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Evidence for a modulating effect of transcutaneous auricular vagus nerve stimulation (taVNS) on salivary alpha-amylase as indirect noradrenergic marker: A pooled mega-analysis

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ABSTRACT

Background: Non-invasive transcutaneous auricular vagus nerve stimulation (taVNS) has received tremendous attention as a potential neuromodulator of cognitive and affective functions, which likely exerts its effects via activation of the locus coeruleus-noradrenaline (LC-NA) system. Reliable effects of taVNS on markers of LC-NA system activity, however, have not been demonstrated yet.
Methods: The aim of the present study was to overcome previous limitations by pooling raw data from a large sample of ten taVNS studies (371 healthy participants) that collected salivary alpha-amylase (sAA) as a potential marker of central NA release.
Results: While a meta-analytic approach using summary statistics did not yield any significant effects, linear mixed model analyses showed that afferent stimulation of the vagus nerve via taVNS increased sAA levels compared to sham stimulation ($b = 0.16$, $SE = 0.05$, $p = 0.001$). When considering potential confounders of sAA, we further replicated previous findings on the diurnal trajectory of sAA activity.
Conclusion(s): Vagal activation via taVNS increases sAA release compared to sham stimulation, which likely substantiates the assumption that taVNS triggers NA release. Moreover, our results highlight the benefits of data pooling and data sharing in order to allow stronger conclusions in research.

1. Introduction

Transcutaneous auricular vagus nerve stimulation (taVNS) has drawn tremendous attention as a promising non-invasive brain stimulation tool for the treatment of clinical disorders [1], such as

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pharmacoresistant epilepsy [2–4], depression [5] and chronic pain [6]. Given its non-invasive nature, taVNS has also more recently been used in non-clinical settings to modulate various affective and cognitive processes, such as emotion recognition, fear extinction, cognitive control, and attention (cf. [7,8]). The effects of taVNS have been suggested to be related to the modulation of distinct brainstem, subcortical and cortical regions, and their associated neurotransmitter systems (cf. [9]). Indeed, previous animal studies have shown that vagal afferents modulate serotonergic [10,11], dopaminergic [12,13], cholinergic [14] and noradrenergic [15,16] signaling. The exact neural mechanisms possibly mediating the effects of taVNS are, however, not fully understood yet.

One of the hypothesized working mechanisms by means of which taVNS may exert some of its effects is through the activation of the locus coeruleus-noradrenaline (LC-NA) system. Afferent fibers of the vagus nerve forward information of the adrenergic release from the adrenal gland to the brain [17], where they project to the nucleus tractus solitarii (NTS) [18,19]. The NTS sends excitatory projections to the nucleus paragigantocellularis (PGi; [20]), which, in turn, is linked to the noradrenergic brainstem nucleus LC [21,22]. The LC-NA system projects to several brain regions through an extended neuronal network including frontal and mediotemporal regions [23] and modulates behavior by tonic and phasic firing [24], thus exerting influence on perception, attention, motivation and memory processes [25]. Impairments in the LC-NA system have further been associated with cognitive decline in aging and some degenerative disorders, such as Alzheimer's disease [26,27].

Evidence for such a modulatory vagal influence on the LC-NA system activity comes from different lines of research. Animal studies showed increased LC-firing rates after invasive vagal nerve stimulation [10,15,16,28,29] and reduced firing after vagotomy [30]. Various processes mediated by the LC-NA system have further been shown to be improved by invasive vagal stimulation in animals, including extinction learning [31,32], memory retention [33] and inhibitory avoidance learning [34], as well as in humans (see for verbal recognition memory [35,36]; but see [37]).

Further evidence comes from studies relating vagal activity to pupil dilation [38–41] and to the attention-related P300 amplitude of the event-related potentials (ERPs) [42,43], both representing physiological markers of LC-NA system activity (see for pupil dilation [44–47]; see for P300 [48,49]; see for review [50]). For instance, invasive vagal stimulation was found to increase resting pupil diameter in epileptic patients [39], an effect also found in animal data [38,40] (see for review [51]). With regard to the P300 amplitude, De Taeye and colleagues [43] observed that epileptic patients who responded favorably to invasive vagal stimulation showed an increase in P300 amplitude during stimulation. This effect was also found in depressive patients in an earlier study by Neuhaus and colleagues [42].

In light of the substantial evidence towards a modulatory role of invasive vagal stimulation on LC-NA system activity (mostly in animals and human clinical contexts), recent studies have investigated whether non-invasive taVNS shows a similar impact on the LC-NA activity in healthy humans. Initial brain imaging studies confirmed enhanced functional LC activation during taVNS compared to active sham stimulation in healthy participants [52–57]. Other studies, but not all, showed a modulatory effect of taVNS on various cognitive and affective processes potentially associated with noradrenergic signaling, with respect to fear extinction (see for positive effects [58–60]; but see for no effects [61,62]), memory (see for positive effects [63–65]; but see for no effects [37,66]), cognitive control (see for positive effects [67–71]; but see for no effects [72]) and attention (see for positive effects [73,74]; but see for no effects [75]).

Despite the promising indications for taVNS-related behavioral improvements, there is current uncertainty regarding the relation between NA markers and taVNS-mediated vagal activation due to a number of non-replicable or merely subtle findings (cf. [7,51]). The modulatory effects of taVNS on pupil dilation [76–78] have not consistently been replicated [71,75,79–81] and studies on the effects of taVNS on the P300 amplitude have also yielded mixed results. Whereas some studies found an increase of the P300 during taVNS compared to sham stimulation [73,82,83], others found an increase only in response to specific stimuli [84], or found no differences between stimulation conditions [70,79,85]. Other attempts of finding reliable physiological markers include for instance vagally-mediated heart rate variability, which, however, did not show to be affected by taVNS (see for review [86]).

