński[3], Anna Borowicz[3] and Antoni Leszczyński[3]*

[1] Faculty of Psychology, University of Warsaw, Warsaw, Poland, [2] Institute of Psychology, Jagiellonian University, Kraków, Poland, [3] Department of Cognitive Science, Faculty of Humanities, Nicolaus Copernicus University in Toruń, Toruń, Poland

Neuromodulatory electroceuticals such as vagus nerve stimulation have been recently gaining traction as potential rehabilitation tools for disorders of consciousness (DoC). We present a longitudinal case study of non-invasive auricular vagus nerve stimulation (taVNS) in a patient diagnosed with chronic unresponsive wakefulness syndrome (previously known as vegetative state). Over a period of 6 months we applied taVNS daily and regularly evaluated the patient's behavioral outcomes using Coma Recovery Scale – Revised. We also took electrophysiological measures: resting state electroencephalography (EEG), heart rate (HR) and heart rate variability (HRV). All these methods revealed signs of improvement in the patient's condition. The total CRS-R scores fluctuated but rose from 4 and 6 at initial stages to the heights of 12 and 13 in the 3rd and 5th month, which would warrant a change in diagnosis to a Minimally Conscious State. Scores obtained in a 2 months follow-up period, though, suggest this may not have been a lasting improvement. Behavioral signs of recovery are triangulated by EEG frequency spectrum profiles with re-emergence of a second oscillatory peak in the alpha range, which has been shown to characterize aware people. However, sustained spontaneous theta oscillations did not predictably diminish, which most likely reflects structural brain damage. ECG measures revealed a steady decrease in pre-stimulation HR combined with an increase in HRV-HR. This suggests a gradual withdrawal of sympathetic and an increase in parasympathetic control of the heart, which the previous literature has also linked with DoC improvements. Together, this study suggests that taVNS stimulation holds promise as a DoC treatment.

Keywords: vagus nerve stimulation, taVNS, disorders of consciousness, coma, unresponsive wakefulness syndrome (UWS), EEG, HRV

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Christopher Austelle, Medical University of South Carolina, United States

****Correspondence:***
Albertyna Osińska
albertyna.osinska@psych.uw.edu.pl
Andrzej Rynkiewicz
rynkiewicz@psych.uw.edu.pl

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INTRODUCTION

Promise of Transcutaneous Auricular Vagus Nerve Stimulation for Disorders of Consciousness

The prolonged disorders of consciousness (DoC) are one of the most severe outcomes of brain damage. They are also notoriously difficult to both diagnose and rehabilitate. For the latter, emerging techniques of brain stimulation have shown promise. With the view of prospective widespread use, of particular interest are especially the non-invasive methods which can be applied

in hospital as well as home settings, e.g., transcranial direct current stimulation (tDCS) or low-intensity focused ultrasound pulse. Recently, encouraging results have come from stimulating the brain via the vagus nerve (see Briand et al., 2020 for an overview). While vagus nerve stimulation (VNS) is mostly known as an adjunct treatment for epilepsy (Ben-Menachem, 2002) and depression (Sackeim, 2001) as well as motor-control recovery after stroke (Dawson et al., 2016), studies are beginning to report improvements in patients with DoCs as well. Traditionally, this type of stimulation was done using a surgically implanted device [referred here as vagus nerve stimulation (VNS)]. However, comparable results can be achieved transcutaneously, simply by attaching an electrode to the left ear, where the current passes to the auricular branch of the vagus nerve [referred here as transcutaneous auricular vagus nerve stimulation (taVNS); Kraus et al., 2013; Capone et al., 2014]. The taVNS may theoretically be a safer option as it selectively stimulates the afferent vagus nerve fibers whereby minimizing potential cardiac risks of efferent fiber activation of the implanted VNS (Kreuzer et al., 2012; Chen et al., 2015; Hakon et al., 2020; Yap et al., 2020).

Vagus nerve (X cranial nerve) is the longest and most widespread of the cranial nerves (*vagus* in Latin means "wandering"). It is in fact a nerve pair (left and right), usually referred to in the singular. It runs from the brain through the face, thorax to the abdomen, where it innervates all the major internal organs. It contains parasympathetic fibers of a mixed type (80% afferent, relaying sensory information from the periphery to the brain; and 20% efferent, motor fibers, carrying impulses away from the central nervous system).

Auricular branch of the vagus nerve (Arnold's nerve) is responsible for somatosensory innervation of the ear. Sensory fibers are located in the central part of the external ear, near the part of the auricle called Cymba Concha. Afferent neurons of the auricular vagus nerve reach the inferior vagal ganglion, and then stimulation is transmitted to the nucleus of the solitary tract in the brainstem. The solitary tract is the main receiver of stimulation from different branches of the vagus nerve and it has numerous output connections, e.g., with the locus coeruleus – this part of the reticular activating system is the main source of adrenergic incentive projections to cortical, subcortical and brainstem circuits. Locus coeruleus activation is probably responsible for many observed therapeutic effects of VNS and taVNS (Butt et al., 2020).

Vagus Nerve Stimulation and Transcutaneous Auricular Vagus Nerve Stimulation in Disorders of Consciousness Treatment

To the best of our knowledge only seven studies so far have looked at vagus nerve stimulation in DoC patients, all reporting at least some encouraging findings. Two of them utilized the invasive, VNS technique, administered through a surgically implanted device, and five the non-invasive counterpart through stimulation of the auricular branch (taVNS), see **Table 1** for details.

Together these studies (presented in **Table 1**) suggest that although promising as a DoC treatment, taVNS may not hold equal promise for all. A study by Yu et al. (2021) presents an important step in determining the characteristics of responders. Out of 10 patients (seven UWS and three MCS) it turned out that only those who reacted to auditory stimuli during the CRS-R examination benefited from taVNS, as evidenced by behavioral and neuroimaging data.

The studies differ not only in patient populations and outcomes, but also devices used and electrode placement on the ear. Auricular branch of the vagus nerve (ABVN) has been shown to innervate the tragus and concha, in particular cymba concha (Peuker and Filler, 2002). Yet there is no consensus on which of these presents the optimal location for stimulation, e.g., Napadow et al. (2012) indicate the concha as the best site, while Kraus et al. (2013) suggest the anterior wall of the auditory canal. Different manufacturers have targeted different locations with their devices, i.e., NEMOS by tVNS Technologies stimulates the ABVN via the cymba concha, while Parasym by Parasym Health, via the tragus. taVNS stimulator can also be relatively easily assembled using transcutaneous electrical nerve stimulator (TENS) devices, commonly used for pain management, with suitable electrodes. Further research is needed to explore electrode placement and stimulation parameters to bring the greatest therapeutic effects to particular conditions.

In terms of outcome measures, previous studies have mostly focused on behavioral assessment, namely CRS-R evaluation. PET or fMRI have been rarely used, as their administration in vulnerable populations such as DoC patients requires highly specialist procedures (e.g., fMRI is carried out under sedation or anesthesia for which some patients may have contraindications). By contrast, EEG and ECG measures can also be taken at bedside and enable additional evaluation of the patient's condition and treatment efficacy. Among the VNS studies in DoC so far only one measured EEG activity (Corazzol et al., 2017) and none of the taVNS interventions. Corazzol et al. (2017) revealed an increase in theta band (4–7 Hz) power, which previous work has indicated as reliably distinguishing MCS from UWS patients (Sitt et al., 2014). Spectra profiles of resting-state EEG have been correlated with DoC severity (Forgacs et al., 2017, 2020; see more in section "Discussion"), but this area has been unexplored yet among the studies of taVNS in DoC.

Mechanism of Action

Because various etiologies can cause DoC, e.g., traumatic brain injury, anoxia, infection, and consciousness may dissociate from motor behavior, it is particularly challenging to pinpoint neural correlates of consciousness and model its recovery. Based on a review of interventional studies on pharmacological and electrical stimulation in patients with longstanding DoCs, as well as functional and structural neuroimaging literature, Schiff (2010) proposed a "Mesocircuit hypothesis" of consciousness recovery. According to the model, one of the key obstacles to functional recovery is widespread deafferentiation and disconnection of neurons located mainly in the central thalamus. As a result, the neuronal circuit involved in cortical activation is disturbed. What follows is that interventions involving

TABLE 1 | A summary of VNS and tVNS studies on DoC patients.

	Participants and diagnoses	Time since injury	Stimulation site and device	Stimulation parameters	Stimulation protocol	Assessment	Outcomes
VNS							
Corazzol et al., 2017	1 UWS Age: 35 Etiology: Traumatic	15 years	Neck level, Cyberonics Inc.	0.25 mA/30 Hz/500 μs; gradually increased to 1.5 mA	30 s stimulation by 5 min rest Duration: 6 months	CRS-R, EEG, PET	CRS-R: 5 → 10 (but not sustained) EEG: ↑ rs theta band (4–7 Hz) power; sources in the DMN; global increase in mean wSMI over theta sources significantly correlated with CRS-R scores PET: ↑ occipito-parieto-frontal and basal ganglia regions ↑ thalamus
Xiang et al., 2020	10 MCS Age: 43.90 ± 15.79 Etiology: Traumatic: 4 Hemorrhage: 5 Ischemic-hypoxic: 1	7.16 ± 2.12 months Range: 5–11.5 months	G112, PINS Medical, Ltd.	0.1–0.3 mA/20–30 Hz/250 or 500 μs, gradually increased to 1.5 mA, then individual adjustments up to 3.5 mA	30 s stimulation by 5 min rest Duration: 6 months	CRS-R	1 month: no sig. total scores vs. baseline 3 months: sig. total scores vs. baseline ($p = 0.0078$) 3/10 – sig. improvement 2/10 – VNS-responders 5/10 – unresponsive to VNS Subscales: visual function more sensitive to VNS ($p = 0.0156$) 6 months: sig. total CRS-R scores vs. baseline ($p = 0.0039$) 1/10 –has emerged from minimally conscious state 2/10 – sig. improvement 3/10 – responsive 5/10 – unresponsive to VNS Subscales: sig visual vs. baseline ($p = 0.0078$) No diff. in CRS-R between 6- and 3-month follow-ups ($p > 0.05$)
taVNS							
Yu et al., 2017	1 UWS Age: 73 Anoxic: 1	50 days	Cymba concha (bilateral) Custom device	4–6 mA/20 Hz/<1,000 μs	Twice daily, for 30 min Duration: 4 weeks	CRS-R, fMRI	CRS-R baseline 6, at 4 weeks: 13, change UWS → MCS; new behaviors in motor and oromotor function ↑FC between posterior cingulate/precuneus and hypothalamus, thalamus, vmPFC, superior temporal gyrus ↓FC between posterior cingulate/precuneus and the cerebellum
Noé et al., 2020	6 UWS 8 MCS Age: 40.2 ± 16.1 Etiology: Traumatic: 7 Anoxic: 4 Hemorrhage: 3	12.1 ± 6.4 months	Left tragus Parasym® CE	1.5 mA/20 Hz/250 μs	30-min twice a day, 5 days a week Duration: 4 weeks + follow-up after 4 weeks	CRS-R	Responders (showing improvement in at least one item in CRS-R): 0/6 UWS 5/8 MCS – in all but one improvement occurred in the 4-week follow-up after taVNS Subscales: - Motor: 3/5 responders - Visual: 1/5 - >1 subscale: 1/5
Hakon et al., 2020	3 UWS 2 MCS Age: Median 46 Range: 21–80 Etiology: Traumatic: 5	Median: 41 days Range: 31–95 days	Cymba concha NEMOS®	0.5 mA for the first 3 days, then 1 mA/25 Hz/250 μs	4 h daily, 30 s on/30 s off Duration: 8 weeks	CRS-R	3/5 patients showed improvement (>3 points); of these: UWS → EMCS (5→22) MCS → EMCS (10→23) UWS → MCS (3→6) Remaining patients: MCS → MCS (12 → 12) UWS → UWS (2 → 3)

(Continued)

TABLE 1 | (Continued)

	Participants and diagnoses	Time since injury	Stimulation site and device	Stimulation parameters	Stimulation protocol	Assessment	Outcomes
Yu et al., 2021	7 UWS 3 MCS Age: 36 ± 15.3 Range: 19–73 Etiology: Hypoxic-ischemic Encephalopathy (HIE): 5 Traumatic: 2 Cerebral hemorrhage: 2 Brainstem hemorrhage: 1	78.5 ± 83.3 Range: 10–300	Cymba concha and cavity concha Custom device	4–6 mA/20 Hz/500 μs	30 min continuously, twice daily Duration: 4 weeks	CRS-R, GOS, fMRI	CRS-R: 5 responded to auditory stimuli (RtAS) 5 did not respond to auditory stimuli (nRtAS) GOS: Only RtAS got favorable prognoses after treatment (GOS > 2) fMRI RtAS: taVNS increased CBF of multiple brain regions nRtAS: taVNS increased CBF only in the left cerebellum

CBF, cerebral blood flow; CRS-R, coma recovery scale-revisited; DMN, default mode network; EMCS, emergence of minimal conscious state; FC, functional connectivity; GOS, Glasgow Outcome Scale; MCS, minimal conscious state; UWS, unresponsive wakefulness syndrome, fMRI, functional magnetic resonance imaging; Hz, Hertz; mA, milliamperes; μs, microseconds; s, seconds; PET, positron emission tomography; taVNS, transcutaneous auricular vagus nerve stimulation (non-invasive, via an electrode attached to the ear); vmPFC, ventromedial prefrontal cortex; VNS, vagus nerve stimulation (via a surgically inserted implant); wSMI, weighted symbolic mutual information.

stimulation of the thalamus should hold particular promise for DoC patients. In terms of the mechanism of taVNS in DoC recovery specifically, a dedicated "Vagal Cortical Pathway Model" has been proposed by Briand et al. (2020). This model describes both the direct and indirect pathways connecting the vagus nerve with, e.g., nuclei of the thalamus and proposes that taVNS stimulates the recovery through activation of the ascending reticular activating system (ARAS), the thalamus, the striatum and through re-establishment of the cortico-striatal-thalamic-cortical loop. It also proposes improvements in activity and connectivity within the default mode network (DMN) and the External Fronto-Parietal Network (ExN) as well as activation of the Salience Network (SN) (see Briand et al., 2020, for details). At a cognitive level of analysis, consciousness restoration through taVNS is consistent with an embodied, interoceptive model of the conscious self where the brain is assumed to constantly evaluate the signal from the physical body (Seth et al., 2012; Seth, 2013; Cleeremans et al., 2020; Paciorek and Skora, 2020; Yu et al., 2021). Stimulating the vagus nerve as the main pathway relaying visceral signals into the brain, therefore, offers a remarkable potential bottom-up approach for DoC therapy.

Need for Multiple Sources of Evidence in Disorders of Consciousness Diagnosis

Finally, and perhaps fundamentally, consciousness itself remains notoriously hard to define and, consequently, diagnose. Wakefulness (arousal) and awareness have been assumed to be its two key components (Laureys, 2005; Briand et al., 2020). Wakefulness pertains to alertness or vigilance while awareness refers to the ability to interact with the environment or the self. Based on the degree of severity DoCs have been classified into: coma – absence of both wakefulness and awareness, UWS – intermittent periods of wakefulness with the absence of awareness of the environment or the self, MCS – variations in wakefulness and minimal, fluctuant but definite signs of awareness (as evidenced by, e.g., visual pursuit or command following). Currently the recommended diagnostic scale is the Coma Recovery Scale – Revised (Giacino et al., 2004), though a full diagnosis is not possible through bedside behavioral observation alone. That is because in DoCs behavior and neurophysiological evidence may dissociate. The best-known example is the study by Owen et al. (2006), in which functional magnetic resonance imaging (fMRI) showed that a patient diagnosed as UWS activated the predicted brain areas when asked to imagine playing tennis or moving around the home. Similarly, Bekinschtein et al. (2009) demonstrated electromyographic evidence of trace conditioning in patients diagnosed as UWS. Such learning involves a temporal gap between the conditioned and unconditioned stimuli and has been known to require explicit knowledge of the temporal contingency. Several independent ERP (event related potential) studies have shown semantic processing in UWS patients, including the presence of the N400 (Schoenle and Witzke, 2005), or reactions to patients' own name, as evidenced by P300 (Perrin et al., 2006) or a stronger theta wave synchronization than other names (Fellinger et al., 2011). All these results therefore provide

evidence for conscious processing not detected by behavioral assessment alone.

In light of this diagnostic challenge, to make as accurate assessment as possible, multiple sources of evidence are usually combined with behavioral evaluation, such as electroencephalography (EEG), functional magnetic resonance (fMRI) or positron emission tomography (PET). In addition, it is possible to use autonomic physiological indicators to assess the general homeostatic and psychophysical state of patients. Autonomic functions are usually regulated by the nuclei located in the brainstem and therefore can remain intact even when the brain is severely damaged. A fairly popular indicator is heart rate variability – HRV (Berntson et al., 1997). Based on the results of a number of studies carried out over the years, a neurovisceral integration model was proposed (Thayer et al., 2012), according to which the higher baseline level of HRV is the index of flexible control over behavior by cortical, subcortical and peripheral neural systems. DoC studies have shown higher levels of resting HRV in MCS than UWS patients, which means that the use of HRV indicators may increase the accuracy of the differentiation between UWS and MCS (Riganello et al., 2018) and possibly be used to detect awareness. The HRV has also been shown to be sensitive to DoC severity measured with Glasgow Coma Scale: HRV amplitude in both low (HRV-LF) and high (HRV-HF) frequency ranges was lower when GCS score was lower (Estévez-Báez et al., 2019).

To this body of literature we contribute here a longitudinal (6-month intervention with a 2-month follow-up), clinical case study of taVNS stimulation of a patient diagnosed with persistent UWS for 6 years following a traumatic brain injury. We combine multiple sources of evidence: behavioral (CRS-R) with neurophysiological analysis of the EEG signal and physiological processes evidenced by HRV indicators. We hypothesized that if transcutaneous auricular vagus nerve stimulation (taVNS) indeed affected the patient's physical and psychological state, we would find improvement in behavioral responses on a Coma Recovery Scale – Revised (CRS-R), increase in resting HRV and in power of oscillations in the theta-alpha range (4–16 Hz) in resting state EEG.

MATERIALS AND METHODS

The research was carried out at the inpatient rehabilitation facility of the "Światło" (eng. "Light") Foundation in Toruń, Poland, from June of 2019 to February of 2020. Ethical approval was obtained from the Ethical Committee at the Faculty of Psychology, University of Warsaw. Written informed consent to participate in this study and to publish the work was signed by the legal guardian of the patient.

Patient

Our patient is a 28 years old woman who suffered traumatic brain injury 6 years prior to the study. She was diagnosed with persistent UWS and her CRS-R score prior to the study's beginning was 4. Her condition had remained stable since the injury. She breathed independently via a tracheostomy tube and was fed through percutaneous endoscopic gastrostomy (PEG). Most of her time she spent in bed. Prior to participating in this study, the patient underwent a standard treatment for the patients with UWS/VS diagnosis, employed in the rehabilitation center. It included daily physical therapy, and 2–3 times a week multisensory stimulation, and speech therapy (training of orofacial reflexes). She was regularly consulted by the medical staff and received 24-h nursing support.

She was selected based on the following inclusion criteria: long time since brain injury (more than 2 years) to ensure any improvement was unlikely to be a result of spontaneous recovery, stable CRS-R results prior to the study, stable day/night rhythm, normal blood pressure level and ECG, lack of significant losses of cortical tissue, autonomic respiratory action without assistance, intact vagus nerve.

Transcutaneous Auricular Vagus Nerve Stimulation

NEMOS® stimulator (tVNS Technologies, Erlangen, Germany) was used to administer taVNS. It comprises a small, portable stimulation unit with an intra-auricular epidermic electrode that is placed in the auricular tract with a contact point at the cymba concha. The settings were fixed at continuous 0.25-ms-duration monophasic square wave pulses at 25 Hz frequency, fixed 25 V voltage, 30 s on/30 s off. The current was changed systematically, starting at 0.2 mA and increasing the intensity by 0.1 mA every week up to 1.5 mA, following the protocol of a previous study (Corazzol et al., 2017). The stimulation was applied for 4 h a day, based on the device recommendations provided by the manufacturer for patients with epilepsy.

Behavioral Measure

A Polish version of the CRS-R was used (Binder et al., 2017). CRS-R is currently the gold standard for DoC diagnosis and monitoring of recovery (Giacino et al., 2004). It consists of six subscales: auditory, visual, motor, verbal communication, and arousal. The maximum score is 23, less than 10 points usually corresponds to UWS. Low score is associated with basic reflexes, whereas high score indicates controlled, intentional behavior. Assessment was conducted weekly, right before the stimulation period, then for a 6-month daily stimulation period, as well as for 9 weeks after the stimulation was ended to assess and monitor any behavioral changes. It was conducted by one of two trained research students. Assessment sessions were recorded and discussed to assure the measurement reliability.

Electrophysiological Measure

Electroencephalography Measurement

The EEG resting-state recording was performed using 64-electrode Active Two (BioSemi, Amsterdam, Netherlands), with a 10–20 system headcap. Electrooculography (EOG) signal was acquired using four electrodes located above and below the right eye and in the external canthi of both eyes. Two reference electrodes were attached on the left and the right mastoid and recorded in parallel. Two electrodes specific to Active Two system, namely "CMS" (common mode sense) and "DRL" (driven

right leg), were placed between "POz" and "PO3" and "POz" and "PO4," respectively. Recording duration was 10 min, eye-opening was maintained during recording. EEG signal was sampled at 1,024 Hz. The recording took place in the isolated room, with the patient sitting in bed in a reclining position, or sitting on the wheelchair in a comfortable position.

The EEG was performed six times with the mean interval of 49 days (range 36–61 days). The first measurement preceded the start of the taVNS stimulation by 1 month, the next four were performed during stimulation and the last one 56 days after taVNS stimulation protocol was completed. The relevant chronological information is presented in **Table 2**.

Electroencephalography Analysis

The off-line preprocessing of EEG data was performed with Brain Vision Analyzer 2.0 software (Brain Products, Gilching, Germany). Noisy channels were interpolated with a spline method, data was filtered using high-pass and low-pass filters at 1–50 Hz range (IIR Filter, Zero phase shift Butterworth filter; order 8), and downsampled to 256 Hz. Raw data inspection excluded massive artifacts before the segmentation, whereas blink and eye movement artifacts were corrected using Independent Component Analysis (ICA). Data was further re-referenced to the common average. Next, signal from each channel was segmented into non-overlapping 2 s intervals, with the noisy segments excluded from the analysis using semi-automatic mode with the following criteria: amplitude limits −150 μV to 150 μV; 150 μV maximum allowed difference in intervals over 200 ms; maximal voltage step of 75 μV/ms. Fast Fourier Transform (FFT, 10% Hanning window, 0.5 Hz resolution) was calculated on the remaining segments and averaged, each channel separately. Then, the single centro-parietal region channel was constructed by averaging the signal from the electrodes "Cz," "CPz," "Pz," "CP3," "CP1," "P3," "P1," "CP2," "CP4," "P2," and "P4." This region was used to calculate the frequency of the highest peak in the EEG spectrum. We decided to exclude other channels due to the frequent occurrence of artifacts of various origins precluding reliable analysis of EEG signal.

The averaged EEG spectra were parameterized using FOOOF algorithm (version 0.1.3). The fooof_mat wrapper (version 0.1.1, Donoghue et al., 2020) was used to analyze data within the MATLAB environment (MathWorks Inc., version 2019b). The FOOOF algorithm transforms the EEG signal spectrum into a set of parameters consisting of exponential two-parameter aperiodic components as well as a series of Gaussian peaks described by parameters referring to their center frequency, amplitude and bandwidth. The goodness of fit metrics are represented by R^2 of the model fit and an error estimate. For the current analyses the default settings for the algorithm were as follows: peak width limits = 2–6, maximal number of peaks = 4, minimum peak height = 0.111, peak threshold = 2 and fixed aperiodic mode. Power spectra were parameterized across frequency range 2–45 Hz.

Based on FOOOF algorithm results, for each EEG measurement in the averaged signal from the centro-parietal region we obtained a Primary Peak Frequency (MaxPeak1) and Secondary Peak Frequency (MaxPeak2) parameters, which were the center frequencies of the peaks with the highest and second highest amplitude found within the search range 3–14 Hz (extending across theta and alpha EEG frequency bands).

TABLE 2 | Chronological information about subsequent EEG measurements.

Measurement ID	Relative day
EEG 1	10
EEG 2	66
EEG 3	95
EEG 4	131
EEG 5	192
EEG 6	248

The days are calculated relative to the day when the stimulation protocol was started.

ECG Measurement

For ECG data acquisition we used BITalino portable toolkit (Batista et al., 2019) and the OpenSignal (r)evolution software. We have used a specific arrangement of electrodes, which in bedside conditions provides comfort and does not require removing the upper part of clothing, and at the same time provides a clear shape of the QRS complex in the recorded ECG. Two active electrodes were attached on the sides of the chest and an inactive electrode was attached at the lower part of the sternum. Self-adhesive Ag/AgCl electrodes and standard ECG jelly were used. Raw ECG signal was transmitted to a portable computer via bluetooth and was visible online in the OpenSignal window. This hardware configuration ensured an appropriate safety standard for measurements as well as an ongoing control of the signal quality.

ECG Analysis

ECG was analyzed in two 5-min epochs – before the start (pre-stimulation) and after the stop (post-stimulation) of a taVNS session. The raw electrocardiogram for each epoch was visually inspected and corrected if necessary. Manual signal correction consisted only in shifting the boundaries of the measuring epoch so that it included the smallest number of artifacts and disturbances. Nevertheless, the correctional shift never exceeded 1 min.

Despite all efforts, we found some epochs with extremely disturbed signal and eliminated them from analysis. This is why the data sometimes contains recordings not from both, but only from the initial or only from the final epoch.

Epochs were cropped and corrected in the MATLAB environment. The same application was used to identify R-waves using a method based on the analysis of the shape of the first derivative of the ECG signal. Then inter-beat intervals (IBI) were calculated. For further analysis we used Kubios HRV (Tarvainen et al., 2014) with a built-in artifact correction algorithm (medium threshold) to reduce the impact of missed and extra beats on HRV. The IBI series was also detrended. As an index of HRV we used the FFT-based power spectrum. For our purpose only the high frequency range of the HRV (HRV-HF) was usable, so we analyzed the range from 0.15 to 0.4 Hz.

Procedure

The research was carried out at the inpatient rehabilitation facility of the "Światło" (eng. "Light") Foundation in the period from June of 2019 to February of 2020. We assumed that the tests would take place in the following cycle: (a) initial CRS-R measurement before taVNS started, (b) initial EEG measurement before taVNS started, (c) taVNS every day, (d) ECG measurement every other day before and after taVNS session, (e) CRS-R measurement once a week, (f) EEG measurement once a month. The entire research program took about 7 months including 6 months of taVNS. Due to unpredictable fluctuations in the health condition of the patient as well as equipment failures and measurement artifacts, the frequency of interventions and measurements sometimes fell below the assumed level. In total, we managed to complete over 100 taVNS sessions, 6 EEG measurements, 37 sets of ECG recordings (before and after stimulation) and 30 CRS-R measurements.

A single taVNS session lasted about 4 h and was always performed continuously at the same time of the day. If there was an ECG measurement on a given day, a trained student researcher connected the ECG electrodes to the patient's chest and checked the correctness of the recorded signal on the computer monitor. The ECG signal was recorded for 11–12 min – the final 5-min period was used as a pre-stimulation level. Then the taVNS electrode was applied to the patient's left ear and the stimulator was turned on. At the end of the 4-h session the researcher turned the stimulator off and started ECG recording. The ECG signal was measured for 11–12 min – the initial 5-min period immediately after taVNS was used as a post-stimulation level.

For safety precautions of the project, every day, when taVNS stimulation was switched on the patient was constantly accompanied by a trained student researcher who watched for any signs of patient discomfort and monitored stimulation stability. Stimulation was paused when the medical staff performed routine daily care activities.

RESULTS

Behavioral Data

The CRS-R measurement was performed approximately every 7 days throughout the 220-day study period. Obtained data creates a time series with irregular distances between measurements (**Figure 1**). Total scores ranged from 4 to 13 points (M = 8.4, SD = 2.46). To illustrate the trend of changes in the measurements over time, we used the regression model. As the non-linear trend of changes is also possible in our conditions, we decided to test linear as well as quadratic models of regression. Both were statistically significant, but the quadratic equation provided a better fit (r^2 = 0.36; $p < 0.01$), than the linear one (r^2 = 0.17; $p < 0.01$). The best fitted regression model is shown on **Figure 1**.

The total CRS-R score is the sum of scores for six subscales. Standard deviation in each of them shows that the Auditory Functions Scale (SD = 0.97) and Visual Functions Scale (SD = 1.14) had the greatest impact on the observed fluctuations in the overall results. In other subscales standard deviations did not exceed 0.6.

Electrophysiological Data

The FOOOF algorithm decomposition of EEG signal from the centro-parietal channel in all six measurements identified

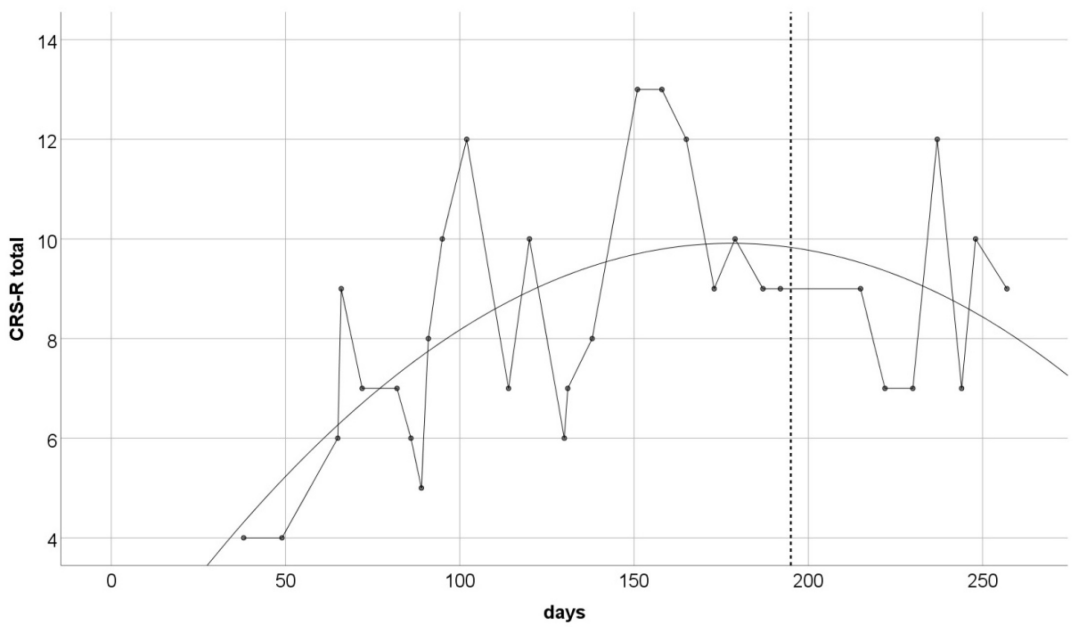

FIGURE 1 | The CRS-R total scores over the study duration. Solid line is the best fitted non-linear regression curve (r^2 = 0.36, $p < 0.01$). Vertical dashed line indicates the day of the last taVNS session.

TABLE 3 | The detailed information about the MaxPeak1 and MaxPeak2.

Meas. ID	MaxPeak1 center frequency (Hz)	MaxPeak1 amplitude (μV^2)	MaxPeak2 center frequency (Hz)	MaxPeak2 amplitude (μV^2)	R^2	Error estim.
EEG 1	6.24	0.70	Not found	Not found	0.9923	0.0411
EEG 2	6.41	0.79	8.99	0.53	0.9985	0.0222
EEG 3	6.50	0.73	9.12	0.28	0.9903	0.0498
EEG 4	6.38	0.53	9.27	0.34	0.9959	0.0267
EEG 5	6.56	0.69	9.23	0.40	0.9969	0.0286
EEG 6	6.11	0.61	8.72	0.23	0.9758	0.0640

peaks within 3–14 Hz search range indicating the presence of spontaneous oscillations within the resting-state EEG signal. The analysis revealed spectral profiles that underwent both qualitative as well quantitative changes over the course of six measurements. In all measurements we observed theta oscillations (MaxPeak1) that were centered around 6.37 Hz (range: −0.26/+0.19 Hz, SD = 0.17). The estimated amplitude of the MaxPeak1 was relatively stable, averaging at 0.68 μV^2 (range: −0.15/+0.12, SD = 0.09). An important qualitative change was the occurrence of the second, smaller peak within the alpha band range. The mean frequency of this second oscillatory peak (MaxPeak2) was 9.07 Hz (range −0.35/+0.20 Hz, SD = 0.22). The MaxPeak2 was not detected by FOOOF algorithm in measurement administered before the taVNS program started. It appeared in the second measurement and its presence was maintained through the remainder of the measurements. The amplitude of the second peak was smaller and more variable, the average value was 0.36 μV^2 (range: −0.13/+0.17, SD = 0.12). The full model goodness-of-fit was high in all measurements, except the last one in which it decreased due to the presence of unmodeled high frequency noise in the signal. The detailed information about the oscillatory peaks is presented in **Table 3**. **Figure 2** shows the spectral profiles from all measurements, and **Figure 3** the changes of the center frequencies of MaxPeak1 and MaxPeak2.