In recent years, salivary alpha-amylase (sAA) has emerged as promising indirect marker of LC-NA system activity based on pharmacological studies showing an involvement of noradrenergic activity in sAA secretion [87–89] (see for review [90]). Although some studies exploring taVNS effects on sAA level changes demonstrated increased sAA levels after taVNS compared to sham stimulation [79,84], supporting sAA as a potential marker of central NA-enhancement modulated by taVNS, others reported no such enhancement [64,65,80,81,91,92]. Ultimately, possible reasons for this lack of replicability regarding physiological markers of LC-NA system activity might be small sample sizes, the heterogeneity of stimulation procedures (e.g., stimulation parameters, stimulation duration [7,8]) or methodological differences in data collection and/or preprocessing across studies (e.g., in saliva collection for sAA level changes [93]).

An opportunity to overcome these limitations and accelerate progress in validating potential relations between reliable NA markers and taVNS-mediated vagal activation is data pooling. By increasing overall sample size, the pooling of several independent studies improves statistical power and the overall generalizability of results (e.g., by distinguishing generalizable findings from false positives that emerge from smaller-samples studies; [94]). It further allows for consideration of within- and between-study variance to possibly explain some of the heterogeneity in the data (i.e., based on differences in study characteristics). Data pooling also enhances the ability to construct predictive models that are more widely applicable and better powered to identify relevant predictive factors [95].

Therefore, the aim of the present study was to overcome the existing limitations by pooling raw data from a large sample of studies that collected sAA levels in the context of taVNS research. Our focus on sAA was primarily due to its widespread use across taVNS laboratories, its inexpensive and non-invasive measurement and ultimately, its potential to become a clinically meaningful and reliable marker that might shed further light on the efficacy of taVNS. In order to explore whether taVNS enhances sAA levels as putative marker of NA activity in the pooled data, and to investigate if, and to what extent, different factors (e.g., stimulation parameters, stimulation duration) may modulate the assumed relation between taVNS and sAA level changes, we conducted linear mixed model analyses based on a hypothesis-driven approach as well as on an exploratory approach. Mixed models allow the specification of fixed and (crossed) random factors (e.g., participants and studies), they further allow the incorporation of continuous variables (i.e., yielding for instance fixed effects of linear and quadratic trends) and their interactions with categorical factors [96]. Mixed models are also optimal to deal with missing data. Thus, conducting mixed model analyses with a sample of pooled sAA data may provide valuable information on the relation between taVNS-mediated afferent vagal activation and sAA as an inexpensive and non-invasive index of central noradrenergic activity.

2. Material and methods

2.1. Sample

Authors of previous and ongoing taVNS studies collecting sAA data were contacted and invited to participate in the project. We received data from twelve studies and included ten studies that applied taVNS as stimulation method [79,84] (Exp. 1b) [79] (Exp. 2) [64,65,80,91], including three unpublished studies [92,97,98] (see Table 1 for details about study characteristics). Two studies that applied auricular acupuncture were excluded from analyses [99,100].

From all included studies, sAA levels were available for a total of 371 healthy participants. All participants provided informed written consent for the experimental protocol, which was approved in accordance with the declaration of Helsinki. Participant characteristics are shown in Table 1. Information on participant pre-selection and data collection for published studies are available in more detail in each individual publication. All data have been made publicly available on the Open Science Framework and can be accessed at https://osf.io/rdpcs.

2.2. Transcutaneous auricular vagus nerve stimulation

In all included studies, taVNS stimulation was conducted using two titan electrodes attached to a mount and wired either to a stimulation unit (NEMOS®, VITOS®; see Table 1 for details) or to a bipolar constant current stimulator (DS5 DIGITIMER; see Table 1 for details). In the active vagus stimulation condition, the stimulator electrodes were placed in the left cymba conchae, an area exclusively innervated by the auricular branch of the vagus nerve [101,102]. For the sham stimulation condition, the electrodes were positioned in the center of the left ear lobe, an area known to be free of vagal innervation [101,102]. All studies applied stimulation on a single day. In studies 1, 2, and 4, stimulation was administered continuously, whereas in studies 3 and 5–10, stimulation alternated between on and off phases every 30 s. Stimulation intensity was either adjusted individually for each participant above the detection threshold and below the pain threshold [101] (studies 1–6 and 10) or was fixed at 0.5 mA for all participants (studies 7–9). Across all ten studies, stimulation intensities varied from 0.1 mA to 5 mA for the sham (earlobe) condition (M_{sham} = 1.20, SD_{sham} = 0.82) and from 0.25 mA to 4 mA for the vagus (cymba conchae) condition (M_{vagus} = 1.03, SD_{vagus} = 0.66). All stimulation characteristics are shown in Table 1.

2.3. Salivary alpha-amylase

Alpha-amylase is a salivary enzyme involved in the digestion of starch in the oral cavity [103]. It can be measured through saliva collection in an inexpensive and non-invasive fashion and, as such, has emerged as a proxy measure of sympathetic arousal, likely reflecting stress-related changes in the body [90,104–107]. It is important to note that sAA levels measured during stress might be influenced by activity of the sympathetic or parasympathetic nervous system or some combination of both [93,108]. In recent years, however, sAA has been accepted as promising marker of sympathetic nervous system activity based on pharmacological studies showing an involvement of noradrenergic activity in sAA secretion [87–89]. For instance, Ehlert and colleagues [87] reported that administration of yohimbine (i.e., an alpha-adrenergic receptor antagonist) activated sAA via adrenergic mechanisms, thus pointing to sAA as marker of the central sympathetic system. More recently, Warren and colleagues [89] administered atomoxetine, a highly selective NA transporter blocker that increases central NA levels, and validated the initial findings by Ehlert and colleagues [87] (see also [88]; see for review [90]).