ECG

The ECG was recorded twice – before and after a taVNS session. Each segment lasted 5 min and was used to calculate mean HR and HRV-HF levels. Over the entire measurement period of 167 days, we collected 37 series covering the pre-stimulation and the post-stimulation segments. Our intention was to take measurements every 2 days. Nevertheless, the measuring equipment we used had two major disadvantages. The first was battery power. In the absence of a battery charge indicator, it was easy to overlook that it was only slightly charged. The second disadvantage is data transmission via Bluetooth, which was sometimes interrupted for unknown technical reasons. Of course, both of the above solutions also have a major advantage – ensuring patient safety. Thus, due to these technical problems and temporary health problems (e.g., viral infections) or physiological state (e.g., blood pressure and

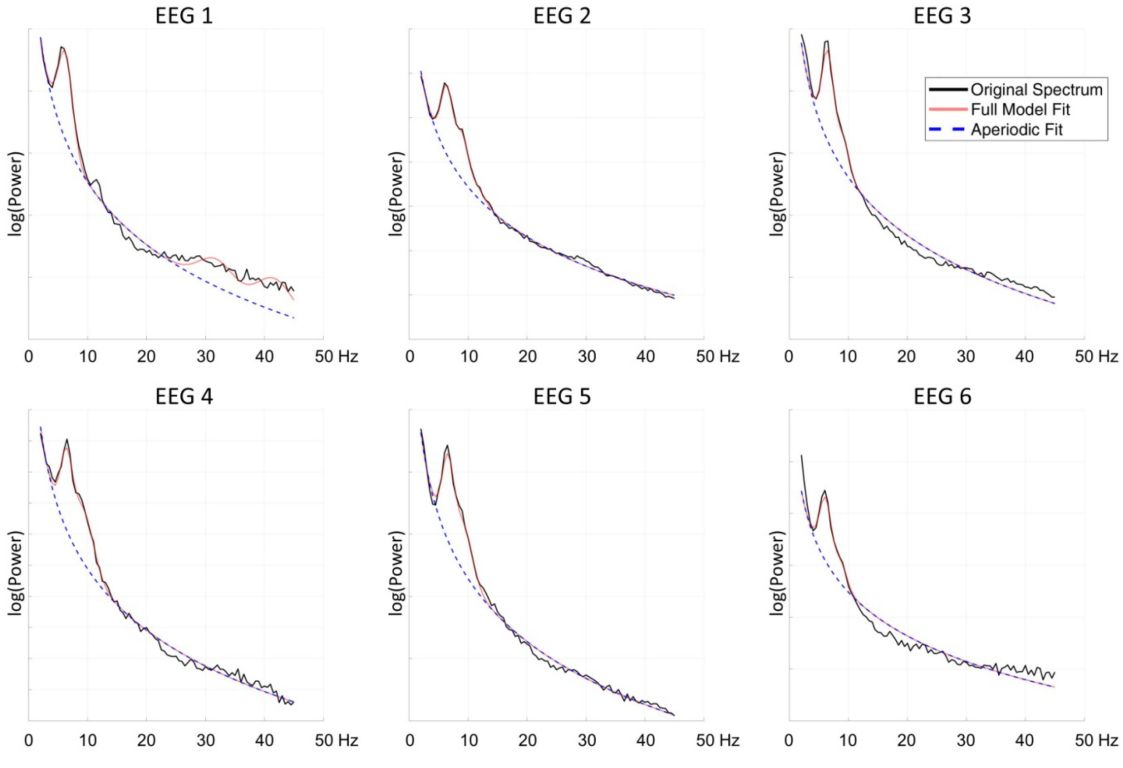

FIGURE 2 | The spectral profiles of all six measurements from the centro-parietal channel. Black line indicates the original spectrum, the red line the fitted function obtained with FOOOF algorithm, and the blue dashed line the fitted aperiodic component.

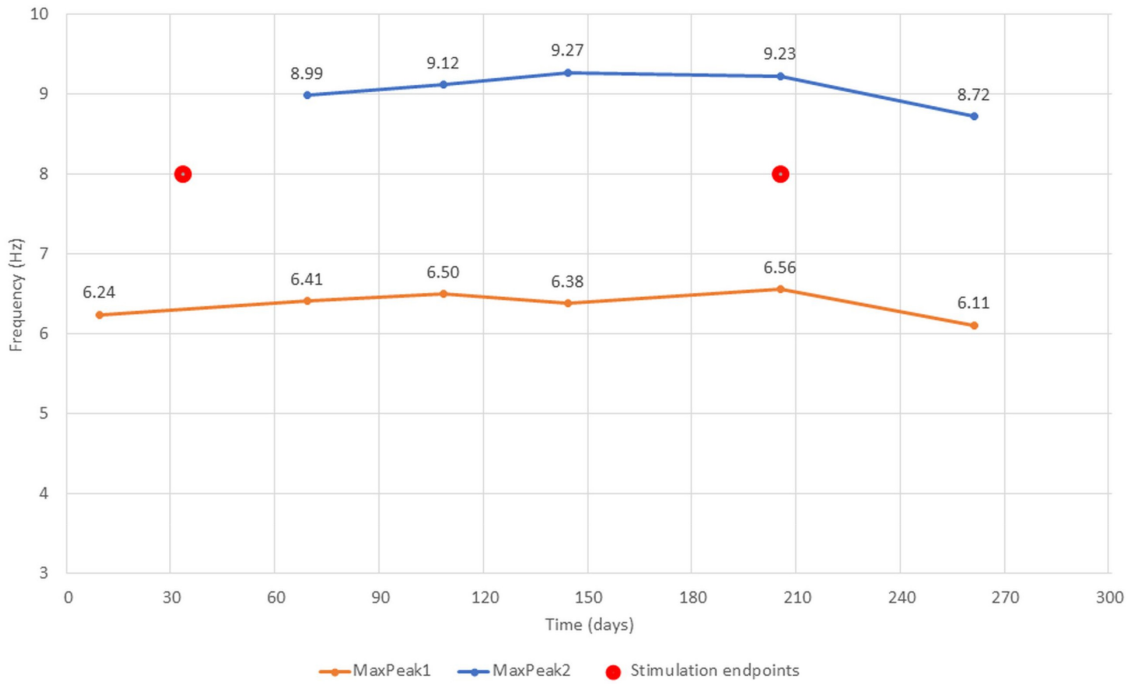

FIGURE 3 | The changes of MaxPeak1 and MaxPeak2 center frequencies across all six measurements. Captions accompanying the score points indicate the center frequency score. Stimulation endpoints (red circles) indicate the first and the last day of stimulation protocol.

temperature changes during menstruation), the frequency of measurements was variable and therefore their time points are not distributed evenly.

The changes in HR during the entire measurement period showed quite a large variance – the HR level was moderately low on some days, and accelerated considerably on others, reaching sometimes over 100 beats per minute (**Figure 4**). The post-stimulation HR level was almost always higher than the pre-stimulation level – the average difference was 5.17 bpm (SD = 7.87). Nevertheless, a systematic decrease of HR was observed throughout the measurement period. Regression analysis showed that only pre-stimulation time series can be approximated by a line with a statistically significant negative coefficient (**Table 4**).

Changes in HRV-HF level also showed limited stability over the course of subsequent measurements. However, similarly to HR, these changes showed a systematic trend throughout the entire measurement period (**Figure 5**). The analysis showed that both pre-stimulus and post-stimulus changes can be approximated with regression lines with statistically significant positive coefficients (**Table 5**).

Additional Observation

After less than 3 months of daily taVNS our patient began menstruating, which had not happened since the brain injury. This is likely to be the result of the intervention since the gonads are innervated by the vagus nerve. The fact that it appeared during the stimulation period is perhaps less crucial than the fact that it remained after the intervention finished, even if only in an on-again, off-again fashion. This implies that the intervention brought a durable change to our patient's physiology.

DISCUSSION

In our study, we applied taVNS daily for 6 months to a patient diagnosed with UWS. Despite the fact that the measurement results were characterized by high instability, we observed a systematic increase of the total CRS-R score, which summarizes the behavioral indicators of consciousness. From the initial score of 4–6 points, the patient progressed to 8–10 points after about 100 days of stimulation, and occasionally the total CRS-R score increased even to 13. This means that the initial UWS diagnosis could be changed to MCS or even MCS+. Behavioral changes that were responsible for this effect were concentrated mainly in visual and auditory functions – the highest variance in results was observed in these two scales of the CRS-R. In the remaining scales, i.e., in motor and oromotor/verbal activity, communication and arousal, the observed changes over time had a much smaller range, so the results were more stable. This is in line with previous reports showing that first signs of spontaneous progression from UWS to MCS usually appear in visual and sometimes also in auditory functions (Bagnato et al., 2017; Bareham et al., 2019).

The apparent improvement in our patient's functioning, however, did not seem to last. As the CRS-R evaluations continued to be performed for over 50 days after the end of the taVNS intervention, we noticed that the total score, as well as

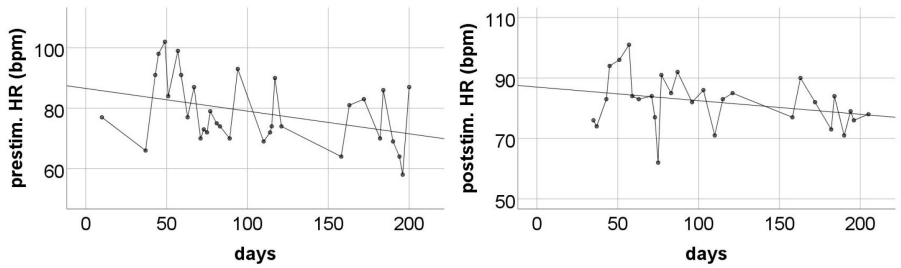

FIGURE 4 | The pre-stimulation **(left panel)** and post-stimulation **(right panel)** HR levels from all measurements during the entire program period. Solid line is the best fitted regression line (mathematical description in **Table 4**).

the regression trend, stopped rising. Still, a definite conclusion that the patient's improvement was only temporary would be premature, as we could only perform these post-intervention evaluations for a limited, quite short, period of time (due to the breakout of the COVID-19 pandemic). CRS-R performed on our patient 1.5 years after the end of our study also revealed significant fluctuations in the total score ranging from 5 (UWS) to even 13 (MCS+). Observations made during testing showed that the patient obtained better results when the test was performed in a sitting position than when lying down in bed, which may be associated with changes in arousal level (Bareham et al., 2019). However, such changes were not demonstrated on the CRS-R arousal scale.

Multiple EEG registration also allowed us to observe interesting effects. We found prominent oscillations in the theta band (average frequency 6.73 Hz) in all six EEG measurements. Interestingly, on the second measurement, 36 days after the beginning of the taVNS stimulation, the spectral profile of the EEG signal changed and FOOOF decomposition suggested that another 9 Hz oscillation in the alpha band was present in the EEG signal. That second oscillatory component was visible until the last measurement. Moreover, between the first and the second measurement we also observed a notable change in the total CRS-R score which increased from 6 to 9 points and during the next four measurements, the total score sometimes even reached the level of 12–13.

On the basis of studies with post-anoxic patients (Forgacs et al., 2017, 2020) have proposed a classification of spectral profiles of resting-state EEG signal associated with various types of thalamocortical dysfunction. They have distinguished four main types of spectral profiles. The first, A-type is dominated by aperiodic activity with the highest power contained within the delta range and apparent 1/f pattern of the signal variability. A-type indicates complete loss of neural networks integrity, without any significant contribution of thalamic nor cortical function. Over the course of recovery another spectral profile may become visible – with significant presence of oscillations within the theta range, indicating only spontaneous cortical activity without thalamic output. In the third division – C-type –spontaneous theta oscillations are accompanied by beta oscillations in the frontal regions. That pattern, according to Forgacs et al. (2017) represents low thalamic activity appearing in bursting mode, and local cortical disinhibition. Finally, the D-type of spectral profile with prominent alpha and beta activity is an indicator of a normal cortical function, and a high, tonic level of thalamic activity. In our study we have observed an apparent transition from the B-type to the D-type of spectral activity. We have not observed the intermediate C-type, probably due to the limitations of our EEG analysis methods. The beta oscillations characteristic of the C-type activity are observed in the frontal regions, and we were not able to obtain artifact-free EEG signal from those locations, thus we are not in position to ascertain the presence of that spectral profile in our subject. However, contrary to Forgacs et al. observations, we have not observed diminishing of spontaneous theta activity in our subject, and these oscillations were present during the whole experiment duration. Thus the spectral pattern observed in the second and later measurements can be viewed as a mixture of a B-type and D-type of resting-state activity. However, we interpret the prolonged presence of theta oscillations as an indication of possible sustained structural damage to the thalamo-cortical system visible in the signal from the centro-parietal region. One may not exclude the hypothesis that the uncertain and variable diagnosis of the patient, as assessed by the repeated CRS-R administration, might have been caused by the coexistence of these two types of prominent neuronal activity. While the presence of local alpha-range oscillations within the centro-parietal region might demonstrate at least partial recovery of normal cortical function, the second theta-range oscillations might be related to the EEG slowing caused by structural brain damage (Synek, 1988). The available three head CT scans acquired within 1 month since the traumatic incident suggested relatively minor lesions of the hemispheres, the first, of hemorrhagic origin, spanning the left lentiform nucleus, as well as lesions of posterior forceps and posterior part of the corpus callosum. The brain stem lesions were also detected, yet they were difficult to the detailed assessment due to the limited CT resolution. We surmise that this relative sparing of

TABLE 4 | Regression coefficients for HR levels (linear regression model was used).

	Reg. coeff.	R^2	*p*
Pre-stimulation level	−0.08	0.14	0.04
Post-stimulation level	−0.05	0.09	0.12

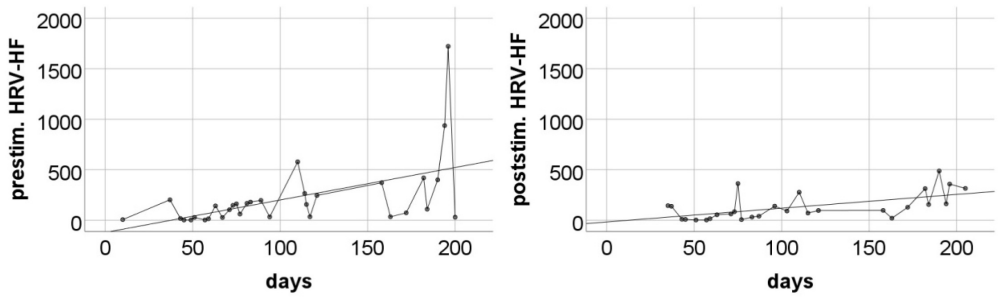

FIGURE 5 | The pre-stimulation **(left panel)** and post-stimulation **(right panel)** HRV-HF power levels from all measurements during the entire program period. Solid line is the best fitted regression line (mathematical description in **Table 5**).

cerebral hemispheres and thalamus might have been a factor facilitating the positive response to tVNS stimulation. However, this conclusion is limited by the fact that the CT scans have been acquired more than 4 years before the tVNS treatment, that later neuronal degeneration might have enlarged the territory of the telencephalic damage.

The similar relation between spectral oscillatory dynamics and condition of the patient was observed in other studies, yet only Forgacs et al. made an attempt to systematize the observed spectral profiles. For example (Lechinger et al., 2013) have observed a strong correlation between dominant posterior oscillatory activity within delta-theta-range and total CRS-R score. Relative increase in delta power, as well as decrease in alpha frequency was more often observed in patients with UWS diagnosis than with MCS in a study by Lehembre et al. (2012). Similar connections between behavioral and spectral measures in the delta-theta-alpha range were observed in a large study of Sitt et al. (2014), and in our study by Drążyk et al. (2022) involving 53 DoC patients. All these studies point at the presence of higher oscillations in alpha range as signs of recovery of consciousness, and at the same time the observed shift of the dominant frequency toward lower frequencies in the theta range was evidenced as a possible marker of disorganized brain dynamics due to sustained functional and/or structural damage.

The systematic ECG measurements showed that almost always post-stimulation HR level was higher than pre-stimulation level. Thus, it seems that the stimulation itself as well as the accompanying treatments could evoke some kind of sympathetically driven physiological arousal. Most likely, this is not a direct result of vagal nerve stimulation, because taVNS either does not cause changes in autonomic arousal (Couck et al., 2017) or causes a decrease in sympathetic arousal and a slower HR (Clancy et al., 2014). In our opinion, the acceleration of post-stimulation HR was rather the effect of mild discomfort associated with electrostimulation or possibly social stimulation related to the presence and activities performed by the student researcher. For this reason, the electrophysiological indicators recorded during the pre-stimulation sessions were more useful for us because they were devoid of situational artifacts.

The pre-stimulation level of HR showed large fluctuations over time, but a statistically significant downward linear trend was visible throughout the entire measurement period. This means that over the course of the study, we observed a systematic decrease in the tonic level of sympathetic arousal. This effect is consistent with the observations made by Clancy et al. (2014). It should be emphasized that the pre-stimulation level of HR was often high, reaching even 90–100 beats per minute in the initial months. This indicates the dominance of the sympathetic system in the regulation of the heart. Thus, the observed long-term decrease in HR should be understood as a gradual withdrawal of sympathetic stimulation and an increase in parasympathetic control. This interpretation is in line with our HRV-HF observations. Resting HRV-HF remained fairly low for the initial few weeks after starting the taVNS program. Later, on some days, this level increased significantly, and in the last period of stimulation, it sometimes reached very high. Regression analysis revealed a clear increasing trend. This is not a short-term reactive effect, as the trend was revealed in the resting data collected each day before the stimulation was started. Thus, we observed a long-term effect, which is most likely a consequence of relatively constant and stabilized regulatory processes.

Increase in resting HRV-HF levels is often associated with neurovisceral integration (Thayer et al., 2012), which in healthy people is an indicator of flexible control over behavior. According to the authors of this model, a high level of parasympathetic heart control means a state in which visceral systems are an important source of information about the body's response to specific environmental influences. So it is an element of awareness of the environment. Some studies directly show that a higher level of HRV-HF in people with DoC often corresponds to a higher level of consciousness on the CRS-R (Riganello et al., 2018). Thus, an increase in the level of HRV-HF may be a signal of cognitive improvement, which cannot be overestimated in DoC patients. The research revealed that the level of resting HRV-HF shows a positive correlation with the capability of brain-computer interface usage (Kaufmann et al., 2012). Such

TABLE 5 | Regression coefficients for HRV-HF levels (linear regression model was used).

	Reg. coeff.	R^2	p
Pre-stimulation level	3.22	0.28	0.002
Post-stimulation level	1.36	0.35	0.001

effects constitute a potential direction for future research. If it could be confirmed that tVNS is able to raise the level of HRV-HF, the question arises whether this stimulation can increase the cognitive abilities of DoC patients large enough to make even minimal contact with them.

CONCLUSION

We observed three positive phenomena accompanying our interventions. First, the behavioral indices of consciousness gradually increased throughout tVNS use. These indices stopped rising after stimulation was interrupted. Secondly, over the course of several months of stimulation, the EEG power in the range of the alpha wave gradually increased, which may be an indicator of a slight neural networks reintegration and strengthening of cortical activity. And thirdly, the systematic intervention was also accompanied by psychophysiological changes, i.e., the resting HR level gradually decreased and the HRV-HF level increased. The changes were not large, but visible in the form of long-term trends. They may be a sign of the increasing influence of the parasympathetic system in autonomic space, which usually helps to improve environmental consciousness.

However, it is clear that the case study does not provide conclusive evidence that the observed electrophysiological and behavioral effects are a consequence of tVNS. It is possible that the actual effect was due to social and physical stimulation during daily interventions, e.g., touch, sounds, changes in light intensity, etc. Thus, the phenomena described above require confirmation in studies with other patients (between-subject comparisons) or with the phase during which tVNS is sham (within-subject comparisons). In this case, the procedure will not be technically simple, because we observed changes in indicators after long-term exposure (at least several weeks of systematic interventions for 4 h a day). This period would extend even more after including the sham phase and some DoC patients may drop out of the program due to changes in physiological parameters (e.g., blood pressure spikes due to tVNS). Nevertheless, such trials could provide a clearer answer to the question of the benefits of using tVNS in the treatment of DoC patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the datasets cannot be anonymized. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The study was reviewed and approved by the Research Ethics Committee at the Faculty of Psychology, University of Warsaw. The written informed consent to participate in this study as well as consent for the publication of any potentially identifiable images or data included in this article was obtained from the legal guardian of the patient.

AUTHOR CONTRIBUTIONS

AO: overall concept of the study, project coordination, ethics application, and article write-up (Introduction). AR: design of the ECG part of the project, ECG data analysis, and article write-up (Introduction, Results, and Discussion). MB: design of the EEG part of the project, EEG data collection and analysis, and article write-up (Results and Discussion). AB and AL: student researchers responsible for taVNS application, CRS-R, ECG, and EEG data collection. TK: project supervision, concept consultation, and data collection coordination. All authors contributed to the article and approved the submitted version.

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Vagus Nerve Stimulation Improves Mitochondrial Dysfunction in Post–cardiac Arrest Syndrome in the Asphyxial Cardiac Arrest Model in Rats

Seonghye Kim[1†], Inwon Park[1,2†], Jae Hyuk Lee[1,2], Serin Kim[1], Dong-Hyun Jang[1] and You Hwan Jo[1,2]*

[1] Department of Emergency Medicine, Seoul National University Bundang Hospital, Seongnam-si, South Korea,
[2] Department of Emergency Medicine, Seoul National University College of Medicine, Seoul, South Korea

Cerebral mitochondrial dysfunction during post–cardiac arrest syndrome (PCAS) remains unclear, resulting in a lack of therapeutic options that protect against cerebral ischemia–reperfusion injury. We aimed to assess mitochondrial dysfunction in the hippocampus after cardiac arrest and whether vagus nerve stimulation (VNS) can improve mitochondrial dysfunction and neurological outcomes. In an asphyxial cardiac arrest model, male Sprague–Dawley rats were assigned to the vagus nerve isolation (CA) or VNS (CA + VNS) group. Cardiopulmonary resuscitation was performed 450 s after pulseless electrical activity. After the return of spontaneous circulation (ROSC), left cervical VNS was performed for 3 h in the CA + VNS group. Mitochondrial respiratory function was evaluated using high-resolution respirometry of the hippocampal tissue. The neurologic deficit score (NDS) and overall performance category (OPC) were assessed at 24, 48, and 72 h after resuscitation. The leak respiration and oxidative phosphorylation capacity of complex I (OXPHOS CI) at 6 h after ROSC were significantly higher in the CA + VNS group than in the CA group (p = 0.0308 and 0.0401, respectively). Compared with the trends of NDS and OPC in the CA group, the trends of those in the CA + VNS group were significantly different, thus suggesting a favorable neurological outcome in the CA + VNS group (p = 0.0087 and 0.0064 between times × groups interaction, respectively). VNS ameliorated mitochondrial dysfunction after ROSC and improved neurological outcomes in an asphyxial cardiac arrest rat model.

Keywords: heart arrest, post-cardiac arrest syndrome, vagus nerve stimulation, mitochondria, cell respiration, reperfusion injury

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***Correspondence:**
Jae Hyuk Lee
hyukmd@gmail.com

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Restoration of cerebral function is the key factor in the successful resuscitation of patients with out-of-hospital cardiac arrest (Laver et al., 2004; Nolan et al., 2008). Although return of spontaneous circulation (ROSC) is achieved in a proportion of patients with cardiac arrest, more than 90% of these patients experience severe neurological injury (Grasner et al., 2016; Kim et al., 2018; Myat et al., 2018). Several mechanisms have been suggested to explain

post–resuscitation cerebral injury and the therapeutic targets (Stub et al., 2011; Sekhon et al., 2017). However, a majority of therapies that target the amelioration of cerebral injury in post–cardiac arrest syndrome (PCAS) have proven unsuccessful, and targeted temperature management remains the only effective treatment (Nolan et al., 2008; Choudhary et al., 2021; Perkins et al., 2021; Sandroni et al., 2021).

Resuscitation results in reperfusion of ischemic tissues, recovery of aerobic metabolism, and organ perfusion; consequently, ischemia–reperfusion injury is inevitable (Madathil et al., 2016). Although mitochondria are known to be key determinants of ischemia–reperfusion injury, cerebral mitochondrial dysfunction during the post–resuscitation period following cardiac arrest is not well understood (Wiberg et al., 2020). Furthermore, most of our understanding of mitochondrial function and energy metabolism is derived from the myocardium (Yeh et al., 2009; Fang et al., 2012) and focal cerebral ischemia models (Li et al., 2012; Madathil et al., 2016). A previous preclinical study in the swine cardiac arrest model reported decreased mitochondrial respiratory coupling and calcium retention capacity with increased reactive oxygen species (ROS) production in the brain (Matsuura et al., 2017). The study also suggested that the current approach of cardiopulmonary resuscitation (CPR) has limited effect on the restoration of the mitochondrial function in the brain; therefore, there is a demand for urgent and novel therapeutic strategies for the restoration of mitochondrial function (Choudhary et al., 2021).

Vagus nerve stimulation (VNS) has been used to treat refractory partial epileptic seizures and treatment-resistant depression (Gonzalez et al., 2019; Lv et al., 2019). VNS has also been proposed to exert anti-inflammatory effects not only in clinical studies but also in preclinical studies on sepsis and stroke (Borovikova et al., 2000; Ay et al., 2016). In a previous study in a rat model of asphyxial cardiac arrest, VNS accelerated the recovery of cerebral blood flow during the post–cardiac arrest period and improved ischemic hippocampal neuronal damage and functional neurological outcomes (Sun et al., 2018; Kim et al., 2019). Furthermore, VNS has been suggested to exert protective effects against mitochondrial dysfunction in the myocardium (Samniang et al., 2016). However, the effects of VNS on cerebral mitochondrial function, particularly during cardiac arrest, have not been fully evaluated.

Therefore, the aim of this study was to investigate the effects of VNS on mitochondrial respiration in an asphyxial cardiac arrest rat model. We hypothesized that VNS could mitigate cerebral mitochondrial dysfunction and exert therapeutic effects to protect against post–resuscitation cerebral injury.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital (protocol No. BA 1803-243/022-01).

Experimental Design

A schematic of the experimental protocol is presented in **Figure 1**. The experiments required two sets of brain harvests for high-resolution respirometry and an evaluation of neurological outcomes.

Animal Preparation

Male 9-week-old Sprague–Dawley rats weighing 290–320 g were used in this study. The animals were housed in a controlled environment with unrestricted access to food and water before the experiments. They were anesthetized with an intramuscular injection of tiletamine/zolazepam (30 mg/kg; Zoletil, Virbac, France) and xylazine (10 mg/kg, Rompun, Bayer, Germany) and intubated with a 16-gauge catheter (Becton, Dickinson and Company, Franklin Lakes, NJ, United States), which was connected to a ventilator (Harvard rodent ventilator Model 645, Harvard Apparatus, Holliston, MA, United States). The ventilator was set to 2.0 mL of tidal volume with a respiratory rate of 45 breaths per minute. The minute ventilation was adjusted to achieve partial pressure of CO_2 ($PaCO_2$) of 35–40 mmHg on arterial blood gas analysis (ABGA). Body temperature was monitored with a rectal probe and was maintained at 36.5°C–37.5°C using an infrared heater. Under aseptic conditions, a 24-gauge catheter (Introcan, B. Braun, Germany) was surgically introduced into the left femoral artery on the left inguinal site to monitor the blood pressure and obtain blood samples for ABGA. Resuscitative drugs were administered *via* the left femoral arterial catheter. ABGA was performed before inducing asphyxial cardiac arrest and after weaning from the mechanical ventilator.

Cardiac Arrest and Resuscitation

To induce asphyxial cardiac arrest, vecuronium (0.2 mg/kg) was injected to paralyze respiration. After the discontinuation of mechanical ventilation, the heart rate (HR) and mean arterial pressure (MAP) increased initially, followed by progressive bradycardia and hypotension. Circulatory arrest was defined by the onset of MAP under 20 mmHg and maintained for 450 s. Following our previously established model (Lee et al., 2013; Kim et al., 2019), cardiac arrest was achieved in less than 120 s of asphyxia. Therefore, if the induction time of cardiac arrest was longer than 120 s, animals were excluded to control the duration of hypoxia. CPR was performed after 450 s of circulatory arrest. It included restarting mechanical ventilation (tidal volume, 2.5 mL; fraction of inspired oxygen (FiO_2), 1.0; respiratory rate, 55/min), administering epinephrine (0.01 mg/kg) and bicarbonate (1.0 mEq/kg), and continuous external chest compressions at a rate of 200 compressions per minute using a mechanical thumper (custom-made device, compressed air-driven, rate 200 cycles/min) until spontaneous pulse was detected on the arterial blood pressure monitor and the mean arterial blood pressure exceeded 50 mmHg. Immediately after the ROSC, the animals were randomized into two groups: the vagus nerve isolation group (CA, $n = 28$) and the VNS group (CA + VNS, $n = 26$).

In the post-resuscitative period, body temperature usually decreased with injection of drugs during the resuscitation period was maintained at 36.5°C–37.5°C using an infrared heater to the extent possible. In the experimental set of brain harvest, additional intramuscular anesthesia was provided to maintain until predefined time of harvest. In the experimental set of neurological outcome assessment, additional anesthesia was not

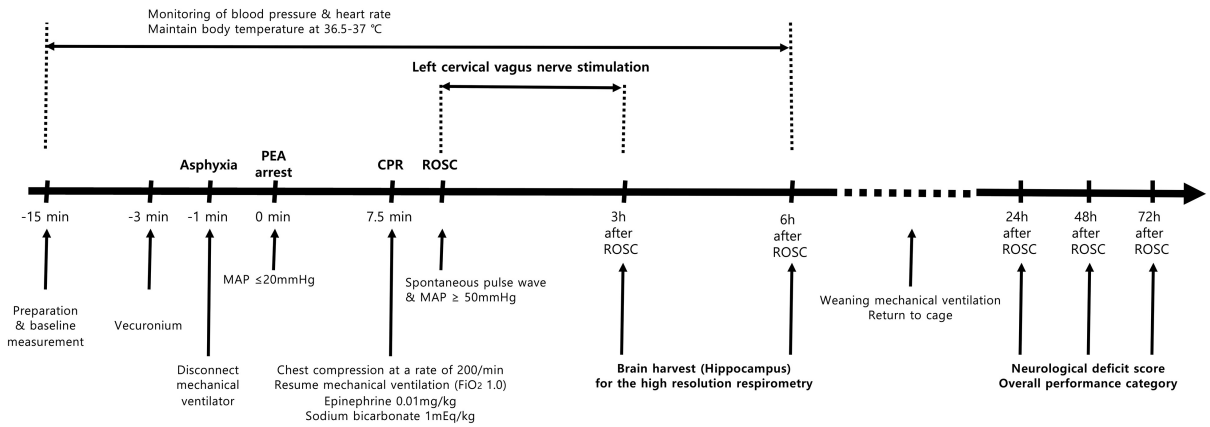

FIGURE 1 | Schematics of the experimental protocol.

provided and weaning from ventilator was performed after spontaneous respiration was recovered. Adequate spontaneous ventilation, defined as no decrease of mean arterial pressure for 5 min with $PaO_2 > 60$ mmHg on room air, was assessed after disconnection of mechanical ventilator. Extubation of the endotracheal tube was then performed and the rats were returned to the cages for neurological observation for 72 h. During the neurological observation period, fluid (5% Dextrose in saline, 50 mL/kg) was subcutaneously administered every 24 h for nutritional support and analgesia (Ketoprofen, 5 mg/kg) was subcutaneously provided if signs of pain were observed.