To assess the effects of taVNS on sAA level changes in our pooled data, in all included studies, sAA levels (U/ml) were collected before (i.e., prior to the application of the taVNS device) and after (i.e., after finalizing the psychological task(s) and removing the taVNS device) stimulation. Four studies also collected sAA levels during stimulation (studies 4, 7–9). Saliva samples were either collected using cotton swabs (i.e., 66.31% of participants were instructed to gently chew the cotton swab in their mouth and then place it into a sample tube) or by spitting (i.e., 33.69% of participants were

Table 1
Overview study characteristics and stimulation parameters.

Study	Reference	N	Task	Design	Stimulation device	Stimulation length	Duty cycle	Stimulation intensity method	sAA collection method
1	Ventura-Bort et al. (2018)	N = 20, 17f, M_{age} = 20.4	visual oddball	within-subject	NEMOS®, tVNS Technologies GmbH	35min	continuous	determined individually	swab collection
2	Ventura-Bort et al. (2021)	N = 37, 20f, M_{age} = 23	passive viewing	within-subject	NEMOS®, tVNS Technologies GmbH	7min	continuous	determined individually	swab collection
3	Ventura-Bort et al. (in prog.)	N = 31, 27f, M_{age} = 21.3	passive viewing	within-subject	NEMOS®, tVNS Technologies GmbH	14min	30s on/30s off	determined individually	swab collection
4	Giraudier et al. (in prep.)	N = 62, 50f, M_{age} = 23.8	visual oddball, serial reaction time	within-subject	NEMOS®, tVNS Technologies GmbH	80min	continuous, 30s on/30s off	determined individually	spitting method
5	Giraudier et al. (2020)	N = 61, 47f, M_{age} = 23.4	lexical decision	between-subject	NEMOS®, tVNS Technologies GmbH	23min	30s on/30s off	determined individually	swab collection
6	D'Agostini et al. (2021)	N = 71, 55f, M_{age} = 23.3	reversal learning	between-subject	NEMOS®, tVNS Technologies GmbH, DS5 DIGITIMER, Welwyn Garden City, UK	40min	30s on/30s off	determined individually	swab collection
7	Koenig et al. (2021)	N = 30, 24f, 14–17 years	morphing faces, emotion recognition, emotional go/nogo	within-subject	VITOS®, tVNS Technologies GmbH	28min	30s on/30s off	fixed at 0.5 mA	swab collection
8	Warren et al. (2019)	N = 20, M_{age} = 23.6	visual and auditory oddball, task switching	within-subject	NEMOS®, tVNS Technologies GmbH	80min	30s on/30s off	fixed at 0.5 mA	spitting method
9	Warren et al. (2019)	N = 17, 0f, M_{age} = 22.1	task switching	within-subject	NEMOS®, tVNS Technologies GmbH	80min	30s on/30s off	fixed at 0.5 mA	spitting method
10	Sommer et al. (in prep.)	N = 27, 16f, M_{age} = 25.6	number categorization based dual task	within-subject	NEMOS®, tVNS Technologies GmbH	61min	30s on/30s off	determined individually	spitting method

instructed to spit out saliva either through a plastic straw or directly without straw into a sample tube). Of note, sAA levels are sensitive to sampling techniques because different salivary glands contribute to different rates of saliva secretion, which influences the quantity of sAA secreted into oral fluids [93,108]. The swab collection method requires chewing (i.e., stimulated saliva secretion), which affects sAA levels independently of central noradrenergic involvement [93]. Therefore, the spitting method is generally favored when collecting saliva samples. For details about sample storage and analysis see each individual publication.

2.4. Statistics

All statistical analyses were carried out in the R environment [109]. Pre- and post-processing of data was conducted using *tidyverse* [110].

2.4.1. Mixed model analysis

To test the effects of taVNS on sAA level changes, we conducted a series of linear mixed models (LMMs) using *lme4* [111]. A Box-Cox distributional analysis [112] indicated that a logarithmic transformation brought the typically skewed sAA data [90] in line with the assumption of normal distribution.

As fixed effects, we specified sequential-difference contrasts (i.e., a priori defined comparisons between specific conditions and/or groups; cf. [113]) for *time* (post vs. pre, post vs. mid), for *stimulation* (vagus vs. sham) and for the interaction between *time* and *stimulation* respectively. We also included the effect of *stimulation length*, the effect of *duty cycle* (continuous vs. 30s on/30s off), the effect of *stimulation intensity method* (fixed at 0.5 mA vs. determined individually), the effect of *sAA collection method* (swab collection vs. spitting method), the effect of *stimulation intensity* (group mean-centered) and their associated interactions (included interactions vary between models). The model predictors *gender* (male vs. female) and *time of day* (i.e., timeslots I-VI based on the time of the sAA measurement) were only included as fixed effects in a separate analysis due to a large amount of missing data (lost or not provided) for those predictors, reducing the total amount of observations drastically when including them (N = 1092).

As random factors, we included *participant* (N = 371) and *study* (N = 10) with a total amount of 1556 observations. The selected random-effect structure included theoretically relevant variance components and correlation parameters and was supported by the data (cf. [114]). We included random intercepts for *participant* and *study* and allowed the effect of *time* (post vs. pre) and the effect of *stimulation* (vagus vs. sham) to vary across subjects (random slope), constraining random intercept and random slope to be independent. We further allowed the effect of *time* (post vs. pre) to vary between studies, constraining uncorrelated random intercept and random slope within studies. The random slope *time* (post vs. mid) did not significantly improve model fit and was excluded from all models. The random-effect structure was identical for all models.

Parsimonious model selection followed the general recommendations by Bates et al. [114] and was performed without knowledge or consideration of fixed-effect estimates. In a maximal to minimal-that-converges modeling process, fitted models were processed with random-effects principal component analysis to obtain loadings of the variance-covariance matrix of the random effects (i.e., an iterative reduction of random-effects structure complexity was performed).

For assessment of relative differences in goodness of fit, we used the log-likelihood and, for model comparisons, the χ^2-distributed likelihood ratio and its associated p-value. P-values for fixed effects were calculated using Satterthwaite's approximations [115]. Final models were estimated with restricted maximum likelihood. Pairwise post hoc comparisons were computed using *lsmeans* [116] with Tukey-adjusted p-values. The report of results followed the recommendations by Meteyard & Davies [117].

2.4.2. Meta-analysis

In addition to LMMs, we performed a meta-analysis of the current studies. We therefore calculated Hedges'g [118] as effect sizes based on standardized mean differences (SMDs) using *metafor* [119] and *meta* [120]. Effect sizes were calculated for the sAA increase under taVNS (Δpost-pre) compared to sham (Δpost-pre) on the log-sAA data. Cohen's d and Cohen's dz [121] have been uploaded as additional effect size estimates on the Open Science Framework and can be accessed at https://osf.io/rdpcs. A statistical power-analysis for the meta-analysis followed the recommendations by Valentine and colleagues [122].

2.4.3. Test-retest reliability

Test-retest reliability of sAA levels (pre vagus vs. pre sham) was tested using an intra-class correlation (ICC) coefficient using *psych* [123] and included all data from studies employing a within-subject design ($N_{participants} = 233$, $N_{studies} = 8$).

2.4.4. Bayesian evidence synthesis

A Bayesian approach (protocol by Scheibehenne et al. [124]) was also performed. Results of this analysis, however, did not reveal additional information and were therefore not included in this paper (results can be found on the Open Science Framework (https://osf.io/rdpcs) where the project was pre-registered on March 2, 2021).

3. Results

3.1. Mixed models

3.1.1. Model selection

Overall, we explored a variety of modeling approaches in order to identify the most appropriate and best-performing predictive models and consequently, specified three models of increasing complexity that were supported by the data. See Supplement A for details about the model selection approaches.

3.1.2. The core model

As fixed effects in M1, we included the sequential-difference contrasts for *time*, for *stimulation* and their associated interaction. The model output from M1 showed no main effect of *time* on sAA, $b = 0.08$, $SE = 0.05$, $p = 0.150$, and no main effect of *stimulation*, $b = 0.06$, $SE = 0.03$, $p = 0.067$. Interestingly, the interaction between *time* and *stimulation* was significant, $b = 0.12$, $SE = 0.04$, $p = 0.005$, showing increased sAA levels for vagus, $b = 0.16$, $SE = 0.05$, $p = 0.048$, as opposed to sham stimulation, $b = 0.03$, $SE = 0.05$, $p = 0.966$ ($M_{vagus_{pre}} = 4.47$ U/ml, $M_{vagus_{post}} = 4.63$ U/ml, $M_{sham_{pre}} = 4.50$ U/ml, $M_{sham_{post}} = 4.52$ U/ml). The model output from M1 is displayed in Table 2.

3.1.3. The full model

As fixed effects in M2, we specified a priori defined comparisons for *time*, for *stimulation* and for the interaction between *time* and *stimulation*. We also included the effect of *stimulation length*, the effect of *duty cycle*, the effect of *stimulation intensity method*, the effect of *sAA collection method*, the effect of *stimulation intensity* and the interaction between *time*, *stimulation* and *duty cycle*. Similarly to the output of M1, the output from M2 showed a significant interaction between *time* and *stimulation*, $b = 0.16$, $SE = 0.05$, $p = 0.001$, revealing increased sAA levels for vagus, $b = 0.19$, $SE = 0.06$, $p = 0.017$, as opposed to sham stimulation, $b = 0.01$,

Table 2
The core model M1 with $N_{observations} = 1556$, $N_{participants} = 371$, $N_{studies} = 10$.

	Est (U/ml)	SE (U/ml)	Fixed Effects 95% CI	t	p
Intercept	4.54	0.12	4.26–4.81	37.26	**<0.001**
Time (post - pre)	0.08	0.05	−0.03 – 0.19	1.58	0.150
Stimulation (vagus - sham)	0.06	0.03	−0.00 – 0.11	1.84	0.067
Time X Stimulation	0.12	0.04	0.04–0.21	2.81	**0.005**
			Random Effects		
			Variance	**S.D.**	**Correlation**
Participant (Intercept)			0.52	0.72	
Study (Intercept)			0.13	0.36	
Time ‖ Participant			0.09	0.30	
Stimulation ‖ Participant			0.10	0.31	−0.39
Time ‖ Study			0.02	0.13	
			Model Fit		
R^2			**Marginal**		**Conditional**
			0.003		0.813

$SE = 0.06$, $p = 0.994$ (see Fig. 1A) (see also Fig. S1 in Supplement B), and a significant interaction between *time*, *stimulation* and *duty cycle*, $b = 0.19$, $SE = 0.10$, $p = 0.050$, showing a stronger sAA increase for vagus than for sham with continuous stimulation as opposed to interval stimulation (see Fig. 1B). No further significant effects were found ($0.05 < ps < 1$). The model output of M2 is displayed in Table 3.