Vagus Nerve Stimulation

To stimulate the vagus nerve, the left cervical vagus nerve (VN) was isolated. A 1-cm incision was made 0.5 cm to the left of the midline of the neck. After retracting the muscles, the left carotid artery was exposed, and the left VN was meticulously dissected from the carotid sheath. VN was stimulated using 1-mA pulses of 10-ms duration at 1 Hz for 3 h after ROSC (Model 2100 isolated pulse stimulator, A-M Systems, Sequim, WA, United States) (Kim et al., 2019). Nerve stimulation was performed with a platinum electrode (Plexiglas-platinum electrode, 73-0336, Harvard Apparatus, Holliston, MA, United States), which offers the advantage of stable electrical delivery and protection from the surrounding fluid. A wet gauze of an appropriate level without water leakage was applied to the VNS isolation site to maintain a moist environment, and the wetness level was repeatedly estimated to determine when the gauze should be replaced. In the vagus nerve isolation group (CA), VNS isolation and placement of electrode without stimulation was performed.

Tissue Preparation

At 3 h (CA_3 h, $n = 8$; CA + VNS_3 h, $n = 8$) and 6 h (CA_6 h, $n = 8$; CA + VNS_6 h, $n = 7$) after ROSC, additional intramuscular anesthesia was provided, and euthanasia was achieved with exsanguination using a femoral arterial catheter in 1 min to prevent tissue swelling. After confirmation of death, a thoracotomy and clamping of the descending aorta were conducted. Then, perfusion with DPBS at 4°C using a 20-gauge needle through the left ventricle was performed, and the brain was harvested. The sham group (Sham, $n = 9$) was used to identify the baseline variables of mitochondrial respiration. In the sham group, intubation and femoral artery catheterization were performed, and before asphyxia modeling, euthanasia as described above was achieved for brain harvest. The hippocampus was carefully isolated from the harvested brain tissue and suspended in buffer X with an ice pack. The right hippocampal tissue was dissected with a microblade, and 10 mg of tissue was transferred into 200 μL of assay buffer (50 mL of buffer Z + 0.1 M EGTA + 0.313 g creatine) and homogenized (Perry et al., 2011).

Measurements of Mitochondrial Respiration

To measure mitochondrial oxygen consumption, high-resolution respirometry was performed (**Supplementary Figure 1**) (O2k, OROBOROS INSTRUMENT, Innsbruck, Austria) (Gnaiger et al., 2000; Burtscher et al., 2015). The resulting homogenates of hippocampal tissue were diluted and transferred into chambers of calibrated Oxygraph-2K. Oxygen polarography was performed under controlled temperature (37 ± 0.001°C) using electronic Peltier regulation. Oxygen concentration (CO_2, μM) and oxygen flux per tissue mass (pmol O_2/s*mg) were measured in real time using specialized software (DatLab, OROBOROS INSTRUMENT). According to the current substrate-uncoupler-inhibitor titration (SUIT) protocol, non-phosphorylating leak respiration was induced by adding the CI-linked substrate glutamate (5 mM, G5889, Sigma-Aldrich, St. Louis, MO, United States), malate (2 mM, G7397, Sigma-Aldrich), and pyruvate (5 mM, P2256, Sigma-Aldrich). The oxidative phosphorylation capacity of complex I (OXPHOS C I) activity was measured after adding a saturating concentration of adenosine 5'-diphosphate (ADP) (5 mM, ADP sodium salt, Sigma-Aldrich A2754). OXPHOS capacity combined with CI and II (OXPHOS CI + II) was estimated by adding succinate (10 mM, sodium succinate dibasic hexahydrate, Sigma-Aldrich

S2378). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5 mM, Sigma-Aldrich C2920) triggers proton leakage over the inner mitochondrial membrane. The capacity of the electron transfer system (ETS), which is the non-coupled state at the optimum uncoupler concentration of the maximum oxygen consumption, was measured. Inhibition of CI by rotenone (1 μM, Rotenone, Sigma-Aldrich R8875) was used to measure OXPHOS CII-linked ETS capacity. To control for other oxygen-consuming processes, ETS was inhibited using malonate (5 mM, malonic acid, Sigma-Aldrich M1296) and antimycin (1 μM/mL, antimycin A from Streptomyces sp., Sigma-Aldrich A8674). The consequent residual oxygen consumption (ROX) represents oxygen consumption from undefined sources and was subtracted from the mitochondrial respiratory states.

Assessment of Neurological Outcomes

Neurological outcomes were evaluated in the CA (n = 12) and CA + VNS groups (n = 11) at 24, 48, and 72 h after ROSC using the neurological deficit scale (NDS) (0–80; normal = 80; brain death = 0) and overall performance category (OPC) (1 = normal; 2 = slight disability; 3 = severe disability; 4 = comatose; 5 = dead) (Jia et al., 2008; Lee et al., 2013). The scores were measured independently by two researchers blinded to the allocation of the experimental groups, and any discrepancy between their measurements was reviewed by the authors.

Statistical Analysis

Normality tests were performed using the Kolmogorov–Smirnov test. Variables are presented as mean ± standard deviation (S.D.) or median (Interquartile range), as appropriate. Analysis of variance (ANOVA) with Bonferroni's *post hoc* multiple comparisons were utilized to compare the variables in the respirometry at each defined period (3 h and 6 h) with the variables in sham. Two-way repeated-measures ANOVA (RM ANOVA) with Bonferroni's *post hoc* multiple comparisons were used to compare the trends and variables in repeated measurement of neurological outcomes. Due to missing variables, mixed-effects model with Bonferroni's *post hoc* multiple comparisons were utilized to compare trends and variables in repeated measurements of hemodynamic variables. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Baseline Characteristics of the Animals

The baseline characteristics of the two groups (CA vs. CA + VNS) demonstrated no significant differences in body weight, pre-ABGA and post-ABGA values, serum lactate levels, or total ischemia time between the induction of cardiac arrest and ROSC (**Table 1**). Trends in the mean arterial pressure over the observation period were not significantly different between the two groups (**Supplementary Figure 2A**; $p < 0.0001$ between times, $p = 0.0701$ between groups, $p = 0.9914$ between times × groups; mixed-effects model). Trends in heart rate over the observation period were significantly different between the two groups (**Supplementary Figure 2B**; $p < 0.0001$ between times, $p = 0.6053$ between groups, $p = 0.0234$ between times × groups; mixed-effects model). From 90 to 150 min after ROSC, the heart rate tended to decrease in the CA + VNS group compared with that in the CA group, which coincides with the VNS period (**Supplementary Figures 2B–D**); however, the difference in heart rate during the VNS period was not statistically significant (**Supplementary Table 1**).

High-Resolution Respirometry

Three hours after ROSC, no significant differences in leak respiration were observed between the sham, CA, and CA + VNS groups (**Figure 2A**). Complex I respiration (OXPHOS CI), Complex I + II respiration (OXPHOS CI + II), and ETS tended to increase at 3 h after ROSC; however, the difference was not statistically significant (**Figures 2B–D**). OXPHOS CI and ETS in the CA + VNS group were significantly higher than those in the sham group ($p = 0.0080$ and $p = 0.0089$, respectively) (**Figures 2B,D**).

Six hours after ROSC, leak respiration was significantly lower in the CA group than in the sham model (**Figure 2E**) (Sham vs. CA_6 h, $p = 0.0291$) and was restored in the CA + VNS group, which was significantly different from that in the CA group (CA_6 h vs. CA + VNS_6 h, $p = 0.0308$). Complex I respiration (OXPHOS CI) tended to decrease at 6 h after ROSC, while a significant increase was identified in the CA + VNS group (**Figure 2F**, CA_6 h vs. CA + VNS_6 h, $p = 0.0401$). Complex I + II respiration and ETS capacity tended to decrease at 6 h after ROSC and slightly increased in the CA + VNS group at 6 h after ROSC; however, there was no statistically significant difference (**Figures 2G,H**).

Neurological Outcomes

The trends in NDS after ROSC were significantly different between the CA and CA + VNS groups (**Figure 3A**; $p < 0.0001$ between times, $p = 0.0300$ between groups, $p = 0.0087$ between times × groups; two-way RM ANOVA). NDS at 48 h and 72 h after ROSC were significantly higher in the CA + VNS group than in the CA group ($p = 0.0221$ and $p = 0.0131$, respectively; Bonferroni's *post hoc* multiple comparisons test). Similarly, the trends in OPC after ROSC were significantly different between the CA and CA + VNS groups (**Figure 3B**; $p < 0.0001$ between times, $p = 0.0271$ between groups, $p = 0.0064$ between times × groups; two-way RM ANOVA). OPC at 48 h and 72 h after ROSC were significantly lower in the CA + VNS group than in the CA group ($p = 0.0221$ and $p = 0.0210$, respectively; Bonferroni's *post hoc* multiple comparisons test), thus suggesting favorable neurological outcomes in the CA + VNS group.

DISCUSSION

In this study, using the asphyxial cardiac arrest model of rats, we identified that VNS might reduce cerebral injury by improving the mitochondrial dysfunction induced by ischemia–reperfusion injury. At 6 h after ROSC, leak respiration was significantly

TABLE 1 | Comparison of hemodynamic and blood gas analysis variables.

Variables	CA (n = 28)	CA + VNS (n = 26)	p-value
Body weight	294.0 [285.0–314.0]	304.0 [295.0–313.0]	0.43
Hemodynamic and blood gas values at baseline			
pH	7.38 [7.35–7.40]	7.38 [7.34–7.40]	0.81
$PaCO_2$ (mmHg)	36.5 [34.5–44.4]	39.5 [35.8–42.2]	0.62
PaO_2 (mmHg)	68.1 [58.4–75.2]	73.7 [59.9–80.1]	0.32
HCO_3^- (mmol/L)	22.0 [20.6–24.9]	22.6 [21.3–24.0]	0.87
Base excess (mmol/L)	–3.0 [–5.5–0.1]	–2.4 [–4.1—1.6]	0.95
Lactate (mmol/L)	0.9 [0.8–1.0]	0.9 [0.7–1.1]	0.94
MAP (mmHg)	109.0 [90.0–140.0]	110.0 [78.0–128.0]	0.48
Heart rate (bpm)	287.0 [258.0–313.5]	293.0 [271.3–312.8]	0.48
Body temperature (°C)	37.1 [36.9–37.4]	36.6 [36.3–37.2]	0.07
Hemodynamic and blood gas values at ROSC period			
ph	7.30 [7.22–7.33]	7.31 [7.22–7.42]	0.25
$PaCO_2$ (mmHg)	74.6 [70.1–82.0]	69.8 [60.1–79.1]	0.22
PaO_2 (mmHg)	115.9 [92.4–157.9]	128.7 [107.7–156.3]	0.25
HCO_3^- (mmol/L)	35.8 [31.4–40.6]	37.1 [32.6–42.2]	0.83
Base excess (mmol/L)	10.1 [4.8–14.0]	11.4 [5.0–16.2]	0.62
Lactate (mmol/L)	7.4 [5.5–7.9]	6.6 [5.9–7.9]	0.78
MAP (mmHg)	112.0 [100.0–131.0]	101.0 [89.8–119.0]	0.08
Heart rate (bpm)	136.0 [116.0–261.0]	128.0 [96.0–165.0]	0.13
Body temperature (°C)	34.5 [34.2–34.9]	33.6 [33.1–34.9]	0.07
Post ROSC 1 h period			
Body temperature (°C)	36.7 [36.4–37.0]	36.3 [35.9–36.9]	0.13
Induction time (s)	73.0 [64.0–79.0]	74.0 [68.0–82.0]	0.41
CPR duration (s)	25.0 [24.0–29.0]	24.0 [22.0–27.0]	0.21
Total ischemia time (s)	550.0 [538.0–556.0]	552.0 [544.0–556.0]	0.21

Variables are presented with median [IQR]. ROSC, return of spontaneous circulation; MAP, mean arterial pressure.

decreased, and CI, CI + II, and ETS were tended to decrease without statistical significance, which were all reversed by VNS. Although the increases in CI + II and ETS were not statistically significant, leak and CI respiration in the CA + VNS group significantly increased at 6 h after ROSC compared to the CA group. Additionally, VNS improved the functional neurological outcomes at 48 and 72 h after ROSC, as measured using NDS and OPC.

Previous preclinical cardiac arrest models in rats and pigs have revealed the decreased CI and CII function in the mitochondria of hippocampus (Yang et al., 2018; Lautz et al., 2019; Marquez et al., 2020). Although the global brain ischemia with reperfusion model was utilized, another study demonstrated CI suppression in mitochondria in the hippocampus and cortex during ischemia (Chomova et al., 2012; Borutaite et al., 2013). A previous study of an 8-min cardiac arrest mouse model with 60 min of reperfusion found decreased CI function but normal CII function in heart mitochondria, which suggests that CI is a more vulnerable site than CII in myocardium (Han et al., 2008). In contrast, a previous clinical study that investigated skeletal muscle biopsy in patients with out-of-hospital cardiac arrest identified significantly lower CI + II and ETS than in age-matched healthy controls, while CI showed no difference in muscle (Wiberg et al., 2020). The discrepancy in types of major complex dysfunction in mitochondria might originate from differences in modeling method and type of species, but the major difference appears to be the difference in target tissues. Mitochondrial diversity, including in the number, volume density, and gene expression profile, has been reported between tissues (Bugger et al., 2009; Kim et al., 2016). Additionally, several studies investigating the mitochondria have reported conflicting results between different tissues (heart vs. brain) for the same subject (Matsuura et al., 2017; Ji et al., 2021). As brain injury is the most devastating complication during PCAS that brings the main cause of mortality and long-term disability in survivors of cardiac arrest, investigation of mitochondrial dysfunction in cardiac arrest should be prioritized in brain (Sekhon et al., 2017; Perkins et al., 2021; Sandroni et al., 2021).

Surprisingly, at 3 h after ROSC, CI and CI + II respiration and ETS were rather increased in the CA and CA + VNS groups compared to the sham group. In addition, every increase in CI, CI + II, and ETS at 3 h was followed by a decrease at 6 h after ROSC. It can be assumed that mitochondrial dysfunction in PCAS represents delayed features. Consistent with our study, a previous study of global brain ischemia–reperfusion injury identified a slight increase in CI activity 1 h after reperfusion compared to controls (Chomova et al., 2012). Although the tissues were different, previous experimental studies on cardiac arrest have also identified that the activity of complex I-III was relatively unaffected and remained or rather than control levels