3.1.4. The iterative model

We specified a final model M3 based on an iterative modeling approach. As fixed effects in M3, we specified a priori defined comparisons for *time*, for *stimulation* and for the interaction between *time* and *stimulation*. We also included the effect of *sAA collection method* and the interaction between *time*, *stimulation* and *duty cycle*. The model showed no main effect of *time* on sAA, $b = 0.08$, $SE = 0.05$, $p = 0.148$, and no main effect of *stimulation*, $b = 0.06$, $SE = 0.03$, $p = 0.051$. As in M1 and M2, we found a significant interaction of *time* and *stimulation*, $b = 0.16$, $SE = 0.05$, $p = 0.001$, indicating increased sAA levels for vagus, $b = 0.19$, $SE = 0.06$, $p = 0.017$, compared to sham stimulation, $b = 0.01$, $SE = 0.06$, $p = 0.994$. No further significant effects were found ($0.05 < ps < 0.09$). The model output of M3 is displayed in Table 4.

3.2. Model comparison

3.2.1. Best-performing model

The comparison between M1, M2 and M3 revealed significant evidence for a difference in goodness of fit, showing that the full model M2 is the best-performing model as opposed to M1, $\chi^2(2) = 7.65$, $p = 0.022$, and M3, $\chi^2(4) = 9.82$, $p = 0.043$.

3.2.2. Effects in the random structure

The random-effect structure was identical for all models and revealed a negative, medium high correlation between slope of

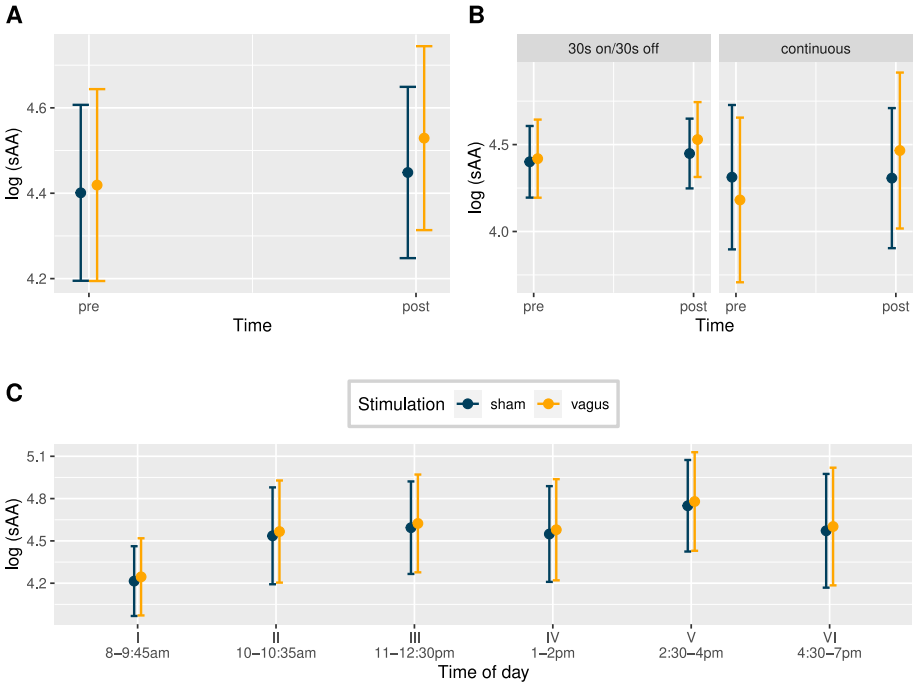

Fig. 1. A: Interaction between *time* and *stimulation*, B: Interaction between *time*, *stimulation* and *duty cycle*, C: Effect of *time of day* for vagus compared to sham stimulation.

Table 3
The full model M2 with $N_{observations} = 1556$, $N_{participants} = 371$, $N_{studies} = 10$.

	Est (U/ml)	SE (U/ml)	Fixed Effects 95% CI	t	p
Intercept	5.04	0.49	3.82–6.26	10.27	**<0.001**
Time (post - pre)	0.08	0.05	−0.03 – 0.19	1.59	0.148
Stimulation (vagus - sham)	0.06	0.03	−0.00 – 0.12	1.91	0.057
Time X Stimulation	0.16	0.05	0.07–0.26	3.38	**0.001**
Stimulation intensity method (fixed at 0.5 mA - determined individually)	0.43	0.23	−0.13 – 1.00	1.84	0.112
sAA collection method (swab collection - spitting method)	0.05	0.50	−1.19 – 1.28	0.09	0.928
Stimulation intensity	−0.05	0.04	−0.13 – 0.02	−1.34	0.180
Stimulation length	−0.01	0.01	−0.03 – 0.01	−1.12	0.308
Duty cycle (continuous - 30s on/30s off)	−0.16	0.15	−0.47 – 0.14	−1.08	0.284
Time X Stimulation X Duty cycle	0.19	0.10	0.00–0.38	1.96	**0.050**
			Random Effects		
			Variance	**S.D.**	**Correlation**
Participant (Intercept)			0.53	0.73	
Study (Intercept)			0.07	0.26	
Time || Participant			0.09	0.30	
Stimulation || Participant			0.09	0.31	−0.38
Time || Study			0.02	0.13	
			Model Fit		
R^2			**Marginal**		**Conditional**
			0.148		0.829

stimulation and slope of time in all models (see Tables 2–4), i.e., participants with higher difference between measurements (pre and post stimulation) over both conditions showed a larger stimulation main effect (higher sAA levels in taVNS session) over both time points.

3.3. Additional model predictors

3.3.1. Gender and time of day in the full model

Adding *gender* and *time of day* to the best-performing model M2 (with a total amount of 1092 observations, 285 participants and 6 studies due to a large amount of missing data for those predictors) did significantly contribute to goodness of fit, $\chi^2(6) = 15.10$, $p = 0.019$. However, neither the associated interaction between *time*, *stimulation* and *gender*, $\chi^2(1) = 0.80$, $p = 0.371$, nor the interaction between *time*, *stimulation* and *time of day*, $\chi^2(5) = 4.92$, $p = 0.426$, significantly improved model fit. Similar to the output of M2, the interaction between *time* and *stimulation* was significant when adding *gender* and *time of day* as fixed effects, $b = 0.18$, $SE = 0.06$, $p = 0.002$, revealing increased sAA levels for vagus compared to sham stimulation. The model further showed a significant main effect of *stimulation*, $b = 0.09$, $SE = 0.04$, $p = 0.029$, which, however, seemed to be driven by the significant interaction between *time* and *stimulation*. Moreover, a significant difference for *time of day*, $b = 0.32$, $SE = 0.12$, $p = 0.007$, showing significantly lower sAA levels for time of day I (i.e., early morning) as compared to later during the day, was significant ($M_{timeofdayI} = 3.98$ U/ml, $M_{timeofdayII} = 4.30$ U/ml, $M_{timeofdayIII} = 4.39$ U/ml, $M_{timeofdayIV} = 4.32$ U/ml, $M_{timeofdayV} = 4.50$ U/ml, $M_{timeofdayVI} = 4.33$ U/ml) (see Fig. 1C). No further significant effects were found ($0.10 < ps < 0.80$).

3.4. Meta-analysis

There was strong evidence for the null hypothesis across studies, $g = 0.13$, 95%*CI* [− 0.07, 0.34], $t = 1.52$, $p = 0.164$, suggesting no effect of vagal stimulation on the sAA increase. There was no evidence for homogeneity, $\tau = 0.265$, 95%*CI* [0.17, 0.51], $I^2 = 92\%$, $p < 0.01$. This meta-analysis, however, was shown to be underpowered to detect potentially meaningful effects significantly different from zero, with a power of 0.21. The forest plot of this analysis is represented in Fig. 2.

Table 4
The iterative model M3 with $N_{observations} = 1556$, $N_{participants} = 371$, $N_{studies} = 10$.

	Est (U/ml)	SE (U/ml)	Fixed Effects 95% CI	t	p
Intercept	4.49	0.11	4.24–4.74	41.20	**<0.001**
Time (post - pre)	0.08	0.05	−0.03 – 0.20	1.59	0.148
Stimulation (vagus - sham)	0.06	0.03	−0.00 – 0.12	1.96	0.051
Time X Stimulation	0.16	0.05	0.07–0.26	3.38	**0.001**
sAA collection method (swab collection - spitting method)	0.42	0.22	−0.08 – 0.92	1.94	0.087
Time X Stimulation X Duty cycle	0.19	0.10	−0.00 – 0.37	1.95	0.051
			Random Effects		
			Variance	**S.D.**	**Correlation**
Participant (Intercept)			0.53	0.72	
Study (Intercept)			0.09	0.31	
Time || Participant			0.09	0.30	
Stimulation || Participant			0.10	0.31	−0.39
Time || Study			0.02	0.13	
			Model Fit		
R^2			**Marginal**		**Conditional**
			0.052		0.815

4. Discussion

Previous work has suggested a modulatory role of taVNS on cognitive and affective functions, which might be mediated by activation of the LC-NA system. Reliable effects of taVNS on markers of LC-NA system activity, however, have not been demonstrated yet (cf. [7]). The present project, therefore, aimed to shed light on this recent controversy by pooling raw data from a large sample of taVNS studies that collected sAA levels as potential marker of central NA release. We explored a variety of modeling approaches and observed that taVNS, compared to sham stimulation, increased sAA levels in all generated predictive models, suggesting a modulatory role of taVNS on sAA. When considering potential confounders of sAA, we further replicated previous findings on the diurnal trajectory of sAA activity with lower levels in the morning and an increase during the course of the day.

The enhancing effect of taVNS (prior compared to post stimulation) on sAA was consistent across all generated predictive models, suggesting that it is a highly relevant predictor. The release of central NA has previously been associated with increased sAA secretion in pharmacological studies [87–89] (see for review [90]). Consequently, sAA has emerged as a promising marker of sympathetic nervous system activity, orchestrated by the LC-NA system [125]. The current findings thus suggest that taVNS, through activation of afferent fibers of the vagus nerve, leads to the activation of the LC-NA system.