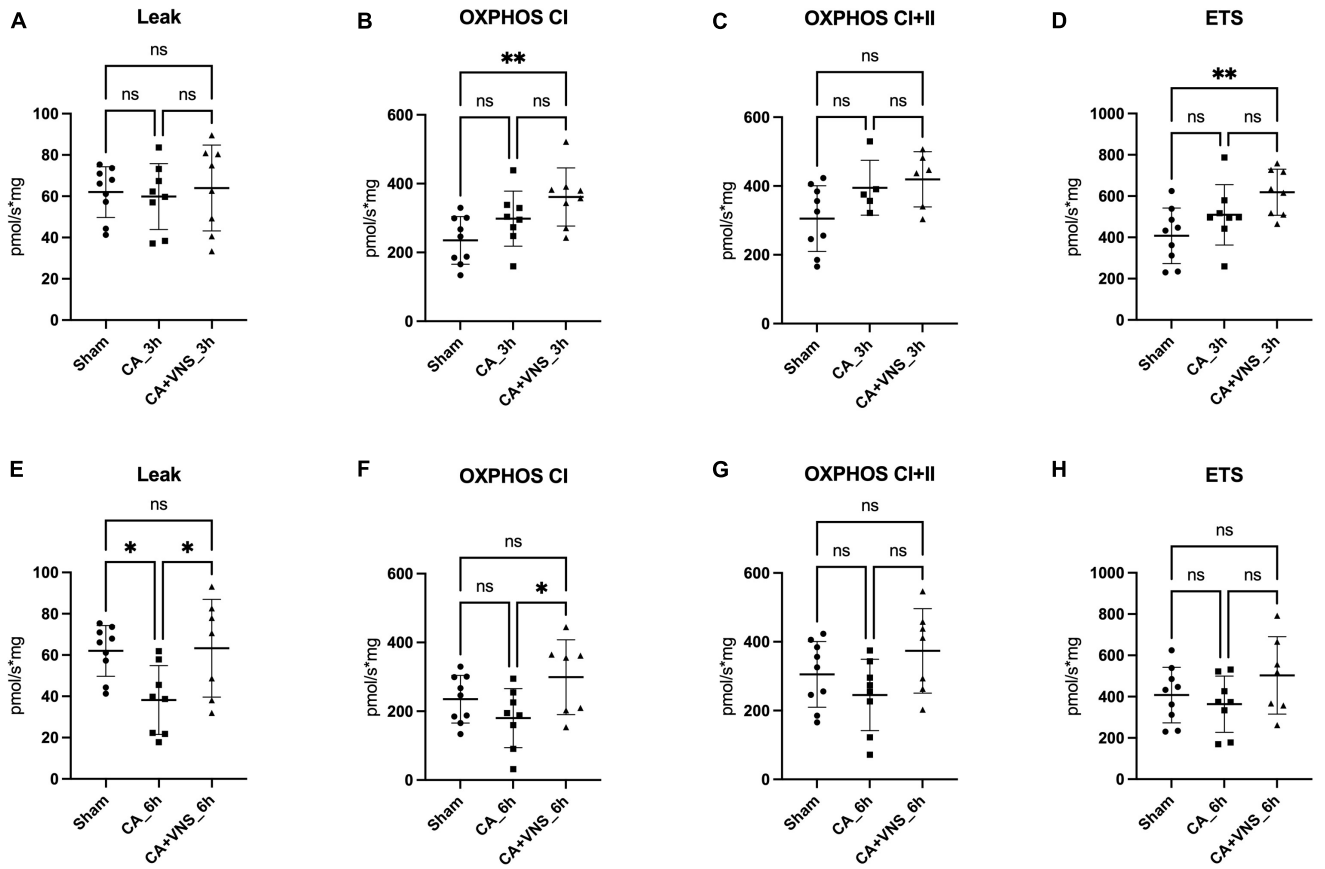

FIGURE 2 | Comparisons of mitochondrial respiration at 3 h and 6 h after ROSC. **(A)** Leak, **(B)** OXPHOS CI, **(C)** OXHPOS CI + II, and **(D)** ETS in the sham group (n = 9), CA_3 h group (n = 8), and CA + VNS_3 h group (n = 8). **(E)** Leak, **(F)** OXPHOS CI, **(G)** OXHPOS CI + II, and **(H)** ETS in the sham group, CA_6 h group (n = 8), and CA + VNS_6 h group (n = 7). Data are presented as the mean ± standard deviation. One-way ANOVA with Bonferroni's post hoc multiple comparisons test was performed (*P < 0.05; **P < 0.01; ns, not significant); CA, cardiac arrest; ETS, electron transfer system; OXPHOS, oxidative phosphorylation capacity; VNS, vagal nerve stimulation.

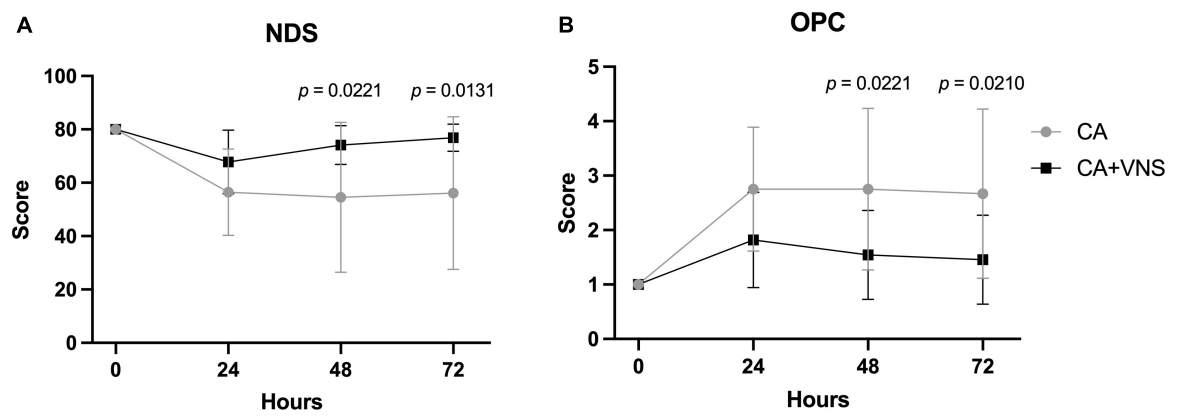

FIGURE 3 | Comparisons of neurological outcomes at 0, 24, 48, and 72 h after ROSC. **(A)** NDS in the CA (n = 12) and CA + VNS (n = 11) groups at 0, 24, 48, and 72 h after ROSC. p < 0.0001 between times, p = 0.0300 between groups, p = 0.0087 between times × groups; two-way RM ANOVA. **(B)** OPC (OPC; 1, normal; 2, slight disability; 3, severe disability; 4, comatose; 5, dead) in the CA (n = 12) and CA + VNS (n = 11) groups at 0, 24, 48, and 72 h after ROSC. p < 0.0001 between times, p = 0.0271 between groups, p = 0.0064 between times × groups; two-way RM ANOVA. Data are presented as the mean ± standard deviation. Only p-values < 0.05 are depicted in the graph and indicate the statistical comparisons of both groups at the marked time (Bonferroni's *post hoc* multiple comparisons test). NDS, neurologic deficit score; OPC, overall performance category; ROSC, return of spontaneous circulation; VNS, vagal nerve stimulation; CA, cardiac arrest.

until 30 min after ROSC (Han et al., 2008). In both global and focal brain ischemia models, the initial decline in mitochondrial respiration fully recovers during the first hour of reperfusion, but then delayed suppression of mitochondrial respiratory capacity is observed after 2–4 h of reperfusion, which supports our findings (Sims et al., 1998; Sims and Anderson, 2002). This deterioration might be attributed to the degradation or inactivation of the pyruvate dehydrogenase complex (Zaidan and Sims, 1993, 1997; Borutaite et al., 2013).

Several studies have demonstrated that VNS reduces mitochondrial dysfunction in ischemia–reperfusion injury by exerting antioxidant, anti-apoptotic, and anti-inflammatory effects (Jiang et al., 2015; Chunchai et al., 2016; Lai et al., 2019). While most studies have focused on mitochondrial dysfunction in myocardial ischemia–reperfusion injury, the present study focused on the brain, specifically the hippocampus, where most ischemia–reperfusion injury is generated during cardiac arrest with devastating sequelae in these patients (Sekhon et al., 2017). A possible mechanism of improvement in mitochondrial dysfunction in PCAS following VNS might be explained by the rapid recovery of cerebral blood flow. As demonstrated in our previous study, the no-reflow phenomenon, characterized by hypoperfusion following cerebral hyperemia, resolved with VNS treatment (Kim et al., 2019).

The current study, which implemented the VNS treatment immediately after ROSC for 3 h, highlights the technical feasibility of VNS in patients with ROSC after cardiac arrest. The approval of VNS by the United States of American Food and Drug Administration (FDA) as a treatment for patients with intractable focal seizures and its worldwide applications are some of its strengths (Johnson and Wilson, 2018; Yang and Phi, 2019; Yap et al., 2020).

This study has several limitations. First, the possible mechanisms of the restoration of mitochondrial dysfunction in the VNS group were not fully elucidated. Further detailed studies investigating the mechanisms of VNS on mitochondrial dysfunction, including the pyruvate dehydrogenase complex, are required. Second, mitochondrial dysfunction in the hippocampus was investigated, while neurological scores reflective of the cerebral cortex were evaluated. Cerebral injury in PCAS is a global injury in which the degree of injury in the various regions is similar and usually depends on ischemia time (Perkins et al., 2021; Sandroni et al., 2021). Due to the severity of our model, there was a limitation for the maze test, as motor function may unintentionally affect the outcome of spatial memory function. In addition, a previous study evaluated the mitochondrial dysfunction of similar CI activities in the hippocampus and cortex that assumes comparable injury in both sites (Chomova et al., 2012). Third, direct observation of nerve signal propagation or long-term stimulation effects, including the refractory period, were unidentifiable. However, the resulting decrease in heart rate in CA + VNS group was identified, which represents stimulation of the vagus nerve, and the VNS setting presented in this study (1-mA pulses of 10-ms duration at 1 Hz for 3 h) is usually an acceptable condition in both preclinical and clinical studies (Yap et al., 2020). Fourth, although body temperature was controlled with infrared heater to the extent possible, injection of drugs during the resuscitation period decreased the body temperature right after the ROSC period which might act as neuroprotective effect. Moreover, the brain temperature was unidentifiable due to the limitations of experimental setup. However, as both CA and CA + VNS group has been treated equally with identical protocol, we believe that the results of this study are still valid. Fifth, only male participants were included in our study. Although there are no differences in the prognosis of CA between men and women, further VNS studies in women might be needed.

CONCLUSION

VNS improved mitochondrial dysfunction and neurological outcomes at 48 and 72 h during PCAS in a rat model of asphyxial cardiac arrest. Further study investigating the mechanisms of VNS on mitochondrial dysfunction in the brain, including the hippocampus, in cardiac arrest and the consequent neurological outcome must be conducted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital.

AUTHOR CONTRIBUTIONS

JL and YJ designed the study and prepared the protocol. SHK, IP, SeK, D-HJ, and JL carried out experiments. SHK, IP, and JL drafted and revised the manuscript. All authors participated in the interpretation of data, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Hypothalamic Neurochemical Changes in Long-Term Recovered Bilateral Subdiaphragmatic Vagotomized Rats

Anna Teresa Kobrzycka[1], Adrian Mateusz Stankiewicz[2], Joanna Goscik[3], Monika Gora[4], Beata Burzynska[4], Roksana Iwanicka-Nowicka[4,5], Krystyna Pierzchala-Koziec[6] and Marek Wieczorek[1]**

[1] Department of Neurobiology, Faculty of Biology and Environmental Protection, University of Łodz, Łodz, Poland, [2] Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, Jastrzebiec, Poland, [3] Software Department, Faculty of Computer Science, Bialystok University of Technology, Bialystok, Poland, [4] Department of Genetics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland, [5] Faculty of Biology, University of Warsaw, Warsaw, Poland, [6] Department of Animal Physiology and Endocrinology, University of Agriculture in Krakow, Krakow, Poland

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R. Alberto Travagli,
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United States

***Correspondence:**
Anna Teresa Kobrzycka
anna.kobrzycka@biol.uni.lodz.pl
Marek Wieczorek
marek.wieczorek@biol.uni.lodz.pl

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Background: Vagus nerve is one of the crucial routes in communication between the immune and central nervous systems. The impaired vagal nerve function may intensify peripheral inflammatory processes. This effect subsides along with prolonged recovery after permanent nerve injury. One of the results of such compensation is a normalized plasma concentration of stress hormone corticosterone – a marker of hypothalamic-pituitary-adrenal (HPA) axis activity. In this work, we strive to explain this corticosterone normalization by studying the mechanisms responsible for compensation-related neurochemical alterations in the hypothalamus.

Materials and Methods: Using microarrays and high performance liquid chromatography (HPLC), we measured genome-wide gene expression and major amino acid neurotransmitters content in the hypothalamus of bilaterally vagotomized rats, 1 month after surgery.

Results: Our results show that, in the long term, vagotomy affects hypothalamic amino acids concentration but not mRNA expression of tested genes.

Discussion: We propose an alternative pathway of immune to CNS communication after vagotomy, leading to activation of the HPA axis, by influencing central amino acids and subsequent monoaminergic neurotransmission.

Keywords: hypothalamus, subdiaphragmatic vagotomy, amino acid neurotransmitters, inflammatory response, microarray, HPA axis

INTRODUCTION

The vagus nerve, due to its extensive peripheral projection range, is a prominent interoceptive pathway (Forsythe et al., 2014). The vagus nerve transmits intraperitoneal immune signals to the CNS (Watkins and Maier, 2005; Quan and Banks, 2007; Dantzer, 2009; D'Mello and Swain, 2016), causing neurotransmission alterations (Wieczorek et al., 2005; Dunn, 2006; Hopkins, 2007), behavioral changes (Wieczorek et al., 2005; Dantzer and Kelley, 2007; Dantzer et al., 2008) and indirect activation of the hypothalamus (Wieczorek and Dunn, 2006; Noori et al., 2017) a first element of the hypothalamic-pituitary-adrenal (HPA) axis of stress. HPA activation results in secretion of glucocorticoids (GCs), which are important regulators of the immune system (Hosoi et al., 2000). Moreover, the parasympathetic part of the vagus nerve can directly influence the activity of immune cells in internal organs exposed to pathogens through the cholinergic anti-inflammatory pathway (Tracey, 2007; Karimi et al., 2010; Pereira and Leite, 2016).

The physiological tonic activity of the vagus nerve (so-called vagal tone) is crucial for maintaining body allostasis. Vagal tone is a critical regulator of the inflammatory response. Low vagal tone results in increased basal plasmatic cortisol, tumor necrosis factor α (TNF-α), and epinephrine levels, which Bonaz et al. (2016) summarized as a pro-inflammatory effect (Pellissier et al., 2014; Bonaz et al., 2016). Vagal tone is also involved in regulating emotional and behavioral responsiveness as well as sickness behaviors – a natural response to ongoing inflammation, which supports the immune system in its fight against the pathogen (Porges et al., 1994; Porges, 1995). The experimental model of abolished vagal tone in context of peritoneal vagal functions is a full subdiaphragmatic truncal vagotomy, which we used in the current experiment.

Vagus nerve integrity is necessary for proper inhibition of inflammatory response. Thus, vagotomy results in highly intensified inflammation and decreased secretion of anti-inflammatory GCs. Surprisingly, these effects diminish after a prolonged period of recovery following vagotomy (Ghia et al., 2007; Mitsui et al., 2014).

Previously, we reported that 30 days after subdiaphragmatic vagotomy LPS-induced increase of plasma corticosterone is similar to the control group (Kobrzycka et al., 2019). At the same time, we reported that monoaminergic neurotransmission is altered in the hypothalamus after a prolonged period of recovery following vagotomy, which might contribute to the restoration of HPA axis activity. These neurotransmission changes may occur due to the presence of compensatory mechanisms for impaired immune sensory and anti-inflammatory functions of the vagus nerve. Wieczorek and Dunn (2006) suggested that function of the damaged vagal nerve is substituted by COX-dependent pathway, where immune signals are carried by blood-borne prostaglandin E2 (PGE2). Corroborating this hypothesis, we previously observed that the concentration of PGE2 under inflammatory conditions is increased in the plasma of vagotomized animals, 30 days after vagotomy procedure (Kobrzycka et al., 2019).

In this work, we present a data set investigated simultaneously with those presented previously (Kobrzycka et al., 2019). Here, we investigate whether transcriptomic changes and/or altered amino acid neurotransmission accompanies monoaminergic alterations in the hypothalamus and, in consequence, may be responsible for the preservation of HPA axis activity after recovery from vagotomy. To test those hypotheses, we performed an RNA microarray with subsequent RT-qPCR analysis and high performance liquid chromatography (HPLC) concentration analysis of excitatory and inhibitory amino acid neurotransmitters.

MATERIALS AND METHODS

The studies were performed on 3-month-old male Wistar rats (300 g ± 25 g, $N = 75$). Animals were individually housed in breeding cages under the following conditions: free access to water and feed (Purina granules), artificial lighting conditions (a 12-h day-night cycle, light on at 7.00 AM), temperature 21–22°C and 60–65% humidity. Before the start of the experiments, the animals were habituated for 7 days to the conditions in the animal facility.

Experiment

The animals were divided into two main groups: sham operated (SH, $n = 31$) and subdiaphragmatically vagotomized (VG, $n = 33$). Animals in the VG group were subjected to surgery under general anesthesia, induced by an intraperitoneal injection of Innovar plus (6 μl/g body weight) and local anesthesia with 2% lidocaine solution (0.5 ml/animal). During operation, a small fragment of gastrointestinal and hepatic branches of the vagal nerve was cut just below the diaphragm. To prevent post-operative infection and to facilitate wound healing, an antibacterial agent (Alu Spray, V.M.D.) was applied to the operation site. Bilateral subdiaphragmatic truncal vagotomy procedure and its validation are described in detail in Kobrzycka et al. (2019). Sham operation (SH) was performed in an analogous way to subdiaphragmatic vagotomy, with exception of cutting the nerves. Following surgeries, the animals were returned to their cages, and after a 30-day-long recovery period, the animals from SH and VG groups were intraperitoneally injected with either saline (0.9% NaCl, 100 μl i.p., SH $n = 15$, VG $n = 15$) or LPS (10 μg *E. coli* 026:B6 in 100 μl 0.9% NaCl, 100 μl i.p., SH $n = 16$, VG $n = 18$). After 120 min, the animals were decapitated.