Single studies, however, produced mixed results. In one study, Ventura-Bort and colleagues [84] reported increased sAA levels after taVNS but not after sham stimulation based on post hoc analysis. Similarly, Warren and colleagues [79] replicated this finding and further found no effects of taVNS on salivary flow rate (i.e., amount of saliva per minute), ruling out parasympathetic influence on sAA release (cf. [93]). Nevertheless, there has also been a growing body of null findings in taVNS studies, challenging the reliability of sAA as potential NA marker and further questioning taVNS efficacy. Most recently, D'Agostini and colleagues [81] reported no evidence for a modulating effect of taVNS on sAA in a sample of 66 healthy participants performing a novelty auditory oddball task. Similarly, five other studies used in the current data pooling have added to the inconsistent evidence for a modulating effect of taVNS on sAA in humans [64,65,80,91,92]. The inconsistency and lack of replicability across taVNS studies may be due to several reasons. First, as shown in our meta-analysis, most included studies had relatively low sample sizes and the investigated effects were small (as indicated by the wide CI in Fig. 2). This can lead to an increase in both false-negative and false-positive findings. Second, our meta-analysis showed a large heterogeneity between studies, which most likely is related to differences in study characteristics, including experimental designs (e.g., experimental tasks), stimulation procedures (e.g., stimulation length, stimulation intensity, stimulation duty cycle), methodological differences in data collection (e.g., sAA collection method), preprocessing and/or statistical analysis. This was further validated by the fact that the meta-analysis was underpowered (i.e., lack of power in meta-analyses has been proposed to be potentially caused by high heterogeneity rather than by the number of studies [126]). It is worth mentioning that our dataset included taVNS studies that collected sAA, which predominantly reported no significant effects of taVNS on sAA. By increasing overall sample size, however, the pooling of these independent studies led to evidence for a modulating effect of taVNS on sAA, suggesting that taVNS increases central noradrenergic release. An important implication of the fact that we find this effect even though most of the included studies reported null findings is that the effects of taVNS on sAA are rather delicate. Interestingly, the overall high variance between participants in sAA levels might suggest that participants tend to react differently to taVNS. The assessment of the distributions of sAA increases and decreases for vagus and sham stimulation, however, did not enable us to conclusively clarify whether we are looking at a small but generalizable effect or if a small percentage of responders drives the observed effect (see Fig. S2 and Fig. S3 in Supplement B). As suggested by the meta-analysis and Fig. S3 in Supplement B, the variability across studies is large. Some studies show an (almost identical) overlap between vagus and sham stimulation conditions (e.g., [97]), whereas others show higher values for one of the conditions (see for vagus condition for instance [65]; see for sham condition for instance [79], Exp. 2). Although not conclusive, we interpret these results as not pointing towards a few responders. When looking closely at single distributions of studies showing the observed effect of vagus stimulation on sAA levels, the effect seems to be due to a general, small effect (see Refs. [65,79,84,98]), rather than being driven by a group of responders. Fig. S2 in Supplement B further highlights that the overall distribution is not characterized by individual outliers. This needs to be further investigated in future studies, which should determine statistically valuable sample sizes in order to confirm meaningful increases of sAA after taVNS compared to sham stimulation. Based on our analyses, however, it is not possible to determine such statistically valuable sample sizes for future studies due to the large heterogeneity of the data. Although a power analysis revealed that statistical power was

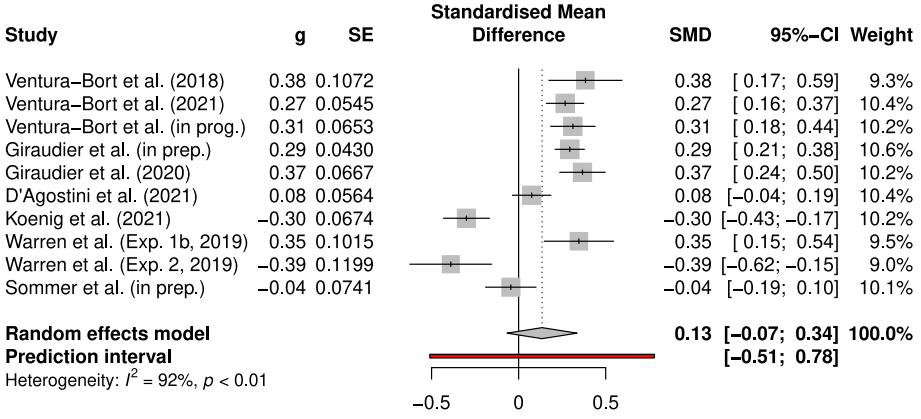

Fig. 2. Forest plot of standardized mean difference for all included studies for the sAA increase under taVNS compared to sham stimulation. The diamond shape represents the average effect and its length symbolizes the confidence interval of the pooled results. The red line below the diamond represents the length of the associated prediction interval. Note: g, effect estimate; SE, standard error; SMD, standardized mean difference; CI, confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sufficient for conducting linear mixed model analyses in the present dataset (see Supplement C), the corresponding estimation of sample sizes to reach an acceptable power only applies to similarly heterogeneous datasets and thus, cannot be transferred to single study designs. The fact that some studies could find significant effects of taVNS on sAA levels with rather small sample sizes, however, suggests that this is generally possible and might depend on specific and possibly yet unknown study characteristics (e.g., stimulation length, task).

In order to identify the most appropriate predictive models, we explored a variety of modeling approaches and consequently, determined our full model as best-performing model out of the three developed models. In addition to the already discussed enhancing effect of taVNS on sAA, this model also showed a significant interaction between *time*, *stimulation* and *duty cycle*, possibly indicating continuous stimulation to be more efficient as opposed to interval stimulation (30s on/30s off). It has been suggested that interval stimulation might lead to unwanted rapid decline in NA activity, thus possibly reducing the modulating effect of taVNS on markers of noradrenergic activity [81]. Although this is in line with some animal research showing such decline in NA after invasive vagal stimulation was turned off [40,127], other electrophysiological studies in rats have reported enhanced firing rates of LC neurons and NA release after invasive vagal stimulation delivered in 30s on/30s off cycles [10,11,28,128]. In humans, the impact of different duty cycles on effects of taVNS is also not well understood yet. Recent studies showed for both, continuous and interval stimulation, an improvement in memory (see for continuous stimulation [65]; see for interval stimulation [64]) and cognitive control (see for continuous stimulation [70]; see for interval stimulation [67–69,71]). In general, however, the majority of taVNS studies delivered stimulation in 30s on/30s off cycles, mostly due to technical reasons (i.e., tVNS Technologies GmbH has embedded this on/off cycle in their commercial device). This imbalance across studies is also reflected in our dataset, with three studies applying continuous stimulation, and eight studies applying stimulation alternating between on and off phases every 30 s. It must be mentioned though that the triple interaction observed in the present data may also be partly driven by differences in experimental designs. Of note, all studies that applied continuous stimulation also used emotionally laden (arousing) material (IAPS images [129]), which also modulates sAA levels (e.g., [130]). Thus, sAA levels may increase particularly under tonic stimulation and in the context of emotional arousal. Considering the heterogeneity of our data and the explorative character of the full modeling approach, the observed advantage of continuous stimulation, however, should be interpreted with caution and requires future verification.

When further considering potential confounders of sAA levels by adding *time of day* and *gender* to the best-performing model, we found decreased sAA levels in the morning as compared to later during the day. This finding is consistent with previous literature suggesting that saliva composition varies rhythmically over the day [106,131,132]. Specifically, animal studies showed that sAA levels are low at the beginning of the day and increase at the end of the afternoon [131,133]. In humans, similar effects have been found [134–136]. More recently, Nater and colleagues [132] investigated the diurnal profile of sAA in a field study with hourly samplings from morning to evening and confirmed a decrease of sAA in the first hour after awakening, along with rising levels towards the afternoon and evening. The authors further examined potentially influencing factors of sAA and found that the diurnal profile of sAA was rather robust against influence factors such as gender. This is consistent with our results showing a similar diurnal course of sAA (i.e., decreased levels in the morning and rising levels throughout the day) and no evidence for an effect of *gender*. These findings invite to consider potential confounders for a reliable measurement of sAA. Even though *time of day* did not seem to directly influence stimulation, researchers should consider scheduling experimental sessions at the same time of day in within-subject designs and preferably avoid the measurement of sAA early in the morning (i.e., before 10am) to control for the effects of circadian influence (cf. [132]). Researchers should also control for other potentially influencing factors of sAA (e.g., age) to further investigate which confounders are statistically associated with the outcome, and if so, these factors should be entered as covariates in statistical analyses [137].

When interpreting the results of the present study, some limitations should be taken into consideration. First, the validity of our findings is limited to the noradrenergic pathway as potential working mechanism of taVNS. Future research may consider alternative pathways targeted by taVNS, such as serotonergic, dopaminergic and cholinergic signaling, and their associated physiological markers (cf. [9]). Ideally, this should include more stable markers with less potentially confounding factors than sAA. Although an acceptable test-retest reliability was found for sAA ($ICC = 0.79$, $CI[0.75; 0.83]$, $p < 0.001$), other NA markers could be further explored such as the P300 ERP component (see for review [50]) or pupil dilation (see for review [75]). Second, although sAA levels can be measured in a non-invasive and inexpensive fashion, some methodological concerns of sAA as index of central noradrenergic activity must be taken into account. Based on the ongoing debate whether sAA levels measured during stress reflect purely sympathetic or parasympathetic activity or some combination of both [93,108], it has been recommended to collect salivary flow rate as measure of parasympathetic activity [137]. In the present study, however, we did not investigate the contribution of salivary flow rate and thus, cannot exclude parasympathetic influence on sAA secretion, as this data was not available for the majority of included studies. Third, all included studies used tasks that might induce additional levels of stress (arousal), possibly interacting with the observed taVNS stimulation effects of sAA. Thus, it remains unclear whether the sole application of taVNS without such engaging task would also lead to similar increases. Future research should therefore investigate if and to what extent the effects of taVNS on sAA levels might be task-dependent. Although our work emphasizes the advantages of data pooling and data sharing (especially of raw data) to overcome limitations of single studies (i.e., small sample size), and to accelerate progress in validating potential relations between reliable NA markers and taVNS-mediated vagal activation, disadvantages and shortcomings of data pooling should also be taken into consideration. Mega-analyses require homogeneous datasets and the establishment of a common centralized database [94]. Methodological differences in study characteristics, stimulation protocols, data collection, preprocessing and/or statistical analysis across studies therefore reduce comparability. Indeed, our meta-analysis showed high heterogeneity in the data, which in turn might explain why we were not able to detect any other effects of stimulation parameters (e.g., stimulation length, stimulation intensity) on sAA levels. Therefore, it is important to emphasize the explorative character of the present approach and further research is certainly necessary.

To summarize, the present findings lead us to conclude that vagal activation via taVNS increases sAA release compared to sham stimulation, which likely substantiates the assumption that taVNS triggers NA release. Future taVNS studies with appropriate sample sizes, collecting sAA levels, along with other potentially confounding factors of sAA, are essential to further validate our findings in other contexts. Given the rather small effect size and the heterogeneity of our data, there are still numerous open questions and concerns that need to be addressed. Importantly, the generalizability of the observed effect of taVNS on sAA release remains unclear. Future studies need to account for the possibility of inter-individual differences of participants (i.e., non-responders) and

should further determine statistically valuable sample sizes in order to confirm meaningful increases of sAA after taVNS compared to sham stimulation. Accordingly, the question arises as to the practicality of sAA as an indirect marker of NA system activation in the context of taVNS research since not all included studies showed a significant effect of taVNS on sAA. This work particularly emphasizes the benefits of data pooling and data sharing in order to publish more meaningful and valuable data in research and to further address these open questions together. In this line, we urge researchers to join forces in the search for essential stimulation parameters and reliable markers that might shed further light on the efficacy of taVNS.

CRediT authorship contribution statement

Manon Giraudier: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Carlos Ventura-Bort:** Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Andreas M. Burger:** Data curation, Writing – review & editing. **Nathalie Claes:** Data curation, Writing – review & editing. **Martina D'Agostini:** Data curation, Writing – review & editing. **Rico Fischer:** Data curation, Writing – review & editing. **Mathijs Franssen:** Data curation, Writing – review & editing. **Michael Kaess:** Data curation, Writing – review & editing. **Julian Koenig:** Data curation, Writing – review & editing. **Roman Liepelt:** Data curation, Writing – review & editing. **Sander Nieuwenhuis:** Data curation, Writing – review & editing. **Aldo Sommer:** Data curation, Writing – review & editing. **Taras Usichenko:** Data curation, Writing – review & editing. **Ilse Van Diest:** Data curation, Writing – review & editing. **Andreas von Leupoldt:** Data curation, Writing – review & editing. **Christopher M. Warren:** Data curation, Writing – review & editing. **Mathias Weymar:** Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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