Immediately after decapitation, hypothalamus (HPT) samples, restricted mainly to the medial part containing the paraventricular nucleus (PVN), were taken. Immediately after decapitation, the brain was cut on a glass plate kept on ice. After the section, samples assigned for HPLC analysis were placed in tubes on dry ice, weighed, and processed in homogenization buffer – the whole procedure took up to 5 min. The samples assigned for RNA analysis were placed in RNA-later solution right after dissection (within 2–3 min after decapitation). HPT samples weighted on average 0.05 g (±0.02 g). Stereotactic coordinates used to locate HPT were: AP = −1.44 to −2.04, $L = \pm 1.5$, $H = -8$ to -10 according to the Paxinos and Watson stereotaxic atlas (Paxinos and Watson, 1998).

Samples Preparation Procedures

For the enzyme-linked immunosorbent assay (ELISA) test of pro-inflammatory cytokines, plasma was obtained by centrifuging

the trunk blood collected on EDTA (1 ml/100 μl of Na_2 EDTA), 4,000 rpm for 10 min at 4°C. The obtained plasma was frozen and stored at −80°C until future analysis. For HPLC analysis of amino acid neurotransmitters, hypothalamuses (SH NaCl n = 6, VG NaCl n = 7, SH LPS n = 7, VG LPS n = 8) were immediately homogenized using an ultrasonic homogenizer (Fisher BioBlock Scientific, France) for 15 s in 150 μl homogenization solution (0.4 mM $Na_2S_2O_5$, 0.6 mM $HClO_4$), and centrifuged at 12,000 rpm for 15 min at 4°C. At least 100 μl of the supernatant was collected from each sample, transferred to chromatographic tubes and frozen at −80°C.

For microarray and PCR analyses, hypothalamuses (naive control CNT n = 11, SH NaCl n = 9, VG NaCl n = 8, SH LPS n = 9, VG LPS n = 10) were stored in −20°C in 200 μl of RNA-later (Thermo Fisher Scientific, United States). Total RNA was extracted from dissected hypothalamuses using a spin column-based Universal RNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. RNA samples were subjected to DNase treatment on-column (RNase-free DNase I, EURx, Poland, 15 min, room temperature) as well as in-sample (TURBO DNA-free Kit, Thermo Fisher Scientific, United States, 30 min, 37°C), to remove any residual DNA contamination. The quantity, purity, and quality of RNA samples were estimated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, United States) and a Bioanalyzer 2100 microcapillary electrophoresis device (Agilent, United States) with RNA 6000 Nano Assay Kit (Agilent, United States). Samples were of high quality (RNA Integrity Number >9) and purity (see **Supplementary Table 1**).

Enzyme-Linked Immunosorbent Assay Test for Tumor Necrosis Factor α

Tumor Necrosis Factor α (TNF-α) concentration (SH NaCl n = 11, VG NaCl n = 10, SH LPS n = 11, VG LPS n = 12) was established as a control variable for confirming ongoing intraperitoneal inflammation. Plasma TNF-α concentration was determined using the Rat TNF-α ELISA Kit, from Diaclone, France (cat. no. 865.000), according to the manufacturer's instructions. The same statistical assumptions and analytical procedure for HPLC data were used.

High Performance Liquid Chromatography of Amino Acids

The concentration of the amino acid neurotransmitters: aspartate (ASP), glutamate (GLU), glycine (GLY), gamma-aminobutyric acid (GABA), tyrosine (TYR), and tryptophan (TRP) was determined in prepared HPT samples with RP-HPLC-ED gradient method with pre-column derivatization with fresh OPT-thiol reagent (0.1 M Borax, 0.5% OPT, 0.9% mercaptoethanol). The Agilent 1100 chromatographic system with Waters, AccQ-Tag for hydrolysate Amino Acid analysis chromatographic column (3.9 × 150 mm) preceded by a ZORBAX SB-C18 pre-column (4.6 × 12.5 mm) was used. The column temperature was set at 37°C and mobile phase flow at 0.4 ml/min. The carbon working electrode was set at +0.5 V, relative to the Ag/AgCl reference electrode. The gradient elution was performed using phosphate buffer (5.5 pH) containing: at 0.05 M $NaH_2PO_4 \times H_2O$, and addition of methanol; in buffer (A) 20% and (B) 80%. At the start, the mobile phase consisted of 100% buffer A. During the first 10 min of analysis, the concentration of buffer B was raised to 10% (in the 10th min: 90% A and 10% B). In the next 30 min, the content of buffer B in the mobile phase was raised from 10 to 85% (in 40th min: 15% A and 85% B). In the next 5 min, the content of buffer B was decreased to 0% (in the 45th min: 100% A and 0% B). After each sample, the system was re-equilibrated for 10 min. The chromatographic data were analyzed using ChemStation, Revision-B.03.02, Agilent software.

High performance liquid chromatography (HPLC) and TNF-α data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Because some data did not meet the assumptions of the parametric test, we performed a Box-Cox transformation. Next, ANOVA with the Bonferroni *post hoc* test was used. Results of statistical tests and Box-Cox transformation formulas are presented **Supplementary Table 5**. The p-values lower than 0.05 were considered statistically significant. These statistical analyses were performed using the STATISTICA software, version 13.3 TIBCO Software Inc (2017).

Microarray and Real-Time qPCR

Microarrays were used to analyze hypothalamic gene expression in all four experimental groups. The results of this analysis were validated using real-time qPCR.

Microarray Preparation

A total of 21 samples (3–5 per experimental group, see **Supplementary Table 1**) were used for microarray analysis. These comprised the entire set of samples available in the experiment at that time. Samples were analyzed individually; 100 ng of the given sample that passed the initial quality control screen (2100 Bioanalyzer, Agilent) was labeled and amplified using GeneChip WT PLUS Reagent Kit and hybridized to an Affymetrix Rat Gene 2.1 ST Array Strip microarray using GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips. GeneChip Poly-A RNA Control Kit and GeneChip Hybridization Controls were used to monitor the process of sample preparation. Hybridization to the microarrays was conducted for 20 h at 48°C. Following the hybridization, the microarrays were washed, stained, and scanned on a GeneAtlas™ System for Academic Customers. All the reagents and equipment for the microarray experiment were provided by Affymetrix, a subsidiary of Thermo Fisher Scientific, United States, and the procedures were performed according to the manufacturer's protocol. The quality of the analyses was verified using Affymetrix Expression Console Software and standard Affymetrix quality metrics.

All Affymetrix.cel files were first imported into Partek® Genomics Suite® software, version 7, build 7.19.1125 (Partek, United States). Prior to any computations, the imported data set was normalized. Background correction was conducted with the use of the RMA method (Bolstad et al., 2003) and was followed by quantile normalization. Probes were logged using base 2, while the median polish (Mosteller and Tukey, 1977) algorithm was applied to probe set summarization. The definition

of all contrasts of interest (comparisons of interest) constituted a base for the detection of differentially expressed genes, which was performed with the use of the ANOVA feature of Partek® Genomics Suite®. As a result, every feature's description contained (among others) *p*-value for a particular comparison accompanied by its FDR-corrected (Mosteller and Tukey, 1977) value and 95% confidence interval for those. The data set was annotated with the Ensembl Transcripts, release 100, genome assembly Rnor_6.0.

qPCR Analysis

The results of the microarray analysis were validated using SYBR Green-based real-time qPCR. A total of 47 samples (including 21 samples used for microarray experiment, 8–11 samples per each experimental group, see **Supplementary Table 1**) were used for qPCR analysis. For each sample, 100 ng of total RNA was reverse-transcripted to cDNA using Smart RT-PCR Kit (EURx, Poland). Expression of 7 genes (*Actn2, Cxcl14, Gabrg1, Gria1, Oxt, Rab1b,* and *Vim*) was studied. Hmbs was used as a reference gene. It was identified as the most stably expressed out of a set of candidate reference genes (*Gapdh, Hmbs, Hprt1, Tbp,* and *Yhwaz*) using the NormFinder tool (see **Supplementary Table 3**). For details on the method of selecting the reference gene, see our previous work (Stankiewicz et al., 2015). For detailed primers description, see **Supplementary Table 2**. Rotor-Gene Q (Qiagen, Netherlands) with Fast SG qPCR Master Mix (2x) (EURx, Poland) were used to perform qPCR. qPCR reaction conditions were as follows: (1) denaturation −95°C for 50 s; (2) amplification, 35 cycles – 95°C for 10 s then 10 s in primer-specific annealing temperature then 72°C for 15 s; (3) melting curve analysis – 72–95°C. All assays were executed in triplicate. Each run included five 4-fold serial dilutions for calculating reaction efficiency. A melting curve analysis was performed to verify the presence of one gene-specific peak and the absence of primer-dimer peaks. The Pfaffl model was used to calculate relative expression ratios of studied genes. Detailed data on qPCR analysis are presented in **Supplementary Table 2**. qPCR was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Shapiro–Wilk test was used to test the normality of qPCR data. As the data were not distributed normally, Mann–Whitney–Wilcoxon test was used to investigate the significance of comparisons, which showed a non-adjusted *p*-value lower than 0.05 in the microarray experiment. As multiple comparisons were made within each analyzed gene, the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) was used to control False Discovery Rate. The R script used for statistical analysis of the results can be found in **Supplementary Presentation 1**.

RESULTS

TNF-α-Based Validation of Inflammatory Response

Increased plasma TNF-α (**Figure 1**) concentration confirms the development of inflammatory conditions in both rat groups injected with LPS [$F(3,34) = 28.191$, $p < 0.001$, SH NaCl vs. SH LPS $p < 0.001$, VG NaCl vs. VG LPS $p < 0.001$]. However, the inflammatory response of vagotomized animals seems to be stunted in comparison to sham control, which is in line with observations made by Ghia et al. (2007).

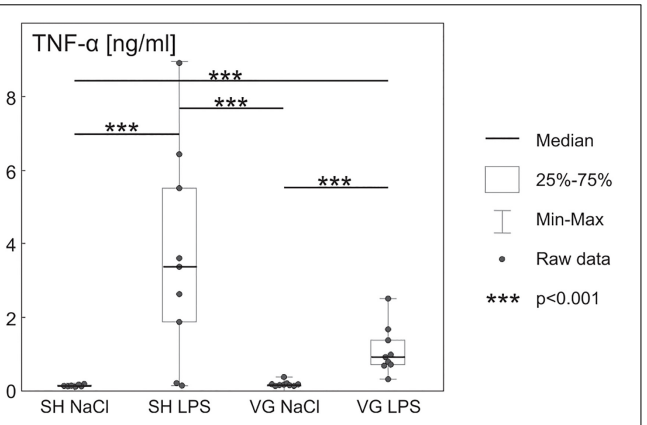

FIGURE 1 | Increased plasma tumor necrosis factor α (TNF-α) concentration after intraperitoneal injection of LPS confirm initiation of pro-inflammatory activity of the immune system. Details in text.

High Performance Liquid Chromatography Analysis

Statistical analysis revealed significant differences between groups in concentrations of all measured hypothalamic amino acids concentration (**Figure 2**) [TRP $F(3,21) = 18.292$, $p < 0.001$, TYR $F(3,21) = 10.419$, $p < 0.001$, ASP $F(3,21) = 15.473$, $p < 0.001$, GLU $F(3,21) = 8.019$, $p < 0.001$, GABA $F(3,21) = 18.020$, $p < 0.001$, GLY $F(3,21) = 20.72$, $p < 0.001$]. Intraperitoneal injection of LPS did not affect hypothalamic TRP, ASP, and GABA, concentrations in SH group (SH NaCl vs. SH LPS: TRP $p = 0.077$, ASP $p = 1$, GABA $p = 0.604$). Subdiaphragmatic vagotomy significantly increased the concentration of these amino acids despite LPS conditions (SH NaCl vs. VG NaCl: TRP $p = 0.001$, ASP $p = 0.011$, GABA $p < 0.001$; SH LPS vs. VG LPS: TRP $p < 0.001$, ASP $p < 0.001$, GABA $p = 0.041$). Worth noting, GABA concentration in VG group is increased after LPS administration (VG NaCl vs. VG LPS $p = 0.006$). On the other hand, intraperitoneal injection of LPS significantly increased hypothalamic TYR, GLY, and GLU concentrations in SH group (SH NaCl vs. SH LPS: TYR $p = 0.024$, GLU $p = 0.025$, GLY $p < 0.001$). Subdiaphragmatic vagotomy significantly increased TYR, GLY, and GLU concentrations in non-septic conditions (SH NaCl vs. VG NaCl: TYR $p = 0.003$, GLU $p = 0.024$, GLY $p < 0.001$). Also, after LPS administration, TYR, GLY, and GLU concentrations are elevated in similar manner as VG NaCl group – significant increased compare to SH NaCl group conditions (SH NaCl vs. VG LPS: TYR $p < 0.001$, GLU $p < 0.001$, GLY $p < 0.001$) and no differences between vagotomized groups (VG NaCl vs. VG LPS: TYR $p = 1$, GLU $p = 0.886$, GLY $p = 1$). Therefore, no significant differences between SH LPS and VG LPS are observed (SH LPS vs. VG LPS: TYR $p = 0.262$, GLU $p = 0.840$, GLY $p = 0.619$).

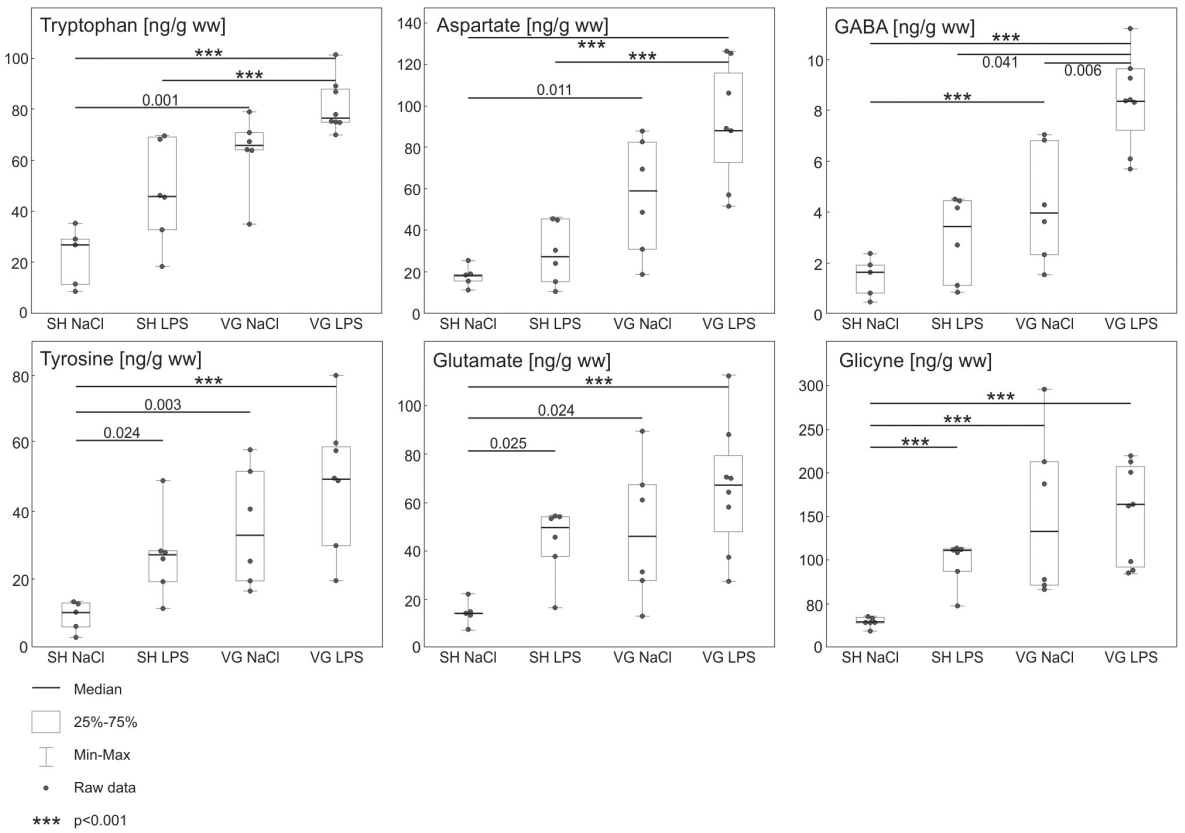

FIGURE 2 | Results of high performance liquid chromatography (HPLC) analysis of amino acids in hypothalamus. Concentrations of monoaminergic neurotransmitter precursors (Tyrosine, Tryptophan) and inhibitory amino acid neurotransmitters (GABA, Glycine) are increased in vagotomized group (VG NaCl vs. SH NaCl). Similar effect is absent in case of excitatory amino acid neurotransmitters (Glutamate, Aspartate). During intraperitoneal inflammation changes in tyrosine and tryptophan concentrations in vagotomized group (VG LPS vs. SH LPS) are even more pronounced. Additionally, we observe a significant increase of inhibitory GABA and excitatory aspartate concentration in that group (VG LPS vs. SH LPS). Details in text.

Microarray Experiment

Using microarrays, we have analyzed transcriptomic patterns induced by experimental conditions in the hypothalamus. None of the observed statistically significant changes in expression of analyzed transcripts survived adjustment for multiple comparisons. Nevertheless, we have selected 7 genes characterized by relatively low non-adjusted *p*-values in some of the comparisons (mean = 0.019, SD = 0.01, also see **Supplementary Table 4**), and analyzed their expression using qPCR (**Figure 3**). The qPCR analysis showed that the studied genes (*Actn2, Cxcl14, Gabrg1, Gria1, Oxt, Rab1b,* and *Vim*) do not respond to experimental conditions, thus supporting data from microarrays (see **Supplementary Table 4**).

DISCUSSION

Vagotomy Affects Precursors of Monoamine Neurotransmitters in the Hypothalamus

Vagal denervation attenuates inflammation-induced increase of plasma corticosterone and ACTH concentrations for up to 2 weeks (Fleshner et al., 1995; Wieczorek and Dunn, 2006). Afterward, this effect of vagotomy is diminished. This indicates that the proper functioning of the anti-inflammatory HPA axis is restored in the long run.

However, even after 1 month following a vagotomy, a monoaminergic neurotransmission in the hypothalamus (a first element of the anti-inflammatory HPA axis), as well as in other limbic system structures, is deregulated (Kobrzycka et al., 2019). We confirmed these results in this work by observing an increase in a number of precursors of the monoaminergic neurotransmitters (tyrosine and tryptophan) in the hypothalamus of the vagotomized animals. In fact, a similar tendency to increase tryptophan concentration was also noticed by Wieczorek and Dunn (2006) in many brain areas of vagotomized mice. CNS may require higher amounts of the precursors because they are necessary for reported increased dopamine and serotonin synthesis and turnover in many brain regions after the subdiaphragmatic vagotomy (Wieczorek and Dunn, 2006; Kobrzycka et al., 2019). It is also worth noting that one of the side effects of subdiaphragmatic vagotomy procedure is a slowed movement of food through the digestive system (Martin et al., 1977), which also may affect the content of tyrosine

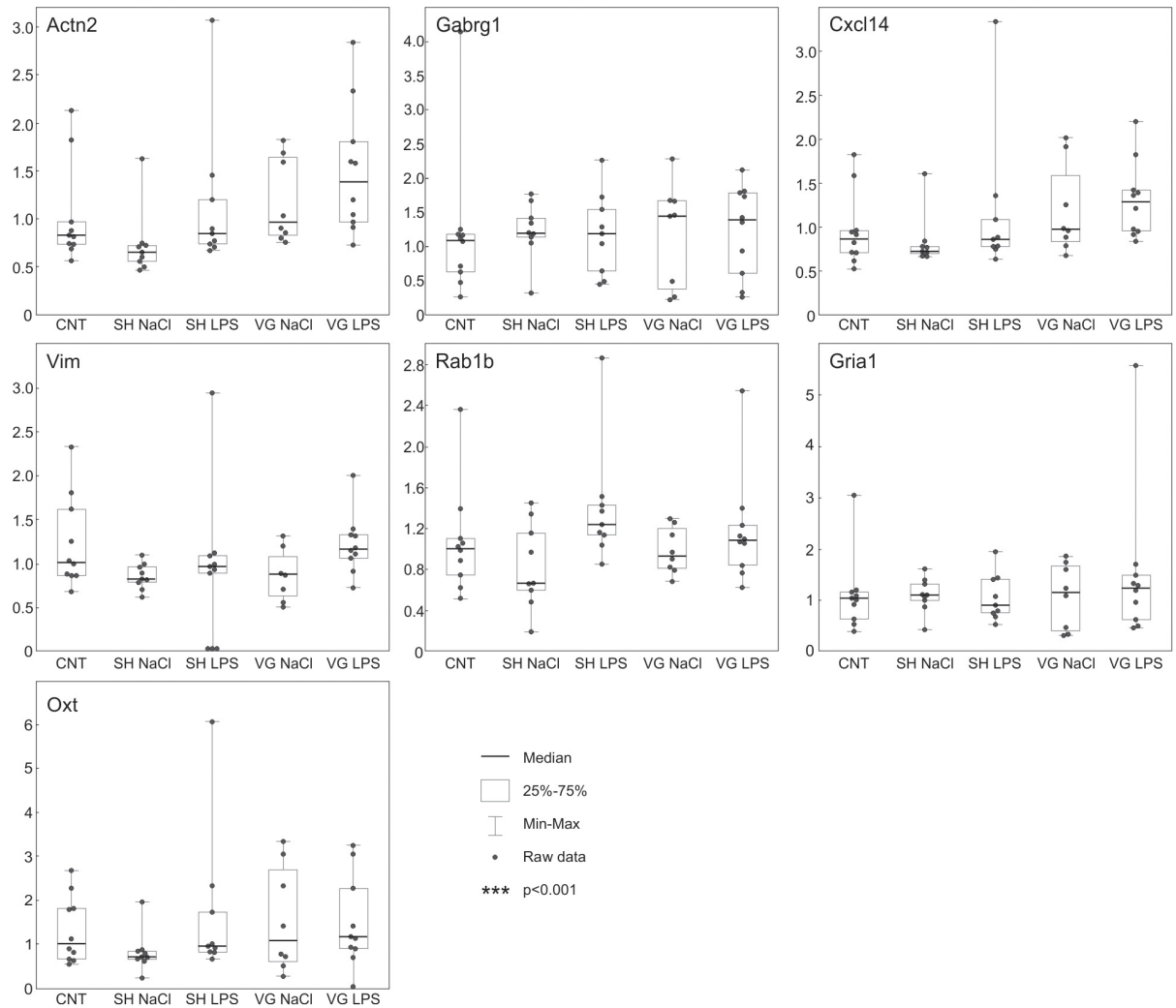

FIGURE 3 | QPCR analysis confirms that experimental procedures did not affect the hypothalamic gene expression pattern. The whiskers extend from the hinge to the smallest/largest value no further than 1.5 × IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). Data beyond the end of the whiskers are called "outlying" points and are plotted individually.

and tryptophan in blood (Fernstrom and Fernstrom, 2007; Kapalka, 2010; Al Mushref and Srinivasan, 2013; Fu et al., 2014).

Vagotomy Affects Amino Acid Neurotransmitters in the Hypothalamus

Hypothalamus is innervated by monoaminergic fibers originating from a number of brain areas directly or indirectly affected by nucleus tractus solitarius (NTS), which distributes the sensory information from the vagus nerve (Rinaman, 2007; Myers et al., 2012; Elson and Simerly, 2015; Miller and Yeh, 2017; Weidenfeld and Ovadia, 2017). Those innervations modulate hypothalamic functions, i.e., by affecting hypothalamic concentrations of amino acid neurotransmitters (e.g., GABA and Glycine) and, subsequently, post-synaptic potentials (Miller and Yeh, 2017). Previously, we observed vagotomy caused changes in monoaminergic neurotransmission in many of those areas (e.g., AM, PAG, and HIP) and the hypothalamus itself. In this work, we show a modest increase in the hypothalamic concentration of amino acid neurotransmitters in full-recovered animals subjected previously to vagotomy as compared to sham procedure. Similar results were reported by Klarer et al. (2014), who studied amino acid neurotransmitters in several other limbic brain structures. Based on these results, we hypothesize that those vagotomy-induced changes in hypothalamic amino acid neurotransmitters may be caused by deregulated monoaminergic signaling originating in limbic structures.

Glutamate, aspartate, and glycine innervation of the hypothalamus originate from the brainstem and internal hypothalamic circuit (Ziegler et al., 2005; Noori et al., 2017; Varga et al., 2019). Glutamate, aspartate, and glycine inhibit CRH release from the hypothalamus *in vitro* (Buckingham and Hodges, 1977; Patchev et al., 1994). In our experiment, we observed increased levels of all of these neurotransmitters in the

vagotomized group regardless of LPS-induced inflammation. Such observation suggests suppression of CRH release and in consequence HPA axis activity. However, such a hypothesis would be in opposition to the previously mentioned restoration of LPS-induced corticosterone increase in long-term recovered vagotomized rats. This apparent incompatibility might be explained by intensified GABA concentration in the hypothalamus.

In basal conditions, the tonic, inter-hypothalamic GABA circuit suppresses the activity of hypothalamic CRH-releasing neurons (Kovács et al., 2004). Our results show that this mechanism is intensified in vagotomized animals compared to sham-operated animals. We propose that this intensification of basal hypothalamic GABA release may underlie reported down-regulation of corticosterone release in animals that underwent vagotomy procedure a few days before measurement (Fleshner et al., 1995; Wieczorek and Dunn, 2006). As we report here, 30 days after vagotomy procedure, hypothalamic GABA concentration is even higher in inflammatory conditions. We think that this may be a result of enhanced external inhibitory signals, especially amygdalic GABA-GABA disinhibitory connections (Herman et al., 2004), which would inhibit the activity of the inter-hypothalamic GABA circuits. Thus, the internal hypothalamic inhibition of CRH release would be suppressed, and in consequence, hypothalamic hormonal activity and further adrenal corticosterone release in response to inflammation would be the same as in sham animals (Kobrzycka et al., 2019). This is indeed what we observe in our studies. However, this hypothesis requires further, more detailed investigation.

Another amino acid crucial for hypothalamic functioning is glycine. Glycine inhibits orexin- mediated arousal, energy homeostasis, and reward-seeking. It also promotes sleep and decreases body temperature during sleep episodes *via* peripheral vasodilation (Kawai et al., 2015). In conscious rats, glycine acts in PVN to enhance the renal excretion of water and sodium and decrease central sympathetic outflow to the heart and kidneys (Krowicki and Kapusta, 2011). Glycine can act allosterically as an excitatory modulator of the NMDA subtype of ionotropic glutamate receptors and through the activation of NMDA receptors in suprachiasmatic nucleus (SCN). Glycine is also involved in oxytocin and vasopressin synthesis in magnocellular neurosecretory cells (MNCs) in the PVN and SON (supraoptic nucleus) and a significant tonic inhibitory effect is mediated through ionotropic GlyR (Choe et al., 2016).

In the context of HPA axis activity, glycine can inhibit hypothalamic vasopressin synthesis (Hussy et al., 2001; Choe et al., 2016) which synergistically to CRH stimulates ACTH secretion (Mazzocchi et al., 1997; Papadimitriou and Priftis, 2009). Glycine also decreased the spontaneous release of CRH from the hypothalamus but does not affect hypothalamic CRH content (Buckingham and Hodges, 1977). This observation comes, however, from a study carried out on isolated hypothalamic tissue with restrictive controlled neurotransmission alterations – that does not consider simultaneously changes of other neurotransmitters content. *In vivo* studies showed that intracerebroventricular (Rundgren et al., 1993) or intravenous (Hahn et al., 1991) glycine infusion may increase vasopressin plasma levels. This difference originating from the different routes of glycine administration might be an important factor in the interpretation of our results.

The raphe magnus and the ventrolateral periaqueductal gray were found to be the exclusive sources of the inhibitory glycinergic innervation in the PVN (Varga et al., 2019). Although sufficient amounts of glycine are synthesized *de novo*, exogenous glycine passively diffuses across the blood-brain barrier and modulates neurotransmission in the CNS (Kawai et al., 2015). Observed after vagotomy increase in hypothalamic glycine concentration might result from altered neurotransmission of other brain structures, but also, as mentioned before, from altered after vagotomy peristalsis.

We do not know how the immune signals reach the CNS in vagotomy conditions in the early stages of inflammation. We think that this may be an effect of an alternative mechanism replacing disrupted vagus-mediated signaling pathway. Wieczorek and Dunn (2006) suggested that this mechanism might be associated with changes in prostaglandin signaling. In fact, Dalli et al. (2017) showed that even unilateral subdiaphragmatic truncal vagotomy significantly alters macrophage lipid profile, which is involved in the metabolism of arachidonic acid during intraperitoneal inflammation. Additionally, they reported that following left trunk vagotomy, the expression of COX2, an enzyme involved in PGE2 synthesis, significantly increased in macrophages (Dalli et al., 2017). We consider that elevation of inflammation-induced PGE2 synthesis may take over immune-to-CNS communication after vagotomy. We think that elevation of inflammation-induced PGE2 synthesis may take over immune-to-CNS communication after vagotomy, as we suggested previously (Kobrzycka et al., 2019). In theory, blood-borne PGE2 reaches the brain *via* the bloodstream, where it acts upon epithelial cells of blood vessels. These vessels preserve intact prostaglandin-related cellular machinery after vagotomy (Sergeev and Akmaev, 2000). Once at the brain, PGE2 activates CVOs, PVN, and NTS (Sekiyama et al., 1995; Ek et al., 1998; Rocca and FitzGerald, 2002; Cai et al., 2014; Le Maître et al., 2015) and subsequently, other brain areas in a way similar to the now non-functional vagus nerve (Sekiyama et al., 1995; Marty et al., 2008).

Some authors proposed an alternative to the vagal, neural route of immune to CNS communication. Splanchnic sympathetic nerves were shown to control inflammation processes, induced by intravenous administration of LPS (Martelli et al., 2014, 2019). It was proposed that splanchnic sympathetic nerves constitute an efferent part of the inflammatory reflex along with vagal afferents or/and bloodstream as sensory routes of immune to CNS communication (Komegae et al., 2018; McAllen et al., 2022). We cannot exclude that after abdominal vagal denervation, part of immune sensory functions is taken over by other sensory fibers. However, we could not find information about such a process occurring during intraperitoneal inflammation. Until such a process is observed, the vagus nerve is still considered the main immune sensory neural pathway from the abdominal cavity to CNS (Pavlov and Tracey, 2017).

Microarray and subsequent Real-Time qPCR analysis suggest that the transcriptome does not affect the activity of the hypothalamus during inflammation in vagotomized rats after a 30-day-long recovery period. It would be interesting to see whether gene expression changes are more pronounced at earlier stages of recovery while later giving way to other compensatory mechanisms such as neurotransmission alterations. On the other hand, we found that amino acid neurotransmitters are deregulated in the hypothalamus of vagotomized animals after prolonged recovery. This, along with our previous finding on deregulated monoamine systems in brain structures affecting the functioning of the hypothalamus, suggests that vagal role in the regulation of HPA axis after a longer recovery period is replaced by changes in external neurotransmitter modulation of hypothalamic activity rather than alteration of basic hypothalamic functionality. Of course, the presented data do not fully describe the observed phenomena of restored HPA axis activity in recovered vagotomized animals. Previously, we presented changes in CNS monoaminergic neurotransmission. Here, we report changes in amino acid components in the hypothalamus. In neuronal tissue, amino acid neurotransmitters play a critical role in electrophysiological processes not investigated in this study. We also pay attention that observed changes might be evolving in time along with prolonged recovery after vagotomy. For future studies, we would like to extend our research to a few time points that allow us to establish the time dynamic of presented processes. For now, we simply report that hypothalamic amino acid neurotransmission is altered 30 days after vagotomy and it is an interesting issue for discussion and further investigation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199231.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethical Committee for Animal Experiments in Łodz, 73/ŁB582/2012.

AUTHOR CONTRIBUTIONS

AK wrote the manuscript, isolated material for the microarray and qPCR analyses, and performed the qPCR and HPLC analysis as well as statistical analysis of the HPLC and ELISA results. AS wrote the manuscript, and oversaw and participated in isolation of the material for the microarray and qPCR analyses and coordinated them. MW designed the experiment, performed the experimental procedures on living animals, and collected the samples. JG performed the bioinformatic and statistical analyses of microarray data. MG, BB, and RI-N performed the microarray analysis. KP-K performed the ELISA tests. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.869526/full#supplementary-material

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